Biochemical and Molecular Approaches to the Study of Iron Nutrition in the Marine Cyanobacterium Synechococcus WH 7803

by

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I dedicate this thesis to the memory of my Grandma;

Marjorie Elizabeth Chadd

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Declaration

I hereby declare that the work described in this thesis was conducted by myself, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the information contained herein has been used in any previous application for a degree.

All sources of information have been specifically acknowledged by means of reference.

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Summary

Field work with natural phytoplankton assemblages using conventional oceanographic techniques, has established the limited ability of such methods to be able to identify the existence of iron-deplete conditions in the North Atlantic Ocean. Such results confirmed the requirement of "novel" detection methods to determine whether phytoplankton are iron limited in non-productive waters.

Carotenoid-containing cell walls from the marine cyanobacterium *Synechococcus* WH 7803 contained two major polypeptides of M_r = 94,000 and 67,000. The larger of the two appeared to be heat modifiable, a characteristic of porins. Western blot analysis showed that the cell walls from different species of cyanobacteria and one prochlorophyte exhibited a high degree of antigenic relatedness.

The effects of iron-deplete conditions upon the outer membrane protein (OMP) profile of *Synechococcus* WH 7803 was simulated by the addition of 15 $\mu g\ ml^{-1}$ EDDA to normal growth medium. Initial SDS-PAGE analysis revealed the presence of specific iron-regulated outer-membrane proteins. The size of the polypeptides present depended upon the type of iron limitation imposed.

Light-stimulated ^{35}S-methionine uptake and incorporation into protein was observed with *Synechococcus* WH 7803. The reproducibility and sensitivity of this technique resulted in its use as a detection system for the synthesis of iron-regulated membrane proteins. After 21-48 hr growth of *Synechococcus* WH 7803 cells in iron-deplete medium a protein of M_r 36,000 was synthesized. Repression of the protein's synthesis occurred after 24 hr growth in iron-replete medium. This protein was localized to both the internal membrane and the cell wall. By contrast, antibodies raised against the 36,000 Da polypeptide were able to detect the protein in internal membrane, cell wall and whole cell fractions of *Synechococcus* WH 7803 cells grown in both iron-replete and deplete medium. This suggests that the protein is expressed constitutively.

Although partial characterization of the 36,000 Da polypeptide has, as yet, been unsuccessful, certain characteristics of the protein were reminiscent of those of TonB. Antibodies raised against a synthetic peptide containing the x-proline dipeptide repeat of TonB were unable to detect a similar protein in *Synechococcus* WH 7803. However, Southern hybridization analysis using both the *S. typhimurium tonb* gene and an oligonucleotide probe constructed against the TonB Lys-Pro dipeptide repeat, suggest the presence of a TonB homologue. Once the blockage has been removed from the N-terminus of the 36 kDa protein the sequence should hopefully yield conclusive evidence as to the protein's function.

Sequestration of iron by *Synechococcus* WH 7803 cells was observed using flame atomic absorption spectroscopy on whole cell samples. Such sequestration suggests the presence of iron storage proteins. Western blot analysis and Mössbauer spectroscopy confirmed the presence of an iron storage protein namely bacterioferritin. The ^{57}Fe associated with the storage protein was in the same form as that found in the bacterioferritins of *E.coli* and *P.aeruginosa*, as determined by Mössbauer spectroscopy. In addition, siderophore production has not been found in this cyanobacterium.

Abbreviations

APC	- allophycocyanin
Ap^r	- ampicillin resistant
ASW	- artificial seawater
BFR	- bacterioferritin
BOFS	- Biogeochemical Ocean Flux Study
cpm	- counts per minute
EDDA	- ethylenediamine di(o-hydroxy-phenylacetic acid)
EDTA	- ethylenediaminetetra acetic acid
Fe	- iron
Fur	- ferric uptake regulon
HEPES	- N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hr	- hours
HRP	- horseradish peroxidase
IM	- internal membrane
IRMPs	- iron-regulated membrane proteins
IrpA	- iron-regulated protein A
kb	- kilo base pairs
μE	- micro Einsteins
M_r	- relative molecular mass
OMP	- outer membrane protein
PAGE	- polyacrylamide gel electrophoresis
PBP	- periplasmic binding protein
PBS	- phosphate buffered saline
PC	- phycocyanin
PCR	- polymerase chain reaction
PE	- phycoerythrin
PEB	- phycoerythrobilin
PUB	- phycourobilin
PVDF	- polyvinylidene difluoride
RFLP	- restriction fragment length polymorphism
rpm	- revs per minute
R.R.S.	- Royal Research Ship
SDS	- sodium dodecyl sulphate
TCA	- trichloroacetic acid
v/v	- concentration, volume by volume
w/v	- concentration, weight by volume

CHAPTER 1
INTRODUCTION

1.1 The cyanobacteria - a general introduction

Cyanobacteriaceae, a phylum of the Eubacteria, are believed to have contributed to the transition in the Earth's atmosphere from its anaerobic to aerobic state during the Precambian Era (Schopf & Walter, 1982). This transition resulted from the ability to use water as the reductant in photosynthesis with the concomitant production of oxygen. This is achieved in cyanobacteria via a two stage photosynthetic process, which is similar to the process in higher plant chloroplasts, but distinct from that of photosynthetic bacteria. Photosynthetic bacteria have only one photosystem which requires an organic molecule or sulphide as the electron donor. Cyanobacteria are diverse both in morphology and in the habitats from which they are isolated. They are found in various niches: terrestrial, marine, freshwater and hypersaline environments as well as in many symbiotic associations (see Carr & Whitton, 1982; Fay & Van Baalen, 1987). The diverse nature of this phylum is emphasized by the ability of its members to exhibit a number of different growth modes, ranging from photoautotrophy, through photoheterotrophy to chemoheterotrophy. Whereas most species are prototrophic some require vitamin B_{12} and many have the ability to fix nitrogen. Although the subject of nitrogen fixation is fascinating and has begun to be analysed at the molecular level, it is somewhat removed from the theme of this thesis and has been extensively reviewed elsewhere (see Haselkorn, 1986; Van Baalen, 1987; Gallon & Chaplin, 1988).

Other aspects of cyanobacteria that have attracted recent attention include the function and formation of gas vesicles (see Walsby, 1987), production of toxins (see Codd & Poon, 1988) and the analysis of photosynthetic mechanisms and structures (see Bryant, 1986).

Cyanobacteria were initially discovered and distinguished from other algae by virtue of their possession of phycobiliproteins which give them their distinctive colours; ranging from green, blue-green, olive-green, to various shades of red to purple and even black (see Rippka, 1988). Further analysis was achieved by microscopy, characterization of growth requirements, oxygen evolution measurements and pigment content determination. These procedures continued to allow their separation from other bacteria such as photosynthetic bacteria or *Prochlorophytes*.

Classification of the Cyanobacteriaceae is a complex issue because of the large degree of diversity within this phylum, as a result a bacteriological approach using morphological characteristics was provisionally suggested by Rippka *et al.* (1979). Five sections were devised.

Section I. unicellular cyanobacteria e.g. *Synechococcus*, and *Synechocystis*. Both vary greatly in their physiological and biochemical properties, reflected by their wide span in mean DNA base composition of 39-71 mol% G+C for *Synechococcus* and 35-45 mol% G+C (Herdman *et al.*, 1979) for *Synechocystis*.

Section II. comprises the pleurocapsalean cyanobacteria, in this group cell division is internal via multiple fission, producing baeocytes (small daughter cells).

Section III. comprises the filamentous and non heterocystous cyanobacteria.

Section IV and V. comprise the branching and non-branching filamentous cyanobacteria capable of heterocyst formation.

Rippka (1988) gives a detailed description of the genera in each section.

All cyanobacteria are able to harvest light energy via light-harvesting pigments such as the phycobiliproteins, chlorophyll *a* and in some instances carotenoids (Prezelin & Boczar, 1986). Phycobilisomes are composed of a core and rod structure, the core structure contains the phycobiliprotein allophycocyanin (APC) whereas the rod structure contains the phycobiliproteins phycocyanin (PC) and sometimes phycoerythrin (PE). All three chromophoric proteins are composed of an α and a β chain, each polypeptide chain carries one or more covalently attached bilin. The phycobiliprotein α β subunits assemble to form disc shaped trimers $(\alpha\beta)_3$, within the phycobilisome two trimers are arranged face to face producing a hexameric molecule $(\alpha\beta)_6$. Each hexameric biliprotein complex is attached to the adjacent complex through its specific linker such that PE hexamers are at the periphery

followed by PC and then APC. The colour of the phycobiliprotein is dependant upon both the chemical nature of the bilin and the conformation and environment imposed upon the bilin by the native protein as well as inter α β interactions. Furthermore, the location of the bilin residue upon the primary polypeptide structure is identical for all phycobiliproteins, however, the chemical nature of the bilin at these positions differs between phycobiliproteins (Glazer, 1989). Unlike higher plants, cyanobacteria contain only chlorophyll *a*, and the most common thylakoid arrangement is peripheral with three to six layers arranged in concentric shells parallel to the cytoplasmic membrane (Golecki & Drews, 1982). One exception to this is the cyanobacterium *Gloeobacter violaceus* which lacks thylakoids and instead maintains its photosynthetic machinery within the cytoplasmic membrane; the phycobilisomes are arranged in an underlying cortical layer (Guglielmi *et al.*, 1981). *Prochloron sp.* a member of the prochlorophyta (Lewin, 1976), closely related to the cyanobacteria contains both chlorophyll *a* and *b* but no phycobilisomes (Lewin, 1984; Burger-Wiersma *et al.*, 1986) and in this respect resembles the chloroplast of higher plants. However, its thylakoid membrane is arranged in the same manner as in cyanobacteria.

The evolutionary relatedness between cyanobacteria and chloroplasts has been a focus of discussion for many years and has been approached by a number of laboratories, often with inconclusive techniques such as fossil record determination and the use of comparative biochemical and physiological data

(see Schopf & Oehler, 1976; Margulis, 1981; Doolittle, 1982). These methods required as Doolittle stated " *ad hoc* assumptions about the course of evolution of these processes and about true homology (at the genomic level) between apparently similar processes in different groups".

The most conclusive way to determine the evolutionary relatedness between different species is to compare their nucleic acid sequences, the results of which form the basis for other phylogenetic markers to be used. The recent development of nucleic acid sequencing has revolutionized the study of phylogenecity and evolutionary relatedness. Highly conserved genes must be chosen when conducting sequencing studies on evolutionary phylogenetic relatedness, otherwise spurious results and incorrect phylogenetic trees will ensue. For example, when interpreting ferredoxin sequences from *Halobacterium halobium*, which contains a 2Fe-2S ferredoxin, it was found to be more similar in sequence to that of cyanobacteria, algae and plant ferredoxin compared to any other photosynthetic or non-photosynthetic bacterial ferredoxin. However, not only is it believed that photosynthesis developed independently in halobacteria (Bayley, 1979), but the halobacterial lineage is one of the archaebacterial lineages and very remote from the true photosynthetic prokaryote lineage (Magrum *et al.*, 1978). The high homology was probably due to inter-specific or inter-kingdom transfer of a required gene (Doolittle, 1982). This result highlights one of the disadvantages when using DNA-DNA hybridization studies in evolutionary relatedness.

For a decade 16S rRNA was characterized by partial sequence analysis using the oligonucleotide cataloguing method described by Fox *et al.* (1977). However the resolving power was increased by full 16S rRNA sequencing. These techniques were subsequently utilized to define ten major divisions of eubacteria (see Woese, 1987).

Woese (1987) coined the phrase "molecular chronometers" to describe genetic material which represents the overall rate of evolutionary change in a line of descendants. Until recently nucleic acid hybridization probes had been used to throw light upon the relationship among cultivated bacteria (Giovannoni *et al.*, 1988_a). However, the disadvantages of working with hybridization probes as a means of elucidating evolutionary relatedness is that, in being highly specific themselves, they will only detect specific organisms. More recently probes have been used to detect groups of organisms which are phylogenetically related (Woese, 1987; Giovannoni *et al.*, 1988_a). A group of evolutionarily-related organisms will embrace a range of shared properties, the scope of which is dependent upon the phylogenetic breadth of that group. The most appropriate phylogenetic probes or "molecular chronometers" are 16S rRNA sequences. Some segments of the rRNA base sequence are invariant in all organisms whereas other segments are unique to a particular organism or genus (Lane *et al.*, 1985). Coupled with the fact that they are ubiquitous, 16S rRNAs are powerful analytical tools with the ability to identify virtually any organism or group of organisms.

The relationship between the cyanobacteria and the green (euglenoids, green algae, higher plants) and red (rhodophyte) chloroplast has been characterized via molecular phylogenetic analysis of c type cytochromes and rRNA sequences (Giovannoni *et al.*, 1988_b). Hybridization studies using two genes, *rbcA* (Curtis & Haselkorn, 1983) encoding the large subunit of RuBisCO and *psbA* (Curtis & Haselkorn, 1984) encoding a 32 kDa integral membrane protein Q_B associated with Photosystem II, have been used to detect homologies between plants, algae and cyanobacteria. The high sequence homology (71-77%, 84-87% respectively) between plant chloroplast DNA and that of cyanobacteria, coupled with the results of the molecular phylogenetic analysis above, contribute to substantiating the evolutionary relationship between the chloroplast and cyanobacteria. Furthermore, Giovannoni *et al.* (1988_b) exploited 16S rRNA sequencing to study the evolutionary relationship among thirty representatives of the cyanobacterial groups and the phytoflagellate *Cyanophora paradoxa*. They concluded that the green chloroplast and cyanobacteria form a coherent phylogenetic group and that the descendancy of the cyanelle of *Cyanophora paradoxa* and the green chloroplast should be viewed as one of the cyanobacterial sublines.

The use of 16S rRNAs for the study of microbial systematics has culminated in the generation of a large computer data base which reflects the phylogenetic diversity found within culture collections (Britschgi & Giovannoni, 1991). To investigate the genetic structure and diversity of

natural marine bacterioplankton populations, Giovannoni and his co-workers have taken these 16S rRNA probes into the natural marine environment, in order to characterize the bacterioplankton population, and as a result have discovered that there were abundant naturally occurring bacteria which have escaped our knowledge due to our inability to culture them. The abundance of rRNA in the cell facilitate its use in ecological studies (Giovannoni *et al.*, 1990; Britschgi & Giovannoni, 1991). Using oligonucleotides complementary to conserved regions of a broad range of eubacterial 16S rRNA, they were able to isolate and clone a number of 16S rRNA genes from Sargasso Sea bacterioplankton genomic DNA via the polymerase chain reaction (PCR) technique. Two clusters were identified upon sequence analysis, one of which (SAR 7), was related to the oxygenic phototrophs:- cyanobacteria, prochlorophytes and chloroplasts. However, on comparison of the 16S rRNA gene sequences of four culturable marine cyanobacteria (*Synechococcus* sp.), a high diversity of 16S rRNA gene lineages among closely related marine cyanobacteria became apparent (Giovannoni *et al.*, 1990). The appearance of novel lineages closely related to marine *Synechococcus* sp. suggests the presence of species as yet unknown to microbiologists. Furthermore, Britschgi & Giovannoni (1991) found that of the total bacterioplankton genes analysed from the Sargasso Sea 47% were from undefined species. Liesack *et al.* (1991) have outlined potential risks of using PCR-mediated amplification of 16S rRNA genes from mixed cultures, their results suggest that amplification of 16s rRNA in the presence of genomic DNA from different origins supports the formation

of hybrid molecules. Therefore, the presence of such hybrid molecules would suggest the existence of organisms which do not exist in the sample investigated. More recently Schmidt *et al.* (1991) have analysed the phylogenetic composition of marine samples from the north central Pacific Ocean. Their findings show the picoplankton to be dominated by cyanobacteria and the proteobacteria. Moreover, the cyanobacterial sequences recognised have been shown to be closely related to the *Synechococcus* strains WH 8103 and WH 7805. The fact that the cyanobacterial sequences obtained from the Sargasso Sea (Giovannoni *et al.*, 1990; Britschgi & Giovannoni, 1991) also belong to the closely related group of marine *Synechococcus* strains, supports earlier work suggesting that these marine strains are the most abundant cyanobacteria in the oceans (Waterbury *et al.*, 1986).

The advance of the study of molecular phylogies of natural populations, in contrast to similar work on cultured bacteria, will provide a greater insight into the genetic structure of the marine microbial population, together with a better representation of the physiological and ecological roles supported by these novel microbial lineages (Britschgi & Giovannoni, 1991).

As discussed earlier there is now confirmation that the higher plant chloroplast is descended from the cyanobacteria. For this reason and because they are more genetically amenable cyanobacteria have become important model organisms for the study of photosynthesis. In 1986 Bryant reviewed the cyanobacterial photosynthetic system, comparing them to higher

plants. Since then, using molecular biological techniques, he and his co-workers have taken a major role in the elucidation of the complex intermediary steps involved in the electron transport chains of Photosystem I (Zhao *et al.*, 1990, Li *et al.*, 1991) as well as Photosystem II (Gingrich *et al.*, 1990). Further studies are aimed towards understanding the regulation at the level of gene expression, of the protein components of the photosynthetic apparatus (Mohamed & Jansson, 1991). Moreover, molecular studies have also been used to characterize phycobilisome assembly. By constructing a mutant in the *cpcC* gene (encoding a 33,000 Da linker polypeptide) of *Synechococcus* PCC 7002, de Lorimier *et al.* (1990) have shown that this 33,000 Da gene product is required for assembly of half the total phycocyanin into phycobilisomes.

The light-harvesting complex of the cyanobacteria, responsible for trapping solar energy, is the phycobilisome (see Gantt, 1980; Cohen-Bazire & Bryant, 1982). Unlike higher plant chloroplasts this light-harvesting complex is extrinsic to the thylakoid membrane, attached to photosystem II via a 92,000 Da linker polypeptide. The phycobilisomes have a complex structure (reviewed by Glazer, 1987, 1989), which allows an adaptation to variations in environmental conditions in certain strains. For example, under high light intensities, fewer phycobilisomes are synthesized and those present are composed of shorter rods. Some strains are capable of complementary chromatic adaptation e.g. *Calothrix* PCC 7601. In this case the composition of the phycobiliproteins present in the phycobilisome is dependent

upon the light quality. Under both red and green light one set of phycocyanin and linker polypeptide genes are constitutively expressed. However, the same organism also contains a set of red-light inducible PC genes and a set of green-light inducible PE and linker polypeptide genes (Tandeau de Marsac *et al.*, 1988; Grossman, 1990).

In response to nitrogen starvation phycobilisome synthesis is repressed and the preformed phycobilisome is used as an alternative nitrogen source (Allen & Smith, 1969; Bou ssiba & Richmond, 1980). Wyman *et al.* (1985) proposed that phycoerythrin was used as a nitrogen reserve in *Synechococcus* WH 7803, being mobilized when exposed to nitrate starvation. Under alternative nutrient starvations such as phosphorus or sulphur, synthesis of the phycobilisome is again repressed (Grossman, 1990; Glazer, 1990). Moreover, under sulphur limitation a third set of phycocyanin genes are expressed (in *Calothrix* sp. PCC 7601). This organism contains a separate set of genes which encode sulphur-depleted amino acid versions of both phycocyanin and three linker polypeptides. The separate set of genes are present upon the *cpc3* operon, this operon has been shown to be highly expressed at low sulphur concentrations. In addition, the PE-encoding operons are switched off under conditions of sulphur limitation (Mazel & Marliere, 1989). Tandeau de Marsac *et al.*, (1990) have reviewed the organisation and regulation of expression of the genes involved in phycobilisome assembly in *Calothrix* PCC 7601, as a response to various environmental factors.

Although a considerable amount is known about environmental effects of nutrient assimilation and light quality on gene expression in cyanobacteria, we lack any detailed mechanistic understanding and infact, no clearly defined regulatory genes have been found.

1.2 The marine *Synechococcus* sp

All the work in this study has been conducted on *Synechococcus* WH 7803, a member of the marine picoplankton population, which are photoautotrophic, phycoerythrin-containing unicellular cyanobacteria, incapable of nitrogen fixation and with an obligate requirement for elevated salt concentrations (see Glover, 1985; Waterbury *et al.*, 1986; Fogg, 1987). A brief discussion of this group of organisms is presented below.

A decade after Guillard (in 1965) isolated a red phycoerythrin-rich cyanobacterium from the coast of South America (Glover, 1985), Waterbury *et al.* (1979) and Johnson & Sieburth (1979) documented the discovery of phycoerythrin-rich, unicellular cyanobacteria inhabiting a range of marine locations. These tiny components of the marine picoplankton (size range between 2 - 0.2 μm) had until then remained largely unnoticed. A major factor in their detection was the introduction of epifluorescence microscopy into oceanography as a means of enumerating picoplankton samples which had been filtered onto a 0.2 μm Nuclepore filter (Hobbie *et al.*, 1977). Owing to their high phycoerythrin content, which has an absorption maximum in the green region of the spectrum at 546

nm (Waterbury *et al.*, 1986), their presence was described as a result of their orange fluorescence. These cyanobacteria were subsequently isolated and cultured (Waterbury *et al.*, 1979; Johnson & Sieburth 1979). The thylakoid membranes in these *Synechococcus* sp. were shown to be arranged peripherally (Waterbury *et al.*, 1986) and they contained chlorophyll *a* only, with phycobiliproteins as accessory pigments (Waterbury *et al.*, 1986). Using the classification system of Rippka (1979) these marine cyanobacteria were assigned to the genus *Synechococcus* owing to their chrococcoidal shape and small size, together with cell division in one plane (Waterbury *et al.*, 1986). Furthermore, these phycoerythrin-containing *Synechococcus* sp. were photoautotrophic and unable to grow at salinities below 14‰, identifying them as intrinsically marine (Waterbury *et al.*, 1979).

The phycoerythrin-containing Synechococci in certain marine locations are more abundant, numerically, than the total eukaryotic phytoplankton (Olson *et al.*, 1990_b). Furthermore, there is evidence to suggest that marine *Synechococcus* sp. contribute significantly to primary productivity; ranging from a few percent to about 50% of the total for a water column (Glover, 1985; Waterbury *et al.*, 1986; Glover *et al.*, 1986; Fogg, 1986; Iturriaga & Marra, 1988; Joint, 1990). Being photosynthetic organisms they are found in the euphotic zone within the water column and mainly associated with the depth of the nitrite maximum (Olson *et al.*, 1990_b). The euphotic zone reaches to a depth of about 100 m. Waterbury *et al.* (1986) documented numerous vertical

profiles of *Synechococcus* sp. distribution within the water column. In some cases the maximum *Synechococcus* sp. concentration was located at the surface whereas in others it was constant throughout the mixed layer. Cell numbers were in the region of 1.5×10^5 cells ml^{-1} to 9×10^4 cells ml^{-1}. The vertical distribution of *Synechococcus* sp. throughout the water column appears to be governed by a number of factors. The most important is the depth to which photosynthetically active light can penetrate giving a 1% transmittance. Below this value cyanobacteria are less competitive in their growth. The depth at which the 1% light level occurs is dependent upon the presence or absence of biomass, especially algal biomass. During algal blooms most of the photosynthetically available light is attenuated (see Kirk, 1986), hence the 1% light level occurs at a much shallower depth, explaining the appearance of *Synechococcus* sp. at the surface. Other factors include the effects of mixing between water masses above and below the thermocline and wind driven mixing, together with the presence of boundary layers between different water masses (Waterbury *et al.*, 1986). These physical processes affect the overall structure of the water column and, therefore, the structure of *Synechococcus* sp. distribution. Glover *et al.* (1988) found that *Synechococcus* sp. accounted for more than 95% of all picoplankton cells in surface waters.

Joint (1986) and Howard & Joint (1989) found maximum *Synechococcus* sp. numbers (ranging from 2.5×10^3 cells ml^{-1} to 1.7×10^5 cells ml^{-1}) in near surface waters in both the Celtic Sea and North Sea respectively. These findings appear to contradict early work by Morris & Glover (1981) who

suggested that, because photosynthesis became saturated at low light intensities in laboratory cultures of *Synechococcus* sp., they would contribute significantly towards primary productivity at the base of the euphotic zone. A further study by Glover *et al.* (1985) in the North Atlantic reported the greatest abundance of *Synechococcus* sp. appearing towards the base of the euphotic zone. Green light (546 nm) has a greater penetration through sea water than red light and the presence of large amounts of phycoerythrin within these *Synechococcus* sp. maximises the existence of these organisms in the region where red light is attenuated. Therefore, it might be expected that these cells would grow preferentially at a depth in the sea where light intensity is low (Joint, 1990). Results with laboratory cultures, on irradiance levels, showed that the optimum growth rate of *Synechococcus* WH 7803 occurred at 45 $\mu E\ m^{-2}\ s^{-1}$ (Morris & Glover, 1981) and enrichment cultures used to isolate cyanobacteria required growth at 10 - 20 $\mu E\ m^{-2}\ s^{-1}$ (Waterbury *et al.*, 1986). This early work on both natural assemblages and laboratory cultures led to the initial belief that oceanic picoplankton should be considered as shade organisms, with a preference for growth at low light intensities, and thus, making a greater contribution to primary productivity at the base of the euphotic zone (Glover, 1985). However, Joint (1990) argues that if marine cyanobacteria are shade organisms, preferentially, if not exclusively found in deeper levels of the euphotic zone (Sournia, 1982), then they should not be able to adapt to differing growth irradiances. Transmission electron micrographs of *Synechococcus* sp. grown under different light

intensities, show differences in thylakoid membrane structure, suggesting that at low irradiances *Synechococcus* sp. are able to adapt by increasing the total photosynthetic membrane per cell (Joint, 1990). Kana & Glibert (1987) found that up to irradiances of 2000 $\mu E\ m^{-2}\ s^{-1}$ there is no photoinhibition of photosynthesis. However, an alteration in the phycoerythrin to phycocyanin ratio per cell from 14 to 2.5 was observed over a light intensity range of 30 - 2000 $\mu E\ m^{-2}\ s^{-1}$. This acts to control the amount of energy flow from the phycobilisome to the photosynthetic reaction centre (Bryant, 1986, Glazer, 1987). The alteration in cell colour from red to yellow at high irradiances is attributable to changes in cellular content of phycoerythrin, chlorophyll and zeaxanthin; zeaxanthin becoming the predominant pigment in the cell at high irradiances (Kana *et al.*, 1988). Cyanobacterial picoplankton therefore, do not appear to be specifically adapted to low light relative to any other phytoplankton (Joint, 1990). If picoplankton do not have an obligate requirement for low light conditions the reason for their high abundance at the base of the euphotic zone poses an interesting question.

Although the introduction of the epifluorescence microscope was instrumental in bringing marine Synechococci into the forefront of marine science, it is unable to differentiate between different *Synechococcus* strains (Waterbury *et al.*, 1986). Applying an immunofluorescence assay against *Synechococcus* strains within the *Synechococcus* WH 7803 serogroup Campbell *et al.* (1983) probed the natural

environment to attempt to detect distinct subgroups of marine *Synechococcus* sp. They found that there was a 20% cross reactivity with the antibody in a warm core eddy, and 5% cross reactivity in neritic waters at the same time. Cells of this serotype were rare in oligotrophic waters. In warmer tropical and subtropical waters numbers exceeded 80% of the total population (Campbell & Carpenter, 1987).

Genetic analysis of marine *Synechococcus* sp. isolates has shown a DNA base ratio ranging from 54-63 mol% G+C; this range spans the gap between two of Rippka's DNA base ratio clusters (Rippka *et al.*, 1979; Rippka & Cohen-Bazire, 1983). This finding led Waterbury *et al.* (1986) to postulate that the phycoerythrin-containing marine *Synechococcus* sp. form a discrete generic unit.

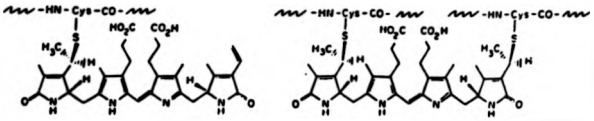
Preliminary studies using analytical tools such as immunology (Campbell *et al.*, 1983; Campbell & Carpenter, 1987; Campbell & Iturriaga, 1988) and flow cytometry (Wood *et al.*, 1985; Olson *et al.*, 1985) have been used as a way of discriminating between *Synechococcus* sp. populations. Also, following studies on fatty acid composition and comparison of the major lipid classes of marine *Synechococcus* isolates with freshwater *Synechococcus* sp. Merritt *et al.* (1991) state that fatty acid compositional groups, defined by Kenyon (1972), used to classify freshwater species may also be applied to marine strains of cyanobacteria.

Olson *et al.* (1990_b) have identified two different populations of *Synechococcus* sp. in the North Atlantic and Pacific oceans using flow cytometry. The cells have been

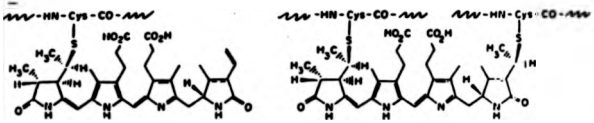
classified depending on the number of phycourobilin tetrapyrrole prosthetic groups covalently attached to phycoerythrin (PE). The absorption maxima (490 nm and 550 nm) for *Synechococcus* sp. PE are a function of the presence of phycourobilin (PUB) and phycoerythrobilin (PEB) prosthetic groups respectively attached to the protein (Ong *et al.*, 1984; Olson *et al.*, 1988). Olson *et al.* (1990_b) have found that the majority of the *Synechococcus* sp. found in the open ocean contain large amounts of phycourobilin (PUB) with respect to phycoerythrobilin (PEB), such cells have been called high-PUB types. Low-PUB *Synechococcus* sp. types have been found in coastal waters only. The structural differences between the phycoerythrobilin (PEB) chromophore and the phycourobilin chromophore is presented in Figure 1.1. Olson *et al.* (1990_b) extended their study to determine the spatial arrangement of these populations. The results confirmed their abundance within the marine environment, being found in every sample taken. However, cell concentrations were higher nearer the coast than in the central oligotrophic regions, probably as a result of low nutrient levels. Furthermore, they found that low-PUB cells, similar to *Synechococcus* WH 7803, were restricted to shallower depths and also occurred in conjunction with high-PUB strains such as *Synechococcus* WH 8103 (Ong *et al.*, 1984). The PUB to PEB ratio in *Synechococcus* WH 8103 is 2.4 as opposed to 0.39 for *Synechococcus* WH 7803. This PUB chromophore is also present in the light-harvesting phycobilisome complex of two macrophytic red algae (Alberte *et al.*, 1984). The knowledge that *Synechococcus* WH 7803 is a low-PUB type organism means

Figure 1.1 **A schematic diagram showing the structural differences between the phycoerythrobilin and the phycourobilin chromophores. (After Glazer, 1987).**

The diagram also shows the different bilin-peptide linkages found.



Peptide-linked PHYCOUROBILINS



Peptide-linked PHYCOERYTHROBILINS

that it is not a true representative of the open oceanic *Synechococcus* sp., unlike the high-PUB containing species *Synechococcus* WH 8103. Even so, it is not appropriate to describe *Synechococcus* WH 7803 as a coastal species. Coastal species are halotolerant species typified by *Synechococcus* WH 5701, *Synechococcus* WH 8101, and *Synechococcus* PCC 7002; none have an elevated salt requirement or contain phycoerythrin, in contrast to *Synechococcus* WH 7803. The absence of phycoerythrin places strains of this type at a distinct spectral disadvantage in seawater (Wood, 1985). It is believed that these strains were washed out from the freshwater environment (Waterbury *et al.*, 1986). As *Synechococcus* WH 7803 appears to fall into a category between coastal and open oceanic species it seems appropriate to categorize it as a marine shelf species.

Douglas & Carr (1988) examined the genetic relatedness of seven commonly studied marine *Synechococcus* clones using the restriction fragment length polymorphism technique (RFLP). They found that *Synechococcus* strains WH 7803 and WH 7802 gave a different RFLP pattern compared to *Synechococcus* strains WH 8008, WH 8018 and WH 7805. All the strains were different from the phycocyanin-rich isolate *Synechococcus* WH 5701. Furthermore the two RFLP similar strains *Synechococcus* WH 7803 and WH 7802 both contained phycoerythrin, but also had near identical PUB to PEB ratios of 0.39 and 0.4 respectively (Waterbury *et al.*, 1986). Strains of *Synechococcus* WH 8008, WH 8018 and WH 7805, shown to be related on the basis of RFLP data, do not contain PUB, however they do contain PEB. In

comparison, all of the strains are different from *Synechococcus* WH 5701 which contains only phycocyanobilin. All the strains above tested have an obligate requirement for salt (except *Synechococcus* WH 5701) and therefore can be regarded as truly marine (Waterbury *et al.*, 1986). Therefore RFLP data in this case supports the apparent species diversity seen when studying differences in phycobiliprotein composition. Wood & Townsend (1990) extended RFLP studies to include eight strains of *Synechococcus* sp. in the WH 7803 serogroup (Campbell & Iturriaga, 1988). Five of these have the *Synechococcus* WH 7803 pigment type and three have the *Synechococcus* WH 8018 pigment type. Their observations confirmed the earlier work by Douglas & Carr (1988). On the basis of RFLP patterns ten clones in the study fell into five groups presented in Table 1.1:-

Table 1.1

Genetically similar groups of *Synechococcus* sp. based upon RFLP data.

group	Organism
I	*Synechococcus* PCC 7942
II	*Synechococcus* WH 8008
	Synechococcus WH 8018
	Synechococcus WH 7805
III	*Synechococcus* WH 7802
	Synechococcus WH 7803
IV	*Synechococcus* WH 8001
	Synechococcus WH 8003
V	*Synechococcus* WH 8002

RFLP patterns obtained were irrespective of pigment composition. The molecular genetic differences between marine Synechococci within the *Synechococcus* WH 7803 serogroup are the same as the differences between marine strains which contain phycoerythrin and freshwater strains which do not (Wood & Townsend, 1990). Furthermore, the fan-shaped phylogenetic tree described by Giovannoni *et al.* (1988_b) for cyanobacteria as a whole, is also applicable to the *Synechococcus* sp. subgroup. This suggests the presence of highly divergent lineages within closely related strains. These results also highlight the difficulties in using other criteria such as biliprotein composition and serogroup analysis as a means of identifying genetically distinct subpopulations of marine *Synechococcus* sp. For example the presence of phycoerythrin and the PUB to PEB ratio for *Synechococcus* WH 8001 and *Synechococcus* WH 8003 is the same as that for *Synechococcus* WH 7803 and *Synechococcus* WH 7802 respectively, however, both fall into a completely different group based on RFLP data. Also *Synechococcus* WH 8002 with a PUB to PEB ratio of 0.48 and very similar to the strains above falls into a group of its own under RFLP determination. Furthermore, despite their differences all these widely divergent lineages of marine *Synechococcus* sp. occur together in natural populations (Wood & Townsend, 1990).

However, analysis of 16S rRNA data for these marine *Synechococcus* strains indicates very similar phylogenacity, in contrast to the results obtained by Wood and Townsend (1990) using RFLP data (J. Waterbury, personal communication). This

result has prompted Waterbury to suggest that RFLP analysis may not be a good technique to determine phylogenecity. It is however, a good technique to determine relationships between organisms.

All the phycoerythrin-containing marine *Synechococcus* sp. belong to marine cluster A (J. Waterbury personal communication). For example; they have a 52-62 mol% G+C ratio, are obligately photoautotrophic, contain phycoerythrin as the primary light-harvesting protein and require elevated salt concentrations for growth.

Further studies of strains from marine cluster A have been carried out to determine at the molecular level the way in which they have optimized their phycobilisomes for the absorption of green light (Ong & Glazer, 1991; Swanson *et al.*, 1991; Wilbanks *et al.*, 1991). These strains are of particular interest as they have the ability to exhibit a wide range of spectral diversity, owing to the range in PEB to PUB ratio within the individual chromoproteins (Kursar *et al.*, 1981; Alberte *et al.*, 1984; Ong *et al.*, 1984).

Ong and Glazer (1991) have discovered that the phycobilisome of *Synechococcus* WH 7803, *Synechococcus* WH 8103 and *Synechococcus* WH 8020 contains two phycoerythrins, PEI and PEII, PEII being the major form. The minor form (PEI) has been sequenced from *Synechococcus* WH 7803 (J. Newman this laboratory, unpublished work), whereas the major form (PEII) has been sequenced from *Synechococcus* WH 8020 (Wilbanks *et al.*, 1991). Both of these phycoerythrins differ in amino acid sequence, bilin composition and content implying the possible

presence of two separate genes. Pycoerythrin II and phycoerythrin I are present in the cell at a weight ratio 2-4:1. Up until now only a single set of phycoerythrin genes have been located (Ong & Glazer, 1991).

Chisholm *et al.* (1988) identified a new abundant group of picoplankters which are barely visible using traditional microscopy techniques, being much smaller than the coccoidal cyanobacteria. They reach concentrations greater than 10^5 cells ml^{-1} in the deep euphotic zone, and are also present though less abundantly at the surface, (Chisholm *et al.*, 1988, Olson *et al.*, 1990_a). Owing to the presence of divinyl chlorophyll *a*-like and a divinyl chlorophyll *b*-like, plus a chlorophyll *c*-like pigment (Olson *et al.*, 1990_a) they fluoresce red under epifluorescence microscopy. Furthermore, they contain α carotene and zeaxanthin and lack phycobiliproteins.
These characteristics were used to assign these prokaryotes to the prochlorophytes, despite the fact that they contain α-carotene rather than ß-carotene plus divinyl chlorophyll *a*-like pigment (Chisholm *et al.*, 1988).

Prochlorophytes of the order Prochlorales were originally believed to share a common ancestry with plant, green algal and euglenoid chloroplasts. Results using 16S rRNA sequence comparisons showed that *Prochlorococcus marinus* (Chisholm *et al.*, 1988) is closely related to the open ocean marine *Synechococcus* cluster A, namely strains WH 7805, and WH 8103 (Urbach *et al.*, 1991; Urbach *et al.*, 1992). Based upon this 16s rRNA data Urbach *et al.* (1992) proposed that the

prochlorophytes should be reclassified in the Cyanobacteriaceae, despite the fact that they do not contain phycobilisomes and have both chlorophyll *a* and *b*.

These novel free living marine prochlorophytes which appear to be well adapted to life at the base of the euphotic zone were identified using flow cytometry (Chisholm *et al.*, 1988; Olson *et al.*, 1990_a).

Other documented marine cyanobacteria include *Trichodesmium* sp., *Synechocystis* sp. and *Richelia* sp.. Both *Trichodesmium* sp. and *Richelia* sp. are filamentous nitrogen-fixing cyanobacteria present mainly in tropical waters, the latter are intracellular symbionts present in several species of diatoms (Fogg, 1982). A recent investigation by Carpenter & Romans (1991) suggests that *Trichodesmium* rather than the picoplankton is the major primary producer in the tropical North Atlantic Ocean. Furthermore, they suggest that *Trichodesmium* is responsible for the introduction of the largest fraction of new nitrogen to the euphotic zone. The marine *Synechocystis* strains are novel unicellular forms capable of aerobic nitrogen fixation (Waterbury & Willey, 1988). Mitsui *et al.* (1986,1988; Leon *et al.*, 1986) documented the discovery of an unicellular *Synechococcus* sp. from a marine environment which displays nitrogenase activity. However, it is important to note that there is no evidence to suggest that this organism has an obligate requirement for elevated salt concentrations, a criterion which must be met for it to be classified as a marine cyanobacterium (Waterbury *et al.*, 1986). Furthermore, the presence of *nif* genes in

marine *Synechococcus* sp. have not been found (J.Zehr -pers. communication, J.Kramer -pers. comunnication, Waterbury *et al.*, 1986). These workers have used enzymatic analysis and molecular genetic probes as a means to locate nitrogenase in marine *Synechococcus* and have as yet been unsuccessful.

It should be noted however, that there is considerable information on the existence and operation of nitrogenase in non-marine unicellular cyanobacteria (Gallon *et al.*, 1974; Mullineaux *et al.*, 1981; 1983; Maryan *et al.*, 1986_a; 1986_b).

1.3 Fe^{+++} and the marine environment.

Presented below is an overview of an area of oceanography which directly relates to much of the experimental work discussed later. It is meant to highlight the relationship and importance of the marine photosynthetic community to the global carbon cycle and potentially, global warming.

The ocean covers 71% of the Earth's surface, and maybe regarded as the last major frontier on earth for the exploration and development of resources to sustain mankind in the future (Couper, 1989). It is thought that, in the future, the oceans will become a much expanded food source for the world's growing population. However, it is not yet known whether man's activities are in danger of causing irreversible damage to such an arguably fragile ecosystem.

The total oceanic surface waters cover an area of about 36×10^6 km^2, reaching to depths ranging from 1000 m to 3000 m

and to the deep ocean trenches below 6000 m. Besides harbouring a multitude of life forms (even though many are uncharacterized, due to the inaccessible nature of the environment) this vast water mass is responsible for playing a critical role in the regulation of the global carbon cycle, due primarily to the presence of the photosynthetic plankton. The oceans are considered to be the major sink for atmospheric carbon dioxide, with estimated values of total oceanic primary production being 20-45 x 10^9 tons C yr^{-1} (Eppley & Peterson, 1979). This primary productivity results in the surface waters being relatively depleted for carbon dioxide compared to other deeper regions. The carbon dioxide concentration gradient between surface and deep waters is derived from the function of the "biological pump"; i.e. the remineralization of organic material in deep waters, as a result of the downward flux of photosynthetically derived organic material from surface waters. As described in section 1.2 the photosynthetic picoplankton communities such as *Synechococcus* sp. are responsible for up to 50% of primary productivity. Therefore, they are clearly important contributors towards global carbon cycling.

The oceanic biological pump is under the control of a number of factors, principally light, temperature and nutrients. Low concentrations of nitrogen, phosphorus and silicon in certain areas of the ocean limit primary productivity and therefore, the export of carbon to the depths (Ryther & Guillard, 1959; Menzel *et al.*, 1963; Owens *et al.*, 1989; Krom *et al.*, 1991). Nutrients usually become limiting during bloom conditions (Goldman *et al.*, 1979). However, in

the Equatorial Pacific, Subarctic Pacific and the Southern Ocean, relatively high levels of these nutrients persist. For example, in the Antarctic, concentrations of nitrate, phosphate, and silicate are typically, 15-30 μM, 1-2 μM and 40-90 μM respectively (Holm-Hansen *et al.*, 1977). These values are three times the concentration for water entering the English Channel from the Atlantic ocean. In contrast, chlorophyll *a* concentrations in the Antarctic Ocean are low, at a value of 0.1-1 $\mu g\ l^{-1}$ seawater (El-Sayed, 1987), compared to the value of 15 μg chlorophyll *a* l^{-1} seawater, which would be expected for the Southern Ocean considering the nutrient levels present. This is assuming the production of 1 g chlorophyll *a* (g atom nitrogen)$^{-1}$ in phytoplankton (Caperon & Meyer, 1972). The apparent lack of available nutrient utilization, low chlorophyll *a* concentration and low carbon fixation rates (El-Sayed, 1988) has led to the belief that productivity in these and similar waters must be restricted by the shortage of another nutrient.

The chemical composition of oceanic phytoplankton is in the approximate proportions of 106:16:1, for carbon, nitrogen and phosphorus respectively. This ratio is known as the Redfield ratio (Redfield, 1958; Redfield *et al.*, 1963). On comparing the elemental composition of plankton with nutrient concentrations found in deep water, the ratios of nitrogen : phosphorus : zinc : manganese in the deep waters were similar to the ratios of these nutrients required to produce plankton biomass. However, the amount of iron required by the plankton

is about twenty times greater than that actually available in the deep ocean, (Table 1.2). This extension of the Redfield ratio indicates that the quantity as well as the chemical form of iron may cause it to be a limiting nutrient to oceanic phytoplankton (Brand, 1986).

Table 1.2 Molar Ratios of Nutrients in Plankton and Deep Water (normalized to Phosphorus)
(after Brand, 1986)

Plankton elemental composition	N	P	Fe	Zn	Mn
Molar ratios (plankton)	16	1	10^{-2}	$4x10^{-3}$	$4x10^{-4}$
Molar ratios (deep water)	15	1	$4x10^{-4}$	$2.8x10^{-3}$	$3.4x10^{-4}$

Although iron is the fourth most abundant element in the Earth's crust, it is one of the least available metals to biological systems. Iron can exist in the metallic (Fe), ferrous (Fe^{++}) and ferric (Fe^{+++}) valency states. In aerobic waters at pH 8.0, 90% of iron is in the Fe^{+++} form (Boyle *et al.*, 1977) as insoluble iron oxides and hydroxides precipitates (Byrne & Kester, 1976). Such insoluble forms will adsorb to particles such as humic substances. "Dissolved iron", specified by it's ability to pass through a 0.45 μm filter, is most likely to be in the colloidal state (Huntsman & Sunda, 1980; Finden *et al.*, 1984; Hudson & Morel, 1989; Wells *et al.*, 1991_b; Wells & Goldberg, 1991). In these Fe^{+++} forms, the iron is unavailable for direct assimilation by

phytoplankton (Wells *et al.*, 1991_b; Rich & Morel, 1990). However, Finden *et al.* (1984) observed the effect of light-induced reduction of naturally occurring iron oxides associated with humic substances in freshwater and Waite & Morel (1984) also found light-induced production of Fe^{++} species in a coastal seawater sample. More recently Wells *et al.* (1991_b) found that light increased the chemical lability of colloidal iron in seawater at pH 8.0. These preliminary results seem to support the hypothesis that the light-induced dissolution of colloidal and particulate Fe^{+++} extends deeply into the euphotic zone. However, both the light source and iron concentrations used were not directly comparable to normal conditions. Even so these results suggest that iron availability in the ocean may be greater than originally estimated.

Iron has a depth distribution qualitatively similar to that of nitrate, phosphate and silicon (Martin & Gordon, 1988). Until recently it was believed that the major source of nanomolar quantities of iron, present in the ocean's mixed layer, was derived from the deeper iron rich waters (Bruland, 1983). However, the upward fluxes of nitrogen and phosphorus were sufficient to account for carbon production in the open ocean, whereas the iron upward flux values were not sufficient to account for the estimated iron to carbon stoichiometric utilization ratios (Duce, 1986). Furthermore, Duce (1986) concluded that the major source of iron supplying the oligotrophic oceans was from long range transport and fall-out of iron rich atmospheric dust. Between 90-95% of iron in

surface North East Pacific oceanic waters is aeolian (Moore *et al.*, 1984; Duce, 1986; Martin & Gordon, 1988). Young *et al.* (1991) found that during periods of high deposition of aeolian iron into the North Pacific Ocean, primary productivity was increased by 60%. This observed increase could be explained by the dissolution of 10% of the iron deposited as part of the atmospheric dust (Young *et al.*, 1991).

As early as 1933 Gran demonstrated that the addition of iron to seawater may stimulate the growth of phytoplankton. Harvey (1937) stated that iron was especially important for most of the open ocean, which is by and large iron poor, due to its important role in the photosynthetic process. Later Menzel & Ryther (1961) showed the response of natural populations of phytoplankton in the Sargasso Sea to additions of nitrate, phosphate, silicon and iron. Further attempts to demonstrate that productivity is limited by reduced trace element concentrations have proved inconclusive. This is due mainly to the problems of obtaining seawater samples which have not been contaminated by the presence of extraneous trace metals. Bruland *et al.* (1979) have in fact shown that some metal concentrations are far less than previously believed, owing to the introduction of anti-contamination techniques into analytical oceanographic measurements.

Recent culture experiments, using specific anti-contamination techniques, by Martin and his co-workers on natural assemblages of phytoplankton from the North East Pacific Subarctic (Martin & Fitzwater, 1988), Gulf of Alaska

(Martin *et al.*, 1989), have shown that phytoplankton respond to small (nanomolar) additions of iron. More recently determination of iron concentrations in the Antarctic Ocean have shown that low productive (0.1 g C m^{-2} d^{-1}) Antarctic waters had endogenous iron concentrations (0.16 nmol Kg^{-1}) such that the phytoplankton were able to use less than 10% of the other available nutrients; most importantly nitrogen (Martin *et al.*, 1990). Productivity in iron rich waters (7.4 nM) reached values of 3.0 g C m^{-2} d^{-1} (Martin *et al.*, 1990). Furthermore, input of iron into these low iron waters comes mainly from melting sea ice, aeolian input being relatively small (Martin *et al.*, 1990).

As a result of these observations Martin has suggested that the build up of atmospheric carbon dioxide in the atmosphere could be reduced by fertilizing the nutrient rich southern oceans with iron (Martin, 1990), being quoted as saying; "Give me half a tanker of iron and I will give you an ice age" (Roberts, 1991). Martin believes that on the addition of iron a more efficient biological pump would draw a larger proportion of carbon dioxide from the atmosphere. This suggestion has raised many fears and doubts within the oceanographic community. The addition of large quantities of iron to a balanced ecosystem may lead to major changes in the ocean's present structure (Weiler, 1991). As a result of a special symposium arranged by the American Society of Limnology and Oceanography a consensus statement was constructed to represent the views of the oceanographic community (Weiler, 1991). This statement called for a

decrease in greenhouse gas emissions, as an initial step for the reduction of atmospheric carbon dioxide levels, as well as further research into the role that iron and other factors play in limiting phytoplankton productivity in the worlds oceans, prior to implementation of an open ocean iron enrichment experiment (Weiler, 1991). However, it is apparent from a recent article in Science that a 100-400 Km^2 region of the Equatorial Pacific is the likely future subject of an iron fertilization experiment (Roberts, 1991). As a result of this, it is clear that the iron controversy is still raging.

1.4 Iron Acquisition Systems

1.4.1 Iron chelation and uptake in Cyanobacteria

In cyanobacteria, as in many other cells iron is required both as a cofactor and a functional component in many major cellular activities such as chlorophyll and phycobiliprotein biosynthesis, photosynthesis, nitrate reduction, dinitrogen fixation, ammonia utilization and electron transport (Guikema & Sherman, 1984; see Boyer *et al.*, 1987; Rueter, 1988). Over the past decade there has been an increasing literature on the elucidation of iron acquisition systems, mainly with respect to enteric bacteria, and this has been reviewed by Bagg & Neilands (1987_a), Cross (1989) and Crichton (1991). A similar acquisition system has been observed in cyanobacteria (Murphy *et al.*, 1976; Armstrong & Van Baalen, 1979; Boyer *et al.*, 1987; Scanlan *et al.*, 1989).

Compared with enteric bacteria very little is known about the mechanism of iron uptake in cyanobacteria. Iron is assimilated in bacteria by both a high- and a low-affinity uptake system (Neilands, 1984). The high-affinity uptake system is expressed under iron-deplete conditions and depends upon the presence of siderophores together with their respective transport and membrane associated uptake proteins. In comparison very little is known about the low-affinity iron uptake pathway except that it does not require specific Fe^{+++} carriers and is believed to be constitutive. Deletion of the high affinity system is not lethal and growth can occur at normal rates, unless the organism is confronted with a ligand that is able to hold the iron in a form unavailable to the cell. In this case a high-affinity uptake system is required (Neilands, 1984).

The term "siderophore" is derived from the Greek words sider meaning Iron and phore meaning bearer. They are low molecular weight (50-100 Da) compounds with very high affinities for Fe^{+++}. A combination of approaches has been used to investigate the presence of siderophores in cyanobacteria. Although several techniques are available no single method is a universal indicator for detection of the presence or absence of these compounds. Siderophores can be detected by two chemical tests:- the Csaky test for bound hydroxylamines (Csaky, 1948), this has given many positive results with cyanobacteria (see Boyer *et al.*, 1987), and the Arnow test for catechols (Arnow, 1937), this test has given consistent negative results with cyanobacteria (Simpson &

Neilands, 1976). Such results suggest that cyanobacteria do not produce catechol-type siderophores, similar to those present in enteric bacteria. Alternatively, hydroxamate siderophores can be detected biologically using the growth response of *Arthrobacter flavescens* JG9, an obligate auxotroph for hydroxamate siderophores (Neilands, 1984).

Hydroxamate siderophore production has been detected in *Anabaena* sp. (Simpson & Neilands, 1976), *Agmenellum quadruplicatum* PR-6 (Armstrong & Van Baalen, 1979) together with *Microcystis aeruginosa, Phormidium autumnale* and *Anabaena flos-aquae* (Murphy *et al.*, 1976). Furthermore Lange (1974) found six cyanobacteria able to grow in defined media without the addition of the external chelator EDTA. Since three species of cyanobacteria produced only low-affinity chelators in response to growth on chelator-deficient media, the chelators described by Lange may not necessarily be siderophores. More recently Kerry *et al.* (1988) identified hydroxamate siderophore production in *Anacystis nidulands* R2 and *Anabaena variabilis*; as measured by the Csaky test for hydroxamate siderophores. Many of the strains of cyanobacteria tested could produce siderophores. Depending on their growth response to the presence of different concentrations of the iron chelator EDDA the cyanobacterial cultures tested were separated into three groups as follows:- 1). Cells with a high-affinity uptake system. 2). Cells with an intermediate iron scavenging ability. 3). Cells without a high-affinity uptake system (Kerry *et al.*, 1988).

As yet the only siderophore to be isolated and characterized from cyanobacteria is schizokinin (Simpson & Neilands, 1976). Although cyanobacterial siderophores are synthesized in response to iron-limited conditions they have also been shown to prevent metal toxicity by displaying the ability to sequester other metals such as copper (Clarke *et al.*, 1987).

In conjunction with the discovery that under iron-limiting conditions some cyanobacteria are capable of producing hydroxamate type siderophores came the assumption that in order for the siderophore to be taken into the cell there must be specific transport and receptor proteins in the cell outer membrane as is the case in *E. coli*. Determination of uptake and transport activity for ferri-schizokinen in *Anabaena* sp. showed kinetics of a protein-mediated process with an apparent K_m of 0.04 μM (Lammers & Sanders-Loehr, 1982), which is similar to the K_m reported for the high-affinity uptake systems of ferri-enterobactin (0.1-0.36 μM) and ferri-chrome (0.15-0.25 μM) respectively (see Boyer *et al.*, 1987). In *E. coli* the driving force for siderophore uptake is via the use of a transmembrane electrical potential (Winkelmann & Huschka, 1984) unlike siderophore uptake in *Anabaena* sp. which requires ATP derived from photophosphorylation as the primary energy source. However, specific iron-regulated outer-membrane proteins (IRMPs) had not been described for cyanobacteria until Scanlan *et al.* (1989) showed the presence of polypeptides Mr 92,000 Da, 48,000 Da, 50,000 Da and 35,000 Da in the outer membrane when

Anacystis nidulans R2 was subjected to iron limitation. The localisation of these polypeptides to the outer membrane had been made possible by methodology which enabled specific fractionation of cyanobacterial cytoplasmic and outer membranes (Resch & Gibson, 1983; Omata & Murata, 1984). Reddy *et al.*(1988) have identified a Mr 36,000 Da protein encoded by the *irpA* (iron regulated protein) gene in *Anacystis nidulans* R2. This protein which is expressed as a response to iron-limited conditions has been localized to both the cytoplasmic membrane (Reddy *et al.*, 1988) and the outer membrane (D. Scanlan-personal communication) via the use of specific antibodies. The localization of this protein to the outer membrane and its regulation by iron suggests its possible involvement in iron acquisition and storage.

As is the case with *E. coli* more than one form of siderophore-mediated iron transport system may exist in cyanobacteria. However, especially in the marine environment where conditions of siderophore release are less favourable, the presence of a non-siderophore-mediated iron uptake mechanism may predominate. For example, Anderson and Morel (1982) examined ^{59}Fe uptake rates in the coastal diatom *Thalassiosira weissflogii* when grown in the presence of iron chelating agents. They concluded that the uptake rate was determined by the extent of iron binding to a phytotransferrin receptor molecule on the outer membrane surface of the cell. Maximum uptake rates were observed in the presence of chelating agents in the light. Anderson and Morel (1982) highlighted the importance of such a system in the marine

environment where humic substances are likely chelators and the iron-ligand complex would be amenable to photoreduction in the euphotic zone (section 1.3). Studies on continuous cultures of *Gomphosphaeria aponina*, where iron was complexed with EDTA, found that iron uptake exhibited biphasic kinetics, with a fast, light-independent initial rate which was the same for both iron deficient and sufficient cells. Eng-Wilmont and Martin (1979) interpreted this as an initial adsorption of iron on to the cell surface followed by a slower uptake rate. An identical iron uptake pattern was observed for *Anabaena flos-aquae* however, unlike *G. aponina* this cyanobacterium had been shown to produce hydroxamate siderophores (Murphy, 1976). Furthermore two strategies of Fe^{+++} acquisition in higher plants have been proposed (see Castignetti & Smarrelli, jr, 1986; Boyer *et al.*, 1987). Strategy I invokes the release of hydrogen ions, reducing compounds and/or organic acids (such as citrate) coupled with a plasma membrane-bound Fe^{+++} reductase present in the cortical cells of roots. Strategy II involves the release of phytosiderophores together with the subsequent assimilation of the ferri-phytosiderophore complex. Fe^{+++} reduction to Fe^{++} at the root membrane has also been reported for fungi (Boyer *et al.*, 1987).

1.4.2 Physiological and ultrastructural effects of Iron limitation on cyanobacteria

Hardie *et al.* (1983), Guikema & Sherman (1983) and Pakrasi *et al.* (1985_{ab}) have documented the effects of iron

deficiency upon two unicellular cyanobacteria, *Anacystis nidulans* R2 and *Agmenellum quadruplicatum* PR-6. Iron deficiency resulted in a breakdown of phycobiliprotein and chlorophyll, together with a reduction in both nitrate and nitrite reductase activities and an increase in glycogen storage granules. There was an observed 7 nm blue-shift in the chlorophyll absorption peak in iron-limited cells from 679-672 nm together with an LDS-PAGE analysis showing a decrease in the high molecular weight chlorophyll-protein complexes. Pakrasi *et al.* (1985_b) suggest that a M_r 36,000 Da polypeptide (isolated from *Anacystis nidulans*), which is the major chlorophyll a-binding protein complex in membranes from iron deficient cells, is part of an intermediate light-harvesting antennae for photosystem II. This protein is believed to act as a replacement for phycobilisomes which have been lost due to iron deficiency. Laudenbach & Straus (1988) suggest that *isiA*, which is expressed under iron stress and has a putative gene product with a number of characteristics in common with other chlorophyll binding-proteins, may be the gene encoding the 36,000 Da chlorophyll a-binding photosystem II polypeptide described by Pakrasi *et al.* (1985_b). Furthermore, within the promoter region of the *isiA* genes are two regions similar to the operator sequences of iron-regulated genes from *E. coli* which bind the *fur* repressor protein (Laudenbach & Straus, 1988).

Flavodoxin is known to replace ferredoxin under conditions of iron limitation in some bacteria. Laudenbach *et al.* (1988) reported upon the transcription of the flavodoxin gene of *Anacystis nidulans* R2 under iron-limited conditions.

Addition of iron to these cells resulted in virtual total loss of detectable mRNA transcripts of flavodoxin within 60 min. Ferredoxin message however, was quantitatively unaffected by decreased iron availability. Pardo *et al.* (1990) have been unable to detect flavodoxin in *Anabaena variabilis* when the cells were grown in the absence of added iron even though the loss of iron in the cells showed a concomitant decrease in the amount of ferradoxin. They concluded that this flavoprotein does not seem to replace ferredoxin in this strain. Upon iron restoration to iron-deplete cultures there was an immediate utilization of the glycogen granules, together with the preferential synthesis of phycobiliproteins over chlorophyll for the first 6 hrs. After 24 hrs the chlorophyll:phycocyanin ratio was the same as for normal cells and the chlorophyll maximum at 679 nm was restored.

1.4.3 Examples of iron acquisition systems in micro-organisms other than cyanobacteria

There follows a general discussion regarding iron acquisition systems utilized by other micro-organisms.

Three strategies of iron acquisition have been identified in both bacteria and fungi. These are:- 1). Siderophore-mediated iron uptake systems (Bagg & Neilands, 1987; Crosa, 1989). 2). Transport of Fe^{++} by reduction of Fe^{+++} at the cytoplasmic membrane coupled to an active Fe^{++} transport e.g. *Streptococcus mutans* (Evans *et al.*, 1986). 3). Non-siderophore-mediated acquisition of iron directly from other

iron-containing proteins such as; transferrin, lactoferrin, ferritin, haemoglobin or haem, via the presence of specific binding proteins on the cell surface e.g. *Haemophilus pleuropneumoniae* (Niven *et al.*, 1989; Weinberg, 1989). Alternatively, iron acquisition may be via a periplasmic binding protein (PBP)-dependent transport mechanism e.g. *Serratia marcescens* (Zimmerman *et al.*, 1989; Angerer *et al.*, 1992). Siderophore-mediated iron uptake and the mechanism employed by *Serratia marcescens* will be discussed in more detail below.

1.4.3.1 Siderophore-mediated iron acquisition systems

The best characterized iron acquisition pathway is that of *E. coli*. Out of the eight systems known to occur in *E. coli* six require siderophores. There are three types of siderophore produced by organisms subjected to iron-limited conditions. These are :- catecholates, hydroxamates (Fig. 1.2) and phytosiderophores, in some instances citrate is also utilized as a siderophore (Bagg & Neilands, 1987; Guerinot *et al.*, 1990). *E. coli* not only produces two endogenous siderophores; catecholate-enterobactin which is chromosomally encoded and the hydroxamate aerobactin encoded upon the COLV-K30 plasmid, but is also able to utilize exogenous hydroxamate-type fungal siderophores:- coprogen, ferrichrome and rhodotorulate as well as having an uptake system for ferri-citrate. In Figure 1.3 (after Bagg & Neilands, 1987) the high-affinity iron uptake pathway together with the specific genes encoding the outer membrane uptake proteins for

Figure 1.2 **Schematic diagrams of catecholate and hydroxamate siderophores**

A shows the hydroxamate siderophore, ferrichrome, containing a central Fe^{+++}. B shows the catecholate siderophore, enterobactin, depicted without Fe^{+++}. (After Miller (1988), quoted by Crichton (1991)).

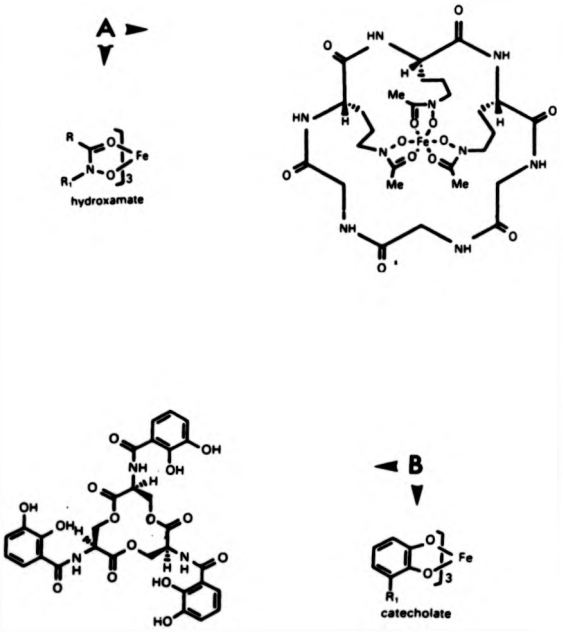
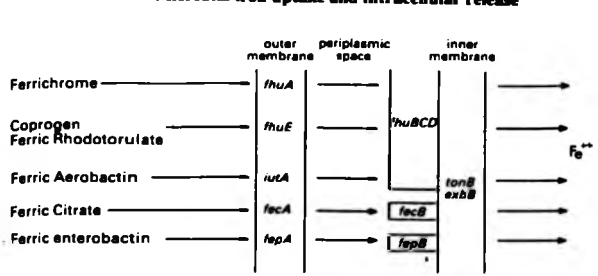


Figure 1.3 The high affinity iron uptake pathways of *E.coli* (after Bagg & Neilands, 1987)



— Relatively well-characterized envelope components of some high-affinity iron transport system in *E. coli* K-12. The organism is equipped to utilize an array of ferric hydroxamate-type siderophores such as ferrichrome, coprogen, and ferric rhodotorulate, all of which are products of other microbial species.

E. coli are depicted. The COLV-K30 plasmid consists of five genes under the control of a single operator. This operon responds to the iron status of the cell (Bagg & Neilands, 1987). Figure 1.4 shows the aerobactin operon and the function of the gene products whereas Table 1.3 summarizes the genes and gene products of the enterobactin system which have been characterized (Crosa, 1989). All siderophore-mediated systems require the participation of specific outer membrane receptor proteins. In all cases so far examined uptake of the siderophore complexes requires a functional *tonB* gene, this is also the case for vitamin B_{12} transport across the cell membrane. The ferri-siderophore complex has been shown to enter the cytoplasm in some cases, while in others the ferri-siderophore is not internalized and the iron is delivered to the cell envelope by an, as yet, undefined process. Release of the Fe^{+++} species from the siderophore is via a poorly understood reductive step (Neilands, 1982).
In the case of *E.coli* seven outer membrane proteins are expressed under iron deplete conditions. Five of these have been shown to contain siderophore receptor functions (Fig. 1.3). The remaining two proteins also derepressed by low iron, Cir and Fiu, are believed to be involved in catechol type siderophore uptake (see Crichton, 1991).

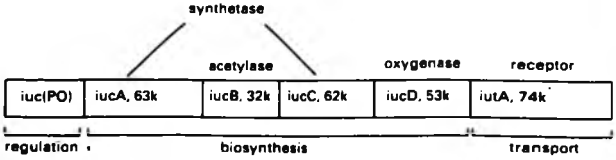
1.4.3.2 Non-siderophore mediated iron acquisition systems

Zimmerman *et al.* (1989) have shown the presence of a novel Fe^{+++} transport system in *Serratia marcescens*, named SFU. The system includes an unusual Fe^{+++} uptake mechanism

Table 1.3 The genes involved in the *E.coli* enterobactin iron acquisition system.

genes	gene product	function
entC	x_1-synthetase	first stage
entB	x_2-synthetase	siderophore
entA	dehydrogenase	synthesis enzymes
entD		second stage
entE		siderophore
entF		synthesis enzymes
entG		
fepA	FepA	outer membrane receptor protein
fes	esterase	cytoplasmic location hydrolyses (ferri) -enterobactin
fep B		periplasmic binding protein
fep C		membrane associated ATP binding protein
fep D,G		cytoplasmic membrane uptake
fep E		uncharacterized

Figure 1.4 **The pColV-K30 aerobactin operon of *E.coli*. (After Bagg & Neilands (1987))**



Organization of the aerobactin operon of pColV-K30. Transcription proceeds from left to right from a promoter (P) and operator (O) sequence. Four *iuc* (iron uptake chelate) genes are required for biosynthesis of aerobactin. The outer membrane receptor for ferric aerobactin is coded by gene *iut* A (iron uptake transport), an integral member of the operon.

across the outer membrane together with a conventional periplasmic binding protein (PBP) uptake mechanism across the cytoplasmic membrane. Fe^{+++} transport did not require siderophores or receptor protein synthesis nor was Fe^{+++} acquisition dependent upon a functional TonB. The SFU system consists of proteins M_r 36,000 Da, 40,000 Da, 34,000 Da and 38,000 Da. The 40,000 Da polypeptide is the precursor form of both the 38,000 Da and the 34,000 Da proteins. The 38,000 Da polypeptide (SfuA) has been localized to the periplasmic space and is probably the primary Fe^{+++} acceptor, whereas the 40,000 Da polypeptide has been localized to the cytoplasmic membrane. In addition the SFU transport system in *S. marcescens* is able to accept Fe^{+++} solubilized with oxaloacetate, sodium pyrophosphate and citrate (Angerer *et al.*, 1992). The sequences of the three transport proteins encoded by *sfuABC* together with their localization in the periplasmic space and cytoplasmic membrane were typical for periplasmic-binding protein (PBP) transport systems (Angerer *et al.*, 1992).

Periplasmic binding proteins (PBP) similar in structure and location to PBP-dependent transport system described for sugars and amino acids have been identified in *E.coli* for the transport of Fe^{+++} via all six siderophore mediated uptake systems described in Figure 1.3. (Pierce *et al.*, 1986; Staudenmaier *et al.*, 1989; Köster & Braun, 1989).

1.5 Aims

The aims of the research described herein are as follows:-

1). To investigate the presence of iron limitation in natural phytoplankton assemblages using conventional oceanographic techniques.

2). To investigate the production of putative iron-regulated outer-membrane proteins involved in iron acquisition in *Synechococcus* WH 7803.

3). To isolate any putative iron-regulated outer-membrane proteins with a view to raising antibodies to be used for potential diagnostic markers against iron limitation in the natural environment.

4). To identify the presence of any other systems in *Synechococcus* WH 7803 pertaining to iron acquisition and storage, including characterization of any proteins isolated in 3, above.

CHAPTER 2
MATERIALS AND METHODS

2.1 Organisms

a) Cyanobacteria

The principal cyanobacterium used in this study was *Synechococcus* WH 7803 (formally DC2) isolated from the Sargasso sea in 1978 (Waterbury *et al.*, 1979). The other cyanobacteria used are listed in Table 2.1a together with their laboratory growth media and natural habitats.

b) *Escherichia coli*

Table 2.1b shows the strains employed.

2.2 Plasmids

The organisms used for genetic manipulation are listed in Table 2.1b, both plasmid DNA and chromosomal DNA data are presented in Table 2.2.

2.3 Chemicals

Lysozyme, ethylene diamine di-(o-hydroxyphenyl acetic acid) (EDDA), phenylmethyl-sulfonyl- fluoride (PMSF), 4-chloro-1-napthol (30 mg pellets), Sigma-104 phosphatase substrate, Tween-20, Commassie Blue R and TEMED (N,N,N',N'-tetramethylethylene diamine), were obtained from the Sigma Chemical Company. Acrylamide, N,N' methylene-bis-acrylamide, 2-mercaptoethanol, SDS-PAGE molecular weight markers and protein assay reagent were obtained from BioRad Laboratories. Ammonium-persulphate, Tris (hydroxymethylmethylamine) base,

TABLE 2.1a. Cyanobacterial Strains.

strain	natural habitat	laboratory media
Synechococcus WH 7803	marine shelf	ASW
Synechococcus PCC 7002	marine coastal	ASW + vit. B_{12}
Synechococcus PCC 7942	fresh-water	BG11
A. cylindrica PCC 7122	fresh-water	BG11
Nostoc sp. MAC PCC 8009	*Macrozamia lucida* (roots of)	BG11

N.B. PCC = Pasteur Culture Collection.

WH = Woods Hole.

TABLE 2.1b. *Escherichia coli* Strains.

strain	phenotype/genotype	source
MC1061	araD139, (ara,leu)7696 lacX74, galK$^-$, har$^-$, hsm$^+$, strA	Casadaban & Cohen (1980)
CH483	pro$^-$, lac$^-$, (trp tonB galU ara)	C. Higgins
K803	r$^-$, m$^+$, gal$^-$, met$^-$	Wood (1966)

The *E.coli* strain CH483 is a TonB mutant of *E. coli* strain NO801 its phenotype is :- pro$^-$ lac$^-$ (trp tonB galU ara)

Table 2.2 DNA

1. Plasmids/vectors

	size kb	comment	source
pCH143	3.0	$Ap^{r(a)}$	C.Higgins, Sheffield.
pKJ110	5.1	Ap^{r}	Reddy *et al.*(1988).
pUC19	2.686	Ap^{r}	Yanisch-Perron *et al.* (1985).

2. Chromosomal DNA

E.coli K12	wild type prototrophic, lysogenic for lambda.	This laboratory

3. Cyanobacterial Gene Libraries[b]

pUC19 :: *Synechococcus* WH 7803 chromosonal DNA

pUC19 :: *Synechococcus* PCC 7942 chromosomal DNA

COMMENT

a). An *Eco*RI-*Bam*HI fragment of *Salmonella typhimurium* chromosomal DNA containing the *tonb* gene cloned into the pBluescript KS^{+} polylinker. The vector encodes ampicillin resistance.

b). Both pUC19 libraries were obtained from D.Scanlan, this laboratory.

and glycine were analytical grade from BDH. Biochemical grade sodium dodecyl sulphate and Aristar grade hydrochloric and nitric acids were also obtained from BDH. Silver nitrate was obtained from Johnson Matthey Materials Technology U.K. Page blue and Ponceau S were purchased from BDH. Nitrocellulose membrane Hybond C, donkey anti-rabbit IgG, ^{32}P αdCTP, $^{32}P\gamma$ ATP, ^{35}S L-methionine and ^{55}Fe were purchased from Amersham International.

2.4 Growth media

All cyanobacteria used were grown photoautotrophically in either Artificial Seawater Medium (ASW) (Wyman *et al.*, 1985) (marine strains) or BG11 medium (Rippka *et al.*, 1979) (fresh water species), as described in Table 2.3. *Synechococcus* PCC 7002 was grown in ASW with vitamin B_{12} (10 μg l^{-1}) supplement. Media were made using analytical grade chemicals and double distiled water further purified by means of a double ion-exchange unit plus carbon filter (Elgastat), and sterilized by autoclaving at 15 lb $inch^{-2}$ for 15 min, (30 min for volumes of 10 l and over). 1 litre volumes, or over, of ASW media were modified by the addition of $NaHCO_3$ 0.5 g l^{-1}. Trace elements were sterilized separately (15 min at 15 lb $inch^{-2}$) for ASW media over 1 litre in volume (to prevent the production of insoluble iron phosphate complexes) and added to the autoclaved media aseptically.

The iron-limited medium as described in the text was ASW, (Table 2.3), except that the normal iron concentration, 11 μM $FeCl_3.6H_2O$ was reduced from 11 μM to either 1.1 μM, 0.11 μM,

Table 2.3 Artificial Seawater Medium

Salt	g l^{-1}	Trace elements	g l^{-1}
NaCl	25	H_3BO_3	2.86
$MgCl_2.6H_2O$	2.0	$MnCl_2.4H_2O$	1.81
KCl	0.5	$ZnSO_4.7H_2O$	0.222
$NaNO_3$	0.75	$Na_2MoO_4.2H_2O$	0.39
$MgSO_4.7H_2O$	3.5	$CuSO_4.5H_2O$	0.008
$CaCl_2.2H_2O$	0.5	$Co(NO_3)_2.6H_2O$	0.0049
Tris-HCl	1.1	$FeCl_3.6H_2O$	3.0
$K_2HPO_4.3H_2O$	0.03	$Na_2MgEDTA$	0.5
trace elements	1ml l^{-1}		

The Medium was adjusted to pH 8.0 using concentrated HCl.

BG11 MEDIUM

Salt	g l^{-1}	Trace elements	g l^{-1}
$NaNO_3$	1.5	H_3BO_3	2.86
$K_2HPO_4.3H_2O$	0.04	$MnCl_2.4H_2O$	1.81
$MgSO_4.7H_2O$	0.075	$ZnSO_4.7H_2O$	0.222
$CaCl_2$ $2H_2O$	0.036	$NaMoO_4.2H_2O$	0.391
trace elements	1ml l^{-1}	$CuSO_4.5H_2O$	0.079
		$Co(NO_3)_2.6H_2O$	0.049
		citric acid	0.006
		$FeNH_4$ citrate	0.006
		$Na_2MgEDTA$	0.001

0.011 μM or zero $FeCl_3.6H_2O$. Growth medium described as chelator-deficient medium contained neither added $FeCl_3.6H_2O$ or chelator, (EDTA). Iron-replete medium plus added chelator were composed of ASW medium and EDDA (15 μg ml^{-1}). All stock $FeCl_3.6H_2O$ solutions used in the iron-limited media were maintained in 0.5 M HCl to prevent precipitation of insoluble iron III complexes. BG11 medium used for siderophore bioassays was modified such that citrate was removed from the trace elements and $FeNH_4$ citrate (20 μM) was substituted for the same concentration of $FeCl_3.6H_2O$, (added as a complex with EDTA).

2.5 Assessment of culture purity

D-glucose (final concentration 2% [w/v]) and yeast extract (final concentration 0.15% [w/v]) were dissolved in either ASW or BG11 media, 1.5% [w/v] LabM agar was added to the medium if plates were required. The medium was sterilized by autoclaving. Stock cultures were incubated at 25°C, the absence of bacterial or fungal growth was determined after 7 days. Contamination of fresh water strains was checked in the same fashion except that stock cultures were plated onto BG11 medium supplemented with D-glucose (2% [w/v] final concentration) and yeast extract (0.2% [w/v] final concentration) and incubated at 30°C. Alternatively contamination could be checked by plating stock cultures onto nutrient agar or into nutrient broth.

2.6 Growth and maintenance of organisms

Stock cultures of *Synechococcus* WH 7803 were maintained at 25°C in a Gallenkamp incubator under continuous irradation at a low light intensity of 4 - 8 $\mu E\ m^{-2}\ s^{-1}$, under warm-white fluorescent light, cultures were occasionally mixed by swirling the flasks by hand. Stock cultures were subcultured into fresh medium at monthly intervals. Small (100 ml) batch cultures for experimental purposes were grown as described above.

Large volumes (10 l) of cells required for bulk membrane or protein preparation were grown with constant stirring and aeration in a 25°C water bath at a light intensity of 36 $\mu E\ m^{-2}\ s^{-1}$. Illumination was from warm-white fluorescent strip lights placed above the growing cultures. Other strains used in this study were grown in a 30°C warm room under constant irradiation from warm-white strip lights at a light intensity of 60-70 $\mu E\ m^{-2}\ s^{-1}$.

Escherichia coli was grown in liquid media (Table 2.3) at 37°C in an incubator shaker, series 25, New Brunswick Scientific Co inc. shaking at 250 revs min^{-1} or on solid media in a 37°C incubator. Both solid and liquid media contained 50 $\mu g\ ml^{-1}$ of ampicillin (25 $mg\ ml^{-1}$ stock) where appropriate. Cultures were stored at 4°C for short term storage.

Table 2.4 *E. coli* growth media

	component	g l^{-1}
Nutrient broth	Oxoid nutrient broth	13
Nutrient agar	Oxoid nutrient agar	28
Luria broth	Bactotryptone	10
	Bacto yeast extract	5
	NaCl	5
SOB medium	Bactotryptone	20
	Bacto yeast extract	5
	1 M NaCl	10 ml
	1 M KCl	2.5 ml
	1 M $MgSO_4$, 1 M $MgCl_2$ filter sterilized	10 ml
Double Difco medium	Bactotryptone	20
	Bacto yeast extract	5
	NaCl	10
	Bacto agar	15
	1 M $MgSO_4$, filter sterilized	10 ml
Top agarose	Double Difco media as above subatituting Bactoagar for 0.7% [w/v] agarose.	

2.7 Growth determination by dry weight estimation.

2.7.1 Optical density verses dry weight.

Cell suspensions were spun for 10 min at 4,500 rpm in a Gallenkamp bench centrifuge and the cell pellet resuspended in a known volume of medium. Diluted samples of the cell suspension were filtered onto pre-dried and pre- weighed 0.2 μm Nuclepore filters. The filters were washed with ELGA water to remove any salt remaining from the medium and then dried in a 64°C oven until no further reduction in weight was recorded. The filters were then placed in a desiccator until cool. The extinction at 750 nm was recorded for each dilution, and a relationship between dry weight and $OD_{750\ nm}$ was obtained.

2.7.2 Dry weight verses cells ml^{-1}

A 10 ml volume of a 1 in 1000 dilution of the diluted samples of cell suspension was passed down a filter tower of a known diameter under negative pressure. The cells were caught on 0.2 μm polycarbonate Nuclepore filters lying over 0.45 μm nitrocellulose Millipore filters - this ensures that the cells are equally distributed over the surface of the filters. The filters were then placed onto microscope slides containing a drop of immersion oil, the slides were stored at -20°C.

2.7.3 Estimation of cell number by epifluorescence microscopy

Cell numbers in laboratory cultures were counted using epifluorescence microscopy. *Synechococcus* WH 7803 cells held on microscope slides (prepared as described above) fluoresced bright orange using a rhodamine filter (excitation wavelength 550 nm). Cells were counted over 10-20 fields. The number of cells ml^{-1} for each dilution was then determined using the method described by Waterbury *et al.* (1986), a relationship between $OD_{750\ nm}$ and cells numbers ml^{-1} and subsequently dry weight was obtained.

2.8 Estimation of growth

Samples (1 ml) of culture were removed aseptically and placed into 1 ml cuvettes. Prior to reading the extinction at 750 nm *Synechococcus* WH 7803 samples were mixed using a 0.5 ml syringe with a bore size of 5/8th of an inch to disperse any cells which may have clumped together. Optical density measurements of a solution were achieved using an LKB Ultraspec II spectrophotometer. Sterilized medium was used as a blank.

2.9 Scanning spectroscopy

Scanning spectroscopy of either whole cells or cell wall samples was achieved using a PU 8720 spectrophotometer. Samples were scanned over a broad spectrum e.g. 350 nm to

700 nm for cell walls, or 500 nm to 700 nm for whole cells. Whole cell samples were normalized either to $OD_{280\ nm}$ or $OD_{750\ nm}$. Chelator-deficient cells were used as the baseline for the difference spectrum, so that it was possible to correlate the change in pigment content, such as phycoerythrin, with an increase in Fe^{+++} in the medium. Buffer (50% [w/v] sucrose, 10 mM Hepes-Mg^{++}, pH 8.0) was used as a baseline for scanning cell wall preparations.

2.10 SDS-PAGE analysis of proteins

6-20% [w/v] gradient gels were used in this study as they were found to give better resolution. The components of the gradient gels used are as shown in Table 2.5 a-d.

2.10.1 Protein gel system

SDS-PAGE was routinely performed on vertical Studier-type slab gel apparatus (Hames & Rickwood, 1981), gels were allowed to set under *iso*-butanol prior to the addition of the stacking gel. Electrophoresis was achieved at a current of 10 mA and the gel was run overnight in 0.025 M Tris - 0.2 M glycine running buffer (Table 2.5e).

In some cases a BioRad gel system was used as described in the text, under these circumstances proportions of the gel components were adjusted to account for the decrease in total gel volume, gels were run at a lower current for the same length of time. In some instances 10-30% [w/v] SDS-PAGE gels were run, these were prepared as described by Scanlan (1988) and run as described above.

Table 2.5a **6% low bis acrylamide**

concentrated stock	per 50 ml
30% [w/v] low bis acylamide	10.0 ml
3 M Tris-HCl pH 8.8	6.25 ml
20% [w/v] SDS	0.25 ml
water	33.375 ml
10% [w/v] AMPS	0.075 ml
100% [v/v] TEMED	0.050 ml

Table 2.5b **20% high bis acrylamide**

concentrated stock	per 20 ml
30% [w/v] high bis acrylamide	13.34 ml
3 M Tris-HCl pH 8.8	2.5 ml
20% [w/v] SDS	0.1 ml
75% [v/v] glycerol	3.73 ml
water	0.282 ml
10% [w/v] AMPS	0.03 ml
100% [v/v] TEMED	0.014 ml

Table 2.5c **Acrylamide stock solutions**

Low bis acrylamide	30% [w/v]	acrylamide
	0.75% [w/v]	bis acrylamide
High bis acrylamide	30% [w/v]	acrylamide
	1.2% [w/v]	bis acrylamide
Stacking gel acrylamide:-	10% [w/v]	acrylamide
	0.5% [w/v]	bis acrylamide.

Table 2.5d **Stacking gel**

concentrated stock	per 10 ml
10% [w/v] acrylamide	3.0 ml
0.5 M Tris-HCl pH 6.8	2.4 ml
10% [w/v] SDS	0.1 ml
water	4.4 ml
10% [w/v] AMPS	0.1 ml
100% [v/v] TEMED	0.005 ml

Table 2.5e

SDS-PAGE running buffer

	($g\ l^{-1}$)
Tris-HCl	3.0
glycine	14.4
SDS	1.0
pH	8.3

Table 2.5f

4x sample buffer

	(per 5ml)
1 M Tris-HCl pH 6.8	1.55 ml
SDS	1.0 g
100% [v/v] glycerol	2.5 ml
100% [v/v] ß-mercaptoethanol	1.0 ml
Bromophenol blue	0.025 g

2.10.2 Sample preparation

Protein samples were denatured by heating at 100°C in sample buffer for 5 min. The composition of 4x sample buffer is shown in Table 2.5f. SDS-PAGE low molecular weight markers (BioRad Laboratories) were run routinely. They comprised (molecular weight in brackets) lysozyme (14,400), soya bean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), phosphorylase B (97,400). For polyacrylamide gels which were to be silver stained, 10 µl of a 1:100 dilution of the markers was loaded. Pharmacia low molecular weight markers were also used.

2.10.3 Visualization of protein banding patterns in polyacrylamide gels

2.10.3.1 Coomassie Blue staining

For protein bands to be visible 100 µg of total soluble cell protein must be loaded per track, sensitivity is approximately 0.2-0.5 µg protein/band.

Gels were stained for 3 hours in 45% [v/v] methanol, 10% [v/v] acetic acid, 0.1% [w/v] Coomassie Brilliant Blue R and destained in several changes of 45% [v/v] methanol 10% acetic acid [v/v], with constant shaking.

2.10.3.2 Silver staining

The method of Wray *et al.* (1981) was employed. The method is sensitive to proteins at the nanogram level, 10 μg of total soluble cell protein can be loaded per track. There is little preparation time required and the staining reaction is both rapid and reproducible. The procedure was carried out using ELGA water and clean boxes. After electrophoresis gels were washed with four changes of 50% [v/v] methanol, the gel was transferred to stain solution C, composed of solution A (3.2 g $AgNO_3$ in 16 ml water) added dropwise to solution B (84 ml 0.36% [w/v] NaOH and 5.6 ml of 14.8 M NH_4OH) with vortexing and made upto 400 ml with ELGA water and left shaking for 15 min. The gel was washed thoroughly in ELGA water for two thirty minute periods, the box had been rinsed in tap water to precipitate any remaining silver ions, then cleaned in ELGA water. The gel was also rinsed in ELGA water prior to both washing steps (it was important to ensure that the gel was devoid of any excess chemicals carried over from the staining procedure). The silver stain was developed for the required length of time in 2.5 ml 1% [w/v] citric acid, 0.4 ml formaldehyde solution made upto 500 ml with ELGA water. The developing reaction could be stopped by transferring the gel into 50% [v/v] methanol 10% [v/v] acetic acid.

2.10.3.3 Autoradiography

Polyacrylamide gels containing ^{35}S L-methionine labelled proteins were viewed using Betamax Hyperfilm (Amersham

International). Gels to be autoradiographed were dried under vacuum for one hour at 80°C and layed down against X-ray film for an appropriate length of time at room temperature.

Films were developed for 1.5 min in Kodak LX-24 developer at a ratio of 1:5, rinsed in water and fixed for 10 min in Kodak FX-40 fixer at a ratio of 1:4.

2.11 Protein determination

2.11.1 BioRad protein microassay

This assay uses the principal that an acidic solution of Coomassie Brilliant Blue G-250 shifts in absorbance from 465 nm to 595 nm when protein binding occurs, as described by Bradford (1976). 0.2 ml of dye reagent concentrate was added to 0.8 ml of appropriately diluted sample, the solution was mixed by inversion. The resulting absorbance was determined at 595 nm after 15-60 minutes, and read against a reagent blank. A standard curve was prepared using 0.8 ml aliquots of bovine serum albumin (BSA) at six concentrations ranging from 1-25 μg ml^{-1}.

2.11.2 Lowry protein assay

The procedure was conducted as described by Lowry *et al.* (1951). The method relies on the production of a coloured substrate which can be measured at 750 nm. The reaction is a two step process involving initially a copper protein

interaction followed by reduction of the folin reagent, maximal reduction occurring at pH 10. Although this method is 10-20 times more sensitive than ultraviolet absorption at 280 nm BioRad protein assay was the preferred method mainly due to its accessibility.

2.11.3 Scintillation counting

Equal counts of ^{35}S-methionine labelled proteins were loaded on gels as specified in the text. 10 µl of sample was diluted with 5 ml of Optiphase Safe scintillation fluid (FSA laboratory supplies) and the cpm determined using an LKB 1216 RACKBETA liquid scintillation counter.

2.12 Detection of protein using western blot analysis

2.12.1 Protein transfer onto nitrocellulose membranes

After electrophoresis gels were placed onto a base of two layers of Whatman 3MM filter paper overlaying a scourer, nitrocellulose was smoothed over the gel ensuring no air bubbles were trapped. The transfer system was complete when two further sheets of Whatman 3MM filter paper and a scourer were placed over the nitrocellulose. The holder was closed and placed into the western transfer tank with the gel nearest to the negative electrode and the nitrocellulose nearest the positive electrode. For ten minutes prior to use both

scourers, filter paper and nitrocellulose were presoaked in transfer buffer. Proteins were transferred for three hours at 300 mA in either 20 mM Tris-HCl, 150 mM glycine, 20% [v/v] methanol pH 8.3, or 48 mM Tris-HCl, 39 mM glycine, 10% v/v methanol, 0.03% [w/v] SDS. This latter buffer was used principally for protein transfer onto PVDF for protein sequencing, (however, it is also believed to promote a higher efficiency of transfer for western blotting).
When transfer was complete, proteins were visualized by incubating nitrocellulose filters in Ponceau S (0.5% [w/v] in 5% [w/v] TCA) for 10 min with shaking. Unbound dye was removed by washing with water, filters were destained on washing in phosphate-buffered saline 0.14 M NaCl, 0.02 M sodium phosphate pH 7.3 (PBS).

2.12.2 Probing filters with antibodies.

Nitrocellulose filters were blocked for one hour with 2% [w/v] Marvel in PBS, then incubated overnight in an appropriate dilution (1:50, 1:300, 1:500 or 1:1000 see text for details) of primary antibody added to 10 ml of a fresh solution of blocking buffer. Filters were stringently washed for three 10 min periods in 50 ml 0.1% [v/v] Tween 20 in PBS prior to the addition of secondary antibody, donkey anti-rabbit horseradish peroxidase (HRP) linked IgG (Amersham), diluted (1:300) in a fresh 10 ml solution of 0.1% [v/v] Tween-20 in PBS. After 1 hour incubation, filters were washed for two 10 min periods in the above solution excluding the

secondary antibody followed by two 10 min washes in PBS alone. All stages required vigorous shaking.

2.12.3 Staining with 4-chloro-1-napthol

Using a solution of the above dye and hydrogen peroxide as catalyst, protein-antibody interaction can be determined. Solution A and B can be prepared upto one hour prior to use.

solution A		solution B	
1.5g	NaCl	30 mg	chloro-napthol pellet
1ml	1 M Tris-HCl pH 7.5	10 ml	100% [v/v] methanol

make up both solutions to 50 ml with ELGA water

50 µl of H_2O_2 was added to solution A then A and B were mixed together. The reaction mixture was added to the filters and incubated up to 1 hour. Purple colouration developed at the site of the HRP and hence the primary antibody, this was confirmed using pre-immune serum. The rate of colour development and intensity of colour depended upon the samples under study. Filters were removed from solution before the reaction had gone to completion and washed for three 30 min periods in ELGA water. Filters were stored wrapped in foil.

2.13 Determination of ^{35}S L-methionine uptake

2.13.1 Light versus dark uptake

Two 50 ml cultures of *Synechococcus* WH 7803 $OD_{750\ nm}$=0.2 (late exponential phase) were "spiked" with 25 µCi of ^{35}S L-methionine. Prior to addition, one culture was completely wrapped in foil, and labelled as the dark control. Both cultures were incubated under normal growth conditions. Three 1 ml sample replicates were removed at time intervals between 0 and 48 hours and filtered onto 0.2 µm polycarbonate Nuclepore filters under negative pressure. Filters were washed with 1 ml 10 mM L-methionine to remove any non-specifically bound ^{35}S L-methionine label and placed into scintillation vials, 5 ml of scintillant (Optiphase Safe) was added, the vials were inverted several times to ensure wetting of the filters prior to placing into the scintillation counter. Radioactivity was measured as described previously. The radioactivity of 1 ml normal ASW, 3 x the volume of cells at final time, 1 cold 0.2 µm Nuclepore filter and scintillation fluid only were also determined as controls.

2.13.2 Trichloroacetic acid (TCA) treatment of ^{35}S L- methionine labelled whole cells

500 µl of labelled cell culture was aliquoted into Eppendorfs and 500 µl of 10% [w/v] TCA was added, and incubated for 30 min at 4°C. The sample was then filtered onto 0.2 µm polycarbonate Nuclepore filters and washed with 1

ml 5% [w/v] TCA, 1 ml absolute ethanol, 1 ml equal parts ethanol:ether. Filters were placed into scintillation vials and 5 ml scintillant was added, each vial was counted for 60 seconds.

2.13.3 Addition of cold carrier and chloramphenicol

A 500 µl aliquot of *Synechococcus* WH 7803 cells was removed from an exponentially growing culture and labelled t_0, the remaining culture was divided into five 20 ml samples containing either 0 nM, 10 nM, 100 nM, 1 µM, L-methionine, or 500 µg ml^{-1} chloramphenicol (30 mg ml^{-1} stock). 20 µCi of ^{35}S-methionine (1000 Ci $mmol^{-1}$) was added to each culture giving a final concentration of 1.0 nM. Cultures were incubated under normal growth conditions. 500 µl samples were taken 30 min after inoculation, labelled t_1, one hour after inoculation, labelled t_2 and then at hourly intervals for five hours in total labelled t_3, t_4, t_5 and t_6 respectively. Samples were treated with TCA as described above.

2.14 ^{35}S L-methionine protein labelling

2.14.1 Heat shock experiments

Time course experiments were conducted in the following ways:-

A) A 4 ml aliquot of a mid exponentially growing culture was removed prior to the addition of ^{35}S-methionine. 100 µCi was added to 100 ml culture which was then split into two 50 ml

samples, one culture flask was placed at normal growth temperature (25°C), as a control, the other culture flask was placed at 33°C. Both flasks were incubated in Gallenkamp incubators at the same light intensity. The cells were heat shocked for six hours and 4 ml samples were removed hourly from both flasks.

Sample preparation

The 4 ml samples were centrifuged at 4,500 rpm in a Gallenkamp bench centrifuge for 15 min. The supernatant was discarded and the pellet washed in 1 ml 10 mM cold methionine in ASW prior to being resuspended in 200 µl of sample buffer. Samples were stored on ice prior to heating at 100°C for 5 min. Analysis of heat shock proteins was carried out by 6-20% [w/v] SDS-PAGE and autoradiography.

B) Prior to the start of the experiment 5 ml samples of a mid exponential phase culture were aliquoted into sterile Universals. These samples were incubated at either 25°C (control) or 40°C in Gallenkamp incubators at the same light intensity. At incubation times 0 min, 30 min, 60 min, 90min, 180 min and 240 min samples were pulsed for 30 min periods with 10 µCi of ^{35}S-methionine.

Sample preparation

Labelling was stopped by the addition of 5 ml 20% [w/v] TCA (final concentration 10%) and centrifuged at 4,500 rpm in a Gallenkamp centrifuge for 15 min. The cell pellet was resuspended in 200 µl of single strength sample buffer and

heated at 100°C for 5 min prior to analysis by SDS-PAGE and autoradiography.

2.14.2 Cold shock experiments

The procedure was the same as for heat shock experiment except that cultures were incubated at 25°C (control) and 10°C, cold shock sample. The second 4 ml sample was removed after 30 min incubation at 10°C. After this, samples were removed at hourly intervals for a total of 5 hours. Samples were prepared for analysis by SDS-PAGE and autoradiography as described in method A above.

2.14.3 Time course labelling experiments for cells grown under different nutrient conditions

In all experiments, unless stipulated, cells grown normally were used as inoculum. The inoculum was centrifuged for 15 min at 4,500 rpm in a Gallenkamp centrifuge and the supernatant discarded, cells to be grown in chelator-deficient medium were further washed in this medium to remove any exogenous iron remaining in traces of the normal medium. Cell pellets were then inoculated into their new growth medium to give an $OD_{750\ nm}$ corresponding to between the early and mid exponential phase of growth. Labelling was then carried out in two ways:- either 50 μCi ^{35}S-methionine was added to 50 ml cultures and samples removed at certain time intervals or 10 μCi added to 5 ml aliquots removed at certain time intervals and incubated over a 24 hr period, unless stated otherwise. Samples were centrifuged at 4,500 rpm in a Gallenkamp

centrifuge for 15 min and the cell pellet resuspended in 40 µl ml^{-1} of sample i.e cell pellet from 5 ml cells was resuspended in 200 µl single strength sample buffer. All samples were heated at 100°C for 5 min prior to SDS-PAGE and autoradiography.

2.14.4 Labelled cells used as a radioactive marker for protein localization and purification

As the 36 kDa low iron induced protein was not an abundant protein the sensitivity of ^{35}S-methionine labelling was required as a way of locating the protein of interest during experimental procedures.
Freshly prepared 100 ml cultures ($OD_{750\ nm}$ 0.05) of either normal growing cells or cells growing in normal media +15 µg ml^{-1} EDDA were labelled with 100 µCi of ^{35}S-methionine. Cultures were grown until late exponential phase ($OD_{750\ nm}$ 0.3-0.4), cells were harvested by centrifugation at 4,500 rpm in a Gallenkamp centrifuge for 15 min and used as markers for localization and protein purification procedures, to be discussed later.

2.15 Determination of phycoerythrin concentration per cell.

Culture volumes between 25-50 ml were centrifuged at 4,500 rpm in a Gallenkamp centrifuge for 15 min and resuspended in 5 ml of ASW medium. Cells were broken by homogenization in a Braun homogenizer (F.T. Scientific Instruments Ltd.) using glass beads in the size range of 0.1-

0.11 mm. Cells were broken over a period of 5 min and cooled constantly with CO_2. Cell debris was removed from the homogenate by centrifugation for 15 min prior to reading the extinction at $OD_{542\ nm}$. Phycoerythrin concentration $\mu g\ ml^{-1}$ was determined at 542 nm using an extinction coefficient E^{1}_{1cm} 106 (Alberte *et al.*, 1984, Barlow & Alberte, 1985). The amount of phycoerythrin per cell was calculated using the cell number and optical density (750 nm) measurements.

2.16 EDDA simulation of iron-restricted media

Using the results obtained in the EDDA bioassay, cultures of *Synechcoccus* WH 7803 were grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. EDDA was used as a means of restricting iron in the medium, therefore promoting a cellular response to iron-limitation without restricting eventual biomass yield.

2.17 EDDA titration curve

EDDA, (ethylenediamine di(o-hydroxyphenylacetic acid), a phenolic analog of EDTA (ethylene diaminetetraacetic acid) gives a red colour in the presence of ferric iron with an extinction at 480 nm, Underwood (1958).
Using this observation the extinction of a series of solutions containing normal ASW and increasing amounts of EDDA $\mu g\ ml^{-1}$ was measured in order to ascertain the concentration of EDDA required to maintain all available iron in a stable chelate complex within the medium. Replicates of appropriate dilutions of a stock solution of EDDA 0.8 mg ml^{-1} were

prepared in normal ASW medium, the medium was autoclaved, 1 ml aliquots removed and the extinction at 480 nm measured.

2.18 Characterization of iron storage mechanisms

2.18.1 Determination of internal iron concentration

Flasks containing 1 litre of normal or chelator-deficient medium were inoculated to an initial $OD_{750\ nm}$ 0.02 and grown for a period of five days in a heated water bath at 25°C with constant aeration and mixing, in warm white fluorescent light at an intensity of 10 $\mu E\ m^{-2}\ s^{-1}$. Cells were harvested by centrifugation at 9,000 rpm for 15 min in a JA10 rotor in a Beckman centrifuge and washed in chelator-deficient medium to remove any exogenous iron. Cell pellets were resuspended in 1 ml of 10 M HNO_3 (Aristar, BDH) and heated at 100°C for 30 min in the fume-hood, cell samples turned brilliant yellow. It was possible to store samples at -20°C in this state. Samples were diluted to 5 ml with 1% [v/v] HCl prior to analysis using a varian AA 1275 flame atomic absorption spectrophotometer with an air acetylene flame mix. Standard solutions of iron sulphate heptahydrate ranging from 10^{-4} M to 5 x 10^{-7} M in 1% [v/v] HCl were used to produce a calibration curve, taking care to wash all glassware involved with concentrated hydrochloric acid, rinsing with 1% [v/v] hydrochloric acid to remove any iron bound to the glassware.

2.18.2 Preparation of cells for Mössbauer spectroscopy

A 6 litre culture of *Synechococcus* WH 7803 was grown in normal ASW medium enriched with ^{57}Fe added in the form of $^{57}FeSO_4$. Samples were prepared for Mössbauer analysis as described by Evans *et al.* (1977).

2.18.3 Sample preparation for bacterioferritin analysis

The presence of putative iron storage proteins was examined in a number of cyanobacterial strains with the use of an antibody against *Escherichia coli* bacterioferritin, kindly sent as a gift from Professor J. Guest at Sheffield University together with the pure bacterioferritin and pre-immune serum. 100 ml cultures of cyanobacterial strains, *Synechococcus* sp. WH 7803, PCC 7002, PCC 8009, PCC 7942 were grown in both normal medium and chelator-deficient medium, and in the case of WH 7803 normal medium + 15 $\mu g\ ml^{-1}$ EDDA. 2 ml and 5 ml samples were removed at different times during exponential growth and the centrifuged cell pellet resuspended in single strength sample buffer. These samples were stored at -20°C for SDS-PAGE and western blot analysis.

2.19 Determination of siderophore production

Two methods were employed to test whether *Synechococcus* WH 7803 produced siderophores when grown under iron-limited conditions.
Initially the universal chemical assay described by Schwyn & Neilands (1987) was used. However, the EDDA bioassay

described by Kerry *et al.* (1988) proved more appropriate. All cultures were grown for a period of 5-7 days in chelator-deficient medium to ensure that cellular iron levels were depleted, prior to inoculation into normal medium containing EDDA concentrations ranging from 0-75 $\mu g\ ml^{-1}$. Cultures were grown under the same conditions as described in Maintenance of Cultures. Growth was determined at $OD_{750\ nm}$.

2.20 Localization studies for the 36 kDa iron stress protein

2.20.1 Periplasmic fraction

50 ml samples of Synechococcus WH 7803 were treated for isolation of the periplasmic fraction in three ways:-
i) osmotic shock as described by Neu & Heppel (1965).
ii) chloroform extraction described as by Ames *et al.* (1984).
iii) osmotic shock described by Thiel (1988).

The osmotic shock method of Neu & Heppel (1965) was as follows. To a bacterial culture, one-tenth volume of 0.5 M Tris-HCl, pH 7.8 was added and incubated for 10 min at room temperature. Harvested cells were resuspended in 800 µl sucrose solution (30 mM Tris-HCl pH 7.8, 40% [w/v] sucrose, 2 mM EDTA), incubated for 10 min in 1.5 ml Eppendorf tubes at room temperature. Cells were pelleted by centrifugation for 5 min in an Eppendorf centrifuge, the supernatant was discarded and the cells resuspended in 500 µl of ice-cold distilled water and incubated on ice for 10 min. Cells were pelleted by centrifugation for 5 min in an Eppendorf centrifuge, the supernatant containing the periplasmic fraction was removed

and stored at -20°C. The remaining cells were resuspended in buffer (10 mM Hepes pH 7.2, 10 mM $MgCl_2$), and treated with Triton-X-100 (2% [v/v] final concentration) for 30 min at room temperature, PMSF, a protease inhibitor, (1 mM final concentration) was added to this suspension. Whole cells were removed from the Triton-X-100 soluble cell lysate by centrifugation in an Eppendorf centrifuge for 5 min. The cell lysate fraction was stored at -20°C. Remaining *Escherichia coli* cells used for control purposes were resuspended in 500 µl 30 mM Tris-HCl pH 7.8 and subjected to two 15 sec bursts at an amplitude of 18 µ in a Jencons sonicator, with a 15 sec cooling time in between pulses. During sonication cells were kept on ice. The cytoplasmic fraction was removed from the cell debris by centrifugation in an Eppendorf centrifuge and stored at -20°C.

A chloroform extraction procedure was also used to obtain periplasmic proteins. Harvested cells (20 ml) were resuspended in residual medium by brief vortexing, 20 µl of chloroform was added, after brief vortexing the suspension was incubated at room temperature for 15 min. Buffer 0.2 ml, (0.01 M Tris-HCl pH 8.0) was added, and the cells were removed from the periplasmic fraction by centrifugation in an Eppendorf centrifuge for 1 min. The supernatant was carefully withdrawn using a Pasteur pipette and stored at -20°C.

The method described by Thiel (1988) was also used to obtain periplasmic proteins. Harvested cells were resuspended in twice the original volume of buffer (10 mM TES, 0.2 M $MgCl_2$

pH 7.3,) and incubated at 25°C for 20 min. The cells were centrifuged at 3,000 rpm in a Gallenkamp centrifuge for 10 min and resuspended rapidly in half the original volume of distilled water, the suspension was maintained at 25°C for a further 20 min. The supernatant obtained after centrifugation as described above was stored at -20°C. The remaining cells were treated with Triton-X-100 as described above.

2.20.1.1 Alkaline phosphatase assay

The method of Brickman and Beckwith (1975) was used. 100 µl of a periplasmic sample was added to 900 µl of 1 M Tris-HCl pH 8.0 in a microfuge tube. 100 µl of either ASW or SOB medium was used as an appropriate blank. 100 µl of freshly prepared 4 mg ml^{-1} Sigma 104 in 1 M Tris-HCl pH 8 was added and the time recorded. All tubes were incubated at 37°C until a faint yellow colour appeared, the reaction was stopped by the addition of 100 µl of 1 M K_2HPO_4 (filter sterile) and the time recorded.
The absorbance at 420 nm was determined and the data was expressed as the change in OD 420 min^{-1} ml^{-1} mg^{-1}.

2.20.2 Cell fractionation procedure

2.20.2.1 Cell breakage

Unless stated otherwise samples were kept on ice during this procedure.

Radioactively labelled [^{35}S-methionine] cells were used as a marker for the 36 kDa low iron induced protein. A culture (100 ml) of *Synechococcus* WH 7803 labelled with ^{35}S-methionine, as described previously was harvested and resuspended in 1 ml of Tris-HCl buffer (20 mM Tris-HCl pH 8), giving a final concentration of 0.2 g cells ml^{-1}. To this cell suspension 3 ml of a 0.2 g ml^{-1} suspension of unlabelled cells, (harvested from a 10 litre volume and grown as described in Section 2.6) were added such that the radioactivity was diluted to one-quarter of the original value. A 200 µl sample was removed for control purposes. The remaining cells were homogenized using a Braun homogenizer (Section 2.15) using a bead size of 0.1 mm - 0.11 mm for 15 min under constant cooling. Up to 2 g of cells could be homogenized per tube, however, larger cell masses required longer times for homogenization. The homogenate was washed from the beads using 20 mM Tris-HCl pH 8 buffer and PMSF was added to a final concentration of 1 mM. Cell debris and unbroken cells were removed from the homogenate by centrifugation at 4,500 rpm for 15 min in a Gallenkamp centrifuge. Any unbroken cells were resuspended in 20 mM Tris-HCl buffer and rehomogenized in order to achieve a maximum yield of cell material.

2.20.2.2 Differential centrifugation and detergent extraction

The membrane fraction was removed from the homogenate by ultracentrifugation at 35,000 rpm for 60 min at 4°C using a Beckman SW40Ti rotor in a Beckman L8 ultracentrifuge. The supernatant, (the cytoplasmic fraction - purple-pink in colour) was removed and stored at -20°C. The membrane pellet, after washing to remove any remaining cytoplasmic fraction, was resuspended by hand homogenization in an equal volume of Tris-magnesium buffer (20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$) and 4% [v/v] Triton-X-100 (in 20 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$). The internal membrane fraction was removed from the cell wall component by extraction in 2% [v/v] Triton-X-100 Tris-magnesium buffer, at 25°C for 60 min with constant stirring. The Triton-insoluble cell wall fraction was removed from the internal membrane fraction by ultracentrifugation at 35,000 rpm for 60 min at 4°C using a Beckman SW40Ti rotor in a Beckman L8 ultracentrifuge. The supernatant (internal membrane fraction) was decanted, and the cell wall fraction was resuspended in 500 µl of 20 mM Tris-magnesium buffer. Both samples were stored at -20°C.

2.20.2.3 Sucrose density centrifugation

Alternatively, the membrane fraction after detergent extraction was layered onto the top of a sucrose gradient consisting of:- 85% [w/v], 80% [w/v], 70% [w/v], 60% [w/v] and 50% [w/v] sucrose in 10 mM Hepes pH 7.2, 10 mM $MgCl_2$. Polycarbonate centrifuge tubes (17 ml) were used in a Beckman

SW28 rotor. The samples were centrifuged at 23,500 rpm for 18 hr at 4°C in a L8 Beckman ultracentrifuge. Cell walls banding in the 70%-80% [w/v] sucrose interface were removed from the gradient by piercing the side of the tube with a syringe and allowing the fraction to flow out under positive pressure. The internal membrane fraction was removed from the top of the 50% [w/v] sucrose fraction.

2.21 Purification of the 36 kDa protein

The 36 kDa protein was purified from the internal membrane fraction by means of a Fast Protein Liquid Chromatography system (Pharmacia) using an anion exchange column (Mono Q HR 5/5; Hiload Q-Sepharose 16/20). The type of column used was dependant upon the total protein concentration of the sample.

The internal membrane fraction was concentrated to a volume of 5 ml containing approximately 20 mg of protein (determined by Biorad protein assay) prior to loading onto the Mono Q anion exchange column (bed volume of 1 ml). The sample was injected via a sample loop onto the top of the column which had been equilibrated in 10 mM Tris-HCl pH 8.0, 0.1% [v/v] Triton-X-100). Protein was eluted from the column with a linear KCl gradient from 0-1M over a period of 20 min. Samples with a total protein content of over approximately 29 mg in 10 ml were loaded onto a Hiload Q anion exchange column (bed volume 50 ml) as described above. The larger column size ensured a higher loading capacity. Protein was eluted from the column in the same way as described above with the same

buffers, except that a flow rate of 5 ml min^{-1} was used. The salt gradient, was formed between 50 ml-100 ml of elution buffer.

All samples were and stored at 4°C prior to concentration using a stirred cell ultrafiltration unit (Amicon) and SDS-PAGE analysis.

2.22 Amicon concentration of experimental samples

Internal membrane samples and fractionated samples from the FPLC purification process were concentrated using a stirred cell ultrafiltration unit (Amicon) with a nitrocellulose filter having a 10,000 molecular weight cut off (Flowgen). Filtration was conducted under pressure from an attached nitrogen source, with rapid stirring.

2.23 Preparation of 36 kDa protein antigen for antibody production

2.23.1 Location of the protein antigen after electrophoresis

This was achieved using a non-fixing Coomassie Brilliant blue stain as described by Harlow and Lane (1988). The sensitivity of staining was approximately 1-2 µg/band. After electrophoresis the gel was washed with three changes in deionized water, then stained for 10 min at room temperature in 0.05% [w/v] Coomassie Brilliant blue R prepared in 500 ml water. The gel was destained by washing in several changes of deionized water for 2 hr. The band of interest was excised

from the gel and stored at -20°C until electroeluted from the gel.

2.23.2 Electroelution of the protein antigen

The protein was removed from the gel by electroelution as follows:-
Dialysis tubing, (18/32 inch in size) prepared by boiling in 10 mM EDTA, was rinsed thoroughly in distiled water prior to use. The protein band of interest was transferred to the dialysis tubing containing 5ml SDS-PAGE running buffer. The tubing was placed horizontally across an electrophoresis chamber containing SDS-PAGE running buffer, and the protein eluted at 70 mA for 3 hr. A reverse pulse for 20 sec after elution moved the protein away from the tubing edge. The eluant was stored at -20°C in a sterile Universal. Prior to use as an antigen for antibody production the sample was concentrated as described in section 2.22.

2.24 Antibody production

The eluted protein sample was used as an antigen to inject Sandy Half Lop rabbits. The immunization schedule was similar to that described by Harlow and Lane (1988). Prior to immunization a pre-immune serum sample was removed from the rabbit.

2.24.1 Immunization schedule

Day 0 400 µg of antigen in 500 µl half strength SDS-PAGE running buffer was emulsified with 500 µl of complete Freund's adjuvant (Difco Laboratories Ltd) and injected subcutaneously at the back of the neck.

Day 14 A second injection of the antigen (580 µg) was emulsified with an equal volume of incomplete Freund's adjuvant and injected subcutaneously as described above.

Day 21 10 ml of blood was removed and the antisera stored at -20°C in 1 ml aliquots prior to use in western blotting.

Day 38 A second 10 ml aliquot of blood was removed three weeks after the second injection. The antisera at -20°C in 1 ml aliquots.

2.24.2 Serum preparation

The procedure was followed as described by Harlow and Lane (1988).

After the blood had been allowed to clot it was removed from the sides of the collection vessel by ringing, using a Pasteur pipette, and placed at 4°C overnight. The serum was separated from the clot by centrifugation at 4,500 rpm in a Gallenkamp

centrifuge for 5 min at 4°C, and stored in 1 ml aliquots at -20°C.

2.25 Preparation of samples for N-terminal sequencing

Proteins were separated using SDS-PAGE followed by transfer to PVDF membranes prior to N-terminal sequence analysis using automated sequential Edman degradation. N-terminal protein sequencing was performed by the SERC protein sequencing facility, University of Aberdeen.
Protein samples were prepared in two ways prior to N-terminal sequencing.

2.25.1 Freeze drying procedure

Samples from the purification procedure (section 2.21) containing the protein of interest were dialysed overnight at 4°C against 50 mM NH_4HCO_3 pH 8.0 in 8/32 inch dialysis tubing. Aliquots (0.6 - 1.0 ml) were frozen at -70°C prior to being freeze dried. The Eppendorf tubes were left open and clingfilm was wrapped around the top. The sample was freeze dried under vacuum for 24 hr. The dried protein pellet was stored at -20°C.

2.25.2 Gyrovapping procedure

Samples from the purification procedure containing the appropriate protein were dialysed as described above (section 2.25.1). The samples were then transferred to Eppendorf tubes in 0.5 ml aliquots prior to being centrifuged at >1000 rpm for

2 hr under vacuum in a Gyrovap attached to a refrigerated solvent trap (Howe). The protein pellet was stored at -20°C.

2.26 General molecular biological techniques

Unless otherwise stated the procedures of Maniatis *et al.* (1982) were employed.

2.26.1 Restriction endonuclease digestion

The appropriate high, medium or low ionic strength buffers (10 x concentrated) were obtained from Amersham International. To 9 µl of a DNA solution 1 µl of 10x buffer was added and mixed by tapping the Eppendorf tube, restriction enzyme was added and the digestion carried out at 37°C for at least 1 hr. Spermidine (4 mM final concentration) was added to restriction digests of chromosomal DNA.

2.26.2 Agarose gel electrophoresis

Restricted DNA fragments were separated using horizontal slab gel apparatus, gels were prepared by boiling agarose, 0.7% [w/v], (Sigma Type I Low EEO) in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). Prior to pouring, the solution was cooled to 50°C and ethidium bromide (0.5 $\mu g\ ml^{-1}$ final concentration) was added. The samples were mixed with loading buffer (Table 2.6) and loaded into the slots of the submerged gel. Electrophoresis was conducted at 80 mA for 1 hr (minigel system) or 30 mA overnight in

Table 2.6 DNA agarose gel loading buffer

	per 10 ml.
glycerol	2.0 ml
1 M Tris-HCl pH8.0	0.1 ml
0.1 M EDTA	1.0 ml
water	6.9 ml
agarose	20 mg
bromophenol blue	10 mg
xylene cyanol	10 mg
orange G	10 mg

Table 2.7 RNA electrophoresis loading buffer

		final concentration ml^{-1}		
formamide	(BDH)	50%	[v/v]	500 µl
formaldehyde	(BDH)	6%	[v/v]	160 µl
10x MOPS				100 µl
glycerol	(Fisons)	10%	[v/v]	100 µl
10 $mgml^{-1}$ xylene cyanol	(Sigma)	0.025%	[w/v]	25 µl
10 $mgml^{-1}$ bromophenol blue	(Sigma)	0.025%	[w/v]	25 µl
10 $mgml^{-1}$ ethidium bromide				10 µl
water				80 µl

electrophoresis buffer (TBE). Visualization of the DNA was via transillumination with short-wave UV light (260 nm) and photography using Polaroid Type 665 film.

2.26.3 Large scale plasmid isolation from *Escherichia coli*

The procedure used was that described by Clewell & Helinski (1970).

An overnight culture of *Escherichia coli* (500 ml), grown in nutrient broth containing the appropriate antibiotic, was harvested by centrifigation at 10,000 rpm for 10 min at 4°C in a Hi-spin 21 centrifuge (MSE) using a 6x300 angle rotor. The supernatant was decanted and the pellet resuspended in 16.5 ml Tris-sucrose (1.51 g Tris-HCl pH 8.0, 62.5 g sucrose, 250 ml). Lysozyme, (Sigma) 5ml of 10 mg ml^{-1} in 0.25 M Tris-HCl pH 8.0 was added and the cells transferred to sterile SS34 Oakridge tubes (Nalgene Labware), the tubes were left on ice for 5 min after which time 4.5 ml of 0.25 M EDTA pH 8.0 was added and left on ice for a further 5 min with occasional swirling. The cells were lysed by the addition of lysis mix (3.03 g Tris-HCl, 11.63 g EDTA, 10 g Brij 58, 2.0 g sodium deoxycholate pH 8.0). The tubes were either inverted until the solution turned clear, or alternatively incubated at 42°C. The cleared lysate was spun at 18,000 rpm, 4°C, for 15 min in a Hi-Spin 21 centrifuge using an 8x50 angle rotor, this removed unlysed cells, cell debris and chromosomal DNA. The supernatant (29 ml) was used to dissolve 29 g of caesium chloride. Ethidium bromide (3 ml of 5 mg ml^{-1}) was added prior to loading the solution into a Beckman polyallomer heat sealed tube. The

preparation was centrifuged at 45,000 rpm for 16-18 hr at 15°C in a VTi50 rotor in a Beckman L8 ultracentrifuge. The plasmid DNA was visualized with the use of a hand held long wave UV light, withdrawn from the side of the tube into a 5 ml syringe using a 21 guage needle and transferred to SS34 tubes. Ethidium bromide was removed by extraction (twice) into water saturated butan-1-ol. To a 3 ml volume of a DNA solution 1.2 ml 1% [v/v] Sarcosyl, 1.2 ml 3 M sodium acetate pH 5.6, 6.6 ml TE, two volumes of 100% [v/v] ethanol was added. The tubes were mixed by inversion and stored overnight at -20°C. DNA was pelleted by centrifugation at 18,000 rpm for 40 min at 4°C in a Hi-Spin 21 centrifuge using an 8x50 angle rotor. The supernatant was removed by aspiration and the pellet vacuum dried. The plasmid DNA was resuspended in 0.25 ml sterile water and stored at -20°C.

2.26.4 Small scale plasmid isolation from *Escherichia coli*

This procedure is a modification of the alkaline lysis method (Birnboim & Doly, 1979) described by Maniatis *et al.* (1982). Yields of 2-3 mg l^{-1} of small plasmids (<10 kb) can be obtained with this method. A single bacterial colony of *Escherichia coli* was inoculated into nutrient broth (5 ml) containing the appropriate antibiotic. After an overnight incubation at 37°C with shaking the cells were harvested by centrifugation at 4,500 rpm for 10 min in a Gallenkamp centrifuge. The pellet was resuspended by vortexing in 150 µl of an ice cold solution of GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0), 200 µl of lysozyme dissolved in GTE (10

mg ml^{-1}) was added to the mixture, which was then incubated on ice for 5 min in Eppendorf tubes. Following this 200 µl of a freshly prepared solution of lysis mix (0.2 M NaOH, 1% [w/v] SDS) was added, the tubes were inverted to mix and stored on ice for a further 5 min. The addition (150 µl) of an ice-cold solution of potassium acetate pH 4.8 (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O) caused the solution to go cloudy after gentle vortexing. The tubes were stored on ice for 5 min and centrifuged for 5 min in an Eppendorf centrifuge.

The supernatant was transferred to a fresh tube and the protein removed by phenol/chloroform extraction. An equal volume of phenol/chloroform was added, followed by vortexing to mix and centrifugation for 4 min in an Eppendorf centrifuge. The upper layer was carefully transferred to a fresh tube and the DNA ethanol precipitated (section 2.26.5).

2.26.5 Ethanol precipitation of DNA

Twice the volume of 100% [v/v] ethanol together with 1/10 the volume of 3 M sodium acetate pH 5.6 was added to the DNA solution. The mixture was placed overnight at -20°C, then centrifuged for 10 min at room temperature in an Eppendorf centrifuge. The subsequent pellet was washed in 70% [v/v] ethanol, centrifuged for 5 min at room temperature and vacuum dried for 20 min. The plasmid DNA was resuspended in sterile water.

2.26.6 Chromosomal DNA isolation from cyanobacteria.

Cyanobacterial chromosomal DNA samples of strains:- *Synechococcus* WH 7805, *Synechococcus* WH 8110, *Synechococcus* WH 8018, were obtained from S. Douglas, DNA from strains *Synechococcus* WH 7803, *Synechococcus* PCC 6301, *Synechocystis* PCC 6803, *Synechococcus* PCC 7002, *Nostoc sp.* MAC PCC 8009 was obtained from this laboratory.

DNA from *Synechococcus* PCC 7002 was extracted by the method described by Lind *et al.* (1985) with modifications.

A late exponentially growing culture was harvested by centrifugation at 5,000 rpm for 15 min in a Gallenkamp centrifuge. The cell pellet was resuspended in 0.5 ml of 0.25 M Tris-HCl pH 8.0, 20% [w/v] sucrose, 10 mg ml^{-1} lysozyme and incubated at 37°C for 1 hr. 16 µl of 30% [v/v] Sarcosyl, 20 µl of 5 mg ml^{-1} proteinase K was added and the cells incubated at 65°C for 1 hr. Protein was extracted by the addition of an equal volume of phenol/chloroform, with vortexing to mix and centrifugation for 5 min at room temperature in an Eppendorf centrifuge. The upper aqueous layer was dialysed overnight against TE buffer followed by ethanol precipitation (section 2.26.5) to concentrate the chromosomal DNA. Prior to ethanol precipitation the DNA solution could be stored at -20°C.

2.26.7 RNA isolation from cyanobacteria

The procedure used was RNA extraction method III described by Alley (1987).

All solutions were prepared using sterilized water containing 0.1% [v/v] diethyl pyrocarbonate (DEPC), a potent RNAse inhibitor. All glassware was baked overnight at 180°C prior to use and solutions were sterilized by autoclaving.

Exponentially growing cells of *Synechococcus* WH 7803 (75 ml) were filtered using a Whatman filtration system onto 0.6 µm pore size polycarbonate filters (Nuclepore) laid over 0.45 µm pore size Millipore HA filters. The cells were washed with 0.05 M EDTA, 0.12 M NaCl, then removed from the filter by vortexing in 1.6 ml of RNA extraction buffer (100 mM LiCl, 50 mM Tris-HCl pH 7.5, 30 mM EGTA, 1% [v/v] SDS) in sterile Universals, then placed immediately at -70°C. Samples were transferred to -20°C for storage prior to RNA extraction. The liquefied cell suspension was boiled for 1 min in a Phillips Cooktronic 8915 microwave oven. Acidic phenol, (1 ml (equilibrated with 50 mM sodium acetate pH 5.6)) was added and the solution vortexed rapidly prior to placing at 65°C for 5 min. The lysates were centrifuged for 5 min in an Eppendorf centrifuge at room temperature and the aqueous phase re-extracted in an equal volume of acidic phenol, followed by further extraction in an equal volume of chloroform : isoamyl alcohol (24:1) prior to ethanol precipitation (section 2.26.5). The pellet was washed in 100 % [v/v] ethanol (1.5 ml), centrifuged for 5 min and dried in a desiccator for 5 min

prior to being resuspended in 400 µl DNAse buffer (100 mM sodium acetate pH 5.6, 10 mM $MgCl_2$). RNAse-free DNAse I (1 µl (Boehringer-Mannheim)) was added and the solution incubated at 37°C for 30 min. The RNA was acidic phenol and chloroform/isoamyl alcohol extracted and ethanol precipitated as described above. The vacuum dried RNA pellets were resuspended in DEPC-treated water and stored at -20°C.

2.26.8 Southern blotting

After agarose gel electrophoresis the gel was photographed and trimmed to the required size then treated in the following way :-
DNA was partially hydrolysed by acid depurination in 0.25 M HCl for 10 min at room temperature. This was followed by alkali denaturation in 0.5 M NaOH, 1.5 M NaCl, for two 15 min periods. The gel was neutralised by transferring to 1.0 M Tris-HCl pH 7.4, 3.0 M NaCl, for a further two 15 min periods.

The transfer of DNA to nitrocellulose was achieved as described by Maniatis *et al.* (1982) based on the procedure described by Southern (1975). Transfer proceeded for a period of about 17 hr, after which time the filter was removed from the gel and soaked in 6x SSC (3 M NaCl, 0.3 M sodium citrate) at room temperature for 5 min then baked for 2 hr at 80°C under vacuum.

2.26.9 Northern blotting

For agarose electrophoresis a formaldehyde gel system was used. However, a different loading buffer (Table 2.7) was used, this enabled the gel to be photographed prior to blotting. All electrophoretic equipment was washed with DEPC-treated water with the exception of the gel tank, this was presoaked for 1 hr in 1 M NaOH prior to washing. After electrophoresis the gel was photographed and washed for 2-3 min in ELGA water then soaked for two 20 min periods in 20x SSC. RNA was transferred to the nitrocellulose membrane as described in section 2.26.8.

2.27 Radiolabelling of DNA fragments

The method described by Feinberg & Vogelstein (1984) was used to label DNA fragments with ^{32}P.

Plasmid DNA, cleaved with the appropriate restriction endonucleases, was separated on a 1% [w/v] low melting point agarose gel (BRL). The required bands were visualized under UV light, excised from the gel and placed into preweighed Eppendorf tubes. Water was added giving 1.5 ml H_2O g agarose^{-1}, the agarose was liquefied and the DNA denatured by heating at 100°C for 7 min, followed by incubation at 37°C for 10-60 min prior to initiating the labelling reaction. DNA stored at -20°C was reheated at 100°C for 3 min and maintained at 37°C for 5 min prior to subsequent use.

Preparation of the radiolabelled gene probe was carried out by addition of the following reagents in the stated order:- 5 µl OLB buffer (Feinberg & Vogelstein, 1984), 1 µl BSA (1 mg ml^{-1} stock), 16 µl (25 ng) DNA fragment, 2.5 µl ^{32}P adCTP (10 µCi μl^{-1}) and 0.5 µl Klenow large fragment DNA polymerase I (Amersham International). The mixture was incubated either overnight at room temperature or for 5 hr at room temperature. Unincorporated radionucleotide was removed by centrifugation of the mixture through a Sephadex G50 column. Salmon sperm DNA (0.1 ml of 10 mg ml^{-1}) was added to the radiolabelled probe which was then heated to 100°C for 10 min and placed on ice for 5 min prior to the hybridization procedure.

2.28 Radiolabelling of oligonucleotide fragments

A synthetic oligonucleotide was synthesized using an Applied Biosystems 380B DNA synthesizer by J. DeBellin (Warwick University). The oligonucleotide was designed on the basis of a highly conserved region of *Salmonella typhimurium* TonB protein, (Figure 2.1).

The oligonucleotide was end-labelled using polynucleotide kinase. The reaction mixture consisted of 8 µl of a 1 in 10 dilution of the oligonucleotide, 4 µl water, 4 µl ^{32}P¥ ATP, 2 µl 10x kinase buffer (Amersham International), 2 µl T4 polynucleotide kinase (Amersham International), and was incubated at 37°C for 1 hr. The reaction was terminated by heating at 65°C for 15 min. Unincorporated radionucleotide

was removed by centrifugation of the mixture through a Sephadex G25 column. The labelled oligonucleotide was used immediately for hybridization reactions.

Figure 2.1

LYS	PRO	LYS	PRO	LYS	PRO
AAA	CCN	AAA	CCN	AAA	CCN
G		G		G	

Legend 2.1 Amino acid and DNA sequence used to prepare an oligonucleotide fragment to *Salmonella typhimurium* TonB protein.

2.29 DNA-DNA, DNA-RNA hybridization

Pre-hybridization and hybridization were conducted in 5x SSPE (3 M NaCl, 0.2 M $NaH_2PO_4.2H_2O$, 20 mM EDTA, pH 7.4), 5x Denhardts solution (5 g Ficoll 400, 5 g polyvinylpyrrolidone-10, 5g BSA (Pentax fraction V) in 500 ml water = 50x stock solution), 0.1% [w/v] SDS. For DNA-RNA hybridization studies 6x SSPE was used. Hybridization solution also contained the ^{32}P labelled denatured DNA probe and 1 mg ml^{-1} denatured salmon sperm DNA. The filters were pre-hybridized for 4-8 hr and hybridized for 12-24 hr.
The hybridization temperatures and washing conditions were variable, these will be discussed in the relevant results chapters.

Probing nitrocellulose filters with radiolabelled oligonucleotide was conducted as for DNA-DNA hybridization except that 100 $\mu g\ ml^{-1}$ poly-A was included in the pre-hybridization and hybridization mixtures as opposed to salmon sperm DNA.

Filters were exposed to X-ray film (Fuji RX) at -70°C with an intensifying screen (Dupont, Cronex). Film was developed in Kodak LX-24 X-ray developer, diluted 1+5 with water, fixed with Kodak FX-40 X-ray liquid fixer, diluted 1+4 with water.

Radioactive filters were stripped by bringing them to the boil in 0.1% [w/v] SDS followed by cooling for 30 min.

2.30 Plaque hybridization

The procedure was performed as described in Maniatis *et al.* (1982), based on the method of Benton & Davies (1977).

2.31 Efficient transformation of *Escherichia coli*

Transformation of a culture of *Escherichia coli* with plasmid DNA was achieved using the method described by Gill (1986).

A 50 ml culture was grown in a New Brunswick series 25 shaking incubator at 38°C, at 275 rpm, until an extinction of 0.3 at $OD_{550\ nm}$ was obtained. The culture was stored on ice for 20 min then harvested at 3,000 rpm in a Gallenkamp centrifuge for 10 min at 4°C. The cell pellet was resuspended

initially in 1 ml of an ice-cold solution of 0.1 M $CaCl_2$, then made upto 20 ml and left on ice for 20 min. The cells were pellated by centrifugation at 3,000 rpm in a Gallenkamp centrifuge for 10 min at 4°C and resuspended in 1 ml 0.1 M $CaCl_2$. The DNA was dispensed into 100 µl aliquots of competant cells, the mixture was maintained on ice for 2 hr then heat shocked for 2 min at 42°C. To each sample 200 µl of SOB was added and the cells incubated for 1 hr at 37°C, followed by pelleting by centrifugation for 10 sec in an Eppendorf microfuge. The pellet was resuspended in residual supernatant then plated out onto nutrient agar plates containing the appropriate antibiotic. The plates were incubated overnight at 37°C.

CHAPTER 3

DETERMINATION OF IRON LIMITATION OF PRIMARY PRODUCTIVITY IN NATURAL PHYTOPLANKTON ASSEMBLAGES

3.1 Introduction

The data discussed within this chapter were obtained during the BOFS 47/90 cruise upon the R.R.S. Charles Darwin (Figure 3.1.1).

The BOFS (Biogeochemical Ocean Flux Study) project was devised to study the flux of inorganic carbon from the atmosphere to its final arrival at the sea floor following sedimentation in particulate matter (Savidge *et al.*, 1992). The field programme was designed to measure a large number of parameters, with a view to developing comprehensive ocean-atmosphere mathematical models, which will be capable of predicting the effect of anthropogenic emission of atmospheric carbon dioxide upon the global climate.

Carbon dioxide adsorption into the ocean is controlled by physical, chemical and biological processes. Since the initiation of the BOFS project observations have been made of some of these processes. This includes the roles of various components of the microbial loop, vertical particle fluxes and gas flux measurements, together with global measurements of critical parameters such as temperature and chlorophyll which control the absorption of CO_2 by the oceans. These observations were achieved in order to determine the function of these controlling factors (Savidge *et al.*, 1992).

The BOFS spring bloom experiment 1990 was conducted in the North-East Atlantic, 46-50°N 14-22°W between 18th April - 25th June 1990, and is described in detail by Savidge *et al.* (1992). The study involved both spatial and temporal surveys

Figure 3.1.1 The Royal Research Ship (R.R.S.) Charles Darwin

conducted by R.R.S. Discovery and R.R.S. Charles Darwin respectively. The spatial survey by R.R.S. Discovery, made observations on the physical structure of the water column, mainly by temperature and salinity profiles; since small differences in water temperature and salinity identify water masses of different origins. During the survey an eddy structure was identified within the survey area. The deployment of a central Langrangian marker drogue within the eddy was used as the reference for the BOFS time series sampling point. Two further concentric arrays of similar drogues were also deployed in order to determine the breakdown of the eddy over the course of the experiment. The temporal studies, conducted by the R.R.S. Charles Darwin followed one patch of water believed to be the eddy identified by the spatial survey. The experiments done during the cruise included serial observations of physical, chemical and biological variables, together with a wide range of biological rate process measurements. All measurements were taken from the upper 300 m of the water column within the eddy structure identified by the major marker buoy.

It was hoped to determine whether productivity in these waters was low as a result of iron limitation. Stimulation of growth and nutrient utilization in low productive Southern Ocean and North Pacific Ocean waters by the addition of trace metals (namely iron) has been demonstrated (Martin *et al.*, 1989; De Baar *et al.*, 1990) and the phytoplankton are believed to be iron-limited. It has been suggested that if this is true for large regions of the worlds' Oceans then addition of iron may be a technological "fix" for the removal of excess

CO_2 from the atmosphere into the water column and potentially the sea floor as particulate matter (see Section 1.3). In order to determine if such an iron limited system existed in this body of water, size fractionated ^{14}C *in-situ* experiments with varying concentrations of added $FeCl_3 . 6H_2O$ in 0.5 M HCl were conducted. If iron limiting conditions were apparent, then increased productivity as a function of increased ^{14}C fixation would be observed upon addition of iron. Also ^{55}Fe tracer experiments were conducted in order to see if natural assemblages were able to internalize the iron added.

The identification of an eddy system as the sampling site meant that there was an expectation that one water mass could be followed during the cruise. The changes in carbon flux throughout the water column were to be followed in that single body of water during the development of the spring bloom from winter through to summer conditions. The track of the central Langrangian marker buoy, released at 49.08°N, 19.25°W within the North-East Atlantic is shown in Figure 3.1.2. The samples taken for the analysis for the effect of iron limitation upon primary productivity of the phytoplankton component of the water column are shown by black diamonds in Figure 3.1.2b and are described in Table 3.1.1.

3.2 Precautions used during experimental procedures

As described in Section 1.3 many practical problems accompany working with iron in natural seawater samples. Firstly, one of the major problems is contamination from the

Figure 3.1.2 **Diagram of the track of the central Langrangian marker buoy between 26/4/90 - 15/06/90**

The marker buoy was tracked by the R.R.S. Charles Darwin.

a) Diagram showing the position of the marker buoy in the North-East Atlantic with respect to the main land.

R = The position at which the buoy rig was released, 49.08°N 19.25°W.

. . . . . = The location of the continental shelf.

b) An enlarged profile of the central buoy track.

◆ = The sampling positions for the experiments discussed in the text (see Table 3.1.1).

●1 = The position at which the central buoy was released.

●2 = The position at which the central buoy was recovered.

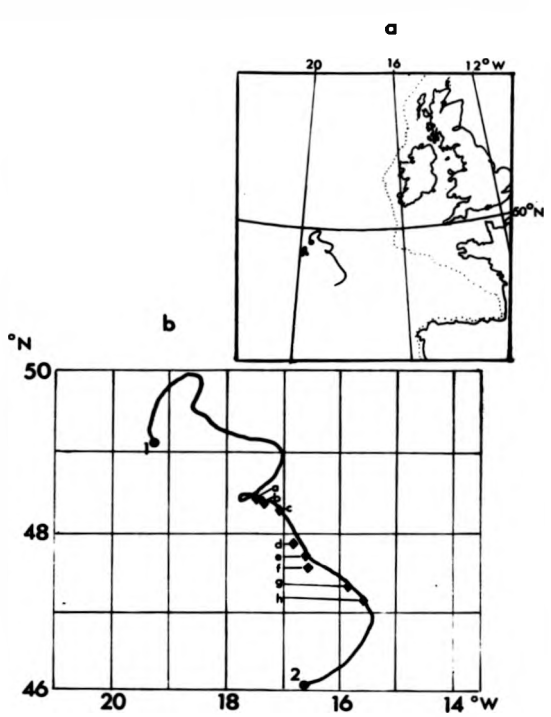

a
20
16
12°W
50°N
b
°N
50
48
46
20
18
16
14 °W
1
2

Table 3.1.1 **Sampling co-ordinates**

Station	latitude	longitude	comments
a	48° 27.37′	17° 28.76′	28/05/90 ^{14}C *in-situ* experiments.
b	48° 24.61′	17° 24.74′	29/05/90 ^{14}C *in-situ* experiments.
c	48° 18.54′	17° 04.13′	01/06/90 ^{14}C *in-situ* experiments.
d	47° 53.64′	16° 44.29′	03/06/90 ^{14}C *in-situ* experiments with 0.5 nmoles Fe^{+++}/ 0.5 M HCl.
e	47° 45.27′	16° 37.33′	04/06/90 ^{14}C *in-situ* experiments with 1 nmoles Fe^{+++}/0.5 M HCl.
f	47° 37′	16° 34.35′	05/06/90 ^{14}C *in-situ* experiments with 0.25 nmoles Fe^{+++} / 0.5 M HCl.
g	47° 19.77′	15° 47.08′	08/06/90 ^{14}C *in-situ* experiments with 0.75 nmoles Fe^{+++} / 0.5 M HCl
h	47° 10.1′	15° 34.8′	09/06/90 ^{55}Fe uptake experiments.

vessels used during the experimental procedure because endogenous iron concentrations in seawater are very low. All glassware and polycarbonate bottles used were thoroughly washed with Aristar HCl (BDH) and rinsed with seawater prior to experimental procedure. Water samples were taken with a 10 litre acid-washed ultraclean GoFlo, which is used by marine chemists from Southampton University for trace metal analysis (see Section 3.5). Sampling was achieved using a non-metal kevlar cable and the GoFlo was submerged closed. Closing the GoFlo before it entered the water, prevented contamination of the seawater samples with water from the surface microlayer, which has higher concentrations of contaminants than bulk seawater. At about 10 metres the GoFlo was triggered by increasing hydrostatic pressure to open, and it was then flushed with seawater as it travelled through the water column to the desired depth. The GoFlo was closed at this depth by triggering a switch by sending down a messenger weight along the kevlar cable. During this procedure gloves were worn to reduce contamination (Figure 3.2.1). Seawater samples for the ^{55}Fe uptake experiments were aliquoted into 1 litre polycarbonate bottles in a clean laminar flow hood. The bottles were sealed in plastic bags, to prevent contamination from iron sources on the ship, and placed in a perspex box, cooled with seawater and incubated under a bank of fluorescent strip lights .

Unfortunately the same rigourous stringency of sampling could not be achieved during the ^{14}C *in-situ* experiments (see Section 3.4) as time was limited and the iron addition experiments had to be comparable to the control experiments

Figure 3.2.1 **Deployment of an acid washed ultraclean 10 l GoFlo**

The GoFlo was attatched to a kevlar cable (arrowed) and deployed to the required depth. Gloves were worn throughout the sampling procedures. All the procedures described were used to minimise contamination from iron or other trace metals.

conducted by A. Pomroy (Plymouth Marine Laboratory). Sampling was achieved using 30 litre GoFlos attached to a kevlar cable. All 60 ml sample polycarbonate bottles were sealed until required.

Other problems arose because, iron added to seawater in an enclosed vessel may be in one of four forms:- 1) it can adhere to the vessel walls. 2) It maybe truly soluble in the seawater within the vessel. 3) It can adhere to the cell surface and other detritus. 4) It maybe metabolic iron present within cells. Davies (1970) gives a comprehensive account of the precautions to be used and the factors to take into account when working with iron and marine phytoplankton. The measurement of iron uptake into natural assemblages is greatly complicated by the fact that iron adheres to any surface in which it comes in contact. As a result ^{55}Fe tracer experiments require controls otherwise results would prove inconclusive; it is particularly important that extraneous iron is removed. In order to do this the titanium III citrate washing method (Hudson & Morel, 1989) was employed during ship board experimental procedure (see Section 3.5). This method was able to remove any non-internalized iron otherwise interfering with results. The validity of this procedure is shown below (Section 3.3).

3.3 The laboratory technique to remove extraneous iron from filters

As all the seawater samples were to be size fractionated by passage through 1 μm and 0.2 μm, 47 mm diameter Nuclepore

polycarbonate filters with precise pore sizes after incubation in the presence ^{55}Fe, a method had to be employed which was able to remove any extracellular iron bound to both the filter and to the cell surface. This was achieved using the method of Hudson & Morel (1989) as follows.

3.3.1 Method

Initial experiments were done with coastal seawater, prior to the cruise.

Seawater samples (28/03/90) collected from Plymouth Sound at a depth of 40 meters were aliquoted into 20, 60 ml acid washed polycarbonate bottles and spiked with 0.2 μCi ^{59}Fe/60 ml bottle. Duplicate samples were prepared for each time point and washing condition, and the bottles were incubated over a time period corresponding to:- t= 0, 1, 2, 3, and 4 hours. At each time point after inoculation four 60 ml samples were filtered under vacuum through 1 μm and 0.2 μm polycarbonate filters using a cascade system. Half of the filters were washed with filtered seawater (FSW) and the remaining filters were prewashed with 5 ml FSW, followed by 5 ml titanium III citrate (see Table 3.3.1) allowed to stand over the filters for 2 min then filtered through slowly under a low vacuum; the filters were further washed with FSW in order to remove any precipitated titanium dioxide. The amount of ^{59}Fe per filter was determined by R. Clifton (Plymouth Marine Laboratory) using a gamma counter.

Table 3.3.1 Preparation of titanium III citrate

Titanium citrate was prepared in the following way:-

Composition		Preparation	
Component	Final concentration (M)	Reagent	Quantity
Ti(III)	0.047	*a*) 0.05 M Na_2EDTA +	
Citrate	0.047	0.05 M Na_3citrate	250 ml
EDTA	0.047	*b*) 1.0 M KCl	2.5 ml
Na	0.61	*c*) $NaCl_s$	3.0 g
Cl	0.35	*d*) 20% [v/v] $TiCl_3$	7.77 ml
K	0.010	*e*) 10 M NaOH	Titrate
pH	8.0		to pH.

Combine chelator solution *a* with salts *b* and *c*. The $TiCl_3$ was added while stirring and pH was attained by adding NaOH (10 ml) dropwise to the mixture. After 30-60 min equlibration time the pH was checked. A clear purple solution should result. Ideally the reagent should be prepared fresh for use. However it could be stored in a brown bottle, with no air spaces present, in the fridge for about 5 days, as titanium III citrate will be oxidized to TiO_2 particles in the presence of oxygen.

3.3.2 Results

Figure 3.3.1 highlights the effect of titanium III citrate washing upon the amount of ^{59}Fe present on both the 1 μm filters and the 0.2 μm filters over a period of four hours incubation in seawater. Unwashed 1 μm filters showed what appeared to be a linear uptake of ^{59}Fe by natural phytoplankton assemblages > 1 μm in size (Figure 3.3.1a). However, the amount of ^{59}Fe on 1 μm pore size filters was greatly reduced after treating with titanium III citrate. This result suggested that the linear uptake on unwashed 1 μm filter was due to adsorption of ^{59}Fe to both particulate matter and the extracellular surface and not direct ^{59}Fe uptake by the phytoplankton. Similar observations were made for the cell populations present upon the 0.2 μm filters (Figure 3.3.1b)

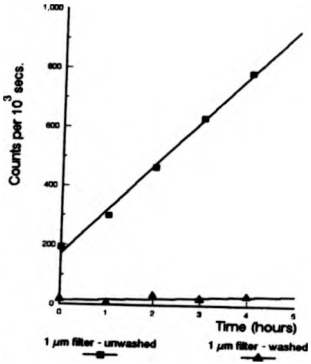
These preliminary laboratory results showed that the majority of ^{59}Fe adsorbed to the both the 0.2 μm and the 1 μm filters and that the amount adsorbed increased with time. Titanium III citrate washing was able to reduce the amount of ^{59}Fe bound to the filter. Furthermore, adsorption of the ^{59}Fe onto the walls of the 60 ml bottles was not instantaneous but increased with time. This would suggest that as soon as the iron is inoculated into seawater samples it is not directly removed by adsorption on to the side of the vessel and therefore, should be available for uptake by phytoplankton.

Figure 3.3.1 The effect of the titanium III citrate washing method upon the removal of non-internalised iron from polycarbonate filters

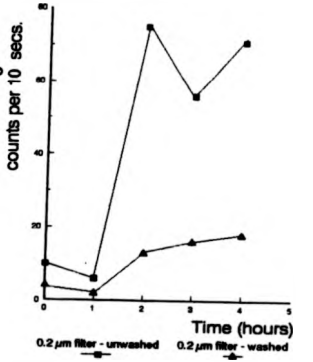
a) Either titanium III citrate washed ▲ or unwashed ■ 1 μm size filters used to filter seawater samples containing added ^{59}Fe.

b) Either titanium III citrate washed ▲ or unwashed ■ 0.2 μm size filters used to filter seawater samples containing added ^{59}Fe.

a


Counts per 10^3 secs.
1,000
800
600
400
200
0
0
1
2
3
4
5
Time (hours)
1 μm filter - unwashed
1 μm filter - washed

b


counts per 10^3 secs.
80
60
40
20
0
0
1
2
3
4
5
Time (hours)
0.2 μm filter - unwashed
0.2 μm filter - washed

These experiments showed that the titanium citrate washing procedure could be used to distinguish between extra- and intracellular iron and was therefore used during the ^{55}Fe uptake experiments at sea.

3.4 ^{14}C *in-situ* experiments

Iron limitation of natural phytoplankton populations is much more likely in the mid-Atlantic than in coastal waters because of the distance from terrestrial and aeolian sources of iron.

Therefore, *in-situ* primary productivity measurements, as determined by ^{14}C fixation, were undertaken in the presence of different Fe^{+++} concentrations to see if iron might result in a stimulation of phytoplankton activity. If such a stimulation occurred then it could be possible that the primary productivity of oceanic natural phytoplankton assemblages was iron limited.

3.4.1 Method

The method used was similar to that described by Joint & Pomroy (1983). Each ^{14}C *in-situ* experiment began before sunrise. Water samples were taken from 9 depths, 2, 5, 10, 15, 20, 25, 30, 35 and 40 metres through the water column, using a 30 litre GoFlo. Aliquots were placed into 60 ml acid washed polycarbonate bottles. To each bottle 10 μCi of $H^{14}CO_3^-$ (Amersham International) was dispensed with a

positive displacement micropipette; in addition, experimental bottles were "spiked" with different concentrations of $FeCl_3$ in 0.5 M HCl or with 0.5 M HCl in order to see if the addition of acid alone had any effect upon productivity. Triplicate control bottles, without iron addition, were used for each experiment to determine the water column productivity at ambient iron concentrations. In some cases duplicate bottles were used for the Fe "spiking" experiments. In all experiments a dark control was included to measure any non-photosynthetic activity. At each depth one sample was incubated in a polycarbonate bottle wrapped in aluminium foil and black masking tape to give an estimate of dark CO_2 fixation. All bottles were incubated for 24 hr from dawn to dawn by suspending the samples at the nine depths from which they were taken. The bottles were held in perspex clamps, fixed to a wire suspended from a light floating frame (Figure 3.4.1). Such a system was constructed to give minimum shading of the samples. The string of incubation bottles were tethered to the ship's stern with 70 meters of line (Figure 3.4.1b). At the end of each incubation the samples were size-fractionated by passing through a cascade of 47 mm diameter Nuclepore filters, of 5, 1 and 0.2 µm pore size, held in separate filter holders. A low vacuum was applied for the 1 and 0.2 µm filters (5 cm Hg and 15 cm Hg respectively). The filters were removed immediately after the sample had passed through and were transferred to a scintillation vial and stored over-night in a desiccator chamber containing active silica gel. Drying of the filters results in complete removal of unfixed ^{14}C. Optiscint (2.5 ml) was added per vial and

Figure 3.4.1 ^{14}C *In-situ* **productivity rigs**

a) The 60 ml polycarbonate bottles containing ^{14}C and Fe^{+++} "spiked" samples were secured to a rope via perspex clamps and lowered to the appropriate depth.
b) The *in-situ* bottles were suspended in the water column at the appropriate depths using a light floating frame, constructed in such a way as to give minimum shading of samples.

a

b

the cpm per filter determined using a LKB liquid scintillation counter. The counting efficiency of the LSC was determined by the external standard, channels ratio method.

Primary productivity (mg C m^{-3} day^{-1}) was calculated using the following formula (Strickland & Parsons, 1968):-

$$\frac{(dpm-bg) \times TCO_2 \times 1.05}{dpm \text{ added} \times t}$$

dpm = disintegrations per minute for each filter.
bg = background counts.
TCO_2 = total CO_2 present in seawater - which has been determined from standard oceanographic tables.
t = time

Chlorophyll fluorescence was determined from discrete samples taken from water bottles at various depths using a Turner Designs fluorometer. Samples were filtered through GF/F filters and extracted over night in 90% [v/v] acetone in the dark at 4°C. The concentrations of chlorophyll *a* and phaeopigment were calculated using the equations given by Strickland and Parsons (1968). The fluorometer was calibrated against pure chlorophyll *a*. Although this method cannot distinguish between the different chlorophylls present in seawater samples it is generally assumed to measure chlorophyll *a* as this is the most abundant chlorophyll.

3.4.2 Results

3.4.2.1 Primary productivity depth profiles obtained from natural phytoplankton assemblages incubated in the presence or absence of additional Fe^{+++}

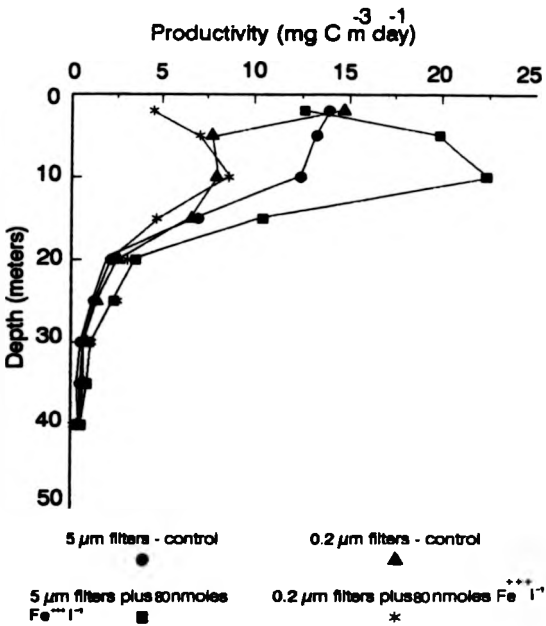
Two sets of depth profiles are shown in Figures 3.4.2 and 3.4.3. Samples were taken at 48° 24.61' N, 17° 24.74' W (see Figure 3.1.2) on the 29/05/90 (Figure 3.4.2) and 48° 18.54' N, 17° 04.13' W (see Figure 3.1.2) on the 01/06/90 (Figure 3.4.3) inoculated with 80 nmoles l^{-1}, 160 nmoles l^{-1}, 1.6 μmoles l^{-1} and 16 μmoles l^{-1} $FeCl_3 . 6H_2O$. The phytoplankton productivity was determined as described in section 3.4.1. Such a large range of Fe^{+++} concentrations were added to "spike" the seawater to give an initial indication of the concentrations required to stimulate photosynthesis, as well as a means of overcoming the Fe^{+++} added adsorbing to the walls of the vessel which would be unavailable to phytoplankton.

Results of productivity in the 5 μm fraction from the 29/05/90 suggest that productivity was stimulated by iron additions of upto 1.6 μmoles l^{-1} (Figure 3.4.2a,b,c) within the first 15 meters of the water column; addition of 16 μmoles l^{-1} of iron did not significantly increase productivity in this size fraction (Figure 3.4.2d). Iron additions had no significant effect upon productivity in the 0.2 μm fraction; in fact, it appeared to inhibit productivity slightly in near surface waters at all concentrations (Figure 3.4.2a,b,c,d). In contrast ^{14}C fixation by the 5 μm size fraction from

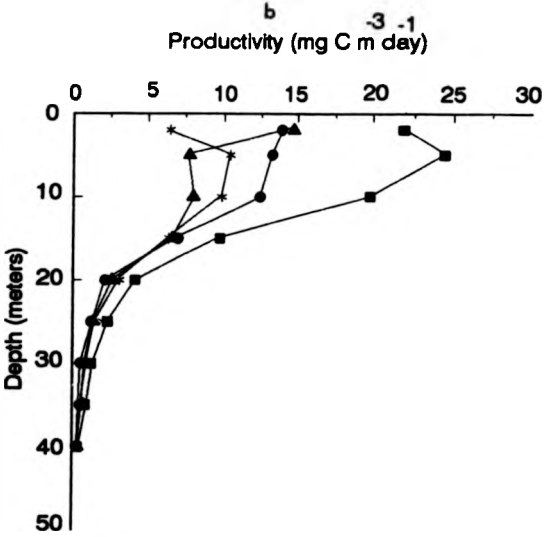
Figure 3.4.2 Productivity depth profiles (mg C m^{-3} day^{-1}). Taken at Station b (see Table 3.1.1)

^{14}C productivity measurements for the 5 μm and the 0.2 μm size fractions were taken in the presence of:-
a) 80 nmoles l^{-1} Fe^{+++}. b) 160 nmoles l^{-1} Fe^{+++} . c) 1.6 μmoles l^{-1} Fe^{+++} d) 16 μmoles l^{-1} Fe^{+++}. Control samples which did not contain any added Fe^{+++} were also included.

a


Productivity (mg C m^-3 day^-1)
0
5
10
15
20
25
Depth (meters)
0
10
20
30
40
50
5 μm filters - control
0.2 μm filters - control
5 μm filters plus 80nmoles Fe+++ l-1
0.2 μm filters plus 80nmoles Fe+++ l-1

b



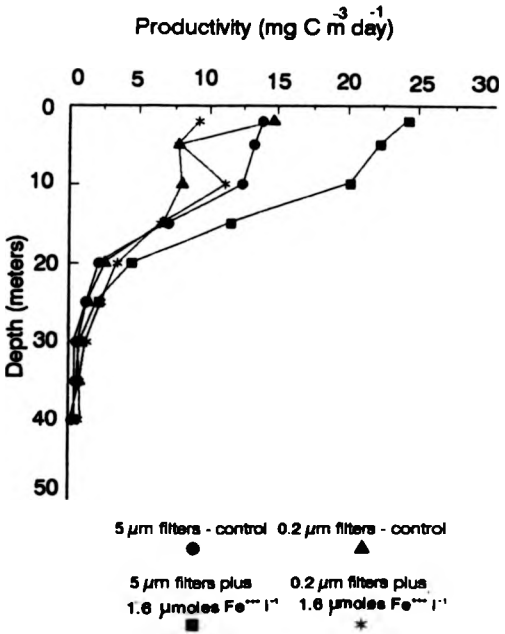
5 μm filters - control ●

0.2 μm filters - control ▲

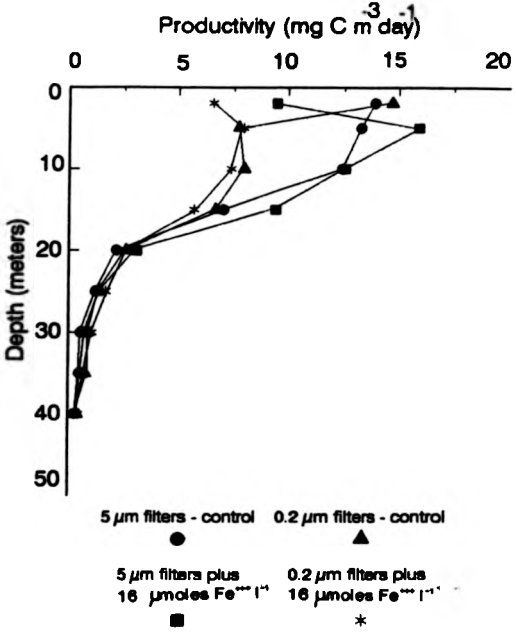
5 μm filters plus 160 nmoles Fe^{***} l^{-1} ■

0.2 μm filters plus 160 nmoles Fe^{***} l^{-1} *

c


Productivity (mg C m^{-3} day^{-1})
0
5
10
15
20
25
30
Depth (meters)
0
10
20
30
40
50
5 μm filters - control
0.2 μm filters - control
5 μm filters plus 1.6 μmoles Fe^{+++} l^{-1}
0.2 μm filters plus 1.6 μmoles Fe^{+++} l^{-1}

d

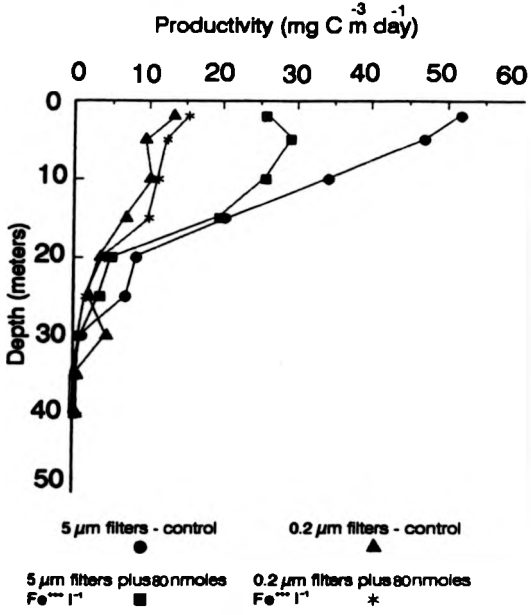

Productivity (mg C m-3 day-1)
0
5
10
15
20
Depth (meters)
0
10
20
30
40
50
5 µm filters - control
0.2 µm filters - control
5 µm filters plus 16 µmoles Fe+++ l-1
0.2 µm filters plus 16 µmoles Fe+++ l-1

01/06/90 was inhibited in the surface 25 m by all concentrations of iron added. Again the 0.2 μm size fraction showed no response to any of the iron concentrations added (Figure 3.4.3a,b,c,d). The same result to the 01/06/90 was observed on the 28/05/90 (results not shown). Temperature and salinity data taken throughout the cruise using a conductivity-temperature-depth profiler (CTD) show the physical structure of the water column present during the period of study (Savidge *et al.*, 1992). As a result it appears that during the period between the 29-31/05/90 samples were taken from a different water mass to those taken on the 28/05/90 and 01/06/90. Productivity as a whole was much lower on the 29/05/90 compared to on the 01/06/90 although the amount of chlorophyll present (mg m^{-3}) in the water column remained virtually constant (Figure 3.4.4a). Movement into different water bodies is shown in Figures 3.4.4b,c due to the variations in the temperature and salinity deep water profiles (Figure 3.4.4 b and c respectively). Unfortunately during the time we appeared to be in an alternate body of water, whose phytoplankton assemblages might have been responding to Fe^{+++} addition, bad weather conditions prevented deployment / reployment of any *in-situ* productivity rigs on both the 30/05/90 and 31/05/90. Therefore, the evidence for iron stimulation of primary productivity in this water body was dependent upon one days result. Lack of space upon the *in-situ* rigs prevented replicate samples of the four iron concentrations from being taken at each depth. The results from these experiments must remain inconclusive.

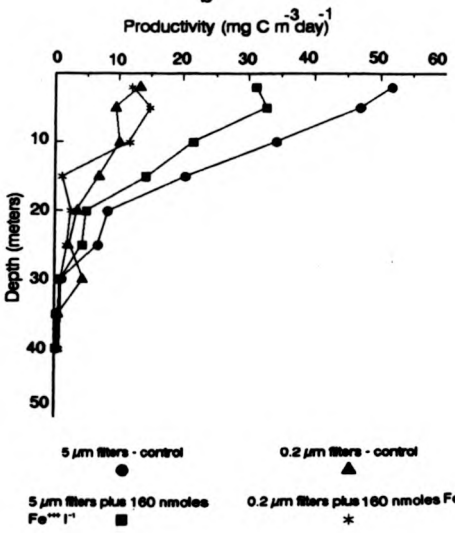
Figure 3.4.3 **Productivity depth profiles (mg C m^{-3} day^{-1}). Taken at Station c (see Table 3.1.1)**

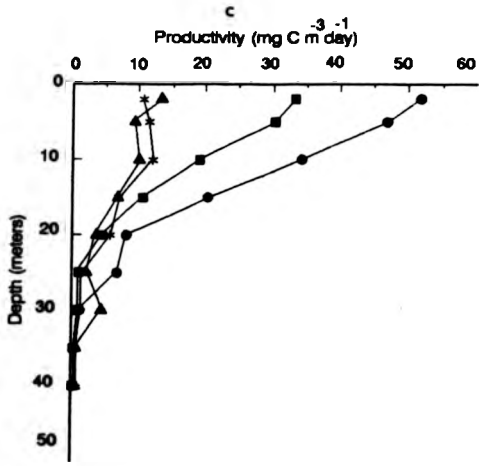
^{14}C productivity measurements for the 5 μm and the 0.2 μm size fractions were taken in the presence of :-
a) 80 nmoles l^{-1} Fe^{+++}. b) 160 nmoles l^{-1} Fe^{+++}. c) 1.6 μmoles l^{-1} Fe^{+++}. d) 16 μmoles l^{-1} Fe^{+++}. Control samples which did not contain any added Fe^{+++} were also included.

a


Productivity (mg C m^-3 day^-1)
0
10
20
30
40
50
60
Depth (meters)
0
10
20
30
40
50
5 μm filters - control
0.2 μm filters - control
5 μm filters plus 80 nmoles Fe*** l^-1
0.2 μm filters plus 80 nmoles Fe*** l^-1

b


Productivity (mg C m-3 day-1)
0
10
20
30
40
50
60
Depth (meters)
10
20
30
40
50
5 μm filters - control
0.2 μm filters - control
5 μm filters plus 160 nmoles Fe*** l-1
0.2 μm filters plus 160 nmoles Fe*** l-1

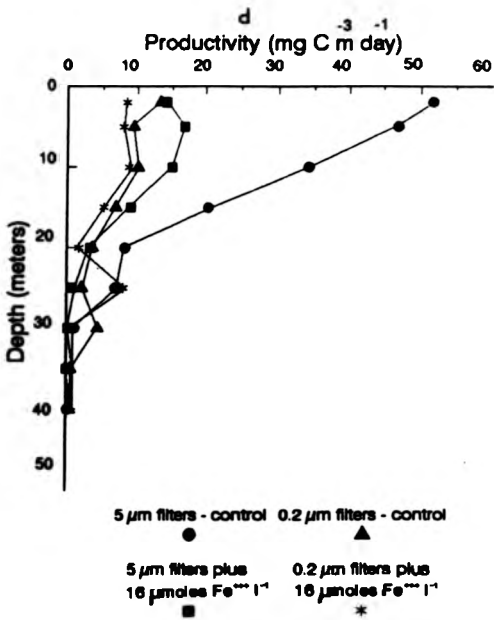


5 μm filters - control ●

0.2 μm filters - control ▲

5 μm filters plus 1.6 μmoles $Fe^{+++}l^{-1}$ ■

0.2 μm filters plus 1.6 μmoles $Fe^{+++}l^{-1}$ *


d
Productivity (mg C m-3 day-1)
0
10
20
30
40
50
60
Depth (meters)
0
10
20
30
40
50
5 μm filters - control
0.2 μm filters - control
5 μm filters plus 16 μmoles Fe+++ l-1
0.2 μm filters plus 16 μmoles Fe+++ l-1

Fig

Figure 3.4.4a **Chlorophyll (mg m^{-3}) measurements**

Measurements were made as described in the text during the 47/90 cruise (after Savidge *et al.*, 1992). The period between the 28/05/90 and 01/06/90 is marked. A = 28/05/90. B = 29/05/90. C = 30/05/90. D = 31/05/90. E = 01/06/90.

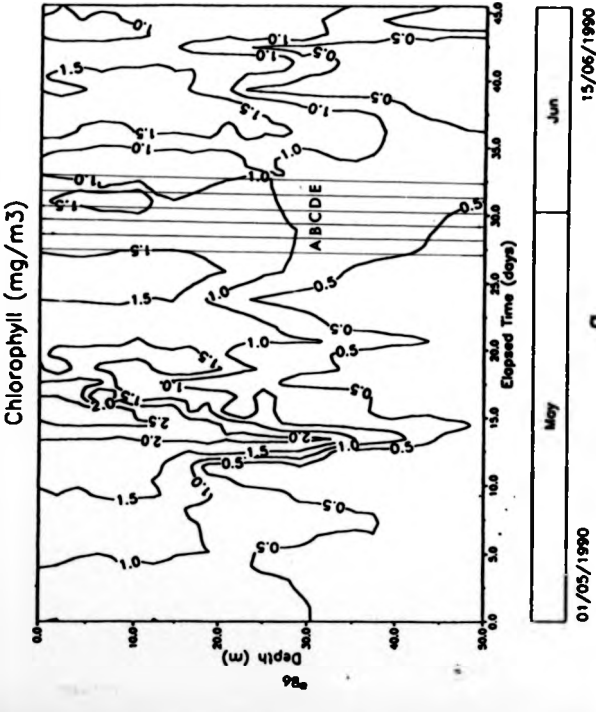
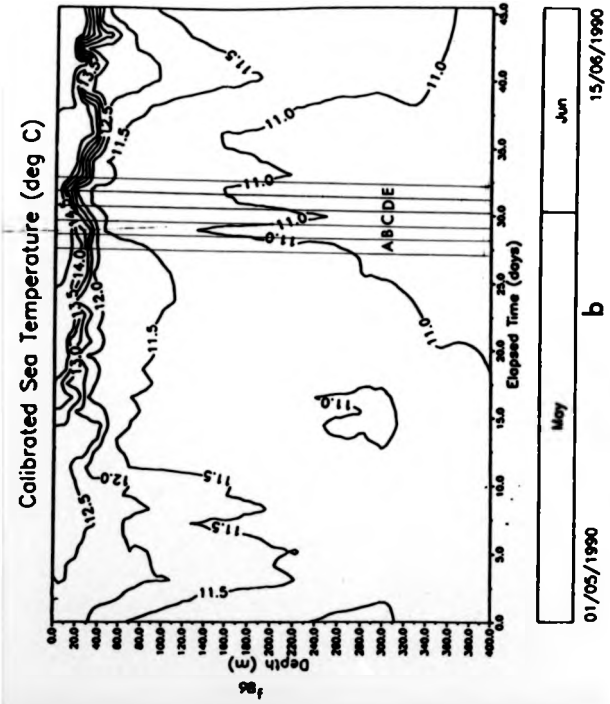
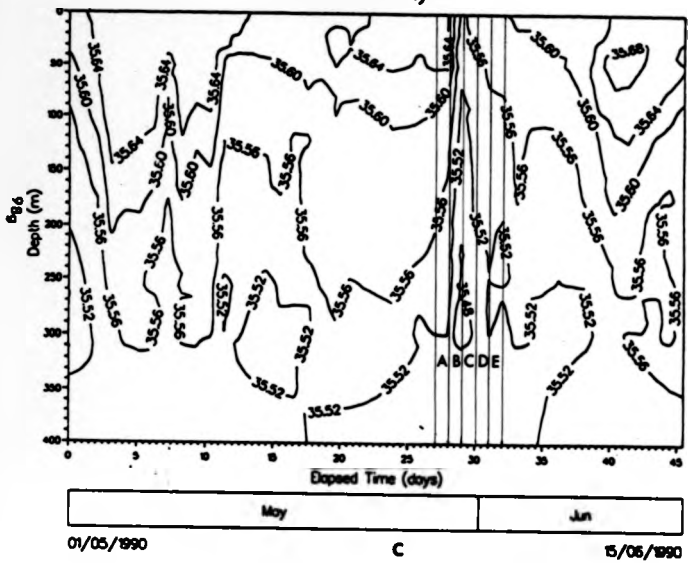


Figure 3.4.4b,c Diagrams showing the physical structure of the water column

Measurements were made during the 47/90 cruise (after Savidge *et al.*, 1992). The period between the 28/05/90 and 01/06/90 is marked. A = 28/05/90. B = 29/05/90. C = 30/05/90. D = 31/05/90. E = 01/06/90.
b) Calibrated sea temperature (°C). c) Calibrated salinity.




Calibrated Salinity
Depth (m)
Elapsed Time (days)
A
B
C
D
E
May
Jun
01/05/1990
C
15/06/1990

As large amounts of added iron appeared to inhibit photosynthetic activity in most of the samples studied, the amount of iron added per 60 ml bottle was reduced to 4 nmoles l^{-1}, 8 nmoles l^{-1}, 12.5 nmoles l^{-1} and 16.6 nmoles l^{-1} in order to determine whether lower concentrations could stimulate productivity. One concentration was studied each day; triplicate samples were used from each depth together with a control of 0.5 M HCl (initial concentration), equivalent to that used for the iron "spiking" experiments. Depth integrated primary productivity values obtained for each concentration are shown in Table 3.4.1, together with the equivalent values obtained when 0.5 M HCl (initial concentration) was added to sea water, compared with control samples. Again the results were inconclusive; the size fraction stimulated by the addition of Fe^{+++}, in comparison to the control samples, varied between the concentration of iron added. Also the presence of the acid alone appeared to have a stimulatory effect which was, in some instances, greater than the samples containing added Fe^{+++}. An example, (Table 3.4.1a) is 5 μm size fraction, 12.5 nmoles l^{-1} Fe^{+++} acid control. Regression analysis was used to compare the effect of iron and acid addition for both the 5 μm and 0.2 μm size fractions from the 16.6 nmol l^{-1} Fe^{+++} "spike" (Figure 3.4.5) and the 4 nmole l^{-1} Fe^{+++} "spike" (Figure 3.4.6). At Fe^{+++} concentrations of 16.6 nmol l^{-1} 82% of the variance in the ^{14}C fixation rate by the > 5 μm fraction in the presence of added iron was explained by the variance in the rate measured when acid alone was added (Figure 3.4.5a). Similarily for the > 0.2 μm fraction 95% of the variance in the ^{14}C fixation rate

Table 3.4.1 Depth integrated primary production (mg C m^{-2} day^{-1})

a

> 5 μm size fraction

Samples	concentration of iron (nmoles l^{-1})			
	4	8	12.5	16.6
control	328.04	554.73	221.29	269.74
+ Fe^{+++}	352.25	228.13	217.13	327.24
+ HCl	339.49	203.25	267.01	266.59

b

> 0.2 μm size fraction

Samples	Concentration of iron (nmoles l^{-1})			
	4	8	12.5	16.6
control	136.97	127.77	129.16	152.9
+ Fe^{+++}	153.46	180.08	139.6	171.6
+ HCl	151.22	164.65	150.64	159.92

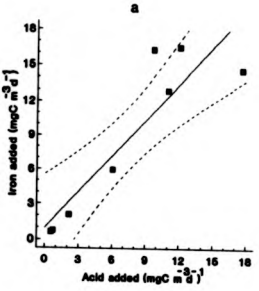
Table 3.4.1 shows the integrated primary productivity values to the base of the euphotic zone, for a) > 5 μm and b) > 0.2 μm size fractionated natural phytoplankton assemblages in seawater samples containing:- no added Fe^{+++} (column 1), 4 - 16.6 nmoles l^{-1} Fe^{+++} added (column 2), 0.5 M (initial concentration) HCl added (column 3). Samples for each set of experiments were taken on different days.

Figure 3.4.5 Regression analysis of the rate of carbon fixation (mg C m^{-3} day^{-1}) in sea-water samples "spiked" with 16.6 nmoles l^{-1} of Fe^{+++} compared with the rate obtained in samples with an equivalent amount of added 0.5 N HCl

The 95% confidence envelopes are shown (- - - - -).

a) 5 μm size fraction. R^2 = 82% (F ratio = 27.34; d.f. = 1,6; P≤ 0.0019).

b) 0.2 μm size fraction. R^2 = 95% (F ratio = 120.61; d.f. = 1,6; P ≤0.00003).


a
Iron added (mgC m-3 d-1)
Acid added (mgC m-3 d-1)
0
3
6
9
12
15
18

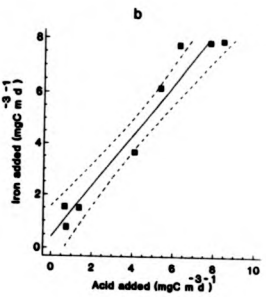
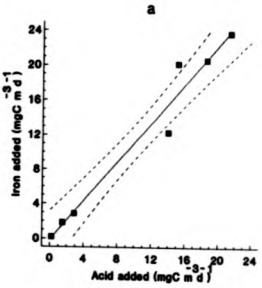

b
Iron added (mgC m-3 d-1)
Acid added (mgC m-3 d-1)
0
2
4
6
8
10

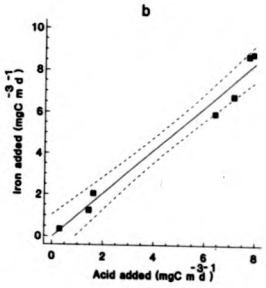
Figure 3.4.6 Regression analysis of the rate of carbon fixation (mg C m^{-3} day^{-1}) in seawater samples "spiked" with 4 nmoles l^{-1} of Fe^{+++} compared with the rate obtained in samples with an equivalent amount of added 0.5 M HCl

The 95% confidence envelopes are shown (.....).

a) 5 μm size fraction. R^2 = 96% (F ratio = 132.3; d.f. = 1,5; $P \leq$ 0.00009.

b)0.2 μm size fraction. R^2 = 98% (F ratio = 199.81; d.f. = 1,5; $P \leq$ 0.00003)


a
Iron added (mgC m-3 d-1)
Acid added (mgC m-3 d-1)
0
4
8
12
16
20
24


b
Iron added (mgC m-3 d-1)
Acid added (mgC m-3 d-1)
0
2
4
6
8
10

in the presence of added iron was explained by the variance in the rate measured when acid alone was added (Figure 3.4.5b). Similarly, for the experiments at 4 nmoles l^{-1} Fe^{+++} R^2 values of 96% and 48% were found for the > 5 μm and > 0.2 μm size fractions respectively (Figure 3.4.6a and b).

These results highlight the difficulty in working with iron at sea. It is possible that even the cleanest commercial grade HCl available contains adequate amounts of trace metal contaminants to affect primary productivity. In addition there are many variables to consider during the experimental procedure, not only effects of contamination from the sampling vessels, the ship, or the form in which Fe^{+++} is to be added to the seawater but also the population of cells which are being sampled. As became apparent after the cruise, sampling traversed from one body of water to another and it is possible the populations of cells present within each water mass had different growth requirements. Such a fluctuation in requirements did not help to verify the presence or absence of iron limiting conditions using a technique which appears to be fraught with so many problems.

3.5 Iron uptake experiments

Given the well known problems of working with iron in seawater, it was decided to include some experiments using ^{55}Fe as a radioactive tracer. There were two aims; to attempt to confirm that the phytoplankton populations were able to

internalize the iron added and also to see whether it would be possible to determine any kinetics of iron uptake.

3.5.1 Method

Samples were collected from a depth of 25 m, 47° 10.1' N, 15° 34.8' W (see Figure 3.1.2) using an ultracleaned acid washed 10 litre GoFlo as described in Section 3.2 (Figure 3.2.1). Samples were aliquoted into 1 litre acid washed polycarbonate bottles as described in Section 3.2. To each 500 ml volume ^{55}Fe tracer was added to give final concentrations of 0.4, 0.8, 1.2, 9.2, 9.6, 10.0 nmoles l^{-1}. In addition a filtered seawater control was included, which was inoculated with 10 nmoles l^{-1} ^{55}Fe. All bottles were incubated as described in Section 3.2. Four 100 ml samples were removed from each bottle over a period of 24 hr at times, 0, 7.5, 17.35 and 24 hr. The seawater was filtered through 1 μm and 0.2 μm pore size filters under vacuum, then washed with titanium III citrate as described in Section 3.3. The filters were dried overnight in a desiccation chamber in the presence of active silica gel. Optiscint (2.5 ml) was added to each vial and the radioactivity counted on the tritium channel of an LKB scintillation counter. The counting efficiency of ^{55}Fe in the scintillation counter was 2.3%.

3.5.2 Results

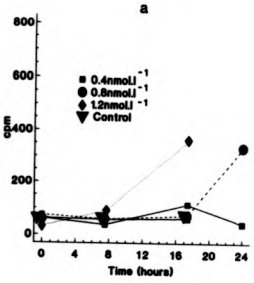
When samples were incubated in the presence of 9.2 and 9.6 nmoles l^{-1} ^{55}Fe for the 1 μm size fraction (Figure 3.5.1) and 0.4, 0.8 and 9.6 nmoles l^{-1} ^{55}Fe for the 0.2 μm fraction

Figure 3.5.1 ^{55}Fe incorporated (cpm) per 100 ml of 1 μm size fractionated seawater samples

a) 500 ml samples were "spiked" with 0.4 nmoles l^{-1}, 0.8 nmoles l^{-1} and 1.2 nmoles l^{-1} ^{55}Fe.

b) 500 ml samples were "spiked" with 9.2 nmoles l^{-1} and 9.6 nmoles l^{-1} ^{55}Fe.

Control samples contained filtered seawater "spiked" with 10 nmoles l^{-1} ^{55}Fe.


a
800
600
400
200
0
cpm
0.4nmol.l⁻¹
0.8nmol.l⁻¹
1.2nmol.l⁻¹
Control
0
4
8
12
16
20
24
Time (hours)

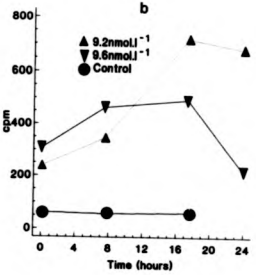

b
800
600
400
200
0
cpm
9.2nmol.l⁻¹
9.6nmol.l⁻¹
Control
0
4
8
12
16
20
24
Time (hours)

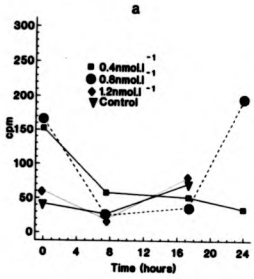
Figure 3.5.2 ^{55}Fe incorporated (cpm) per 100 ml of 0.2 μm size fractionated seawater samples

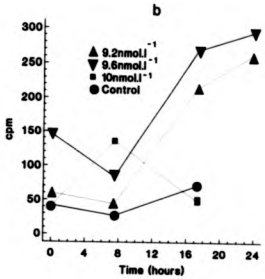
a) 500 ml samples were "spiked" with 0.4 nmoles l^{-1}, 0.8 nmoles l^{-1} and 1.2 nmoles l^{-1} ^{55}Fe.

b) 500 ml samples were "spiked" with 9.2 nmoles l^{-1}, 9.6 nmoles l^{-1} and 10 nmoles l^{-1} ^{55}Fe.

Control samples contained filtered seawater "spiked" with 10 nmoles l^{-1} ^{55}Fe.

$q_1\alpha$


a
0.4nmol.l -1
0.8nmol.l -1
1.2nmol.l -1
Control
cpm
Time (hours)


b
9.2nmol.l -1
9.6nmol.l -1
10nmol.l -1
Control
cpm
Time (hours)

(Figure 3.5.2 a,b), there was a high initial value of ^{55}Fe on the filters which did not wash off with titanium citrate treatment. This suggested rapid incorporation into the phytoplankton. Eng-Wilmont & Martin (1979) observed iron uptake to be biphasic in cultures of *Gomphosphaeria aponina*, and the results were interpreted as an initial adsorption of iron on to the cell surface followed by a slower uptake rate. This may also have happened in these experiments, if the iron was adsorbed to the cell surface in such a way that it could not be removed by titanium III citrate washing. The filtered seawater control showed little to no incorporation, suggesting that the washing procedure was adequate to remove non-internalized iron from the filters. Filters containing 2% [v/v] gluteraldehyde fixed seawater samples also gave low cpm values. However, although there appears to be some uptake of ^{55}Fe by natural phytoplankton assemblages it is not linear and for this reason it was not possible to conduct any kinetic analysis of the data. In addition this technique was limited by very low counting efficiency of ^{55}Fe in the scintillation counter. Most the cpm obtained from the uptake experiments were low (< 100 cpm) and therefore I have little confidence in the significance of these results.

3.6 Conclusions

In conclusion, the above experimental results show that the techniques used routinely in oceanography cannot be applied with any reliability to determine whether natural phytoplankton assemblages are limited by the availability of

iron which is present at low concentrations in the ocean. In accordance with this De Barr *et al.* (1990) were able to show that iron addition stimulated chlorophyll *a* synthesis and nutrient assimilation by natural phytoplankton assemblages in the Southern Ocean. However, the controls, containing ambient iron concentrations, were also found to produce chlorophyll *a* and particulate organic carbon concentrations at higher values than those found in ambient waters. As a result De Barr *et al.* (1990) suggested that Fe is not the single limiting factor controlling phytoplankton in the Weddell/Scotia Seas.

Apart from the problems of contamination with extraneous iron throughout all the experimental procedures, natural populations of phytoplankton appear to be far too dilute in numbers for the sensitivity of existing methods. This is demonstrated by the inability to use ^{55}Fe as a tracer in uptake experiments. Also, because the seawater samples are being incubated within bottles the added problems of bottle effects, such as selection for organisms able to grow under these abnormal conditions and inclusion of grazers within an enclosed area will also result in non-representative results. The experiments done on the 47/90 cruise not only could not demonstrate iron limitation but also were not sensitive enough to determine whether iron limitation actually existed in this area of the ocean. The techniques were further complicated because the experiments conducted each day were not comparable, since it could not be assumed that the natural assemblages present within the seawater samples collected daily were from the same body of water, or that the growth requirements for these organisms remained the same from day to

day. Therefore, it was not possible to repeat the experiments and the only way to achieve a reasonable degree of replication, would have been to do many experiments each day on the same water sample. This would have required impossibly large quantities of water samples, equipment and chemicals.

These experiences confirmed me in the view that alternate methods were required to determine whether natural picoplankton populations are limited by the availability of iron. The rest of the thesis is concerned with the elucidation of the response of cultures of *Synechococcus* sp. (a major component of the marine picoplankton) to iron limitation, with a view to the development of novel methods, in the form of diagnostic markers, for determining the occurrence of iron limitation in natural assemblages. Such an approach would hopefully be able to give both temporal and spatial indications on the presence of iron limitation.

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CHAPTER 4

ISOLATION AND CHARACTERIZATION OF CELL WALLS FROM *SYNECHOCOCCUS* WH 7803

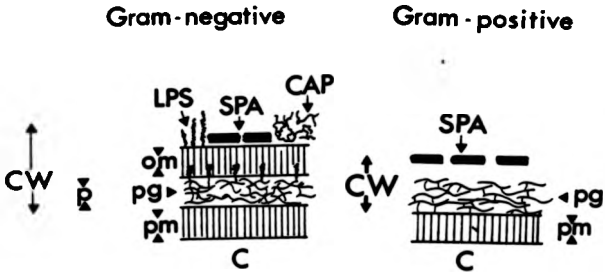
4.1 Introduction

In contrast to animal cells, bacterial cells are surrounded by cell walls which determine and maintain their cell shape. The cell envelope of Gram-negative bacteria is responsible for control of both import and export mechanisms of the cell and also contains receptors for cyanophage adsorption (Samimi & Drews, 1978). The cyanobacterial cell envelope is comprised of three distinct layers: cytoplasmic membrane, peptidoglycan layer and outer membrane. The latter two are the cell wall components (see Jurgens & Weckesser, 1985). In some cases there is also an outer sheath layer.

Cyanobacteria have a complex cell wall which contains structural elements of both Gram-positive and Gram-negative bacteria (Figure 4.1.1). For this reason Jurgens & Weckesser (1985) suggested that a simple classification of cyanobacteria as either Gram-negative or Gram-positive could not be justified. For example, the cell walls of *Synechocystis* PCC 6714 have been shown to contain a thick peptidoglycan layer (10 nm average) which is highly cross linked (56%) and contains polysaccharides covalently bound by phosphodiester bridges to the peptidoglycan. Such a structure is characteristic of cell walls from Gram-positive bacteria. All cyanobacteria studied have been found to contain an outer membrane consisting of lipopolysaccaride, lipids and proteins - characteristic of Gram-negative bacteria. Only a few major proteins dominate the outer membrane and these are of a higher molecular weight than the porins of Gram-negative bacteria (Resch & Gibson, 1983; Scanlan, 1988; this study). The most

Figure 4.1.1 **Schematic diagram of a section through the cell envelope of a typical Gram-negative and a typical Gram-positive bacterium (after Pugsley & Schwartz, 1985)**

C - cytoplasm; PM - plasma membrane; PG - peptidoglycan; P - periplasm; OM - outer membrane; LPS - polysaccharide chains of lipopolysaccharide; SPA - surface protein assay; CAP - capsule; CW - cell wall.



striking feature is the unusual presence of carotenoids in the outer membranes (Omata & Murata, 1984; Jurgens & Weckesser, 1985). Suggestions have been made as to their role in either a structural function or in protection against high light intensities (see Siefermann-Harms, 1987), so far their exact function in the outer membrane is unknown.

Under conditions of iron limitation specific outer membrane proteins have been located in the cell walls of *Anacystis nidulans* R2 (Scanlan, 1988; section 1.4). For this reason a method to isolate cell walls from *Synechococcus* WH 7803 was developed, in order to investigate the possible existence of such iron-regulated outer-membrane proteins in *Synechococcus* WH 7803

4.2 Cell wall isolation procedures

The main approach undertaken to isolate cell walls from *Synechococcus* WH 7803 was via the use of discontinuous sucrose density centrifugation as described by Resch & Gibson (1983). This method has been effective in obtaining cell walls from *Anacystis nidulans* R2 (Resch & Gibson, 1983; Scanlan, 1988). However the procedure had to be modified for *Synechococcus* WH 7803.

4.2.1 The original method (Resch & Gibson, 1983) Method 1

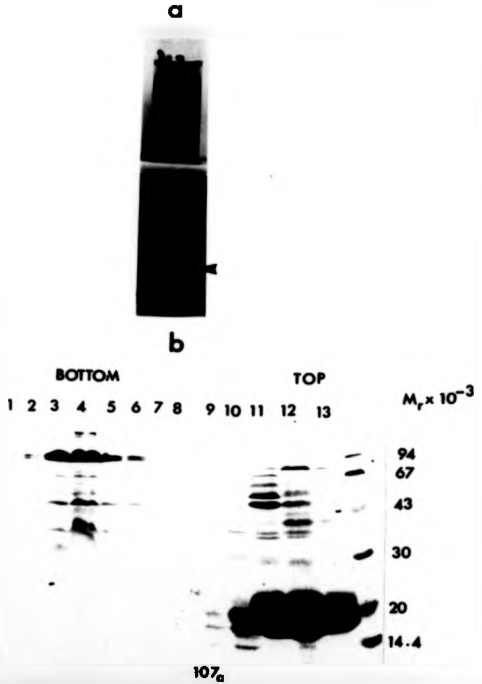
Cells harvested by centrifugation at 8,000 rpm, in a Beckman JA10 rotor, at 25°C for 15 min in a Beckman centrifuge were broken in 10 mM HEPES buffer (Sigma) (pH 7.2) by two passes through a French pressure cell at 20,000 psi. The cell lysate containing 1 mM PMSF was spun for 5 min at 3,000 rpm in a Gallenkamp bench centrifuge, at room temperature, to remove unbroken whole cells. This was then layered onto a 50-85% [w/v] discontinuous sucrose density gradient prepared in 10 mM HEPES pH 7.2. The gradients were centrifuged for 18 hr, 4°C at 25,000 rpm in a Beckman SW27 rotor. The sucrose gradients were constructed as follows, 3 ml 85% [w/v] sucrose, 2 ml 80% [w/v], 2 ml 70% [w/v], 2 ml 60% [w/v] 2 ml 50% [w/v] sucrose in 17 ml polyallomer centrifuge tubes.

4.2.1.1 Results

Sucrose gradients from this preparation (Figure 4.2.1a) did not exhibit a diffuse yellow band in the 72%-80% [w/v] sucrose fraction indicative of cell walls from *Anacystis nidulans* R2 (Resch & Gibson, 1983; Scanlan, 1988). Instead there was a faint rust-red band which was heavily masked by the presence of a dark green pigment. This contamination could possibly have been due to the presence of thylakoid membranes containing chlorophyll a which may be co-migrating with the cell wall fraction. However, the soluble cytosolic proteins appeared to be separated from the membrane proteins

Figure 4.2.1 *Synechococcus* **WH 7803 outer membranes isolated using the method described by Rasch & Gibson (1983), method 1**

a) The possible presence of the cell wall fraction (arrowed) heavily masked due to contamination with inner membranes, isolated from *Synechococcus* WH 7803 by sucrose density centrifugation 50-85% [w/v]. b) Coomassie stained 6-20% [w/v] exponential gradient SDS-PAGE of fractions from the sucrose gradient shown in Fig.a.



and had not entered the gradient. This is indicated by the pink/orange colouration due to phycoerythrin at the top of the gradient. Phycoerythrin is easily released from the phycobilisome and is often present in the cytoplasmic fraction upon cell disruption.

SDS-PAGE analysis (Figure 4.2.1b) and Coomassie staining show a major polypeptide around 94 kDa in size in the putative cell wall fraction (tracks 3 and 4). This fraction corresponded with the faint rust/red band visible just below the contaminating green pigment of chlorophyll *a*. Fractions 11-13, removed from the top of the gradient, show the presence of soluble cytoplasmic proteins, the major band in these fractions is due to phycoerythrin M_r 22,000. Phycoerythrin constitutes upto 25% total cell protein. The gels also contained green pigmentation running ahead of the protein bands due to the presence of chlorophyll *a*. This is not visible here.

Although the method of Resch & Gibson (1983) appears to be able to separate cell walls from the cytoplasm, it is unable to separate them from the thylakoid and possibly the cytoplasmic membranes. Further treatment with Triton-X-100 (Sigma) solubilization, which selectively solubilizes the inner membranes (Resch & Gibson, 1983), was shown to be necessary to achieve a cleaner cell wall banding pattern upon sucrose density centrifugation.

4.2.2 Removal of the inner membranes contaminating the cell wall fraction of the sucrose gradient

The ability to purify the cell wall fractions from contaminating internal membranes was investigated in two ways using Triton-X-100 solubilization. As detergent was being used to solubilize membranes, 10 mM $MgCl_2$ was added to the 10 mM HEPES buffer (pH 7.2) in order to maintain the integrity of the cell walls (Woitzik *et al.*, 1988).

1 ml aliquots of putative cell wall fractions 1-4 (Figure 4.2.1a) were made up to 8 ml with 10 mM HEPES buffer (pH 7.2), 10 mM $MgCl_2$, 1% [v/v] Triton-X-100. The suspension was thoroughly mixed and centrifuged in a MSE centrifuge for 90 min at 4°C, 45,000 rpm in a 10 x 10 fixed angle rotor.

The Triton-insoluble pellets obtained were rust/red in colour whereas the supernatants varied from clear to a deep olive-green depending on the degree of contamination by the inner membranes. The largest Triton-insoluble pellet was obtained from fraction 4 (track 4, Figure 4.2.1b), this fraction also contained the deepest pigmented supernatant. Although this procedure was able to separate the putative cell wall fractions from the internal membranes, extreme difficulty was encountered in recovering and maintaining the insoluble cell pellet in a solution of 10 mM HEPES-Mg^{++} (pH 7.2).

4.2.2.1 Method 2

An alternative method was utilized in order to overcome the problem with the insoluble nature of the cell wall pellet. The broken cell lysate was treated with 2% [v/v] Triton-X-100 in 10 mM HEPES-Mg^{++} buffer (pH 7.2) prior to sucrose gradient centrifugation. As described previously the Triton-insoluble putative cell wall fraction was collected by centrifugation in a MSE centrifuge at 45,000 rpm, 4°C for 90 min in a 10 X 10 fixed angle rotor. The method was modified by centrifuging the sample over an 85% [w/v] sucrose cushion to prevent pelleting of the cell wall fraction. The 85% [w/v] sucrose cushion plus the brown-orange band at the interface were diluted 3 fold in 10 mM HEPES-Mg^{++} pH7.2 prior to sucrose density centrifugation. The Triton-soluble fraction containing the inner membranes was also loaded onto a sucrose gradient. Samples were centrifuged as described in section 4.2.1.

4.2.2.2 Results

A diffuse rust-red band could be observed in the 70-80% [w/v] sucrose fraction (Figure 4.2.2a, gradient B) of the gradient containing the Triton-insoluble cell wall sample. The solubilization appeared to prevent the entry of contaminating inner membranes into the gradient (Figure 4.2.2a, gradient A). The initial centrifugation step over an 85% [w/v] sucrose cushion was not able to remove contamination by phycobiliproteins, mainly PE. However, these were

Figure 4.2.2 a ***Synechococcus*** **WH 7803 outer membranes isolated using method 2**

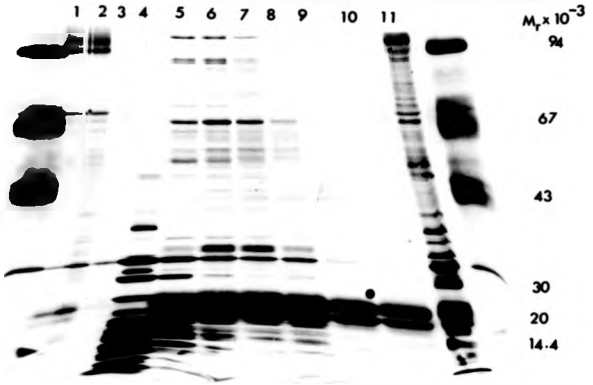
A comparison of sucrose density centrifugation 50-85% [w/v] of both the Triton-soluble (gradient A) and Triton-insoluble fractions of the (gradient B) cell lysate, after treatment with 2% [v/v] Triton-X-100. The presence of the cell wall fraction in the Triton-insoluble sample (arrowed), gradient B and loss of contaminating Triton-soluble inner membranes are shown, gradient A.

separated from the cell wall fraction upon sucrose density centrifugation (Figure 4.2.2a, gradient B). There was no corresponding cell wall band on gradient A containing the Triton-soluble cell lysate.

Sucrose gradient samples were fractionated by puncturing the side of the polyallomer tube with a syringe just below the putative cell wall band. The samples, allowed to flow through the syringe under positive pressure, were collected in 1 ml aliquots. Small sample volumes were used such that the cell wall fractions collected were not contaminated with other proteins present in the gradient.
SDS-PAGE analysis of the fractions and silver staining (Figure 4.2.2b) shows a distinct protein pattern for the putative cell wall fraction (tracks 1 and 2) with major polypeptides of M_r 94,000 and 67,000. These polypeptides are also apparent in Figure 4.2.1b. The cell wall fractions also appear to be very clean with only slight PE contamination. At the top of the gradient, fraction 11, track 10, the phycobiliproteins are the major proteins present. The appearance of phycoerythrin bands in the middle of the gradient (M_r 22,000, tracks 5-8) may have arisen from contamination due to a syphoning effect which sucks the protein from the top of the gradient down through the sucrose. Track 11 contains the Triton-insoluble fraction of the cell lysate (putative cell wall fraction) recovered from the sucrose cushion prior to sucrose density centrifugation. This sample highlights the effect of sucrose density centrifugation on further removing non-cell wall proteins from the cell wall sample.

Figure 4.2.2 b Polypeptide profile of the 50-85% [w/v] sucrose density gradient B

Silver stained 6-20% [w/v] exponential gradient SDS-PAGE of fractions from sucrose gradient B shown in Fig. a.



Although this method is able to give a clean putative cell wall preparation, the yield is poor. A very small amount of protein in the cell wall fraction (69 $\mu g\ ml^{-1}$) was obtained, 137.5 μg total protein from 0.3 g wet weight of cells. Therefore a lot of cell wall material was being lost through this procedure. A more efficient cell wall preparation had to be achieved in order to isolate cell wall proteins which may possibly be expressed at low levels under iron limitation for antibody production. Non-fixed Coomassie preparative gels have a detection limit of 1-2 μg protein per track.

4.2.3 Techniques to increase cell wall yield from sucrose density centrifugation

As the cell wall yield from the Triton-insoluble fraction of the cell lysate was low, the presence of cell walls in the unbroken cell pellet sample was investigated. Unbroken cells were removed from the cell lysate sample by centrifugation at 3,000 rpm in a Gallenkamp bench centrifuge, for 5 min at room temperature. During this process larger cell wall fragments may also have been pelleted.

In order to investigate this, the whole cell pellet was resuspended in 2% [v/v] Triton-X-100 in 10 mM HEPES-Mg^{++}, pH 7.2 and loaded onto a 50%-85% [w/v] sucrose gradient. Samples were centrifuged as described in section 4.2.1. The gradient after centrifugation showed the presence of a major rust-red band at the 80%-70% [w/v] sucrose interface, with a minor pink band at the 80%-85% interface (Figure 4.2.3, gradient C).

Figure 4.2.3 Comparison of *Synechococcus* WH 7803 outer membranes isolated from both the cell lysate fraction and the whole cell pellet fraction using method 2

Gradient B contains cell walls in the 80-70% [w/v] sucrose fraction (arrowed), isolated from the cell lysate after 2% [v/v] Triton-X-100 extraction and sucrose density centrifugation. Gradient C contains a major cell wall band in the 80-70% [w/v] sucrose fraction (arrowed) and a minor band just below at the 80-85% [w/v] sucrose interface, isolated from the whole cell pellet fraction after 2% [v/v] Triton-X-100 extraction and sucrose density centrifugation.

cell Pellet

Comparison of gradient C with gradient B (Figure 4.2.3) shows the major cell wall band to be the same for both gradients, however, the band from gradient C appeared to be a darker orange in colour. This difference in colour could be due to a greater amount of PE contamination or a higher cell wall density. The total protein in the cell wall fraction of gradient C was 199 μg, with a protein concentration similar to that for gradient B at 66 $\mu g\ ml^{-1}$.

The appearance of a putative cell wall fraction from the Triton-treated cell pellet suggested that a large proportion of the cell wall sample was being lost in this fraction during the clearing spin to remove unbroken cells.

4.2.3.1 Method 3

As a putative cell wall band was identified in the whole cell lysate fraction as well as the unbroken cell pellet fraction centrifugation to remove the unbroken cells was omitted (section 4.2.1). Alternatively, the cell lysate containing unbroken cells was incubated in 2% [v/v] Triton-X-100, 10 mM HEPES-Mg^{++} for 30 min at room temperature, then loaded on to a 50%-85% [w/v] sucrose gradient and centrifuged as described in section 4.2.1.

4.2.3.2 Results

Centrifugation resulted in a diffuse orange-yellow band present at the 80%-70% [w/v] sucrose interface (Figure 4.2.4a). The appearance of the cell wall fraction is similar

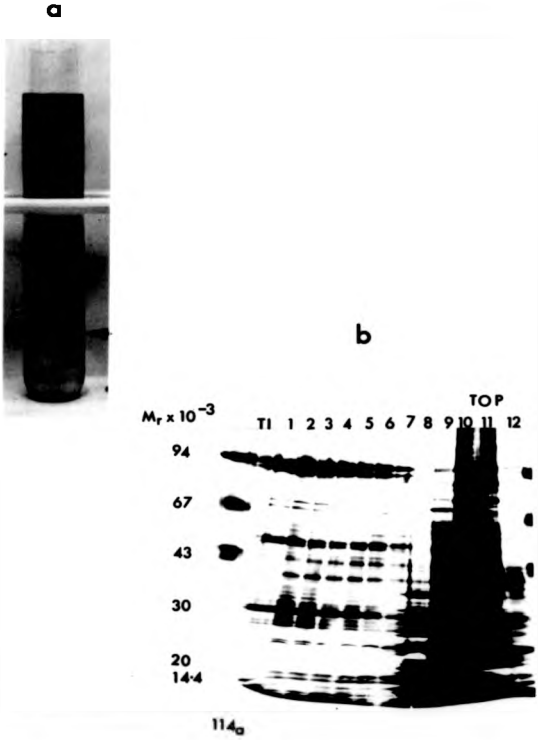
to that described by Scanlan (1988) for *Anacystis nidulans* R2, except that the cell wall was yellow rather than orange. A large batch culture (6 litres) yielding 0.5 g wet weight of cells was required to produce a cell wall protein concentration of 186 $\mu g\ ml^{-1}$ (1.3 mg total protein). The putative cell wall protein yield was approximately three times higher than that obtained by method 2 (section 4.2.2.1). SDS-PAGE analysis of the gradient fractions (Figure 4.2.4b) after silver staining showed the cell wall sample to extend between fractions 1-7. This was determined by the presence of the major polypeptide doublets M_r 94,000 and 67,000. Two other major doublets not previously identified were also apparent at approx M_r 30,000 and 53,000. The reduction in PE contamination, determined by the absence of polypeptide bands at M_r 22,000, within the cell wall fractions could be attributed to the fact that iron-depleted cells were used in this preparation (Chapter 5). At the top of the gradient (fractions 8-12) the different polypeptide pattern is due to the presence of large amounts of Triton-soluble cytoplasmic and inner membrane proteins. The track labelled TI is a control track containing Triton-insoluble cell walls isolated using method 2.

The above results show that method 3 produces the greatest yield of cell wall protein compared to the other methods studied. However, large volumes of cells were still required in order to achieve this.

Unfortunately the results obtained using method 3 were not reproducible, despite many attempts. One of the major

Figure 4.2.4 ***Synechococcus*** **WH 7803 outer membranes isolated using method 3**

a) Cell wall fraction (arrowed) from *Synechococcus* WH 7803 isolated by Triton-X-100 extraction and sucrose density centrifugation 50-85% [w/v]. b) Silver stained 6-20% [w/v] exponential gradient SDS-PAGE of fractions from the sucrose gradient shown in Fig.a.



problems encountered was poor cell breakage which resulted in both a reduced cell wall yield and contamination of the putative cell wall fraction with partially or unbroken whole cells. As a result different attempts of cell breakage were used in order to find a method capable of producing a reproducibly high level of cell breakage.

4.2.3.3 Methods of cell breakage

It was imperative to find a method which gave maximal cell breakage, such that future cell wall isolation from low biomass yield iron limited cells would not be impeded. Two alternative cell breakage methods were investigated, these were:- a) flash freezing. b) Braun homogenization.

Unfortunately flash freezing did not cause adequate cell breakage. Three cycles of freezing in liquid nitrogen and thawing at 55°C was incapable of lysing more than 1% of the total cells visualized under microscopic examination.

Braun homogenization proved more hopeful when upto 0.2 g wet weight of cells per tube were homogenised using 0.1-0.11 mm glass beads over a 15 min period with constant cooling. Microscopic examination after this time period showed that a large proportion of the cells had been disrupted. Any unbroken cells were usually re-homogenised in order to obtain maximum cell disruption.

4.2.4 Cell wall isolation by differential centrifugation (Woitzik *et al.*, 1988)

4.2.4.1 Method 4

Synechococcus WH 7803 cells were broken by Braun homogenization in 20 mM Tris-HCl pH 8.0 (section 4.2.3.3). The homogenate was washed from the beads and centrifuged at 3,000 rpm for 40 min in a Gallenkamp bench centrifuge to remove unbroken cell debris. The supernatant was re-centrifuged initially at 3,000 rpm in a Gallenkamp bench centrifuge, for 5 min, to remove any contaminating whole cells, then at 18,000 rpm, for 30 min in a MSE HiSpin centrifuge using an 8x10 fixed angle rotor. The deep purple supernatant contained the cytoplasmic fraction, the purple colouration was due to the phycobilisomes. The supernatant was decanted and the pellet containing the cell envelopes was washed twice in 20 mM Tris-HCl pH 8.0, by centrifugation at 18,000 rpm in an 8x10 fixed angle rotor in a MSE HiSpin centrifuge for 30 min. The cell envelopes were resuspended initially in the residual volume of 20 mM Tris-HCl (pH 8.0) buffer then loaded (5 ml volume) onto a discontinuous sucrose gradient [4 ml 55, 50, 40% [w/v] sucrose and 2 ml 30% [w/v] sucrose in 20 mM Tris-HCl buffer pH 8.0]. The tubes were centrifuged at 16,900 rpm for 12 hr at 4°C using a swing out bucket Beckman SW28 rotor, in a Beckman L8 ultracentrifuge. Cell walls were isolated from the 55% [w/v] sucrose band by resuspension in 20 mM Tris-HCl (pH 8.0) buffer followed by centrifugation in a MSE centrifuge at 33,000 rpm in a 10x10

rotor for 30 min at 4°C. The cell wall pellet was purified further by discontinuous sucrose density centrifugation, and the putative cell wall fraction recovered from the 55% [w/v] sucrose fraction as described previously. The putative cell wall pellet was resuspended in 5 ml 20 mM Tris-HCl (pH 8.0) buffer containing 10 mM $MgCl_2$. An equal volume of 4% [v/v] Triton-X-100 in 20 mM Tris-HCl (pH 8.0) buffer was added and the Triton-soluble components extracted from the cell walls by stirring for 20 min at 23°C. Triton-insoluble cell walls were isolated by centrifugation at 33,000 rpm for 60 min in a 10x10 rotor in a MSE centrifuge. The resulting supernatant was olive green in colour, similar to the Triton-soluble supernatant described in section 4.2.2 and the cell wall pellet was a similar rust-red colour. The putative cell wall pellet was washed in extraction buffer [20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$] excluding Triton-X-100 and resuspended in 500 µl 20 mM Tris-HCl buffer pH 8.0.

4.2.4.2 Results

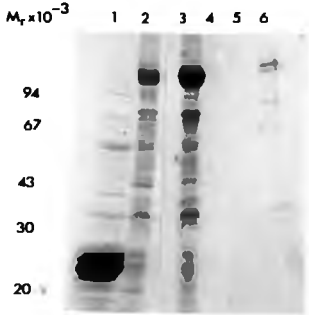
A rust-red cell wall pellet was obtained by this alternate method similar to that described in section 4.2.2. This suggests that the 70%-80% [w/v] sucrose band obtained previously were also cell walls. Cell wall colour can vary between orange, red or yellow once devoid of thylakoid and cytoplasmic membranes (Weckesser & Jurgens, 1988). SDS-PAGE analysis and Coomassie staining (Figure 4.2.5) of samples taken throughout the isolation procedure showed the separation of the cytoplasmic and membrane fractions by

centrifugation after cell disruption (track 1 and 2). However, a lot of the cell wall protein present in the cell envelope fraction (track 2) was lost during discontinuous sucrose density centrifugation. This was indicated by the decrease in the major polypeptide bands in the 55% [w/v] sucrose gradient (track 4). The majority of the cell wall material had been pelleted, this is highlighted by the sample in track 3. Track 3 contains protein taken from the pellet obtained after the initial sucrose density purification step, and subsequently treated in exactly the same way as the putative cell wall fraction in track 5.
Comparison of cell wall fractions obtained by this method (track 5 and 3) compared with method 3 (track 6) indicates that method 3 is able to give a greater cell wall yield (track 6 vs track 5) and that the cell wall fraction contains less contaminating protein (track 6 vs track 3).

Apart from the fact that the cell breakage technique and the buffers employed in method 4 were more favourable, the more efficient cell wall isolation procedure of method 3 was preferred. However, a compromise was eventually decided upon which incorporated aspects of both methods 3 and 4. This method (see Materials and Methods) was eventually used to isolate internal membranes and cell walls from iron-depleted cultures of *Synechococcus* WH 7803 (Chapter 6).

Figure 4.2.5 ***Synechococcus*** **WH 7803 outer membranes isolated by differential centrifugation and detergent extraction (method 4)**

6-20% [w/v] exponential gradient SDS-PAGE after silver staining. Tracks: 1) Cytoplasmic fraction. 2) Cell envelope fraction prior to discontinuous sucrose density centrifugation 55-30% [w/v] sucrose. 3) Cleaned cell wall fraction obtained from the pellet after the initial discontinuous sucrose density centrifugation, and treated as described for the putative cell wall fraction recovered from the 55% [w/v] sucrose band. 4) Cell wall sample from the 55% [w/v] sucrose band after the initial sucrose density centrifugation procedure. 5) Putative cell wall fraction obtained using method 4. 6) Putative cell wall fraction obtained using method 3.



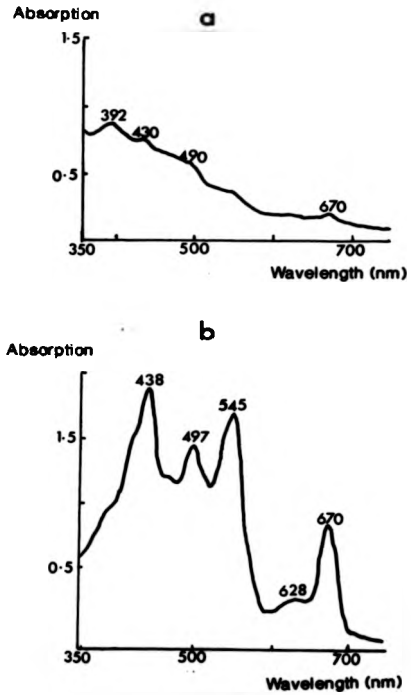
4.3 Cell wall characterization

4.3.1 Use of scanning spectroscopy to identify the cell wall fraction

The absorption spectrum of the isolated cell wall fraction (Figure 4.3.1a) showed absorption maxima at 392 nm, 430 nm, 490 nm, which is indicative of carotenoids. However, there was little or no contamination with chlorophyll *a* (670 nm) or phycobiliproteins (545 nm, 620 nm, 650 nm) in these cell wall preparations. The presence of carotenoids in the cell walls of cyanobacteria is now accepted (Jurgens & Weckesser, 1985). Extraction of the inner membrane fractions with Triton-X-100 failed to remove the absorption maxima indicative of carotenoids, suggesting that carotenoids were a true component of the cell wall and not due to contamination by the cytoplasmic membrane. The absorption spectrum of fractions taken from the top of the sucrose gradient (Figure 4.3.1b) showed absorption maxima at 438 nm, 497 nm possibly due to cytochromes and carotenoids associated with the thylakoid and cytoplasmic membranes, and also at 545 nm, 628 nm - due to phycobiliproteins and 670 nm - due to chlorophyll *a*, both of which are associated with the thylakoid membrane. Carotenoids extracted from the cell wall can also be identified by their R_f values by thin-layer chromatography (Weckesser & Jurgens, 1988).

Figure 4.3.1 Absorption spectrum of fractions from the sucrose gradient 50-85% [w/v]

a) Isolated *Synechococcus* WH 7803 cell wall fraction banding at the 80-70% [w/v] sucrose interface. b) Fraction from the top of the gradient, above the 50% [w/v] sucrose layer.



4.3.2 Use of antibodies to characterize cell wall proteins.

Antibodies raised against cell walls isolated from *Anacystis nidulans* R2 (Scanlan, 1988) cross reacted with cell walls isolated from six other cyanobacterial species, one of which was the marine *Synechococcus* WH 7803, and one prochlorophyte. Cross reactivity was observed when a 1:500 antibody dilution was used (Figure 4.3.2a). The major polypeptides M_r 94,000 and 67,000 from *Synechococcus* WH 7803 were identified in the 70-80% [w/v] sucrose fraction containing cell walls isolated from both the whole cell lysate and the whole cell pellet samples (track a and b). However, a greater loading of cell wall material (track c,d) present in the 70%-80% [w/v] sucrose fraction from the Triton-treated samples described in method 2, (section 4.2.2) would be required in order to obtain a detectable antibody response. The unique polypeptide patterns of each of the respective cyanobacterial cell walls isolated are shown in Figure 4.3.2b. Although the proteins which are present within the cell walls of these species are different in terms of size, western blot analysis has shown that they are antigenically related. This antigenecity extends across ecological barriers, shown by a cross reactivity with marine *Synechococcus* WH 7803 (Figure 4.3.2a track a,b), and wide taxonomic distances, shown by a cross reactivity with the prochlorophyte *Prochlorothrix hollandica* (Figure 4.3.2a track j). In fact, a cell wall fine structure, lipopolysaccharide and peptidoglycan-polysaccharide complex similar to that described for cyanobacteria has been

Figure 4.3.2 a Characterization of cell wall proteins using western blot analysis

Tracks: a-d) *Synechococcus* WH 7803. e) *Synechocystis* PCC 6803. f) *Synechocystis* PCC 6714. g) *Anacystis nidulans* R2 (*Synechococcus* PCC 7942). h) *Nostoc sp. Mac.* i) *Synechococcus* PCC 7002. j) *Prochlorothrix hollandica.* k) *Synechocystis* PCC 6308.
Western blot analysis using *Anacystis nidulans* R2 cell wall antibody. Cross reactivity was observed for all strains studied, using a 1:500 dilution of the primary antibody and a 1:300 dilution of the secondary antibody (donkey anti-rabbit horseradish peroxidase linked IgG).

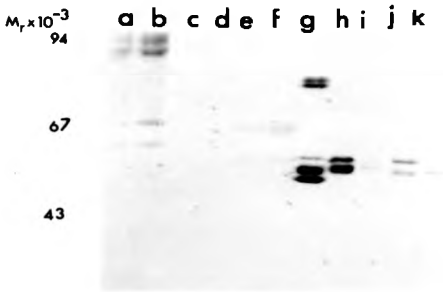
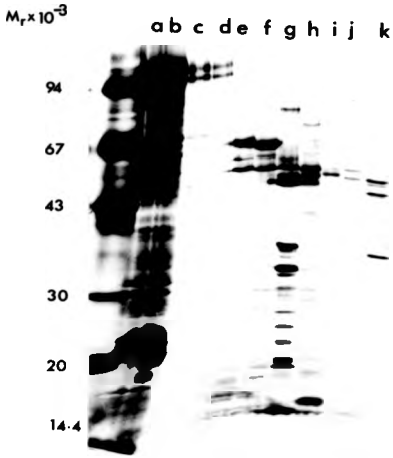


Figure 4.3.2 b **6-20% [w/v] exponential gradient SDS-PAGE profile of cell walls isolated from seven species of cyanobacteria and one prochlorophyte**

Tracks : a-d) *Synechococcus* WH 7803. e) *Synechocystis* PCC 6803. f) *Synechocystis* PCC 6714. g) *Anacystis nidulans* R2 (*Synechococcus* PCC 7942). h) *Nostoc sp. Mac.* i) *Synechococcus* PCC 7002. j) *Prochlorothrix hollandica*. k) *Synechocystis* PCC 6308.

The silver stained gel shows the unique polypeptide pattern of the cell walls from each of these species. Western blot analysis of these cell wall fractions is shown in Fig. 4.3.2a.



identified in *Prochlorothrix hollandica* (Burger-Wiersma *et al.*, 1986; Jurgens & Burger-Wiersma, 1988; Jurgens, 1989).

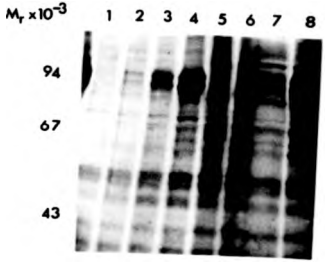
4.3.3 Effect of solubilization temperatures on the polypeptide pattern of cell walls isolated from *Synechococcus* WH 7803

On altering the temperatures used to solubilize cell wall proteins for SDS-PAGE analysis, changes in polypeptide patterns were observed for cell walls isolated from cells grown in the absence of added Fe^{+++} (tracks 5-8, Figure 4.3.3) and in the absence of added Fe^{+++} and EDTA, (chelator-deficient medium) (tracks 1-4, Figure 4.3.3). The major M_r 94,000 doublet polypeptide of the cell wall became visible mainly at solubilization temperatures of 65°C or over (tracks 3,4,7,8). The M_r 72,000 polypeptide was visible at temperatures as low as 25°C (tracks 1-8), a similar mobility was also observed for a polypeptide M_r > 94,000 present in cell walls isolated from cells grown in medium without any added Fe^{+++} (tracks 5-8). The mobility of any other cell wall proteins was not noticeably affected by solubilization temperature.

Heat modifiable proteins have also been identified in the cell wall of *Anacystis nidulans* R2 (Scanlan, 1988). The fact that these major cell wall proteins are heat modifiable corresponds with the observations made by Nakamura & Mitzushima (1976), where proteins from *E. coli* which act as porins were also heat modifiable. The alteration in polypeptide mobility with

Figure 4.3.3 **Effect of solubilization temperature on the electrophoretic mobility of *Synechococcus* WH 7803 outer membrane proteins.**

Silver stained 6-20% [w/v] exponential gradient SDS-PAGE. Tracks 1-4 *Synechococcus* WH 7803 cell wall fraction (chelator-deficient medium) solubilized at: track 1 - 25°C, track 2 - 50°C, track 3 - 65°C, track 4 - 100°C. Tracks 5-8 *Synechococcus* WH 7803 cell wall fraction (ASW medium containing no added Fe^{+++}) solubilized at: track 5 - 25°C, track 6 - 50°C, track 7 - 65°C, track 8 - 100°C.



solubilization temperature was attributed to a conformational change in the protein β - structure to a more mobile α helix. Furthermore, Omata & Murata (1984) described major outer membrane proteins from *Synechocystis* PCC 6714 which could only be solubilized at high temperatures. Weckesser & Jurgens (1988) found that peptidoglycan-associated outer membrane proteins were removed when solubilized in SDS above 70°C whereas extraction of minor proteins occurred at 37°C. Although porins have been found to be heat modifiable proteins it cannot be said that the reverse is necessarily true. Permeability measurements upon outer membrane proteins of *Anabaena variabilis* have shown porin activity, however no data was provided on whether these proteins were heat modifiable (Benz & Bohme, 1985). Permeability measurements with the proteins of interest reconstituted into black lipid bilayer membranes, or the liposome swelling test (Luckey & Nikaido, 1980) are required before any porin function may be ascribed to these heat modifiable outer membrane proteins of *Synechococcus* WH 7803.

Alteration of the cell wall buoyant density by lysozyme digestion of the peptidoglycan proved inconclusive. The incomplete digestion of the peptidoglycan was probably due to its structural features rather than inaccessibility of the enzyme (Resch & Gibson, 1983).

Furthermore, the presence of cell walls in the 70%-80% [w/v] sucrose fraction can be confirmed by a series of chemical analysis. For example, lipopolysaccharide (LPS) determination and identification of the rare sugar 2-keto-3

deoxy octonate (KDO), known to be a component of the LPS found in the outer-membranes of Gram-negative bacteria and many cyanobacteria (Resch & Gibson, 1983; Weckesser & Jurgens, 1988). Chemical analysis of the peptidoglycan and amino acids, such as *meso* diaminopimelic acid from the peptidoglycan, coupled with electron microscopy showing the c-shaped fragments indicative of cell walls, would substantiate the data already obtained on the cell walls isolated from *Synechococcus* WH 7803.

4.4 Conclusions

This is the first documentation of cell wall isolation from a marine *Synechococcus* sp.
The cell walls isolated from *Synechococcus* WH 7803 have been shown to contain carotenoids as a result of scanning spectrophotometric analysis, as well as being antigenetically similar to cell walls isolated from other strains of cyanobacteria, although the respective polypeptide patterns are different. The major cell wall proteins of *Synechococcus* WH 7803 (M_r 94,000 and 67,000) are of a considerably higher molecular weight than those comprising the major cell wall polypeptides in species of the Enterobacteriaceae (M_r 37,000-41,000), and cyanobacterial species (ranging from M_r 52,000-78,000). The 94,000 Da polypeptide is heat modifiable, being solubilised mainly at temperatures of 65°C and over, (The same may be true for the 67,000 Da polypeptide, however this is not so obvious), suggesting that it may be peptidoglycan associated (Weckesser & Jurgens, 1988). It is impossible to

suggest that it may function as a porin without the results of permeability measurements.

Further analysis of the cell wall needs to be conducted to see whether they contain similar structural characteristics as those described for other cyanobacteria (Jurgens & Weckesser, 1985; Woitzik *et al.*, 1988; Jurgens *et al.*, 1989).

A high cell wall yield from *Synechococcus* WH 7803 was impeded due to difficulties in cell breakage, probably due to the presence of a highly structured outer envelope observed using electron microscopy (Kursar *et al.*, 1981). The most favourable breakage procedure in order to obtain a high cell wall yield was Braun homogenization. Recovery of the cell wall fraction was still from the 70%-80% [w/v] sucrose interface after centrifugation, showing that homogenization had no effect on the buoyant density of the cell wall sample. The peptidoglycan associated with the outer membrane allows its separation from the inner membranes as a result of a higher buoyant density. Further separation from the inner membranes was also achieved using Triton-X-100 extraction prior to sucrose gradient centrifugation or differential centrifugation.

CHAPTER 5

BIOCHEMICAL ANALYSIS OF THE RESPONSE OF *SYNECHOCOCCUS* WH 7803 TO IRON LIMITATION

5.1 Introduction

Iron limitation manifests itself in a number of ways in cyanobacteria (see Section 1.4). This chapter details the biochemical approach undertaken with iron-replete/deplete cultures of *Synechococcus* WH 7803, to investigate the response(s) to iron limitation. The parameters studied include the effect of reduced iron in the culture medium on the amount of PE per cell, PE mRNA and the induction of putative iron transport systems. Phycoerythrin and phycoerythrin mRNA have been investigated by spectrophotometric analysis, difference spectrum determination and northern hybridization studies. As described in Section 1.4 iron depletion in some cyanobacteria results in the production of siderophores and/or specific iron-regulated high affinity uptake systems. The presence of such a system has been investigated in *Synechococcus* WH 7803.

Extension of the Redfield ratio (Table 1.2) shows that marine plankton have been found to contain one hundred times the concentration of iron available to them in the oceans. They appear to exhibit the ability to concentrate and possibly store large amounts of iron from the surrounding environment. In order for such concentration to be of benefit to the cell the internalized iron must be stored in a soluble and potentially usable form. Bacterioferritin, so called because of its resemblance to ferritin, was isolated from *E. coli* by Yariv *et al.* (1981) and has since been shown to be identical to cytochrome b_1 (Smith *et al.*, 1988). The protein acts as a storage molecule for Fe^{+++} held as high molecular weight inorganic complexes encapsulated within the protein shell and

may contain from 0 to 3,000 iron atoms per molecule (Mann *et al.*, 1987). Similar proteins have also been identified in *Azotobacter vinelandii* (Stiefel & Watt, 1979), *Rhodospirillum rubrum* (Bartsch *et al.*, 1971), *Pseudomonas aeruginosa* (Mann *et al.*, 1986) and *Synechococcus* PCC 6803 (Laulhere *et al.*, 1992). The presence of such a protein in marine *Synechococcus* WH 7803 was investigated in the course of this study.

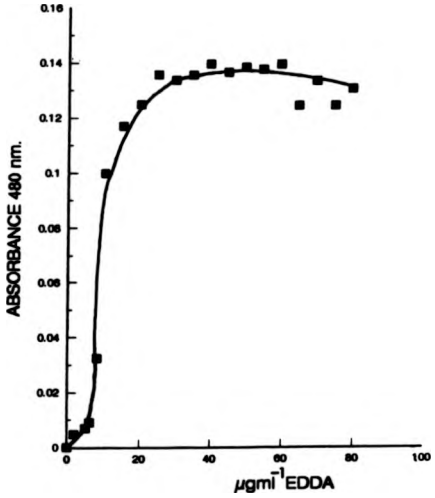
5.2 Phycoerythrin in iron-replete/deplete *Synechococcus* WH 7803 cultures

5.2.1 Simulation of iron limiting conditions without limiting culture biomass

Although iron limited cells could be obtained from chelator-deficient medium (ASW medium containing no Fe^{+++} and no EDTA), it proved very difficult to obtain large quantities of cells for experimental purposes. Therefore, iron limitation was simulated by the use of a synthetic iron chelator, ethylene-diamine di-(o-hydroxyphenyl acetic acid) (EDDA), and as a result large quantities of cell biomass grown under iron deplete conditions were obtained (see below). The dissociation constant of EDDA for Fe^{+++} has been determined to be 10^{-33} M at pH 8.0 which is eighteen orders of magnitude greater than that for EDTA (10^{-15}). The use of EDDA as a means of restricting freely available iron has been documented for cyanobacteria (Kerry *et al.*, 1988; Scanlan, 1988). From the EDDA titration curve (Figure 5.2.1) it is clear that there is near maximal chelation of all available iron in

Figure 5.2.1 EDDA titration curve

The chelation of iron in normal medium by the synthetic chelator, EDDA, is shown by an increased extinction (red colouration) of the solution at 480 nm. Near maximal absorbance occurs at a concentration of 15 $\mu g\ ml^{-1}$ EDDA (see Materials and Methods).



normal ASW medium, at an EDDA concentration of 15 $\mu g\ ml^{-1}$. Figure 5.2.2 shows that *Synechococcus* WH 7803 is able to grow in ASW medium which contains up to 15 $\mu g\ ml^{-1}$ EDDA. Above this value EDDA appears to have an inhibitory effect upon cell growth. EDDA therefore represents a valuable tool for restricting freely available iron within the growth medium. Although the cell is unable to obtain iron directly from the medium, the presence of a high affinity uptake system (Section 1.4) would enable the cells to scavenge the iron from the EDDA. As a result, the cells grow at a generation time similar to that of cells in iron-replete culture, but will also express a high affinity uptake system in order to maintain their growth. This allows for a normal growth generation time and greater cell biomass yield, with respect to the chelator-deficient culture which is unable to grow to $OD_{750\ nm}$ much above 0.1. The growth curves of cells grown in normal medium, chelator-deficient medium and normal medium containing 15 $\mu g\ ml^{-1}$ EDDA are shown in Figure 5.2.3. The growth curve of *Synechococcus* WH 7803 in the presence of EDDA is similar to that of cells grown in normal medium, excepting the occurrence of a 50 hr lag phase. This lag phase is possibly attributed to the response period of *Synechococcus* WH 7803 to reduced iron levels in the medium; this will be discussed later. In comparison, chelator-deficient cells grew normally for the first 50 hr, entering stationary phase after this period, but were unable to display a growth phase similar to that of cells grown in either normal medium or medium containing EDDA. The lack of growth of cells in chelator-

Figure 5.2.2 **Growth of *Synechococcus* WH 7803 in normal medium containing increasing amounts of EDDA**

Flasks:- a) 0 $\mu g\ ml^{-1}$ EDDA. b) 5 $\mu g\ ml^{-1}$ EDDA. c) 10 $\mu g\ ml^{-1}$ EDDA. d) 15 $\mu g\ ml^{-1}$ EDDA. e) 20 $\mu g\ ml^{-1}$ EDDA.
Growth of *Synechococcus* WH 7803 occurs in normal medium containing upto 15 $\mu g\ ml^{-1}$ EDDA.

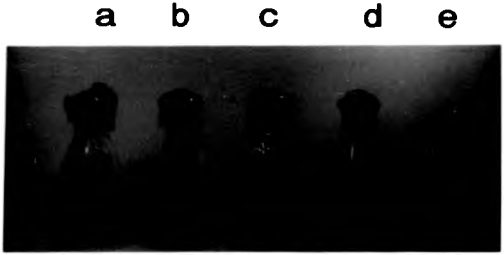
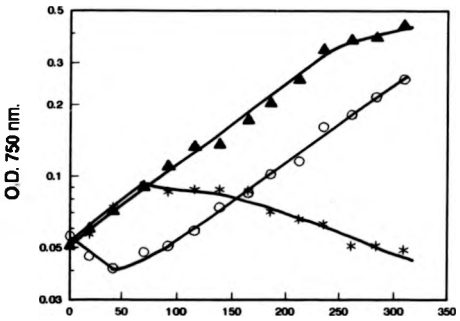


Figure 5.2.3

Growth curves of Synechococcus WH 7803 under different nutrient conditions



a. Normal medium ▲

b chelator-deficient medium *

c. Normal medium + 15 $\mu g\ ml^{-1}$ EDDA ○

deficient media is probably due to the absence of added iron in the growth medium.

5.2.2 Phycoerythrin concentration in iron-replete and -deplete cells

Cultures of *Synechococcus* WH 7803 were grown in ASW medium containing different concentrations of added iron (as $FeCl_3.6H_2O$): 0.11 μM, 1.1 μM, 11 μM (normal), normal ASW plus 15 μg ml^{-1} EDDA or chelator-deficient ASW (no Fe^{+++}, no EDTA). Cells were analysed for their phycoerythrin (PE) content as described in Materials and Methods, the values obtained were normalized to $OD_{750\ nm}$ 0.1 and are presented in fg $cell^{-1}$ in Table 5.2.1. As can be seen from Table 5.2.1 a decrease in iron in the growth medium resulted in a decrease in phycoerythrin per cell. This is to be expected since iron is required for phycobilisome and chlorophyll synthesis (Hardie *et al.*, 1983). Furthermore, iron deficiency in *Anacystis nidulans* and *Agmenellum quadruplicatum* results in a breakdown of phycobiliproteins (Hardie *et al.*, 1983; Guikema & Sherman, 1983; Pakrasi *et al.*, 1985_a). Nutrient starvation is known to affect phycobilisome synthesis (Allen & Smith, 1969; Bouissiba & Richmond, 1980; Wyman *et al.*, 1985; Mazel & Marliere, 1989; Grossman, 1990). Nitrogen, phosphorus and iron limitations in *Synechococcus* WH 7803 manifest themselves initially by a decrease in the pink colouration of the cells as a result of phycoerythrin degradation and decreased biosynthesis. Although the final result is that the PE levels per cell are reduced, the factors leading to the reduced biosynthesis

Table 5.2.1 Phycoerythrin concentrations in iron-replete and iron-deplete cells

GROWTH CONDITIONS	PE fg cell^{-1}				MEAN	SE
	1	2	3	4	x	
NO EDTA NO Fe^{+++}	2.29	3.08	2.88	-	2.75	0.19
0.11 μM Fe^{+++}	-	-	-	5.3	N.D.	N.D.
1.1 μM Fe^{+++}	-	-	-	8.53	N.D.	N.D.
11 μM Fe^{+++}	15.7	15	12.1	11.2	13.5	0.9
11 μM Fe^{+++} +15 μg ml^{-1} EDDA	15.7	15.2	-	-	15.45	N.D.

N.B. 11 μM Fe^{+++} is the concentration of iron in normal medium.

Table 5.2.1 shows the phycoerythrin content per cell as a function of the increase in Fe^{+++} added to the growth medium. *Synechococcus* WH 7803 cells were analysed for their phycoerythrin content following the procedure described in Materials and Methods. All PE values have been normalized to a culture $OD_{750\ nm}$ of 0.1.

/increased breakdown are varied. Table 5.2.1 shows that the addition of 15 $\mu g\ ml^{-1}$ EDDA to normal medium does not cause a reduction in PE levels per cell with respect to normal cells. This suggests that the cells grown in EDDA are able to obtain iron from the chelator, thus not only exhibiting an exponential growth phase similar to iron replete cells, but also similar levels of PE per cell .

A recent paper (Ruetar & Unsworth, 1991) studying the decrease in PE concentration under different iron regimes is in partial agreement with the results described here. However, they observed a 50 fold increase in phycobiliprotein level as opposed to a 5 fold increase shown here between cells grown in chelator-deficient medium (2.75 fg PE $cell^{-1}$) and cells grown in normal medium (13.5 fg PE $cell^{-1}$). Furthermore Ruetar & Unsworth (1991) also observed a decrease in the relative PE content from 39% to 24% of the total protein, with increasing iron. Such results cannot be compared as total protein estimations were not conducted in this study. The methodology used in order to determine these values was not clear and as a result it proves difficult to make an informative comparison and comment upon their work.

5.2.3 Effect on phycoerythrin concentrations per cell after "spiking" *Synechococcus* WH 7803 cultures with iron

The addition of $FeCl_3$ to a final concentration of 11 μM to a chelator-deficient culture (culture 1), and a culture containing 0.5 mg l^{-1} EDTA but no Fe^{+++} (culture 2), (Table

5.2.2) resulted in a four fold and a two fold increase in the cellular phycoerythrin concentrations, respectively. Comparison of PE concentrations per cell between culture 1 and culture 2 prior to the addition of Fe^{+++} suggests that the absence of EDTA (culture 1) may have an effect upon PE levels per cell, possibly due to the lack of chelation of another trace metal such as copper, resulting in its toxicity. However, the addition of Fe^{+++} to the medium results in an increase in PE per cell to 10.72 fg, approaching the amounts found in culture 2 after the addition of Fe^{+++} (15.7 fg $cell^{-1}$). This observation suggests that it is the absence of Fe^{+++} and not the absence of EDTA which is the major cause for reduced PE per cell in cultures grown in chelator-deficient medium.

5.2.4 Difference spectrum of iron-replete and iron-deplete *Synechococcus* WH 7803 cells

The alteration in phycoerythrin concentrations in three culture conditions was observed by obtaining a difference spectrum as described in Materials and Methods. Exponential phase *Synechococcus* WH 7803 cells grown in normal medium, chelator-deficient medium and seven subcultures into normal medium containing 15 μg ml^{-1} EDDA were used. All samples were normalized to an $OD_{750\ nm}$ of 0.13. The cells grown in chelator-deficient medium were used as the baseline and a scanning spectrum was run between 500 nm and 750 nm for the remaining samples. Figure 5.2.4 shows two absorption maxima of 0.064 at a wavelength of 553 nm for phycoerythrin and 0.039

Table 5.2.2 The effect upon phycoerythrin content per cell after "spiking" iron deplete cultures of *Synechococcus* WH 7803 with 11 μM $FeCl_3$

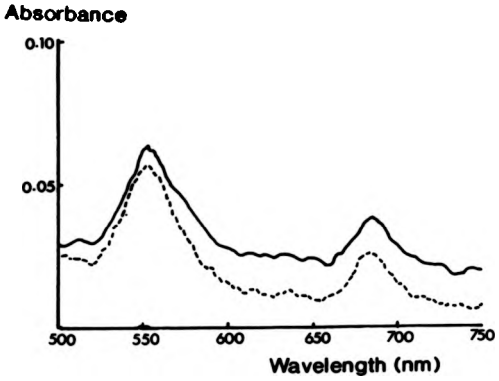
	GROWTH CONDITIONS	PE fg $cell^{-1}$
A	NO Fe^{+++}, NO EDTA (CULTURE 1).	2.88
B	NO Fe^{+++} + 0.5 mg l^{-1} EDTA (CULTURE 2).	7.96
C	CULTURE 1 + 11 μM $FeCl_3$	10.72
D	CULTURE 2 + 11 μM $FeCl_3$	15.7

Table 5.2.2 A) *Synechococcus* WH 7803 cells grown in ASW medium without any added iron or EDTA. B) *Synechococcus* WH 7803 cells grown in ASW medium without added iron, however 0.5 mg l^{-1} EDTA was added. C) Culture 1 (A) "spiked" with $FeCl_3.6H_2O$ to a final concentration of 11 μM D) Culture 2 (B) "spiked" with $FeCl_3.6H_2O$ to a final concentration of 11 μM. Samples from culture A and B were removed prior to "spiking" with iron. All PE values per cell have been normalized to a culture $OD_{750\ nm}$ of 0.1.

Figure 5.2.4 Difference spectrum of iron replete and iron deplete *Synechococcus* WH 7803 cells

———— cells grown in normal medium.
- - - - - - - cells grown in normal medium plus 15 $\mu g ml^{-1}$ EDDA.

Cells grown in chelator-deficient medium were used as the baseline.



at a wavelength of 685 nm for chlorophyll in normal cells with respect to chelator-deficient cells. Cells grown through seven transfers into normal medium containing EDDA have slightly lower absorption maxima for chlorophyll and phycoerythrin compared to cells grown in normal medium, however, the amount of photosynthetic pigment present is still higher than in chelator-deficient cells. This is probably due to the cells being able to obtain the chelated iron from the EDDA in the medium as a result of the expression of a high affinity uptake system. Although such a system is expressed in the cells grown in chelator-deficient medium the cells are unable to sequester the same amount of iron as those growing in the presence of EDDA, hence the reduced phycoerythrin levels.

5.2.5 Northern hybridization analysis of RNA from cells grown under different Fe^{+++} regimes

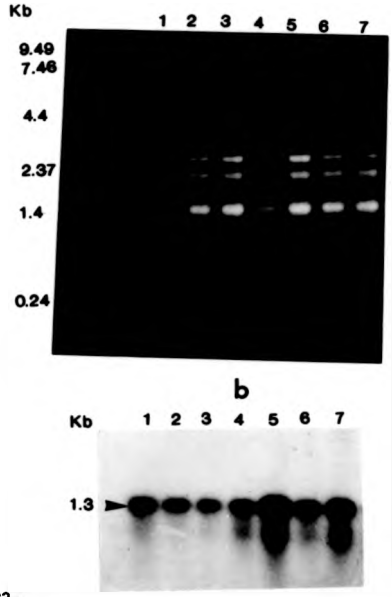
To investigate whether the effect of iron limitation on phycoerythrin synthesis was regulated at the transcriptional level, as is the case under nitrogen starvation (Newman, 1990) RNA was isolated from cultures grown under different Fe^{+++} regimes identified in Table 5.2.1 using the method described in Materials and Methods. Equal amounts of RNA (using standard spectrophotometric measurement) were loaded onto a formaldehyde agarose gel. RNA samples were analysed qualitatively by northern hybridization using the phycoerythrin α-subunit gene from *Synechococcus* WH 7803 (provided by J. Newman this laboratory). Figure 5.2.5 shows

the presence of a 1.3 Kb transcript in all of the iron regimes studied. Unfortunately the differences in RNA loading per track (Figure 5.2.5a) is not explained by the differences in the hybridization intensity observed in Figure 5.2.5b. If this was the case a reduced transcript abundance for chelator-deficient cells (track 1) would be apparent compared to tracks 2 and 3 which contain more total RNA per track. However, qualitative analysis of such samples is not effective at producing reliable data. Therefore, it is impossible to determine whether or not PE synthesis is regulated at the transcriptional or translational level under these conditions of iron limitation. Consequently, further northern hybridization using equal RNA loading needs to be repeated. Quantitative northern dot blot analysis would be a more preferable method to use in order to measure PE transcriptional changes as a result of altered iron concentrations in the growth medium. Even so the use of both northern blots and northern dot blots to measure transcriptional changes is limited to observing the changes in the overall steady state level of mRNA, and does not determine the rates of synthesis and degradation.

Figure 5.2.5 **The study of phycoerythrin transcription in cells grown under different Fe^{+++} regimes, by Northern (RNA-DNA) hybridisation analysis**

a Agarose gel electrophoresis of RNA from cells grown in :- Tracks 1) chelator-deficient medium. 2) ASW with no added iron. 3) ASW plus 0.011 μM $FeCl_3$. 4) ASW plus 0.11 μM $FeCl_3$. 5) ASW plus 1.1 μM $FeCl_3$. 6) ASW plus 11 μM $FeCl_3$ (normal ASW medium). 7) ASW plus 110 μM $FeCl_3$.

b Autoradiogram of the Northern blot of the above agarose gel. The same tracks apply.



5.3 A putative iron storage mechanism in *Synechococcus* WH 7803

5.3.1 Cellular iron concentrations in iron-deplete/replete cells

Cellular iron concentrations were analysed by flame atomic absorption spectroscopy (see Materials and Methods). Both cells grown in normal medium and cells from chelator-deficient medium were analysed. Large volumes (1 litre) were used so that the iron concentrations present in the cell samples could be measured more accurately. The results obtained are presented in Table 5.3.1. There is a large difference between the amount of iron per cell for cells grown in normal ASW medium (culture A and B) and chelator-deficient ASW medium (culture C and D). The iron concentration in culture A was 6×10^{-2} fg cell^{-1}, 2.3% of the iron added to the medium had been internalized by this 1 litre culture. In comparison, the iron concentration in culture B was 80×10^{-2} fg cell^{-1}, 9.8% of the iron added to the medium had been internalized. The difference in iron concentrations between the two cultures growing in normal ASW medium could be due to the expression of a high affinity iron uptake system in culture B, which has failed to grow either as a result of a toxic contaminant in the medium or absence of another medium component (as a result of human error). Alternatively, the results with culture B may show a normal sequestration of external iron by the cells prior to the onset of normal growth which in this case had been prevented. The internalization of iron from the surrounding growth medium has been previously observed with *Chlorogloea fritschii* and

Table 5.3.1 culture A and B contain *Synechococcus* WH 7803 cells which have been grown in normal medium. Culture C and D contain *Synechococcus* WH 7803 cells which have been grown in chelator-deficient medium. Normal ASW* is a *Synechococcus* WH 7803 culture that exhibited unusually poor growth in normal ASW medium (culture B). This poor growth may be due to the presence of a toxic substance in the medium or the absence of an essential nutrient from the medium (due to human error). Culture D also exhibited poor growth in comparison to culture C. The poor growth of culture D may be attributed to the reasons described for culture B.

Cellular iron concentrations were analysed by flame atomic absorption spectroscopy.

Synechococcus WH 7803

CULTURE	MEDIUM	GROWTH PERIOD (Hr)	CULTURE DENSITY $OD_{750\ nm}$	Fe fg cell^{-1} x 10^2	INTERNALIZED Fe PER LITER CULTURE (μg)	% Fe INTERNALIZED
A	normal ASW	120	0.256	6	14	2.3
B	normal ASW *	137	0.085	80	60	9.8
C	chelator-deficient ASW	120	0.088	1	0.78	–
D	chelator-deficient ASW	137	0.021	1.9	0.34	–

Anacystis nidulans (Jones *et al.*, 1977). Finally the high concentrations of internalized iron measured for culture B may be an artifact resulting from adsorption of iron onto the cell surface. Cells from culture C, grown in chelator-deficient medium contained 16% of the amount of iron per cell ($1x10^{-2}$ fg) present compared to culture A. The internalized iron levels per litre culture for culture D (0.34 μg) were lower than those of the growing (albeit slowly) culture C (0.78 μg). However the amount of iron per cell was greater for culture D ($1.9x10^{-2}$ fg) than culture C ($1x10^{-2}$ fg). There was greater iron internalized per litre culture of growing culture C because there was a greater total cell number present. Considering the amount of iron per cell in the poorly growing culture D it seems that iron sequestration may have occurred prior to growth, in a similar fashion to that described above for culture B. Except in this case there was far less iron in the medium to be sequestered. The ability of *Synechococcus* WH 7803 to sequester iron in the way described above must require these cells to have a mechanism where by the internalized iron can be stored prior to mobilization and use within the cell.

5.3.2 Iron storage proteins

Samples of *Synechococcus* WH 7803 grown under different iron regimes were taken during the early, mid and late exponential phases of growth and analysed for the presence of a bacterioferritin (BFR)-like protein by western blot analysis. The amount of protein loaded per track is shown in Table 5.3.2. Transfer of protein onto nitrocellulose was

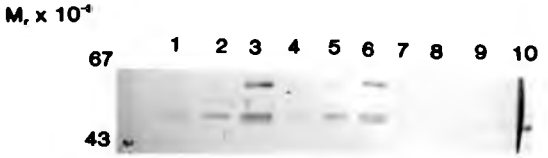
Table 5.3.2 Samples of *Synechococcus* WH 7803 grown under different iron regimes, taken at different stages of growth

TRACK	GROWTH MEDIUM	$OD_{750\ nm}$	μg PROTEIN LOADED PER TRACK
1	NORMAL ASW	0.103	4.107
2	NORMAL ASW	0.163	6.357
3	NORMAL ASW	0.343	13.37
4	NORMAL ASW + 15 $\mu g\ ml^{-1}$ EDDA	0.089	3.471
5	NORMAL ASW + 15 $\mu g\ ml^{-1}$ EDDA	0.132	5.148
6	NORMAL ASW + 15 $\mu g\ ml^{-1}$ EDDA.	0.331	12.9
7	CHELATOR-DEFICIENT ASW	0.082	3.198
8	CHELATOR-DEFICIENT ASW	0.116	4.524
9	CHELATOR-DEFICIENT ASW	0.134	5.226

Table 5.3.2 shows the whole cell protein samples loaded on the gel used for western blot analysis with the anti-BFR antibody (Figure 5.3.1). Cell samples were taken during the early mid and late exponential phases of growth.

Figure 5.3.1 **Detection of cross reacting bacterioferritin-like iron storage proteins in *Synechococcus* WH 7803, using western blot analysis**

The sample tracks shown in Table 5.3.2 were probed with a 1 in 1000 dilution of the anti-BFR rabbit serum and detected using a 1 in 300 dilution of horseradish peroxidase linked secondary antibody. Track 10 contains *Synechococcus* WH 7803 cell lysate from cells grown normally then probed with preimmune serum.



conducted as described in Materials and Methods. Filters were probed with a 1 in 1000 dilution of the anti-bacterioferritin rabbit serum (Figure 5.3.1). Track 10 is a control to detect if any proteins cross react with the pre-immune serum (1 in 1000 dilution). Two proteins within the whole cell extract show a positive cross reaction with the antibody at M_r 52,000 and 60,000 in size. The smaller of the bands also appears with the pre-immune serum. The presence of the 60,000 Da protein on the western blot suggests that *Synechococcus* WH 7803 contains a cross reacting BFR-like protein.

Results tentatively suggest that the cellular level of the BFR-like protein responds to the amount of iron in the medium and therefore internalized into the cell. Comparison of tracks 2, 5 and 9 where similar cell protein is loaded per track shows a reduced amount of the 60,000 Da band when the cells were grown in chelator-deficient medium (track 9) compared to normal medium (track 2) and in the presence of EDDA (track 5). Laulhere *et al.* (1992) observed a constant concentration of BFR in *Synechocystis* PCC 6803 which was independent of the iron status of the cell. This is a conflicting result to those described here and for the synthesis of plant and animal ferritins which are known to be induced upon intracellular increases in iron concentration (Thiel, 1987). However, experiments were not conducted here to observe the increased synthesis of the BFR-like protein upon the addition of iron to medium containing iron-deplete cells.

The presence of a cross-reacting protein in three other cyanobacteria was determined (Figure 5.3.2). Samples used for

Table 5.3.3 Samples of cyanobacterial strains grown in different iron regimes and used for western blot analysis with the anti-BFR rabbit serum.

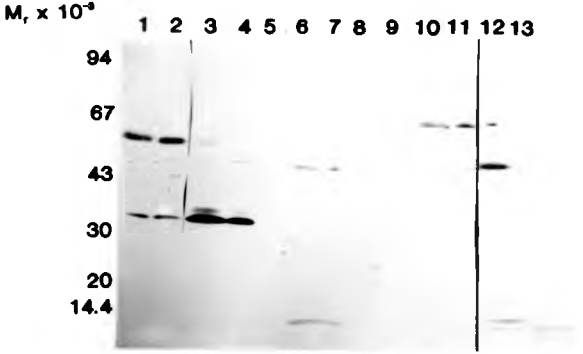
SAMPLE	$OD_{750\ nm}$	CELL MATERIAL LOADED PER TRACK ($OD_{750\ nm}$)
Synechococcus PCC 7002 (CDM)	0.213	4.32
Synechococcus PCC 7002 (NM)	0.455	4.32
Synechococcus PCC 7942 (CDM)	0.071	6.97
Synechococcus PCC 7942 (NM)	1.328	6.97
Nostoc sp. Nac PCC 8009 (CDM)	0.127	5.49
Nostoc sp. Nac PCC 8009 (NM)	0.560	5.49

Table 5.3.3 shows the whole cell protein samples used for western blot analysis in Figure 5.3.2. Equal cell material, normalized to $OD_{750\ nm}$, was loaded per strain. Cell samples were taken from different Fe^{+++} growth regimes. NM = Normal medium, CDM = chelator-deficient medium.

Figure 5.3.2 Detection of cross reacting bacterioferritin-like iron storage proteins in three other cyanobacterial strains using western blot analysis

Tracks:- 1-2) *Synechococcus* PCC 7002 grown in chelator-deficient medium. 3-4) *Synechococcus* PCC 7002 grown in normal medium. 5) *Synechococcus* PCC 7942 grown in chelator-deficient medium. 6-7) *Synechococcus* PCC 7942 grown in normal medium. 8-9) *Nostoc* sp. mac PCC 8009 grown in chelator-deficient medium. 10-11) *Nostoc* sp. mac PCC 8009 grown in normal medium. 12) *Nostoc* sp. mac PCC 8009 grown in normal medium. 13) *Synechococcus* PCC 7002 grown in normal medium.

Tracks 1-11 were probed with a 1 in 1000 dilution of anti-BFR rabbit serum. Tracks 12 and 13 were probed with a 1 in 1000 dilution of the pre-immune serum. The cell material loaded for each track is shown in Table 5.3.3.



SDS-PAGE analysis are shown in Table 5.3.3, equal amounts of cells were loaded per track (with regard to $OD_{750\ nm}$). The cross reaction seen with *Nostoc sp.* MAC (*Synechococcus* PCC 8009) in tracks 8-11 was also apparent with the pre-immune serum. Two cross reacting bands were identified in *Agmenellum quadruplicatum* (*Synechococcus* PCC 7002), at M_r 33,000 and 54,000 (tracks 1-4). There is a noticeable increase in the 33,000 Da band when cells are grown in normal medium (tracks 3,4). This would be expected for an iron storage protein. Unexpectedly, however, the 54,000 Da protein is present mainly in cells grown in chelator-deficient medium (tracks 1,2). Modification of the 33,000 Da protein, via such a process as glycosylation, may occur under conditions of iron depletion affecting its mobility on SDS-PAGE. Alternatively there may be transcription of a second gene similar to this protein under iron deplete conditions. Why such processes may exist in this strain and none other so far studied is unknown. *Anacystis nidulans* R2 (*Synechococcus* PCC 7942), has a cross-reacting protein at a M_r-46,000. This protein is not present under chelator-deficient growth conditions (track 5).

Bacterioferritin isolated from *E.coli* is composed of 24 subunits with a total M_r=500,000 (Yariv, 1983). Each subunit is approximately M_r=18,000 (Andrews *et al.*, 1989). The sizes of the cross-reacting polypeptides within the cyanobacterial strains studied is shown in Table 5.3.4 together with the size of the BFR isolated from *Synechocystis* PCC 6803 (Laulhere *et al.*, 1992). Comparison of the sizes of the cross-reacting polypeptides with those of *E. coli* BFR suggests the

Table 5.3.4 Summary of the sizes estimated by western blot of polypeptides cross-reacting with anti-BFR rabbit serum in three strains of *Synechococcus* sp

SYNECHOCOCCUS SP.

	WH 7803	PCC 7002	PCC 7942	PCC 6803[2]
ESTIMATED M_r (kDa)	60.25	32.7 53.7[1]	45.71	19
RATIO OF SIZE TO *E. COLI* BFR SUBUNITS.	3.26	1.76	2.47	1

N.B. 1.) This band appears mainly under growth in chelator-deficient medium.

2.) Laulhere *et al.* 1992.

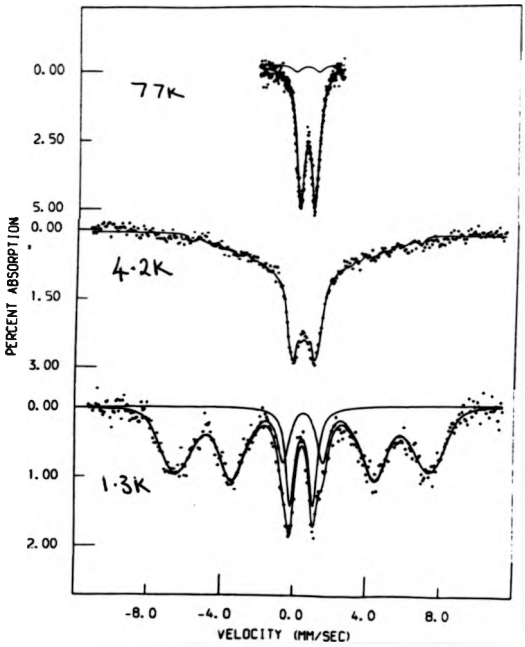
possibility of a gene fusion event whereby either two or three subunits are transcribed from one mRNA molecule, as opposed to one mRNA molecule for each 18 kDa protein as is the case for *E. coli* and possibly *Synechocystis* PCC 6803. This assumes that the cross-reacting species are indeed BFR-like proteins. Isolation and N-terminal sequence analysis of these proteins would confirm their identification as BFR homologues. Northern hybridization studies either with an oligonucleotide synthesized to a conserved region of the cyanobacterial BFR or utilizing the gene from *E. coli*, would help to elucidate whether gene fusion had occurred by analysis of the transcript size.

5.3.3 Identification of an iron storage protein using Mössbauer Spectroscopy

The results obtained from Mössbauer spectroscopy (conducted by Dr D.P.E. Dickson, Liverpool University) upon membrane fractions of *Synechococcus* WH 7803 whole cell extract, after growth in the presence of ^{57}Fe (see Materials and Methods), confirm the presence of bacterioferritin in this organism (Figure 5.3.3). The predominant form of the ^{57}Fe is very similar to that found in the BFRs of *E.coli* and *Pseudomonas aeruginosa*, probably being present as an amorphous iron oxide with considerable amounts of associated phosphate (D.P.E. Dickson - personal communication).

Figure 5.3.3 ^{57}Fe Mössbauer spectra from ^{57}Fe labelled *Synechococcus* WH 7803 cells

K = Kelvins.



5.4. Detection of iron acquisition systems

5.4.1 Siderophore assays

Inconclusive results were obtained for *Synechococcus* WH 7803 and *Agmenellum quadruplicatum* using the universal siderophore assay of Schwyn and Neilands (1987), even though a siderophore has been isolated from the latter cyanobacterium (Armstrong & Van Baalen, 1979). Negative results using this assay to detect siderophore production in cyanobacteria have also been obtained by Scanlan (1988) and Kerry *et al.* (1988). An alternative approach to detecting siderophore-mediated Fe^{+++} acquisition is the EDDA bioassay of Kerry *et al.* (1988). The results obtained by this method are shown in Table 5.4.1. All four strains used for the identification of siderophore production fell into group II of the three groups described by Kerry *et al.* (1988). Group II includes organisms with an enhanced, but intermediate affinity iron scavenging system. Group I contains organisms with a strong, high affinity iron scavenging system and group III contains organisms which lack a high affinity iron uptake system. *Anacystis nidulans* R2 (*Synechococcus* PCC 7942) was able to grow in medium containing 50 $\mu g\ ml^{-1}$ EDDA, although growth was poor, as opposed to 40 $\mu g\ ml^{-1}$ described by Kerry *et al.* (1988). *Agmenellum quadruplicatum* (*Synechococcus* PCC 7002) grew in medium containing 50 $\mu g\ ml^{-1}$ EDDA which is the same as the value obtained by Kerry *et al.* (1988) for this organism. *Anabaena cylindrica* PCC 7122 grew in medium containing 20 $\mu g\ ml^{-1}$ EDDA, however, growth was poor. *Synechococcus* WH 7803 was able to grow in medium containing 15 $\mu g\ ml^{-1}$ EDDA, no growth occurred

Table 5.4.1 Siderophore production determined by the EDDA bioassay of Kerry *et al.* (1988)

Synechococcus sp.	$\mu g\ ml^{-1}$ EDDA
PCC 7002	50
PCC 7942	50
PCC 7122	20
WH 7803	15

Table 5.4.1 shows the maximum concentration of EDDA ($\mu g\ ml^{-1}$) in the culture medium allowing growth of the respective *Synechococcus* sp.

at 20 $\mu g\ ml^{-1}$ EDDA. The EDDA concentrations in which these four cyanobacteria were able to grow suggested that they contain an enhanced, but intermediate affinity iron scavenging system. The fact that *Anacystis nidulans* and *Agmenellus quadruplicatum* were able to grow in higher concentrations of EDDA suggests that their iron scavenging systems are of a higher affinity compared to those of *A. cylindrica* and *Synechococcus* WH 7803. Further analysis of siderophore production can be achieved using the Csaky test (1948) for hydroxamate siderophores, or Arnow's reagent (Arnow, 1937) for catecholate siderophores. Kerry *et al.* (1988) have identified a hydroxamate siderophore from *A. nidulans* by the Csaky test. In cultures where siderophore production is low, concentration of the culture supernatant would probably be required in order to see a positive reaction (C.Trick- personal communication.). Alternatively it may well be that *Synechococcus* WH 7803 does not contain an iron uptake system like that described for *E. coli*, but instead contains a system independent of siderophores. Indeed, Rueter & Unsworth (1991) have not been able to observe siderophore production in *Synechococcus* WH 7803.

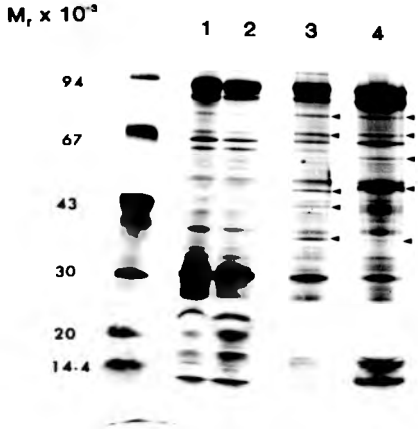
5.4.2 Cell envelope protein patterns as a function of iron replete/deplete growth conditions

Under conditions of iron deprivation proteins required for high affinity iron transport systems are usually present within the membranes of the cell envelope (see Section 1.4). Therefore cell walls isolated by methods described in Chapter

4, were analysed for altered polypeptide profiles following growth under iron-deplete conditions using SDS-PAGE. Growth of *Synechococcus* WH 7803 in chelator-deficient medium and medium containing EDTA but no added Fe^{+++} resulted in the appearance of several apparently iron-regulated outer-membrane proteins (Figure 5.4.1). The majority of the additional polypeptides expressed under Fe-deplete growth were minor in contrast to the abundant polypeptides found under these conditions in *A.nidulans* R2 (Scanlan *et al.*, 1989). The cell envelope polypeptide patterns exhibited by *Synechococcus* WH 7803 under the two growth regimes differed from each other as well as from those of cells grown in normal medium (Figure 5.4.1). Chart *et al.* (1986) noted a variation in the expression of iron-regulated membrane proteins of *E. coli* depending upon the chelator used to restrict the iron availability. It may be that *Synechococcus* WH 7803 has a number of different iron acquisition systems which operate under different levels of iron depletion as is the case in *E. coli* (Bagg & Neilands, 1987). The presence of EDTA in the medium may facilitate the chelation of some of the residual iron in the medium thereby making it more available to the cells. These cells, therefore, would be experiencing a different degree of iron depletion in comparison to cells grown in chelator-deficient medium (no Fe^{+++}, no EDTA). The polypeptides induced under iron-deplete conditions have the following sizes on SDS-PAGE. 1) For cells grown in the absence of added Fe^{+++}: M_r 78,000, 70,000, 50,000, 45,000, and 37,000. 2) For cells grown in chelator-deficient medium M_r 78,000, 72,000, 63,000, 53,000 and 37,000. There is no

Figure 5.4.1 6-20% [w/v] exponential SDS-PAGE gel of *Synechococcus* WH 7803 outer membranes

Outer membranes were obtained from cells grown under different iron regimes. Tracks 1-2) Normal ASW. 3) ASW with no added Fe^{+++}. 4) Chelator-deficient ASW medium.



protein of M_r 92,000 which is present in *A. nidulans*. The majority of the proteins expressed in *Synechococcus* WH 7803 are larger in size unlike those observed under conditions of iron limitation for *A.nidulans* (*Synechococcus* PCC 7942) at M_r 50,000 and 36,000 (Scanlan *et al.*, 1989). However, there are several polypeptides also induced in the higher molecular weight range > 94 kDa in size. The absence of phycoerythrin (M_r 22,000) is noticeable in these iron-deplete cells. An alternative method for protein identification was sought owing to problems of reproducibility and sensitivity when using silver staining coupled with the large quantities of cells required to isolate cell walls from iron-deplete cultures. Moreover, the iron regulated proteins were minor polypeptides present in small amounts and their identification was inhibited by the poor cell yield obtained from iron-deplete cultures.

5.5 ^{35}S L-Methionine protein labelling for the study of stress induced proteins

In order to characterize the protein synthesis profile of cells growing under iron limitation, ^{35}S-methionine protein labelling was used. This method had many valuable attributes. For example, it was able to identify specific protein synthesis over the period of time in which the cells were subjected to iron limitation. The ability to label proteins easily, proved to be a sensitive detection system and only small cell volumes were required for labelling studies. Finally the results obtained were reproducible. ^{35} S-

methionine protein labelling has been used for the identification of heat shock proteins in freshwater species of cyanobacteria for example, *Synechococcus* PCC 6301 (Borbely *et al.*, 1985; Borbely & Suranyi, 1988). The use of ^{35}S-methionine as opposed to $^{35}SO_4$ to characterize protein synthesis in marine cyanobacteria has not been documented before. More recently Paerl (1991) has shown that marine picoplankton show light-stimulated incorporation of tritiated amino acids. Although light-stimulated utilization of amino acids has been documented for fresh water phytoplankton, this is the first time it has been shown for marine picoplankton. This discovery also poses some important implications for how marine cyanobacteria may be able to overcome nitrogen deficiency in the ocean.

5.5.1 Determination of ^{35}S-methionine uptake into cells

Using the protocol described in Materials and Methods the ability of *Synechococcus* WH 7803 to incorporate ^{35}S-methionine was demonstrated. Figure 5.5.1a shows the incorporation of ^{35}S-methionine into the cells over a period of 48 hr. Uptake appears to be rapid for the first 24 hr followed by a slower rate of uptake over the following 24 hr. On comparison with cells placed in the dark for the same length of time it can be concluded that there is light-stimulated uptake of the radiolabelled amino acid as described by Paerl (1991). Furthermore light-stimulated incorporation of ^{35}S-methionine into the cells is evidence for the ability of *Synechococcus* WH 7803 to utilize this free amino acid and against the presence

Figure 5.5.1 Incorporation of ^{35}S L-methionine into *Synechococcus* WH 7803 cells

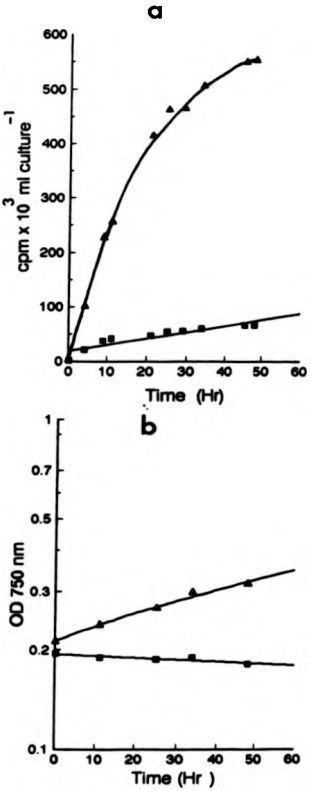
Incorporation was monitored over a 48 hr period.

a) Comparison of ^{35}S-methionine uptake between cells incubated in the light (normal growth conditions) and incubated in the dark. Results indicate light stimulated uptake of ^{35}S-methionine.

b) Growth curve measurements of the ^{35}S-methionine labelled *Synechococcus* WH 7803 cultures, taken during the 48 hr period. No growth was observed for cells incubated in the dark, whereas a small amount of growth was observed for the light incubated culture.

▲ *Synechococcus* WH7803 cells incubated in the light.

■ *Synechococcus* WH 7803 cells incubated in the dark.


a
600
500
400
300
200
100
0
cpm x 10^3 ml culture^-1
0 10 20 30 40 50 60
Time (Hr)
b
1
0.7
0.5
0.3
0.2
0.1
OD 750 nm
0 10 20 30 40 50 60
Time (Hr)

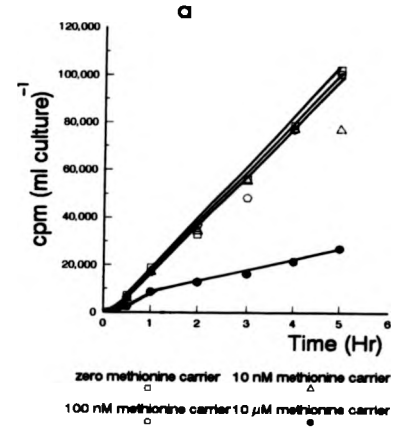
of contaminating heterotrophic bacteria. The difference in the uptake of ^{35}S-methionine between the two cultures is not due to excessive growth of the light incubated culture. Over the experimental period (Figure 5.5.1b), those incubated in the dark did not show any growth, those incubated under normal conditions exhibited a small degree of growth. At this time the cultures were in late exponential/stationary growth. Uptake may be higher in cells undergoing exponential growth.

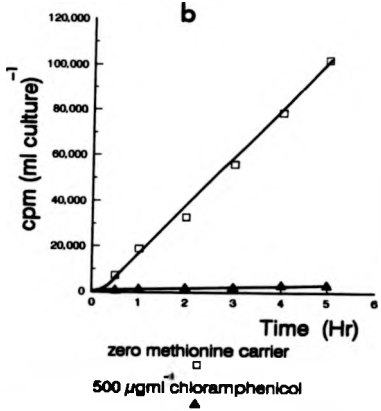
Trichloroacetic acid precipitation of protein in whole cells and chloramphenicol inhibition of protein synthesis showed that the ^{35}S-methionine taken up by the cells was being incorporated into cell protein. After labelling (1 μCi ml^{-1} ^{35}S-methionine, 1.0 nM final concentration) for 6 hr approximately 100,000 cpm of the radiolabel was incorporated into total protein in 1 ml of cell culture (Figure 5.5.2a). Addition of cold L-methionine carrier had no effect on incorporation into protein at concentrations up to 100 nM. The addition of 10 μM cold methionine carrier reduced incorporation of ^{35}S-methionine into protein by 73.6% (Figure 5.5.2a) after 6 hr incubation, at this concentration cold methionine out competed radiolabelled methionine uptake (this is useful to know for possible future pulse chase experiments). The addition of cold carrier did not enhance the degree of ^{35}S-methionine uptake in *Synechococcus* WH 7803 cells, the uptake system was probably already saturated. The addition of 500 μg ml^{-1} chloramphenicol resulted in 97% reduction in the amount of ^{35}S-methionine incorporated into protein (Figure 5.5.2b).

Figure 5.5.2 ^{35}S L-methionine incorporation into total protein after TCA precipitation of whole cell samples

a) ^{35}S-methionine incorporation over a 6 hr period in the presence of 0 nM unlabelled methionine, 10 nM unlabelled methionine, 100 nM unlabelled methionine and 10 μM unlabelled methionine. The presence of upto 100 nM unlabelled methionine carrier showed no effect on ^{35}S-methionine incorporation. However addition of 10 μM unlabelled methionine reduced incorporation of radiolabel into protein by 73.6% after 6 hr incubation.

b) ^{35}S-methionine incorporation into total protein was reduced by 97% when cells were incubated in the presence of 500 μg ml^{-1} chloramphenicol over 6 hr.


a
120,000
100,000
80,000
60,000
40,000
20,000
0
cpm (ml culture)$^{-1}$
0
1
2
3
4
5
6
Time (Hr)
zero methionine carrier
10 nM methionine carrier
100 nM methionine carrier
10 μM methionine carrier


b
120,000
100,000
80,000
60,000
40,000
20,000
0
cpm (ml culture)$^{-1}$
0
1
2
3
4
5
6
Time (Hr)
zero methionine carrier
500 μg/ml chloramphenicol

5.5.2 Time course labelling experiments of cells grown in iron-replete and iron-deplete conditions

Two 50 ml flasks of cells grown in normal medium and medium containing 15 $\mu g\ ml^{-1}$ EDDA were labelled with ^{35}S-methionine (see Materials and Methods). Over a time course of 8-44 hr, after inoculation into the above growth media, it is possible to see the appearance of a protein band M_r=36,000 in whole cell lysates from cells grown for 21 hr in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA (Figure 5.5.3). This 36,000 Da polypeptide can be clearly seen in Figure 5.5.4 where it is also possible to see the presence of higher molecular weight proteins similar in size to those identified in Figure 5.4.1. Even though the protein pattern of the two cultures is different the growth of the two cultures is the same. Therefore the alteration in the protein pattern is not due to differences in growth rate but can be attributed to the absence of available iron in the medium. ^{35}S-methionine labelled cells grown in chelator-deficient medium also exhibited the appearance of the 36 kDa protein after 48 hr growth (Figure 5.5.5). Even though the protein was apparent after 48 hr growth, protein labelling was not sufficient to detect the presence of the polypeptide prior to this time. The middle track contains cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. The presence of the 36 kDa protein in cells from both iron-deplete conditions suggests that the synthesis of this protein is derepressed under conditions of iron limitation.

Figure 5.5.3 Use of ^{35}S-methionine to identify the presence of iron regulated proteins

The presence of the 36,000 Da polypeptide (arrowed) in cells grown in iron deplete medium is apparent between 21-44 hr growth in this medium.

Samples were taken at growth time intervals of :- Track 1 and 6) 0 hr. Track 2 and 7) 8 hr. Track 3 and 8) 21 hr. Track 4 and 9) 29 hr. Track 5 and 10) 44 hr.
Tracks 1-5 contain total cell protein from cells grown in normal medium. Tracks 6-10 contain total cell protein from cells grown in normal medium containing 15 $\mu g ml^{-1}$ EDDA. Equal counts per track were loaded.

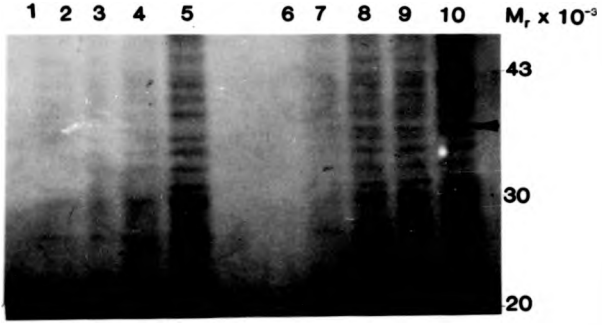


Figure 5.5.4 Comparison of ^{35}S-methionine labelled whole cell lysate from *Synechococcus* WH 7803 grown in iron replete and deplete medium

a) Track a) cells from normal medium, after 44 hr growth.
Track b) cells from normal medium plus 15 $\mu g ml^{-1}$ EDDA, after 44 hr growth. 100 μg protein loaded per track.
The 36,000 Da polypeptide (arrowed) is visible in the protein pattern of track b. Other higher molecular weight proteins are also visible in track b.

b) Enlarged section of a above.

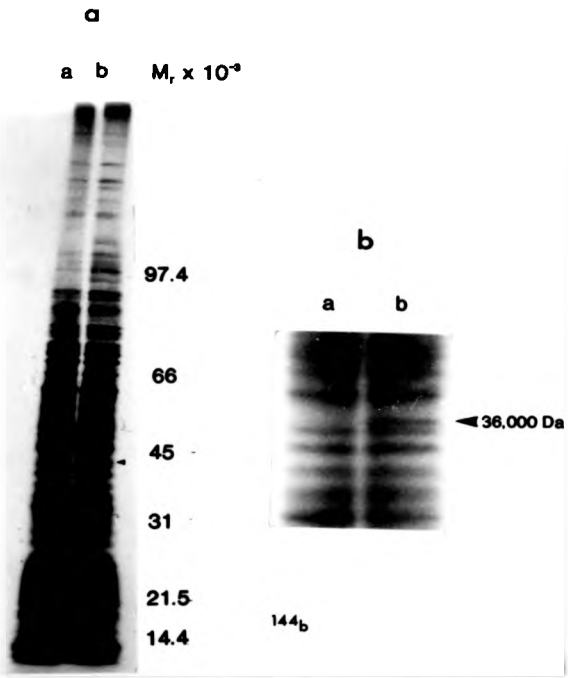
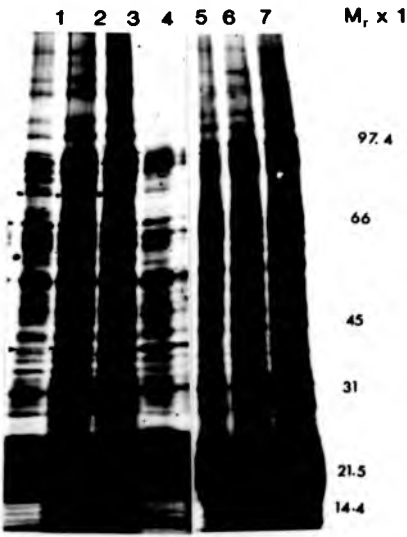


Figure 5.5.5 **Pulse ^{35}S-methionine labelled total cell protein from cells grown in chelator-deficient medium**

Track 1-3) *Synechococcus* WH 7803 grown in chelator-deficient medium. Track 4) *Synechococcus* WH 7803 grown in normal medium plus 15 $\mu g ml^{-1}$ EDDA. Track 5-7) *Synechococcus* WH 7803 grown in normal medium.
Cells were labelled for 6 hr periods at the following times after inoculation. Track 1 and 3) 48 hr. Track 2 and 6) 72 hr. Track 3 and 7) 120 hr. 75 μl of total cell protein was loaded per track.

The 36,000 Da polypeptide (arrowed) is apparent in the total cell protein after 48 hr growth. The control track 4 also contains the 36,000 Da protein (arrowed).



On inoculating cells which have been grown through three transfers into normal medium containing 15 $\mu g\ ml^{-1}$ EDDA back into normal medium lacking EDDA, the synthesis of the 36 kDa protein was repressed after 24 hr growth (Figure 5.5.6). There was no other dramatic change in protein profile observed. The fact that this 36 kDa protein appears to be synthesized under all the iron limiting regimes studied (Figures 5.4.1, 5.5.4, 5.5.5), and that its synthesis appears to be repressed within 24 hr after iron deplete cells were transferred to iron replete medium suggests that it may play a central role in iron acquisition in *Synechococcus* WH 7803. Although the preliminary results based on cell wall isolation suggest that this protein is associated with the outer membrane (Figure 5.4.1), further studies have been conducted in order to confirm this location (see Chapter 6).

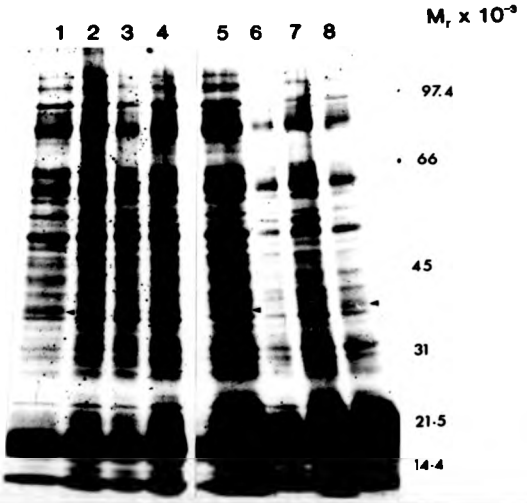
Expression of the *flavodoxin* gene in *Anabaena* PCC 7119 has been shown to occur under iron deplete conditions (Fillat *et al.*, 1988). Unfortunately the induction of this protein in *Synechococcus* WH 7803 was impossible to detect due to masking by the phycobiliproteins which have the same mobility upon SDS-PAGE.

The results presented here also show the presence of other high molecular weight proteins which appear to be synthesized under iron deplete conditions and may be involved in iron acquisition. If this is the case, isolation of these proteins will help in the elucidation of the iron uptake system(s) utilized by this cyanobacterium. It may well be that future work will establish that *Synechococcus* WH 7803 has more than one iron acquisition system, as is the case in *E.*

Figure 5.5.6 **Pulse ^{35}S-methionine labelled total cell protein from cells previously grown in medium containing 15 $\mu g ml^{-1}$ EDDA which have been inoculated into normal growth medium**

Track 1-4) *Synechococcus* WH 7803 grown through four subcultures into normal medium containing 15 $\mu g ml^{-1}$ EDDA. Track 5-8) *Synechococcus* WH 7803 previously grown through three subcultures into normal medium containing 15 $\mu g ml^{-1}$ EDDA which has been inoculated into normal medium.
Cells were labelled for 24 hr at the following times after inoculation. Track 1 and 5) 24 hr. Track 2 and 6) 48 hr. Track 3 and 7) 72 hr. Track 4 and 8) 96 hr. 75 µl of total cell protein was loaded per track.

Synthesis of the 36,000 Da polypeptide is repressed after 24 hr growth in normal medium (arrowed). However, after between 96-120 hr growth in the same medium the presence of this protein reappears (arrowed).



coli (Bagg & Neilands, 1987_a). Alternatively, iron acquisition may be manifest via a novel system.

5.5.3 Determination of proteins synthesized in response to temperature shocks

Heat shock and cold shock experiments were carried out in order to determine whether the 36 kDa protein identified in cells grown under iron deplete conditions was involved in iron acquisition or was in fact a general stress protein. Figure 5.5.7 illustrates the ^{35}S-methionine labelled protein pattern obtained when cell samples were exposed to a temperature of 33°C for 6 hr (see Materials and Methods). After 6 hr incubation four polypeptides appeared to be preferentially synthesized. The sizes were as follows:- M_r 86,000, 76,000, 62,000 and 60,000 . These proteins appeared between 1-2 hr after the cells were heat shocked and persisted within the cell up to 3 hr after the culture had been placed back at the normal growth temperature (25°C). The doublet around 97.4 kDa, of which the lower band appears as a response to increased temperature are the major polypeptides associated with the cell wall. In order to reduce the background labelling, cells were pulse labelled with ^{35}S-methionine for 30 min periods while being incubated at 40°C (see Materials and Methods). It is possible to see a maximal presence of five major bands (Figure 5.5.8) M_r 86,000, 76,000, 62,000, 60,000 and 19,000 within 30-60 min of the cells being exposed to 40°C. After 90-120 min exposure all heat shock proteins were no longer synthesized except for the 19 kDa polypeptide.

Figure 5.5.7 Autoradiograph of ^{35}S-methionine labelled *Synechococcus* WH 7803 cells exposed to heat shock conditions

Synechococcus WH 7803 was incubated at 33°C for 6 hr in normal growth medium. Samples were removed at hourly intervals and identified by 6-20% [w/v] exponential SDS PAGE followed by autoradiography. 75 µl total cell protein was loaded per track.

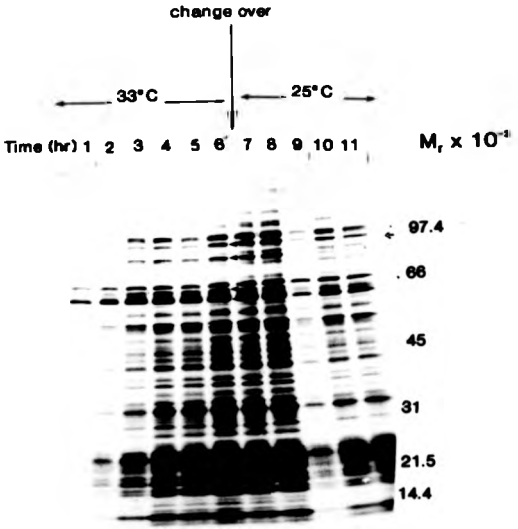
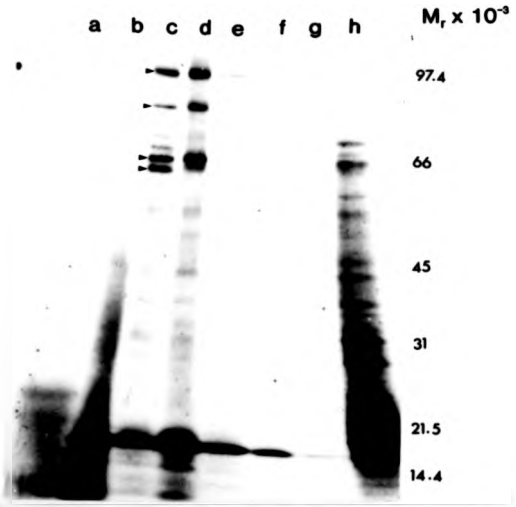


Figure 5.5.8 Autoradiograph of ^{35}S-methionine pulse labelled *Synechococcus* WH7803 cells exposed to lethal heat shock conditions

Cells were incubated at 40°C in normal growth medium and pulse labelled for 30 min periods. The polypeptide pattern was determined by 6-20% [w/v] exponential SDS-PAGE followed by autoradiography.
Tracks a) 0-30 min at 25°C. b) 0-30 min at 40°C. c) 30-60 min at 40°C. d) 60-90 min at 40°C. e) 90-120 min at 40°C. f) 180-210 min at 40°C. g) 240-270 min at 40°C. h) 240-270 min at 25°C. Tracks a and h are control tracks. 75 µl total cell protein was loaded per track. The major polypeptides responding to heat shock are shown by arrows.

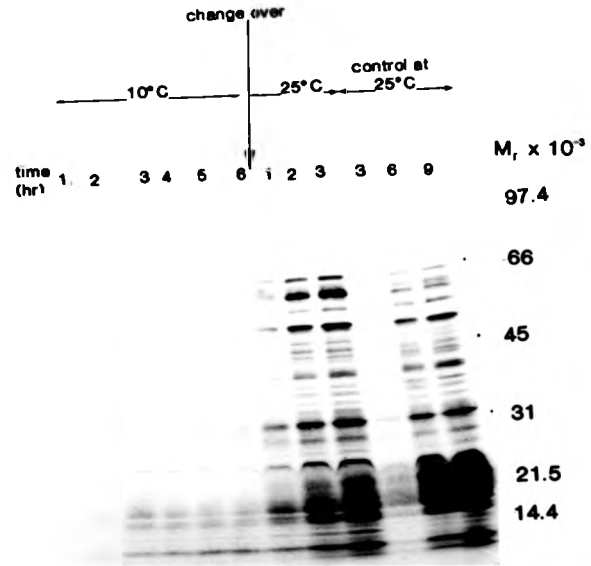


The 19 kDa protein ceased to be synthesized after 240-270 min incubation at 40°C. None of the proteins synthesized as a response to heat shock had a M_r-36,000. Borbely *et al.* (1985) showed with *Synechococcus* PCC 6803 a maximal appearance of heat shock proteins after 30 min incubation at the higher temperature which consequently decreased. This is similar to the situation described for *Synechococcus* WH 7803. Two of the proteins identified by Borbely *et al.* (1985) were 78 and 18 kDa in size, similar to those identified after 30 min at 40°C for *Synechococcus* WH 7803 (Figure 5.5.8). However, ^{35}S-methionine labelling may not give an adequate indication of the proteins involved in the heat shock response as many may contain low levels of sulphur-containing amino acids (Borbely *et al.*, 1985; Borbely & Suranyi, 1988). An alternative label such as sodium ^{14}C bicarbonate could be used. This compound gives an identical protein labelling pattern to that obtained using radioactive protein hydrolyzate when labelling heat shock proteins from *Synechococcus* PCC 6301 (Borbely *et al.*, 1985; Borbely & Suranyi, 1988).

Four proteins were identified 1-2 hr after transferring *Synechococcus* WH 7803 cells from 6 hr incubation at 10°C to the normal growth temperature at 25°C (Figure 5.5.9). The procedure used is described in Materials and Methods. The approximate protein M_r are 64,500, 58,000, 50,000 and 33,000, as determined by mobility through an SDS gel. The shift up in temperature induced the synthesis of these proteins as opposed to the induction of specific cold shock proteins. However, none of these polypeptides resemble, in terms of size, the heat shock proteins previously described. In the three

Figure 5.5.9 Autoradiograph of ^{35}S-methionine labelled *Synechococcus* WH 7803 cells exposed to cold shock conditions

Cells were incubated for 6 hr at 10°C in normal growth medium. Samples were removed at hourly intervals and identified by 6-20% [w/v] exponential SDS PAGE followed by autoradiography.



75 µl total cell protein loaded per track.

control samples taken at 3 hr, 6 hr and 9 hr incubation at 25°C there is no over-production of the four polypeptides identified in the cold shocked cultures even though the proteins are present. Therefore an increase in temperature to a more favourable growth condition may have resulted in the increased synthesis of four essential proteins.

5.6 Conclusions.

The use of ^{35}S-methionine labelling has been shown to be a valuable tool for the study of the polypeptides synthesized as a response to iron depletion. As a result, a polypeptide M_r=36,000 has been observed after 21-48 hr growth in iron-deplete medium. This response time coincides with the 50 hr lag phase exhibited by cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA (Figure 5.2.3). After this period, growth continues normally. It can be postulated therefore, that during this time period the *Synechococcus* cells are responding to the severe EDDA simulated decrease in external iron concentrations, by the synthesis of (amongst others) the 36,000 Da protein, as part of a high affinity iron transport system. Once the system is functional growth rate is the same as for cells grown in normal medium. In contrast, *Synechococcus* WH 7803 cells grown in chelator-deficient medium were able to grow for the first 50 hr after which time growth ceased. ^{35}S-methionine polypeptide patterns show the synthesis of the same 36,000 Da protein 48 hr after the start of growth in this medium. Although the low external iron levels have resulted in the induction of a high affinity iron

scavenging system(s), the residual iron available in the surrounding medium cannot be sufficient to sustain growth. The difference in the initial response between cells grown in normal medium plus 15 $\mu g\ ml^{-1}$ EDDA and chelator-deficient medium may be a result of the presence of EDDA, strong iron chelators have been known to leach iron from inside cells (A.Davies - personal communication).

It is not clear how the response to low environmental iron concentrations is communicated to the cell. The depletion of iron stores within the cell which are unable to be replenished may result in a cellular signal for synthesis of a high efficiency iron uptake system. Recognition of the anti-BFR serum by polypeptides in a series of cyanobacterial strains (including *Synechococcus* WH 7803) indicates the presence of an iron storage protein. Furthermore, the application of Mössbauer spectroscopy has infact confirmed the presence of bacterioferritin molecules in *Synechococcus* WH 7803. Future isolation and characterization of the putative BFR protein may allow for the study of such a response.

So far there has been no evidence to suggest that *Synechococcus* WH 7803 is able to synthesize and secrete siderophores. The lack of siderophore production in an organism naturally found in the open ocean is not surprising. In the natural marine environment physical mixing is such that siderophores would be transported away from the cell surface from which they were secreted. Such an effect is economically unfavourable for the cells. However, the ability of *Synechococcus* WH 7803 to use siderophores secreted from other organisms cannot be ruled out, such an experiment has not been

conducted here. Alternatively this organism may utilize a novel high-affinity iron uptake system unlike those described for *E.coli* (Bagg & Neilands, 1987). Failure, as yet, to locate an homologous ferric uptake regulation gene (*fur*) from *E.coli* in marine *Synechococcus* sp. (Rueter & Unsworth, 1992) suggests that the regulation in response to iron limitation may also be manifest via a novel system.

CHAPTER 6
LOCALIZATION AND PARTIAL CHARACTERIZATION OF THE 36 KDA IRON-REGULATED PROTEIN

6.1 Introduction

Once a putative iron-regulated protein had been identified in *Synechococcus* WH 7803 cells grown in iron-deplete cultures the next step was to attempt to localize, isolate and partially characterize the protein. In order to facilitate localization and purification studies, radiolabelled cells were used as markers to identify the protein. This method was used routinely as the 36 kDa protein was not an abundant protein and could not be identified on Coomassie-stained gels. Cell walls and inner membranes were obtained by detergent extraction and differential centrifugation of the cell envelope, this method was preferred as it was reproducible and simple to use. The cell wall could, alternatively, be separated from the inner membranes by sucrose density centrifugation (see Materials and Methods). Cell walls banded in the 70-80% [w/v] sucrose fraction, whereas the inner membranes remained at the top of the gradient. Partial characterization of the protein was hoped to be achieved by amino-terminal sequencing, western blot analysis and Southern hybridization studies.

6.2 Localization of the iron-regulated protein to the periplasm

Release of the periplasmic contents from *Synechococcus* WH 7803 had not previously been achieved.

Alkaline phosphatase (Pho A) is a useful marker for identifying the periplasmic fraction in *E. coli* and has been used as such in this study. Alkaline phosphatase has previously been observed and purified from *Anacystis nidulans* (Block & Grossman, 1988). The three procedures investigated were all successful in releasing the periplasmic contents from the cell. The periplasmic fraction was identified by PhoA assays (see Materials and Methods) and the values obtained are shown in Table 6.2.1. The highest activity for alkaline phosphatase being found mainly in the putative periplasmic fraction. In all three isolation procedures a small amount of phycoerythrin contamination was apparent, suggesting a slight leakage of this phycobiliprotein through the cytoplasmic membrane.

Periplasmic fractions isolated from radiolabelled *Synechococcus* WH 7803 cells did not show the presence of the 36 kDa protein. The probable absence of this protein in the periplasm rules out its function as a periplasmic binding protein involved in Fe^{+++} transport across the periplasmic space. The high activity of alkaline phosphatase in the supernatant of normal cells isolated by the method of Thiel (1988) (1.105 420 nm min^{-1} ml^{-1} mg $protein^{-1}$) and normal cells isolated by osmotic shock (1.034 420 nm min^{-1} ml^{-1} mg $protein^{-1}$) could possibly be attributed to the fact that these cells were harvested from the stationary phase of growth. At this time the cells were probably

Table 6.2.1 Periplasmic isolation procedures

ISOLATION METHOD	GROWTH CONDITIONS	420 nm min^{-1} ml^{-1} mg $protein^{-1}$		
		LYSATE	PERIPLASM	SUPERNATANT
Chloroform extraction[1]	ASW + 15 $\mu g ml^{-1}$ EDDA	< 100%	0.0828	< 100%
	NORMAL ASW	0.0117 (2%)	0.621 (98%)	-
Osmotic shock[2]	ASW + 15 $\mu g ml^{-1}$ EDDA	0.059 (2.6%)	1.65 (74%)	0.52 (23%)
	NORMAL ASW	0.051 (3%)	0.596 (35%)	1.034 (61%)
	E. coli NORMAL SOB	0.077 (16%)	1.046 (82%)	0.156 (12%)
T. THIEL[3]	NORMAL ASW	0.143 (9%)	0.3 (20%)	1.105 (71%)

N.B. 1) Ames et al. (1984).
2) Neu & Heppel (1965).
3) Thiel (1988).

Table 6.1.1 shows the results of PhoA assays conducted on the cell periplasm, cell lysate and the supernatant after periplasmic extraction. Included in the table is a control experiment with E. coli in order to test the viability of the method.

phosphorus limited and therefore secreting PhoA into the culture medium. More recently *Synechococcus* WH 7803 has been shown to contain an outer membrane protein involved in phosphate acquisition. The gene for this protein has been cloned and sequenced and has a 51% homology to a gene encoding the phosphate-binding protein isolated from *E. coli* (D. Scanlan, personal communication, this laboratory).

6.3 Localization of the iron-regulated protein within the cell membranes

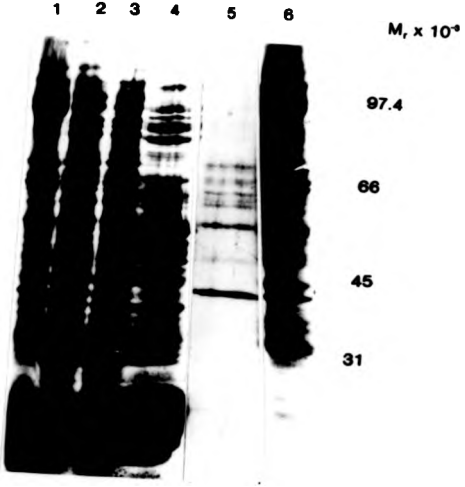
Radiolabelled *Synechococcus* WH 7803 cell membranes were fractionated by differential centrifugation and detergent extraction (see Materials and Methods). Figure 6.3.1 shows the separation of the cell membrane sample into cell wall and internal membrane samples after 50-85% [w/v] sucrose density centrifugation at 23,500 rpm for 18 hr at 4°C using a SW 28 Beckman rotor in a L8 ultracentrifuge. The Triton-soluble inner membrane (IM) sample was unable to enter the 50% [w/v] sucrose fraction. Alternatively, centrifugation (35,000 rpm for 60 min at 4°C using a SW 40Ti Beckman rotor in a L8 Beckman ultracentrifuge) of the cell membrane fraction after Triton-X-100 extraction (25°C, 30 min) resulted in the inner membrane remaining in the soluble supernatant and the Triton-insoluble cell wall being pelleted. The cytoplasmic membrane isolation

Figure 6.3.1 50-85% [w/v] sucrose density gradient

Cell membranes were separated into cell wall and internal membrane fractions by sucrose density centrifugation after detergent extraction (see Materials and Methods). The Triton-insoluble cell walls (arrowed) band in the 70-80% [w/v] sucrose interface, whereas the Triton-soluble internal membranes remain above the sucrose gradient.

Figure 6.3.2 Localization of the 36 kDa polypeptide after 6-20% [w/v] SDS-PAGE and autoradiography

Tracks:- 1) Whole cell lysate prior to fractionation. 2) *Synechococcus* WH 7803 whole cells grown for 44 hr in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. 3)*Synechococcus* WH 7803 whole cells grown for 44 hr in normal medium. 4) Cytoplasmic fraction. 5) Internal membrane fraction. 6) Cell wall fraction.



procedure for *Synechocystis* PCC 6714 (Omata & Murata, 1984) did not yield isolated cytoplasmic membranes from *Synechococcus* WH 7803. Therefore, the alternative procedure described above was used. Samples from the fractionation procedure were analysed by SDS-PAGE and autoradiography. Figure 6.3.2 shows the presence of the 36 kDa protein in the internal membrane fraction (track 5) and the cell wall fraction (track 6), the protein was not apparent in the cytoplasmic fraction (track 4). Track 2 and 3 are control samples of *Synechococcus* WH 7803 cells grown for 44 hr in normal medium containing 15 μg ml^{-1} EDDA and normal medium respectively. Track 1 contains the whole cell lysate sample prior to fractionation. The internal membrane fraction (IM) was green in colour due to the presence of chlorophyll from the solubilized thylakoid membrane.

6.4 Purification of the 36 kDa protein from the internal membrane fraction

The internal membrane (IM) fraction was chosen for further purification of the 36 kDa protein as it was the major polypeptide in this sample, there were fewer contaminating proteins compared to the cell wall fraction, and the membranes required no further solubilization treatment prior to loading onto either the MonoQ/HiloadQ anion exchange columns.

The 36 kDa protein was purified from the IM fraction by anion exchange chromatography (see Materials and Methods), and the protein eluted from the MonoQ column over a salt concentration gradient of about 20-40% [w/v] (Figure 6.4.1a) and 44-50% [w/v] from the HiloadQ column. SDS-PAGE analysis and autoradiography show the protein to be present in fractions 15-19 from the MonoQ column (Figure 6.4.1b).

Comparison of the same elution fractions of protein isolated from cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA and normal medium were made (Figure 6.4.2). Figure 6.4.2 shows that constitutive levels of the 36 kDa protein, were present in cells growing in normal medium (track 1). However, there was more of this protein present when the cells were grown under iron-deplete conditions (normal medium containing 15 $\mu g\ ml^{-1}$ EDDA) (track 2), therefore, it is likely that synthesis of this protein is derepressed under conditions of iron limitation.

The presence of the 36 kDa polypeptide in both the IM fraction and the cell wall fraction (Figure 6.3.2) could be attributed to the following reasons: the cell wall location may be an artifact as a result of incomplete Triton-X-100 solubilization of the internal membranes, the protein may in fact be loosely associated with the peptidoglycan layer of the cell wall being partially removed by Triton-X-100 extraction, or alternatively, the protein may span the

Figure 6.4.1 Purification of the 36 kDa polypeptide from the internal membranes of *Synechococcus* WH 7803 cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA

a) Protein elution profile obtained when the internal membrane fraction was loaded onto a MonoQ column (see Materials and Methods). b) 6-20% [w/v] SDS-PAGE and autoradiography of fractions from the MonoQ column which coincide with the peak in a above. Tracks 1) Internal membranes. 2) Fraction 15. 3) Fraction 16. 4) Fraction 18. 5) Fraction 19. Equal volume loaded per track.

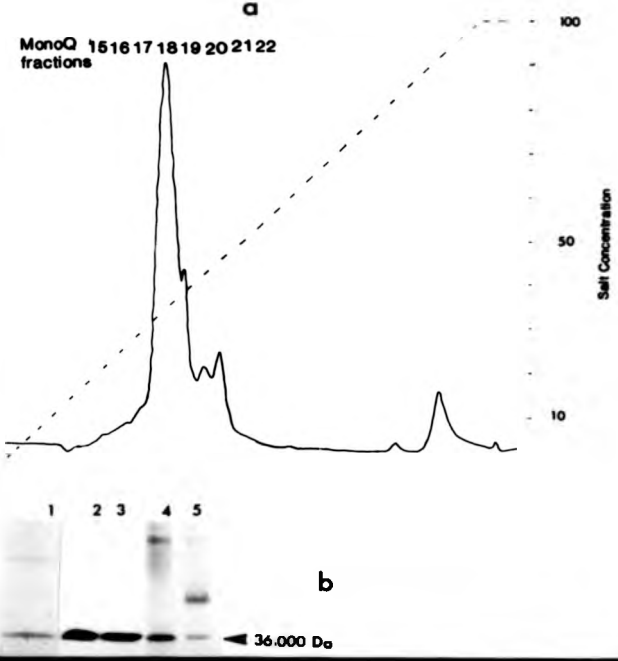
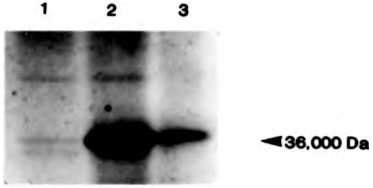


Figure 6.4.2 **A comparison of equivalent purification fractions containing protein isolated from *Synechococcus* WH 7803 cells grown in iron -replete and iron-deplete medium**

Tracks:- 1) Fraction 15 containing protein isolated from cells grown in normal medium. 2) Fraction 15 containing protein isolated from cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. 3) Internal membranes from cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. Equal volume loaded per track.



periplasmic space being attached to both the cytoplasmic membrane and the cell wall.

The gene in *E. coli* and *S. typhimurium* for such a protein, TonB, has been cloned and sequenced (Postle & Good, 1983; Hannavy *et al.*, 1990).

6.5 Putative function of the 36 kDa iron-regulated membrane protein

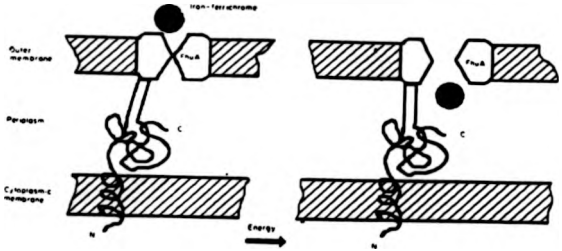
TonB has been shown to be required for vitamin B_{12} transport, the action of several colicins, the irreversible binding step in bacteriophage T1 and ϕ80 infection and iron-siderophore uptake (Hancock & Braun, 1976; Reynolds *et al.*, 1980; Postle & Good, 1983). The expression of *tonb* is controlled by iron availability mediated by the Fur repressor (Bagg & Neilands, 1987_b). TonB has a M_r= 26,000, predicted by its nucleotide sequence, however, its mobility through SDS-PAGE has resulted in a variation in reported M_r ranging from 36,000-40,000 (Plastow & Holland, 1979; Postle & Reznikoff, 1979). This variation is probably due to an extremely high x-proline region within the TonB protein which forms a highly rigid structure, being inflexible under denaturing conditions (Brewer *et al.*, 1990). The N-terminus of the TonB protein is anchored within the cytoplasmic membrane and the signal sequence remains uncleaved, possibly to aid in anchorage (Dalbey & Wickner, 1985; Postle & Skare, 1988; Hannavy *et al.*, 1990). The remaining portions of the protein spans the

periplasm interacting specifically with the FhuA iron-siderophore receptor protein in the outer membrane (Brewer *et al.* 1990). Interaction with the FhuA protein requires the highly rigid lysine-proline repeat. Brewer *et al.*(1990) have found that the presence of the glutamate-proline and lysine-proline repeats adopt a rigid conformation which is resistant to enzymic cleavage and denaturation.
The rigid nature of this section of the TonB molecule plus the fact that these two repeats are contained within a highly conserved region of the amino acid sequence suggests that this conformation has a functional role. Hannavy *et al.* (1990) proposed a model whereby TonB is able to act as an energy transducer between the outer membrane transport receptors and the inner membrane. This is facilitated by a conformational change within the highly conserved and rigid x-proline region of the polypeptide. It is in this way that ferri-siderophore complexes are transported across the outer membrane into the preriplasm in a TonB-dependent fashion (Figure 6.5).

Ton B is not an abundant protein, the protein cannot be observed on SDS-PAGE after Coomassie staining without prior concentration of the membranes. Postle & Skare (1988), have shown the protein to have a half life of 10 min at 42°C and Hannavy *et al.* (1990) observed that it is present at only a few copies per cell.

Figure 6.5 **A schematic model for TonB function. (After Hannavy *et al.* 1990)**

TonB is anchored to the cytoplasmic membrane via the N terminus, the bulk of the protein, including the rigid X-Proline region, spans the periplasm interacting with the outer membrane receptors (e.g FhuA). Conformational changes at the inner membrane, induced by energization of the cell, are transmitted to the outer membrane receptor via the rigid X-Proline region. This results in a conformational change in the outer membrane receptor enabling irreversible adsorption of certain phage and facilitates uptake of iron-siderophore complexes. C = carboxy terminus, N = amino terminus.



The 36 kDa protein identified in *Synechococcus* WH 7803 has certain characteristics similar to that of TonB isolated from *E. coli* and *S. typhimurium*, being shown to be located in both the cytoplasmic membrane and cell wall and cannot be identified on Coomassie stained gels without prior sample concentration. ^{35}S-methionine labelling allows for a more sensitive method of detection for this non-abundant protein. Whether this 36 kDa iron-regulated membrane protein is TonB has been investigated in the following three ways:-

6.5.1 Amino terminal sequencing

Samples containing the 36 kDa iron-regulated membrane protein from either unpurified internal membrane fractions or fractions from the anion exchange column were prepared for N-terminal sequencing (see Materials and Methods). Initial sequence analysis of the purified 36 kDa protein has been prevented by blockage of the amino terminus. As a result no conclusions could be made as to whether the N-terminal sequence shows any homology to TonB. A blocked N-terminus prevents the labelling of the amino terminal residue with phenylisothiocyanate, the initial step in the Edman degradation reaction. However, enzymic cleavage using endoproteinase Glu-C (V_8 protease) EC 3.4.21.19, from *Staphylococcus aureus* V_8, which cleaves at either Glu-Y or Asp-Y residues would allow for

further sequence analysis, either by the Edman degradation reaction or mass spectroscopy which would also identify the nature of the modified residue.

Transport of proteins across the cell membrane is usually accompanied by cleavage of the signal peptide. However, as is the case for TonB in *S. typhimurium* and *E. coli*, the signal sequence of this protein may not have been cleaved (Dalbey & Wickner, 1985).

6.5.2 Western blot analysis

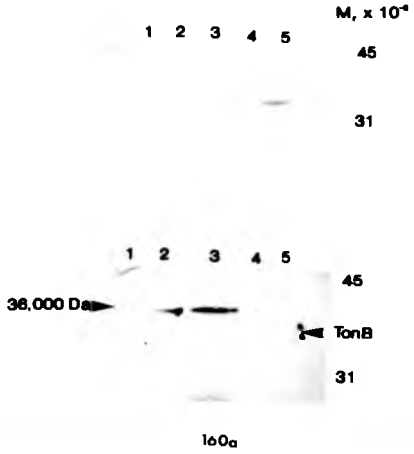
Western blot analysis was utilized in order to ascertain whether the 36 kDa protein was similar to the TonB protein identified in *S. typhimurium*. The antibody used had been raised against a 33 residue synthetic peptide incorporating the two x-proline dipeptide repeats of the TonB protein (Hannavy *et al.* 1990). The presence of a cross-reacting band was investigated in crude IM samples and purified samples known to contain the protein of interest isolated from *Synechococcus* WH 7803. In addition two control samples were included, (Figure 6.5.2.1a) track 5 contains whole cell protein from *E. coli* MC 1061 which has been transformed with plasmid, pCH143, containing the *S. typhimurium tonb* gene. The *tonb* gene was present upon an *Eco*RI-*Bam*HI fragment which had been cloned into the Bluescript ks$^+$ vector (Hannavy *et al.*, 1990), (gene and

vector constitute pCH143). Likewise, track 4 contained whole cell protein from *E. coli* CH 483 also containing pCH143. The plasmid, pCH143, being a high copy number plasmid, resulted in over expression of the *tonb* gene within these strains. Consequently, there were large amounts of this protein produced which gave a positive result with the anti-TonB antibody at a 1:500 dilution. Tracks 1 and 2 consist of anion exchange fractions 18 and 15 respectively containing purified protein from IM of cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. No cross-reacting protein to the anti-TonB antibody was apparent in the samples from *Synechococcus* WH 7803 (tracks 1-3). In addition autoradiography of the nitrocellulose filter showed the 36 kDa protein of interest to have a slightly slower mobility through the original SDS-PAGE (Figure 6.5.2.1b) compared with the TonB protein identified by the antibody in the two control tracks. However, this does not mean to say that the protein of interest is not TonB, the fact that the antibody did not recognise a similar protein in *Synechococcus* WH 7803 may be due to either:

a) a low antibody titre as a result of being raised to a synthetic polypeptide, prepared from part of the TonB protein rather than the whole protein. This antibody is unable to identify TonB expressed at the chromosomal level in *S. typhimurium* (K. Hannavy, personal communication), over expression of the *tonb* gene in *E. coli*, however, allowed effective use of the antibody.

Figure 6.5.2.1 Western blot analysis using an antibody raised against the x-proline dipeptide repeat of the TonB protein

a) Tracks :- 1) MonoQ fraction 18 from *Synechococcus* WH 7803 cells grown in normal medium plus 15 $\mu g\ ml^{-1}$ EDDA. 2) MonoQ fraction 15 from *Synechococcus* WH 7803 cells grown in normal medium plus 15 $\mu g\ ml^{-1}$ EDDA. 3) Internal membrane samples from *Synechococcus* WH 7803 cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. 4) Whole cell protein sample from *E.coli* CH 483 containing pCH143. 5) Whole cell protein sample from *E.coli* MC 1061 containing pCH143. Equal volume per trac loaded. b) Autoradiogram of the nitrocellulose filter after western blot analysis. Tracks 1-5 are as described for a above. The presence of the cross-reacting TonB protein from both *E.coli* strains is arrowed.



b) Not enough putative protein being present in order to allow detection by the antibody. Unfortunately, as the majority of the protein was required for N-terminal sequencing and antibody production the requirement of a greater protein loading for western blot analysis was not investigated.

6.5.3 Southern hybridization analysis

A) Oligonucleotide probing

A seventeen-mer oligonucleotide was synthesized to the conserved lysine-proline dipeptide repeat of the TonB protein known to be present in *S. typhimurium* and *E. coli* (Brewer *et al.*, 1990). The oligonucleotide sequence was :-

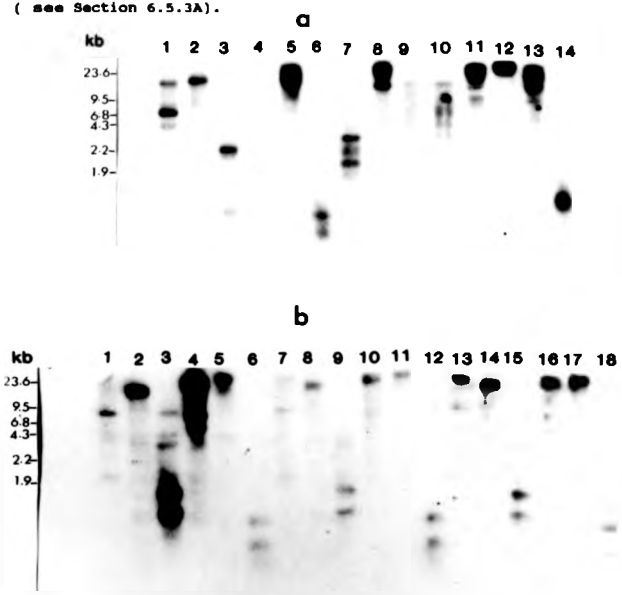
LYS	PRO	LYS	PRO	LYS	PRO
AAA	CCG	AAA	CCG	AAA	CC
	A		A		
	T		T		
	C		C		

This oligonucleotide was radiolabelled (see Materials and Methods) and hybridized at 41°C for 18 hr. The filters contained chromosomal DNA from various marine *Synechococcus* sp. which had been restricted with a number of different enzymes (Figure 6.5.3.1a and b).

Figure 6.5.3.1 Detection of the *tonb* gene in *Synechococcus* sp. using an oligonucleotide synthesized to the conserved lysine-proline dipeptide repeat of the TonB protein

a) Tracks 1-8 :- *Synechococcus* WH 7803 chromosomal DNA restricted with:- *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Kpn*I, *Hin*fI, *Xho*I, *Xba*I respectively. Tracks 9-14:- *Synechococcus* PCC 7002 chromosomal DNA restricted with:- *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Kpn*I, *Hin*fI respectively.

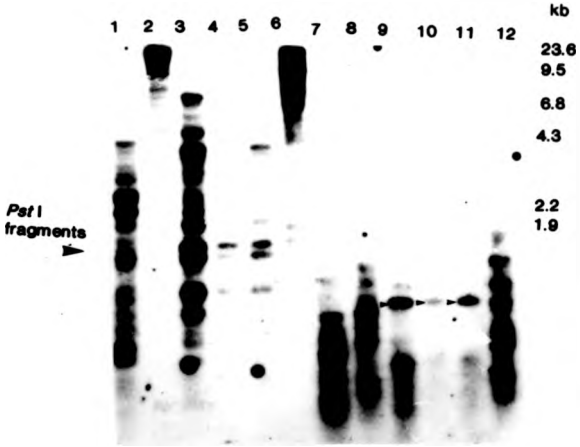
b) Tracks:- 1-6: *Synechococcus* WH 8008, 7-12: *Synechococcus* WH 7805, 13-18: *Synechococcus* WH 8018 restricted with:- *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Kpn*I, *Hin*fI respectively. The hybridization and washing conditions are specified in the text (see Section 6.5.3A).



The oligonucleotide recognised specific fragments in all the strains studied. The most obvious being two doublets of different sizes (< 1.9 Kb in size) when restricting with *Pst*I and / or *Hinf*I (Figure 6.5.3.1a and b). The *Pst*I fragments obtained from *Synechococcus* WH 8008 chromosomal DNA gave a strong response. On examination of comparable tracks (after hybridization at 42°C for 18 hr) the doublet obtained by restriction with *Pst*I could again be observed for all the marine Synechococcus sp. except *Synechococcus* WH 7803 and *Synechococcus* PCC 7002 (Figure 6.5.3.2). The chromosomal DNA of all the strains used in this Southern blot analysis, except *Synechococcus* WH 7803 and *Synechococcus* PCC 7002, showed similar RFLP patterns, this is in accordance with the RFLP data of Douglas & Carr (1988). However, *Synechococcus* PCC 7002, not included in Douglas and Carr's study, does not contain PE, is found in coastal waters and is not obligately marine. Therefore, it is not surprising that this strain shows a different banding pattern with the oligonucleotide. The doublet, originally seen in all the cyanobacteria studied (Figure 6.5.3.1a and b), on restriction with *Hinf*I has been replaced by a single band in tracks 9-11 (arrowed) (Figure 6.5.3.2) for *Synechococcus* WH 8008, WH 7805, WH 8018, although the doublet has also disappeared for *Synechococcus* WH 7803 and PCC 7002 the cross-reacting bands in comparison to the other three strains are also of a different size. The doublet obtained on restriction with *Pst*I (Figures:

Figure 6.5.3.2 Detection of the *tonb* gene in *Synechococcus* sp. using an oligonucleotide synthesized to the conserved lysine-proline dipeptide repeat of the TonB protein

Chromosomal DNA from *Synechococcus* WH 7803, PCC 7002, WH 8008, WH 7805, WH 8018 respectively was restricted with *Pst*I, tracks 1-5, and *Hinf*I, tracks 7-11. Tracks 6 and 12 contain *E.coli* K12 chromosomal DNA restricted with *Pst*I and *Hinf*I respectively. Hybridization and washing conditions are specified in the text (see Section 6.5.3A).



6.5.3.1a and b, 6.5.3.2) could be due to the oligonucleotide recognising more than one sequence containing a lysine-proline dipeptide repeat, or alternatively, there may be two genes present. The presence of non-specific binding on the filters was due to non-stringent washing conditions, which were:- two washes in 6xSSC (3 M NaCl, 0.3 M Na citrate = 20xSSC), 0.1% SDS over 55 min. at 41°C (Figures 6.5.3.1a,b) and two washes in 6xSSC, 0.1% SDS over 40 min at 42°C (Figure 6.5.3.2).

Although plaque lifts were attempted in order to obtained the gene fragment of interest using the oligonucleotide probe, attempts proved unsuccessful as the washing conditions were such that non-specific binding to the filters prevented identification of possible clones.

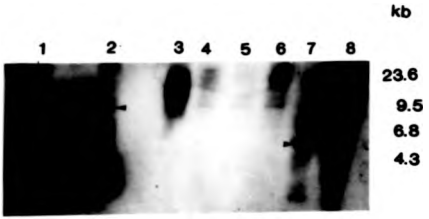
B) Probing with the *tonb* gene

The plasmid, pCH143, containing the *tonb* gene was isolated from *E. coli* MC 1061 via the alkaline lysis method (see Materials and Methods). This strain was used to obtain pCH143 as a greater yield was obtained compared to *E. coli* CH483. Restriction of the plasmid with *Bam*HI and *Eco*RI followed by agarose gel electrophoresis resulted in isolation of the *tonb* gene. The gene was prepared for radiolabelling prior to DNA-DNA hybridization studies.

Filters containing chromosomal *Synechococcus* sp. DNA (Figure 6.5.3.3) restricted with *Hind*III were hybridized at 55°C in 5xSSPE, 5x Denhardt's and 0.1% SDS for 18 hr, and washed with increasing stringency to find the conditions required to remove non-specific binding in the following fashion :- a) 6xSSC, 0.1% SDS, 2 x 30 min, room temp. b) 2xSSC, 0.1% SDS, 15 min, room temp and then 20 min, 55°C. c) 1xSSC, 0.1% SDS, 30 min, room temp and then 30 min, 50 °C. The results after autoradiography show a possible hybridizing, 4.9 kb, *Hind*III fragment, from *Synechococcus* PCC 7002 (track 7). There may also be potential hybridizing *Hind*III fragments from the Puc19 libraries of *Synechococcus* PCC 7942 (8.2 kb) and *Synechococcus* WH 7803 (8.9 kb), tracks 1 and 2 respectively, however, a greater washing stringency is required to remove the non-specific binding which would allow for a better detection of these bands. The same situation is apparent for the hybridizing fragments from chromosomal DNA from four strains of marine *Synechococcus* sp (tracks 3-6). In addition, the *tonb* gene also showed hybridization with the pUC19 fragment of the pUC19 libraries (tracks 1 and 2), this is probably because the bluescript ks^+ vector from which the gene was isolated probably contains homologous sequences to pUC19. Track 8 (Figure 6.5.3.3, control track) contains *E. coli* K12 chromosomal DNA, a 10.4 kb *Hind*III fragment (arrowed) was identified with the *tonb* gene.

Figure 6.5.3.3 Detection of the *tonb* gene in *Synechococcus* sp using the *tonb* gene from *S. typhimurium*

Filters were probed with a 1.3 kb *Bam*HI, *Eco*RI fragment containing the *tonb* gene. All chromosomal DNA samples were restricted with *Hind*III. Tracks:- 1) *Synechococcus* PCC 7942 chromosomal DNA :: pUC19. 2) *Synechococcus* WH 7803 chromosomal DNA :: pUC19. 3) *Synechococcus* WH 7803 chromosomal DNA. 4) *Synechococcus* WH 7805 chromosomal DNA. 5) *Synechococcus* WH 8008 chromosomal DNA. 6) *Synechococcus* WH 8018 chromosomal DNA. 7) *Synechococcus* PCC 7002 chromosomal DNA. 8) *E.coli* K12 chromosomal DNA. Hybridization and washing conditions are specified in the text (see Section 6.5.3B).



Southern hybridization studies need to be repeated in order to optimise the washing stringencies required. When filters containing chromosomal DNA from *Synechococcus* WH 7803, *Synechococcus* PCC 7002 were washed initially in 1xSSC, 0.1% SDS for 30 min at 50°C, the conditions were found to be too stringent. The previous inability of this washing stringency to remove non-specific binding from the filter (see above) may have been due to irreversible binding of the probe to the nitrocellulose.

As a 4.9 kb *Hind*III fragment of *Synechococcus* PCC 7002 chromosomal DNA shows cross hybridization with the *E. coli tonb* gene, isolation of a *tonb* gene fragment from *Synechococcus* PCC 7002 could then be used to probe marine *Synechococcus* chromosomal DNA and may therefore be more successful in identifying an homologous gene.

6.6 Is the *irpA* gene of *Synechococcus* PCC 7942 also present in *Synechococcus* WH 7803 ?

A 36 kDa iron-regulated membrane protein has also been identified in *Synechococcus* PCC 7942 (Reddy *et al.*, 1988). Triton-X-114 fractionation has shown it to be an intrinsic membrane protein (Reithman & Sherman, 1988). However, western blot analysis has also localized it to the cell wall fraction (D. Scanlan- personal communication). This *irpA* gene has also been cloned and sequenced (Reddy *et al.*, 1988). The 2.4 kb

irpA gene probe was isolated from pKJ110 in the same way as described for the *tonb* gene present in pCH143, (see Section 6.5.3.3) and used to probe nitrocellulose filters containing chromosomal DNA from *Synechococcus* WH 7803, *Synechococcus* PCC 7002 and *Synechocystis* PCC 6803. Despite the use of variable hybridization (5xSSPE, 65°C, 18 hr; 5xSSC, 55°C, 18 hr; 5xSSPE, 55°C, 18 hr) and washing conditions (2xSSPE, 30 min, room temp then at 65°C, 15 min; 5xSSPE, 60 min, 55°C and then 1xSSPE, 10 min, 55°C) it proved impossible to obtain a DNA fragment recognised by the *irpA* gene in the marine *Synechococcus* WH 7803, WH 8008, WH 7805 and *Synechococcus* PCC 7002. A cross reacting *Pst*I fragment was identified in *Synechococcus* PCC 6301 about 1.7 kb in size (Figure 6.6.1) which is identical to that described for *Synechococcus* PCC 7942 (Reddy *et al.*, 1988). This is not surprising, however, as both of these strains are very closely related. The 36 kDa protein from *Synechococcus* WH 7803, may posses the same function as the 36 kDa iron-regulated protein A from *Synechococcus* PCC 7942 but have a different amino acid sequence. If this protein does exhibit the same functional role as that of the protein identified in *Synechococcus* PCC 7942 it may well be that the gene would also contain a sequence homologous to the *fur* operator of *E. coli* similar to that found in *Synechococcus* PCC 7942 (Reddy *et al.*, 1988). It is to this sequence that the Fur repressor-Fe^{++} complex would bind. Identification of such a sequence would be an

Figure 6.6.1 **Detection of the *Synechococcus* PCC 7942 *irpA* gene in *Synechococcus* sp**

Filters were probed with a 2.4 kb *Eco*RI fragment containing the *irpA* gene. Tracks 1-8) *Synechococcus* WH 7803 chromosomal DNA restricted with:- *Eco*RI, *Hind*III, *Sal*I, *Bgl*II, *Xba*I, *Xho*I, *Pst*I, *Kpn*I respectively. Tracks 9-12) *Synechococcus* PCC 6803 chromosomal DNA restricted with:- *Eco*RI, *Hind*III, *Sal*I, *Pst*I respectively. Track 13) *Synechococcus* PCC 7942 restricted with *Hind*III. Hybridization and washing conditions used are as specified in the text (see Section 6.6).

initial indication that a response to low iron levels in marine *Synechococcus* WH 7803 may be regulated by a Fur operon. However, as yet no *fur* genes have been identified in marine Synechococcus sp. (Rueter & Unsworth, 1992)

6.7 Western blot analysis using anti-36 kDa protein antibodies

Antibodies were raised against the 36 kDa polypeptide with a view to eventually using them as diagnostic markers for the detection of iron-limited *Synechococcus* sp. cells in natural marine environments. Hopefully, in the long term it should be possible to obtain both temporal and spatial data on the occurrence of iron limitation within these cell populations in the ocean.

Western blot results using the antibody raised against the 36 kDa iron-regulated protein from *Synechococcus* WH 7803 showed that it was able to detect the protein in both whole cell samples, internal membrane samples and cell wall samples (Figures 6.7.1, 6.7.2), at a 1:300 dilution from *Synechococcus* WH 7803. The antibody was able to detect the protein in both the cell envelope and *Synechococcus* WH 7803 whole cells grown in iron-replete and deplete medium (including cells grown in the presence of 15 $\mu g\ ml^{-1}$ EDDA) (Figure 6.7.1, 6.7.2), but not in *E. coli* cells containing the

over expressed TonB protein. The antibody did not cross react with any similar size proteins from whole cell extracts from the freshwater cyanobacteria *Synechococcus* PCC 7942, *Nostoc sp.* MAC PCC 8009 or the marine *Synechococcus* WH 8103 grown in normal medium (Figure 6.7.2), however, cross reacting protein bands were apparent in whole cell extracts from *Synechococcus* PCC 7002 (*Agmenellum quadruplicatum*) (Figure 6.7.2). The high molecular weight bands (>45 kDa in size) are present in the pre-immune serum. Further analysis needs to be achieved as to whether any cross-reacting proteins are also present in any other marine *Synechococcus* sp. This is an important piece of information if the antibody is to be used as a diagnostic marker for marine *Synechococcus* sp. The fact that the antibody was able to detect the 36 kDa protein in cell walls, internal membranes (equal protein loaded) (Figure 6.7.1) and whole cells from *Synechococcus* WH 7803 grown in iron-replete/deplete medium (Figure 6.7.1., 6.7.2) suggests that the use of this antibody to monitor iron limitation may not be practical. Whereas ^{35}S-methionine labelling was able to show a difference in protein synthesis between conditions of iron-replete/deplete growth the antibody was not. ^{35}S-methionine was able to show alterations in the amounts of the protein being synthesized in iron-deplete conditions compared to the amount synthesized at a constitutive level under conditions of normal growth, however, an increase in synthesis of the

Figure 6.7.1 **Detection of the 36 kDa polypeptide in *Synechococcus* WH 7803 internal membrane and cell wall samples using western blot analysis**

Tracks:- 1) Internal membranes from cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. 2) Internal membranes from cells grown in normal medium. 3) Cell walls from cells grown in normal medium containing 15 $\mu g\ ml^{-1}$ EDDA. 4) Cell walls from cells grown in normal medium. Equal protein loaded per track.

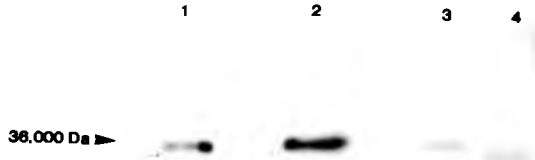
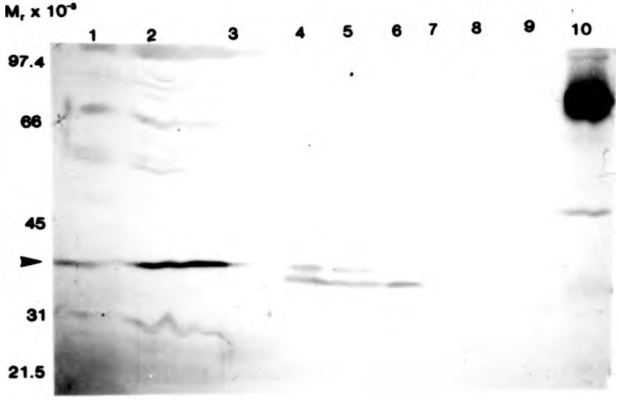


Figure 6.7.2 **Detection of the 36 kDa polypeptide in whole cell samples of different cyanobacteria grown in iron replete and iron deplete medium using western blot analysis**

Tracks:- 1) *Synechococcus* WH 7803 grown in normal medium. 2) *Synechococcus* WH 7803 grown in chelator-deficient medium. 3) *Synechococcus* WH 7803 grown in normal medium containing 15 μg ml^{-1} EDDA. 4) *Synechococcus* PCC 7002 grown in normal medium. 5) *Synechococcus* PCC 7002 grown in chelator-deficient medium. 6) *Synechococcus* PCC 7002 grown in normal medium containing 50 μg ml^{-1} EDDA. 7) *Synechococcus* PCC 7942 grown in normal medium. 8) *Nostoc sp. MAC* PCC 8009 grown in normal medium. 9) *Nostoc sp. MAC* PCC 8009 grown in chelator-deficient medium. 10) *Synechococcus* WH 8103 grown in normal medium. Equal protein as a function of $OD_{750\ nm}$ was loaded per track.



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36 kDa polypeptide was most noticeable in the late exponential growth phase in cells growing in normal medium. The antibody appears to provide a sensitive detection method for this protein, however, the point of a cells growth must also be taken into consideration when using the antibody to discriminate between cells grown in iron-replete and deplete medium. This may be impractical when studying natural populations. Such an experiment has not been conducted whereby the antibody has been used to monitor the presence of the protein during *Synechococcus* WH 7803 growth cycle in iron-replete/deplete medium, in order for this to be achieved large quantities of cell material may be required. This is because a faint positive band was obtained when using the antibody to detect a 36 kDa protein band in cell wall samples containing 50 µg total protein from iron-replete and deplete cells (Figure 6.7.1a).

6.8 Conclusions

Localization of the 36,000 Da polypeptide has shown it to be present in both the internal membranes and the cell walls, using ^{35}S-methionine labelling, detergent extraction and differential centrifugation, but not the periplasm, suggesting that it does not function as a periplasmic binding protein. The protein appears to be constitutively expressed in cells grown

in normal medium in addition to being present in cells growing in iron-deplete medium. This has been highlighted by both western blot analysis (Figure 6.7.1) and ^{35}S-methionine labelled protein patterns of internal membranes purified by anion exchange chromatography (Figure 6.4.2). However, ^{35}S-methionine labelling suggests that the synthesis of this polypeptide is derepressed under iron-deplete conditions. Iron-deplete conditions in normal medium when growing cells approach stationary growth may explain the synthesis of this protein at this time. Such a response is seen to occur with TonB (Postle, 1990). The use of an equilibrium dialysis chamber together with a radiolabelled iron tracer may help to determine the effect of iron availability on these organisms during their period of growth.

Experiments to determine whether the 36 kDa polypeptide is a TonB homologue have as yet proved inconclusive. Probing with the *tonb* gene may indicate possible cross reacting fragments in the strains studied although further work to optimize the experimental conditions using the *tonb* gene probe is required.

Oligonucleotide probing using a conserved region of the *tonb* gene has also suggested the presence of a *tonb* homologue in *Synechococcus* sp., although the use of a conserved region such as this proves difficult to

determine if it is a *tonb* homologue and not another DNA fragment exhibiting this particular motif. Hopefully probing with the *tonb* gene will provide an answere.

Once the N-terminal blockage has been removed, analysis of the remaining fragment will yield sequence information which should contribute significantly to the elucidation of the function of the 36 kDa polypeptide. Furthermore, use of a synthetic pentapeptide (Glu-Thr-Val-Ile-Val) which is known to be a recognition TonB-box, present in TonB-dependent processes (Tuckman & Osburne, 1992), may establish whether growth of *Synechococcus* WH 7803 in iron-deplete medium requires a TonB-dependent process. It would not show whether the 36 kDa polypeptide was TonB, only that a TonB-dependent process was in effect.

Western blot analysis using antibodies raised against a synthetic peptide of the conserved Glu-Pro, Lys-Pro dipeptide repeats was unable to identify a cross reacting protein in *Synechococcus* WH 7803. This was probably due to the low antibody titre, which is unable to detect TonB expressed at the chromosomal level in *S. typhimurium*. Therefore, a larger protein loading for western blot analysis is probably required.

No homology was seen with the *irpA* gene, encoding a 36 kDa polypeptide localized to both the cell wall and internal membrane of *Synechococcus* PCC 7942 (Reddy

et al., 1988) on Southern blots containing chromosomal DNA from marine *Synechococcus* sp. Using *Synechococcus* WH 7803 chromosomal DNA to complement a *Synechococcus* PCC 7942 *irpA* mutant (Reddy *et al.*, 1988) may show that two different genes encompass the same protein function.

Unfortunately western blot analysis of the whole cell samples using the antibody raised against the *Synechococcus* WH 7803 36 kDa polypeptide has shown that the use of this antibody to monitor iron limitation in natural picoplankton assemblages may not be as straight forward as initially thought. Mainly because it is able to detect protein in normal cells and iron-deplete cells, visually to a similar degree. High cell numbers were required to give positive results and because of this cultures in late exponential growth were harvested, explaining the presence of the protein in normal-grown cells. Further work needs to be conducted to determine if this is the fact. Even so, such a large cell number requirement ($8x10^9$ cells) would make the use of this antibody difficult at sea. *Synechococcus* sp. cell numbers are about $5x10^7$ cells l^{-1} during a bloom when nutrient conditions are favourable, under these circumstances approximately 100 litres of seawater would need to be filtered for antibody analysis. However, samples of picoplankton cells from non-productive waters would require even larger volumes of seawater.

CHAPTER 7
FINAL CONCLUSIONS

Concluding Remarks

This thesis has been concerned with the elucidation of the mechanisms by which the marine cyanobacterium *Synechococcus* WH 7803 is able to sequester iron under iron-deplete conditions. During the course of this study ^{35}S-methionine labelling was used as a means to successfully label proteins *in vivo*. ^{35}S-methionine uptake and protein labelling was developed as a means of detecting specific protein synthesis. This technique has not been previously documented for marine cyanobacteria and is frequently not applicable to freshwater cyanobacterial species. Its valuable use as a sensitive tool for protein labelling is apparent. However, the fact that marine *Synechococcus* sp. show light stimulated incorporation of amino acids (Paerl, 1991) suggests important implications for the role of amino acids or peptides as possible nitrogen or even carbon sources in the natural environment.

Iron-regulated membrane proteins have been observed in the cell wall of *Synechococcus* WH 7803 under iron-deplete conditions. Such proteins are assumed to be involved in iron acquisition mechanisms. The majority are of a higher M_r than those described for the freshwater species *Synechococcus* PCC 7942. However, one polypeptide M_r=36,000 has both a similar size and location to the IrpA protein found in *Synechococcus* PCC 7942 (Reddy *et al.*, 1988), although no *irpA* homologue has been identified in marine *Synechococcus* sp. However, complementation analysis of the *Synechococcus* PCC 7942 *irpA*

mutant with *Synechococcus* WH 7803 chromosomal DNA may show that a different gene encodes a polypeptide with a similar function.

The 36,000 Da polypeptide of *Synechococcus* WH 7803 may play a central role in iron acquisition as it has been shown to be derepressed between 21-48 hr growth in iron-deplete medium and repressed after 24 hr growth in iron-replete medium. ^{35}S-methionine labelling highlights the increased synthesis of this protein under conditions of iron deprivation. Its putative function as a TonB homologue is still to be clarified. The process of iron uptake is largely unknown in cyanobacteria and may be a TonB dependent process. The use of an oligonucleotide to establish if such a gene is present in *Synechococcus* sp. was facilitated by the presence of a highly conserved x-proline rigid motif in the TonB proteins of both *E. coli* and *S. typhimurium*. An oligonucleotide probe constructed to this motif has enabled the detection of a putative *tonb* homologue in various cyanobacterial species. Whether a functional TonB protein would be required for a non-siderophore-mediated high affinity iron uptake system would require further investigation once the mechanism of iron uptake in this organism has been determined. The use of a synthetic pentapeptide containing a TonB consensus box sequence would help to determine if TonB is involved in iron acquisition in *Synechococcus* WH 7803. The presence of a functional TonB protein is more likely in *Synechococcus* PCC 7002, this cyanobacterium synthesizes and secretes siderophores under iron-deplete conditions as well

having a requirement for vitamin B_{12}. The uptake mechanisms of both compounds have been shown to require a functional TonB.

Bacterioferritin has recently been isolated from the freshwater cyanobacterium *Synechococystis* PCC 6803 (Laulhere *et al.*, 1992). Furthermore, Mössbauer spectroscopy and western blot analysis has shown that not only do *Synechococcus* WH 7803 cells sequester iron but that they also contain an iron storage protein similar to bacterioferritin.

The presence of high affinity iron-transport systems and iron storage proteins in natural assemblages of *Synechococcus* sp. would be crucial to both the existence and maintenance of an iron concentration gradient especially when the concentration of available iron in the surrounding seawater is so low. However, siderophore production as a means of iron sequestration would result in the unfavourable loss of cell material into a turbulent environment. Therefore, iron sequestration may be manifest in a system which contains an iron-reductase protein with the ability to remove Fe^{+++} directly from colloidal particles or debris associated with the cell wall, by its reduction to Fe^{++}. The existence of such a system still remains to be established.

Unfortunately, the antibody produced against the polypeptide of M_r 36,000 could not be used as a diagnostic marker in the natural environment against iron limitation. Identification of the possible presence or absence of iron

storage proteins under iron-replete/deplete conditions may be an alternative approach. Research has shown that iron storage proteins are synthesized in response to increased iron regimes. Characterization of the genes involved in iron acquisition in *Synechococcus* WH 7803 would allow for the identification of further proteins to be used as diagnostic markers in the natural environment.
The study of mRNA transcription of the appropriate iron acquisition genes would be an alternative approach to studying the response of natural assemblages to iron-deplete conditions. However, application of northern hybridization techniques to natural assemblages would as yet prove difficult to conduct. An abundant message would be required and even then the sampling and filtration times would have to be greatly reduced. Such reduction may be impractical depending upon the quantity of cells required to give the correct yield of mRNA.

The use of diagnostic markers for a range of nutrient limitations would help to divulge how natural assemblages are able to respond to low external nutrient concentrations, as well as ending the controversy upon what limits phytoplankton productivity in the open ocean. Furthermore, of additional significance would be to show the presence of two component sensory systems. Such systems show a stimulus-response coupling where external information from sensory receptors is transferred to a circuitry of regulatory proteins. These regulatory proteins transfer high energy phosphoryl groups from histidine to aspartate side chains (Stock *et al.*, 1990). Identification of such systems would be the first step in the

elucidation of how changes in the natural environment is communicated to a cell which then responds to that change.

I feel that the research described here has illustrated the limitation of conventional techniques used routinely to determine nutrient limitation in natural phytoplankton assemblages. Moreover, this work has established that molecular biology may provide an alternative and more conclusive route to identifying the nature of nutrient limitation at sea. It is hoped therefore, that in future the application of such powerful techniques will be used routinely in the natural marine environment.

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