Diversity and Production of Phytoplankton in the Offshore Mississippi River Plume and Coastal Environments

by

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DEDICATION

To all those people who search
  Who ask
  And who wonder
Why all of the things that are
  Are the way they are
  And in their wonder
  Can’t help but ask
Why they are searching
And why they are asking
And why they wondering
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Diversity and Production of Phytoplankton in the Offshore Mississippi River Plume and Coastal Environments

Boris Wawrik

ABSTRACT

River discharge leads to extensive phytoplankton blooms often observed in ocean color satellite images to extend far into the open ocean as high chlorophyll plumes. We investigated diversity, distribution and ecology of phytoplankton populations in the Mississippi River plume, both spatially and in the water column using molecular tools. A method was developed for the quantification of diatom/pelagophyte \( rbcL \) (large subunit of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase) mRNA using quantitative PCR and applied to cultures and in the plume. The vertical structure of phytoplankton species in the Mississippi River plume was described by flow cytometry, pigments, \( rbcL \) mRNA and \( rbcL \) cDNA libraries. High productivity in the plume was associated with a large population of \textit{Synechococcus} and elevated levels of cellular form IA \( rbcL \) mRNA. \( rbcL \) cDNA libraries indicated two vertically separated clades of \textit{Prochlorococcus} (high-light and low-light adapted) in addition to a diverse group of prymnesiophytes and a microdiverse clade of prasinophytes, which may have dominated the SCM (Subsurface Chlorophyll Maximum). \textit{In situ} sampling and satellite image analysis were used to estimate that the plume accounted for 41% and 13% of all surface water column...
productivity in the oligotrophic Gulf of Mexico, while covering less than 3% of its area. Coastally the plume is dominated by diatoms, which are replaced by a bloom of *Synechococcus* as the plume moves offshore. Diatoms as indicated by pigments and *rbcL* clone libraries again dominated the offshore, least productive plume. 15N uptake measurements indicated that rapid recycling of ammonium despite higher levels of nitrate primarily drives production in the offshore plume. *rbcL* mRNA levels and photosynthetic capacity displayed strong diel patterns in three out of four time series sampled during the GRIST (Geochemical Rate/mRNA Integrated Study). In addition it was demonstrated that transcriptional regulation of the global nitrogen regulatory protein NtcA in *Synechococcus* WH7803 may involve a small cis-encoded anti-sense mRNA. Methods for the generation of large insert BAC (Bacterial Artificial Chromosome) from cultures and the environment were refined. Partial sequencing and genomic comparison of an *ntcA* containing BAC clone obtained from *Synechococcus* WH7803 indicated that *ntcA* is not part of a larger nitrogen assimilation operon in cyanobacteria.

(total word count:350)
INTRODUCTION

In recent years it has become evident that concerns about global warming are justified. Direct and indirect effects of climate change on society include such pressing issues as public health, changes in agriculture patterns, the stability of ecosystems and the destruction of personal and public property. Impacts resulting from climate change have the potential to dramatically alter our way of life and even threaten human civilization.

The United States of America alone is responsible for the greatest proportion of total green-house gas emissions of any nation on the planet. In 1995 emissions in the USA totaled more than $1.3 \times 10^9$ tons of CO$_2$ (Department of State Publication 10496, 1997). At this time, emissions were projected to rise to $1.4 \times 10^9$ tons by the year 2000. According to the Environmental Protection Agencies own ‘Climate Action Report 2002’ greenhouse gas emission in the United States are projected to amount to $8.237 \times 10^9$ tons CO$_2$ equivalent by the year 2020 AD. If such projections are reasonably accurate the same report projects “that temperatures in the contiguous United States will rise (by) 3-5°C on average during the 21$^{\text{st}}$ century. A wider range of outcomes, including a smaller warming is also possible”.

Understanding the sinks of the atmospheric carbon pool, which is believed to amount to ca. $660 \times 10^9$ tons CO$_2$ (Olson et al. 1985), has thus become a particularly important field of study. Permanent removal of CO$_2$ from the atmosphere/oceanic system
is referred to as ‘carbon sequestration’. It is thought, that through a better understanding of terrestrial and oceanic sequestration processes it might be possible to foster, augment and intensify these in order to avert some of the potentially disastrous effect of global warming. This argument contends that additional sequestration could lead to a reduction in the accumulation of atmospheric CO$_2$ and the resulting green-house effect.

By far the most important sink for global CO$_2$ (but not the only one) is photosynthetic carbon fixation (ca. 223 x 10$^9$ tons year$^{-1}$, (IPCC 2001)), almost half of which is thought to occur in the oceans (Chisholm 2000). Phytoplankton productivity in turn can mediate the removal of carbon from the atmosphere on both long and short timescales, making it a major sink in the global sequestration budget. Oceanic carbon sequestration occurs primarily by means of burial and storage of organic carbon in sediments (Karl et al. 1998, Legendre & Michaud 1998). This is thought to occur primarily in sediments along continental margins (Hedges & Keil 1995), where the influx of large amounts of river-borne nutrients leads to high rates of primary productivity in the water column. Particularly important in this process are large eukaryotic algae such as diatoms and phytoflagellates, which are thought to dominate coastal productivity (Marshall & Nesius 1996, Webber & Roff 1996). These organisms, due to their size, tend to sink rapidly upon cell death. They also form the nutritional basis for higher trophic levels and are most likely to be packaged into larger organic particles such as fecal pellets.

On shorter timescales carbon can be sequestered into the deep ocean via the ‘biological pump’. Through downward flux of dead cells, fecal pellets and marine snow organic matter can move below the mixed layer where it remains until upwelled. This
type of carbon sequestration occurs primarily in the oligotrophic regions of the ocean. Carbon is rapidly remineralized in the deep ocean however and returned to the atmosphere within decades to centuries in regions of upwelling. In addition the open ocean is dominated by picoplankton (0.2-2 µm sized organisms, Waterbury et al. 1979, Li et al. 1983). Small cyanobacteria, such as *Prochlorococcus*, numerically dominate the majority of the photic zone, often outnumbering other algal groups by one or two orders of magnitude. Red fluorescing cells (interpreted as picoeukaryotes) are present throughout the photic zone, but numerically only outweigh picocyanobacteria at the subsurface chlorophyll maximum (SCM). Open ocean productivity is thus mainly recycled and not characterized by communities conducive of carbon export.

One possible exception to these generalizations is the occurrence of nutrient rich river plumes originating from the worlds major river deltas. Such plumes have been observed by means of satellite imagery to retain large populations of phytoplankton even as they are carried hundreds of kilometers into the open ocean (Müller-Karger et al. 1991, Müller-Karger et al. 1995, Wawrik et al. 2003).

Figure 1 shows a SeaWiFS (Sea-viewing Wide Field-of-view Sensor) image of the Mississippi River plume. Under normal circumstances the plume is found to hug the Texas and Louisiana coastlines, where it dominates productivity on the northern Gulf of Mexico shelf regions. Occasionally however, as seen in Figure 1, the plume is found to extend dramatically into the otherwise oligotrophic portion of the Gulf. Little is known about its impact in this region.
Figure 1.1 – Shown is a SeaWiFS image of the Gulf of Mexico taken on July 18 2001. Seen is a coastal plume of Mississippi water entrained by the loop current and carried southward along the Florida shelf. (Image courtesy of Frank Müller-Karger, Douglas Myhre)

The purpose of this thesis was to develop and apply molecular biology techniques in order to describe and understand the phytoplankton community dynamics in the off-shore Mississippi River plume, both vertically and horizontally. These findings are compared to results from a similar study in a highly eutrophic environment along the coast of New Jersey. Further I set out to understand the plumes importance to basin wide carbon fixation and its potential implications to carbon sequestration in the oligotrophic Gulf. In this context I analyzed patterns of nitrogenous nutrient cycling in the plume and investigated the regulation of a global nitrogen regulatory gene in *Synechococcus*. 
CHAPTER 1
LITERATURE REVIEW

The composition of the picoplankton

The importance of small (<2 µm) autotrophic plankton to the productivity of the oceans was first recognized in 1983 by demonstrating that in the tropical Pacific Ocean 25-90% of Chlorophyll \( a \) as well as 20-80% of all primary productivity passed through a 1 µm screen (Li et al. 1983). Previous studies had relied on the use of glass-fiber filters with effective pore sizes of 1-2 µm to collect biomass. It is now believed that biomass and productivity of oligotrophic and mesotrophic oceanic ecosystems on earth are largely dominated by autotrophic picoplankton (Guillou et al. 1999a). Picoplankton is operationally defined as cells that are found within the size range 0.2 to 2 µm in diameter. The three main constituents of the picoplankton are two individual species, \textit{Synechococcus} and \textit{Prochlorococcus}, as well as a phylogenetically diverse group of eukaryotic algae (Campbell et al. 1994, Li 1994, Guillou et al. 1999a). Due to their importance to global biogeochemical cycles of nutrient and non-nutrient elements as well as food web structure, much attention has in the past been paid to the distribution of these organisms on a global scale. Considerable progress in understanding the ecology of the picoplankton has in particular been made with respect to its prokaryotic component.
(Waterbury et al. 1986b, Partensky et al. 1999b). Less is known about the taxonomic composition and biology of picoeukaryotes.

Community structure of picoplankton has been systematically studied using flow cytometry and epifluorescence microscopy (Blanchot & Rodier 1996, Blanchot et al. 2001). From these and other studies several characteristic patterns have emerged. *Prochlorococcus* is generally the most abundant organism in the euphotic zone of the tropical and subtropical ocean and usually numerically exceeds other groups by one to two orders of magnitude (Blanchot et al. 2001). Picoeukaryotes are generally found throughout the euphotic zone, independent of light level. They are also the dominant population at the subsurface chlorophyll maximum (SCM), which usually coincides with the nitricline. *Prochlorococcus* only out-numbers picoeukaryotes at the SCM if the SCM is above the nitricline (Blanchot & Rodier 1996). *Prochlorococcus* and picoeukaryotes generally outnumber *Synechococcus* in poorly lit environments. *Synechococcus* counts are usually highest where *Prochlorococcus* is least abundant (Olson et al. 1990a, Partensky et al. 1996). Most of these observations however have been made in the western tropical Pacific Ocean under oligotrophic or HNLC (high nutrient low chlorophyll) conditions and may not hold in other environments.

**Distribution and ecology of *Prochlorococcus***

*Prochlorococcus* is now thought to be the most abundant component of the autotrophic picoplankton in the worlds oceans and has been described as a marine photosynthetic prokaryote of truly global significance (Partensky et al. 1999b). The
organism is typically found in the euphotic zone up to a depth of 200 m in all subtropical and tropical oceans between 40° N and 40° S. Cell concentrations of about $1-3 \times 10^5$ ml$^{-1}$ are common down to a light level of as little as 0.1% of surface irradiance and this genus can sometimes account for as much as 40-50% of all photosynthetic biomass in the water column. *Prochlorococcus* is of particular importance in the subtropical oligotrophic gyres of the Atlantic and Pacific oceans, where other phytoplankton are much less abundant. Despite a large abundance, its existence had been completely overlooked before the advent flow cytometry as a technique for the study of phytoplankton community composition. *Prochlorococcus* is thought to be the smallest of all photosynthetic organisms and exhibits only relatively faint auto-fluorescence (Raven 1994, Partensky et al. 1999b). As a result, cells used to be included in DAPI (4',6-diamidino-2-phenylindole) counts of heterotrophic bacterioplankton.

Following the discovery of picoplankton in the late 1980s (Chisholm et al. 1988, Chisholm et al. 1992) our perception of phytoplankton distribution and dynamics has dramatically changed and we now acknowledge the tremendous importance of these small yet pervasive autotrophs to both phytoplankton ecology and global biogeochemical cycling of carbon, nitrogen and other elements.

*Prochlorococcus: Pigments*

*Prochlorococcus* are small, spherical cells with an approximate diameter of 0.5-0.7 μm (Morel et al. 1993). Cells are non-motile and lack phytobilliproteins that make up the phycobillisomes typically found in *Synechococcus* (Chisholm et al. 1988).
Prochlorococcus contains a unique pigment complement that has been described in detail (Goericke & Repeta 1992a). Most importantly Prochlorococcus is characterized by containing the diagnostic pigments divinyl-chlorophyll a ($a_2$), divinyl-chlorophyll b ($b_2$) (Goericke & Repeta 1992b). Also found in this organism are a chlorophyll c-like pigment, $\alpha$-carotene, zeaxanthin, an unknown carotenoid (Goericke 1990) and chlorophyll b. Not found in Prochlorococcus are phycoerythrin (Johnson & Sieburth 1979), chlorophyll c and $\epsilon$-cyclic carotenoids such as $\alpha$-carotein. The absence of these pigments is apparently characteristic of oxygenic photoautotrophic prokaryotes (Goodwin & Britton 1988).

**Isolates of Prochlorococcus in culture**

Prochlorococcus is notoriously difficult to isolate and only few labs are equipped to maintain and grow cultures. Cells are particularly sensitive to trace metal contamination (especially copper). For this reason strains used in this thesis were not grown in house, but kindly provided to us by Dr. Frederick Partensky in France.

The first Prochlorococcus isolate grown in culture was nonetheless obtained soon after the initial description of the organism (Palenik & Haselkorn 1992). Since then several groups have successfully obtained isolates from virtually all oceanographic regions where Prochlorococcus is though to be abundant. Typical isolation media are either derived from urea enriched seawater with the addition of minimal trace-metal solutions and a chelator (Chisholm et al. 1988) or derivatives of K-media (Keller et al.}
with diluted trace-metal stock without copper. Extinction dilution has been used to obtain clonal cultures and axenic strains.

**High-light and low-light ecotypes**

One of the most striking observations about *Prochlorococcus* is its ability to inhabit and numerically dominate the entire euphotic zone, occurring sometimes at depth greater than 150 m, where light flux is less than 1 µM quanta m$^{-2}$ s$^{-1}$ (Partensky et al. 1999b). Figure 2 shows a typical vertical distribution of *Prochlorococcus* in the oligotrophic ocean.

![Figure 2](image_url)

Figure 1.2 - Typical vertical distribution of *Prochlorococcus* in the Gulf of Mexico. Profile was obtained during a cruise on the R/V Pelican during July of 1999 at an oligotrophic site west of Tampa Bay and outside the Florida Shelf. Cells are abundant throughout a light range of as much as four orders of magnitude (Moore et al. 1998) and can often be found well below the mixed layer.
This remarkable ability to proliferate through such a great range of irradiance is primarily due to adaptations of individual cell populations to particular light ranges. Using flow cytometry it was shown that natural populations of *Prochlorococcus* are indeed divided into two ecotypes. One occurs at shallower depth, only dimly fluoresces and contains a relatively low ratio of chl $b_2$ to chl $a_2$. The other population is more brightly fluorescing, occurs at deeper depth and contains a high ratio of chl $b_2$ to chl $a_2$ (Moore et al. 1998). In particular Moore also demonstrated the simultaneous occurrence of isolates in the North Atlantic, which were either light inhibited or grew at very high light intensities. These two populations were later shown to be genetically distinct and are now referred to as the ‘high-light’ and ‘low-light’ adapted subspecies of *Prochlorococcus*. Ena Urbach (Urbach & Chisholm 1998, Urbach et al. 1998) used 16S ribosomal RNA sequences as well as sequences for *petB/D* and *psbB* loci and demonstrated congruent phylogenetic inference for marine type A *Synechococcus* and both *Prochlorococcus* clades. *Prochlorococcus* and *Synechococcus* appear as sister taxa, while the high-light adapted clade of *Prochlorococcus* appears to be in-group to the low-light clade, implying its more recent evolutionary origin. A PCR and flow cytometric sorting based approach taken by the same group later revealed a similar pattern in natural environments and demonstrated that high-light and low-light populations drew their membership from individual gene pools. West and Scanlan (1999) developed group specific 16S ribosomal RNA gene probes and used these in combination with PCR based cloning to investigate populations in the eastern North Atlantic. Group specific gene probing clearly demonstrated the niche-partitioning of the high-light and low-light clades
and was in full agreement with the pattern of obtained 16S rDNA clones obtained from different depth.

**Synechococcus**

The other important component of the prokaryotic autotrophic picoplankton in the temperate to tropical oceans is *Synechococcus*. *Synechococcus* was first described 1979 (Johnson & Sieburth 1979, Waterbury et al. 1979). The genus was originally defined to include “small unicellular cyanobacteria with ovoid to cylindrical cells that reproduce by binary traverse fission in a single plane and lack sheaths” (Rippka et al. 1979). Unfortunately the genus *Synechococcus* contained organisms of considerable genetic diversity and was thus originally only subdivided into two subgroups, those lacking and those containing the accessory pigment phycoerythrin.

Marine *Synechococcus* are generally coccoid cells between 0.6 and 1.6 μm in size. They are gram negative cells with highly structured cell walls that may contain projections on their surface (Perkins et al. 1981). Electron microscopy frequently reveals the presence of phosphate inclusions, glycogen granules and more importantly highly structured caboxysomes.
Figure 1.3 - Electron micrograph of *Synechococcus* WH7803 grown under 12:12 light:dark cycles at 23ºC. Cells were fixed in 2% gluteraldehyde and 1% OsO₄. Grids were stained with 5% alcoholic uranyl acetate and sTable lead. Magnification = 12000X. Cell wall (PM), DNA (N), thylacoids (T) and a number of carboxysomes (V) are seen.

Cells are known to be motile by a gliding type method (Castenholz 1982) and a novel uncharacterized, non-phototactic swimming method (Waterbury et al. 1985) that does not involve flagellar motion. While some cyanobacteria are capable of photoheterotrophic or even chemoheterotrophic growth, all marine *Synechococcus* strains appear to be obligate photoautotrophs (Waterbury et al. 1986b) that are capable of supporting their nitrogen requirements using nitrate, ammonia or in some cases urea as a sole nitrogen source. Marine *Synechococcus* are traditionally not thought to fix nitrogen (This perception may be changing. See below in ‘The oceanic nitrogen cycle’ section).
Pigments in *Synechococcus*

The main photosynthetic pigment in *Synechococcus* in chlorophyll *a*, while its major accessory pigments are phycobiliproteins (Waterbury et al. 1979). The four commonly recognized phycobilins are phycocyanin, allophycocyanin, allophycocyanin B and phytoerythrin (Stanier & Cohen-Bazire 1977). In addition *Synechococcus* also contains zeaxanthin but no diagnostic pigment for this organism is known. Zeaxanthin is also found in *Prochlorococcus*, rhodophytes and as a minor pigment in some chlorophytes and eustigmatophytes (see appendix A: Distribution of pigments in algal classes). Similarly phycoerythrin is also found in rhodophytes and some cryptomonads (Waterbury et al. 1986b).

**Phylogeny of *Synechococcus***

Phylogenetic description of *Synechococcus* is difficult. Isolates are morphologically very similar, yet exhibited a G+C content ranging between 39 and 71 % (Waterbury et al. 1986b) illustrating the large genetic diversity of this provisional taxon. Initially attempts were made to divide the group into three sub-clusters, each with a specific range of genomic G+C content (Rippka & Cohen-Bazire 1983). The observation that open-ocean isolates alone nearly span the complete G+C spectrum however indicates that *Synechococcus* is composed of at least several species.

Bergey’s Manual (Herdman et al. 2001) now divides *Synechococcus* into five clusters (equivalent to genera) based on morphology, physiology and genetic traits.
Cluster one includes relatively large (1-1.5µm) non-motile obligate photoautotrophs that exhibit low salt tolerance. Reference strains for this cluster are PCC6301 (formerly *Anacycstis nidulans*) and PCC6312, which were isolated from freshwater in Texas and California respectively (Rippka et al. 1979). Cluster 2 also is characterized by low salt tolerance. Cells are obligate photoautotrophs, lack phycoerythrin and are thermophilic. The reference strain PCC6715 was isolated from a hot spring in Yellowstone National Park (Dyer & Gafford 1961). Cluster 3 includes phycoerythrin lacking marine *Synechococcus* that are euryhaline i.e. capable of growth in both marine and fresh water environments. Several strains, including the reference strain PCC7003 are facultative heterotrophs and require vitamin B$_{12}$ for growth. Cluster 4 contains a single isolate, PCC7335. This strain is obligate marine (Waterbury & Stanier 1981). This strain contains phycoerthrin and was first isolated from the intertidal zone in Perto Penasco, Mexico (Rippka et al. 1979). The last cluster contains what had previously been referred to as ‘marine A and B clusters’ of *Synechococcus*. These cells are truly marine and have been isolated from both the coastal and the open ocean. All strains are obligate photoautotrophs and are ca. 0.6-1.7 µm in diameter. This cluster is however further divided into a population that either contains (cluster 5.1) or does not contain (cluster 5.2) phycoerythrin. The reference strains are WH8103 for the phycoerythrin containing strains and WH5701 for those strains that lack this pigment (Waterbury et al. 1986b).

More recently Badger (Badger et al. 2002) proposed the division of the cyanobacteria into a $\alpha$- and a $\beta$-subcluster based on the type of *rbcL* (large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase) found in these organisms. $\alpha$-cyanobacteria were defined to contain a form IA (for a discussion of the *rbcL* forms see
below), while β-cyanobacteria were defined to contain a form IB of this gene. In support for this division Badger analyzes the phylogeny of carboxysomal proteins, which appear to support this division. Also, two particular bicarbonate transport systems appear to only be found in α-cyanobacteria, which lack carboxysomal carbonic anhydrases.

Ecology and distribution of *Synechococcus*

*Synechococcus* has been observed to occur at concentrations ranging between a few cells per ml to $10^6$ cells per ml in virtually all regions of the oceanic euphotic zone except in samples from the McMurdo Sound and Ross Ice Shelf in Antarctica (Waterbury et al. 1986b). Cells are generally much more abundant in nutrient rich environments than in the oligotrophic ocean and prefer the upper well lit portion of the euphotic zone (Partensky et al. 1999a). For a typical abundance profile in the oligotrophic Gulf of Mexico see Figure 1.4. *Synechococcus* has also been observed to occur at high abundances in environments with low salinities and/or low temperatures. *Synechococcus* is usually far outnumbered by *Prochlorococcus* in all environments, where they co-occur. Exceptions to this rule are areas of permanently enriched nutrients such as upwelling areas and coastal watersheds (Partensky et al. 1999a). In the nutrient deplete areas of the oceans, such as the central gyres, *Synechococcus* is apparently always present, although only at low concentrations ranging from a few to $4 \times 10^3$ cells ml$^{-1}$ (Olson et al. 1990b, Blanchot et al. 1992, Campbell & Vaulot 1993, Li 1995, Blanchot & Rodier 1996). Vertically *Synechococcus* is usually relatively equitably distributed throughout the mixed layer and exhibits an affinity for the higher light regime. Below the mixed layer cell
concentrations rapidly decline. Vertical profiles are however strongly influenced by hydrologic conditions and can be very variable both seasonally and spatially. Overall *Synechococcus* abundance often parallels that of *Prochlorococcus* in the water column. In the Pacific HNLC (High Nutrient Low Chlorophyll) zone and in temperate open seas where stratification was recently established both profiles parallel each other and exhibit abundance maxima just about the SCM (Olson et al. 1990b, Li 1995, Landry et al. 1996).

Figure 1.4 - Typical vertical distribution of *Synechococcus* in the Gulf of Mexico. Profile was obtained during a cruise on the R/V Pelican during July of 1999 at an oligotrophic site west of Tampa Bay and outside the Florida Shelf. Cells are abundant throughout the mixed layer (here ca. 40 m thick) yet concentrations rapidly decline below.

The factors controlling the abundance of *Synechococcus* still remain poorly understood, especially considering that even in the most nutrient deplete regions of the central gyres, where cell abundances are often very low, population growth rates are often high and not very drastically limited (Partensky et al. 1999a). Factors such as
grazing, viral mortality, genetic variability, grazing, light adaptation, temperature as well as nutrients are certainly involved, but remain to be investigated on a rigorous and global scale. Despite the uncertainties it has been suggested that there is at least a relationship between ambient nitrogen concentrations and *Synechococcus* abundance (Blanchot et al. 1992, Partensky et al. 1999a) and an inverse relationship to *Prochlorococcus* (Campbell & Vaulot 1993) in the upper euphotic zone, where light is not limiting.

It should also be noted that *Prochlorococcus* is thought to be at least 100 times more abundant than *Synechococcus* in warm oligotrophic waters (Partensky et al. 1999a). Assuming average cellular carbon concentrations it has thus been estimated that *Prochlorococcus* accounts for at least 22 times more carbon in these waters and may thus be of much greater significance to the global carbon cycle than *Synechococcus*.

**Picoeukaryotes**

The least well studied and understood component of the autotrophic picoplankton is a diverse array of small eukaryotic algae collectively referred to as picoeukaryotes. These small algal cells may be the most abundant eukaryotes on earth and occur at concentrations between $10^2$ to $10^4$ cell ml$^{-1}$ in the photic zone throughout the oceans (Diez et al. 2001). Their diversity, distribution as well as their ecology however remain largely unknown (Partensky et al. 1997). Much of the difficulty associated with studying these algae results from the lack of morphological variation when viewed under conventional light microscopy. Cells often merely appear as little green balls (Potter et al. 1997) and display few distinguishing features (Thomsen 1986, Simon et al. 1994, Caron 1997).
et al. 1999). Often cells are referred to as LRGTs (Little Round Green Things). Epi-fluorescence microscopy yields little additional clues and cells appear as red-fluorescing spheres with no distinguishing features. Electron microscopy and cell culture may alleviate some of these limitations, but are too expensive, too difficult and too time consuming to be feasible as routine oceanographic techniques. Similarly flow cytometry can be used to estimate bulk cell abundance, but completely lacks phylogenetic resolution.

Despite limitations progress has been made in several ways over the past decade. At least three novel algal classes have been identified and newly described. Moestrup (Moestrup 1991) described the new class Pedinophyceae composed of the two green algae *Pedinomonas micron* and *Pedinomonas minor* in 1991. Shortly thereafter *Pelagomonas calceolata* was first identified and described in the marine ultraplankton (Andersen et al. 1993). 18S rDNA sequence data placed this species at an unresolved position among other chromophytic algae illustrating at the time how little was known regarding the phylogenetic diversity of heterokont algae (yellow-green algae with flagella of unequal length) in the environment. Pelagophytes are now thought to be an important and prolific component of the autotrophic picoplankton. Most recently the algal class Bollidophyceae, has been identified and described in cultures and the environment (Guillou et al. 1999a, Guillou et al. 1999b). Although the Bollidophyceae are not thought to be a major component of the picoplankton their discovery nonetheless illustrates that numerous novel lineages of picoeukaryotes may yet be identified.

To circumvent some of the limitations of traditional microscope-dependent techniques for the description of picoplankton communities, molecular techniques have
been widely used. Using group specific 18S rDNA probes in order to probe for the prymnesiophyte fraction of an 18S rDNA PCR (Polymerase Chain Reaction) amplicon generated using universal eukaryotic 18S primers, Moon-van der Staay (Moon-van der Staay et al. 2000) found that this group accounted for less of the amplified DNA than would be expected based on pigment ratios determined by HPLC. While these data should be interpreted with caution due to the biased nature of PCR (for a discussion see below), the same group also demonstrated the presence of several novel prymnesiophyte lineages in their 18S rDNA clone libraries, which had no equivalent among cultured species. Using a similar PCR based approach in order to amplify and clone picoplankton \textit{rbcL} gene sequences the diversity of picophytoplankton in the oligotrophic Gulf of Mexico has also been studied (Pichard et al. 1997b, Paul et al. 2000b). In the first study only five unique \textit{rbcL} gene-sequences were recovered. These sequences however demonstrated the presence of algae spanning the diversity of almost the entire form I clade. In the later study the presence of a diverse array of eukaryotic algae in a low salinity coastal plume was detected. Eukaryotic \textit{rbcL} DNA sequences were related to prasinophytes, prymnesiophytes, diatoms and pelagophytes and shared between 85 and 99\% similarity with cultured representatives in GenBank. In addition this study illustrated how unknown levels of genetic diversity within the picoplankton may not only exist in the form of divergent and novel lineages, but also in the form of small micro-diverse clades of closely related sequences.

Several other studies have since also addressed the diversity of picoeukaryotes in the environment using 18S PCR based clone libraries (Diez et al. 2001, Moon-van der Staay et al. 2001, Vaulot et al. 2001). These studies further support the notion that the
oceanic picoplankton (both heterotrophic as well as autotrophic) is genetically very diverse and yet contains a considerable number of undescribed lineages. In order to identify and characterize some of the classified and non-classified species in the environment FISH (Fluorescent In-Situ Hybridization) and DGGE (Denaturant Gradient Gel Electrophoresis) in particular has been useful. Using FISH several novel groups have been observed both in the Pacific Ocean and in coastal waters indicating their ubiquity (Vaulot et al. 2001). Two basal stramenopile lineages have been studied in field samples and enrichments in the northwestern Mediterranean Sea using FISH (Massana et al. 2002). Cells were 2-3µm in diameter and bacteriovorus. One particular lineage may have accounted for as much as 46% of the heterotrophic flagellates suggesting the tremendous importance of this unclassified stramenopile as a bacterial grazer.

**Rubisco, the Key to Carbon Fixation**

The first chlorophyll $a$ containing, photosynthetic organisms hypothesized to have existed in ancient oceans were likely similar to cyanobacteria (Bold & Wayne 1978). Evidence from stromatolitic deposits suggests that these organisms may have existed as early as 3 billion years ago (Echlin 1970, Schopf 1970) and dominated the fossil record until the late Precambrian. The photosynthetic processes responsible for the fixation and reduction of $\text{CO}_2$ as well as regeneration of the carbon dioxide acceptor in these primordial cyanobacteria were likely the same used by these organisms today and have been highly conserved throughout evolution (Tabita 1988). The only significant mechanism for the reductive assimilation of $\text{CO}_2$ in higher plants and algae is thought to
be the Calvin-Benson-Bassham (CBB) pathway. Other pathways such as acetyl-CoA pathway (Wood et al. 1986, Ragsdale 1991), 3-hydroxypropionate cycle (Herter et al. 2002) and the reductive tricarboxylic acid pathway (Buchanan & Arnon 1990) for the assimilation of inorganic carbon are known, but their occurrence is limited to certain autotrophic prokaryotes and not prevalent in the phytoplankton.

While the Calvin cycle involves a total of 13 individual enzymatic reactions, only two enzymes are unique to this pathway: ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and phosphoribulokinase (PRK). All other enzymes involved also perform functions in heterotrophic metabolism. PRK catalyzes the phosphorylation of ribulose-monophosphate to ribulose-1,5-bisphosphate (RUBP). RUBP in turn is the substrate for RubisCO, which catalyzes the actual carbon fixation reaction. The RubisCO enzyme alone as a result represents the most important pathway by which inorganic carbon enters the biosphere. It has also been described as the most abundant protein on earth (Ellis 1979). It is thought as much as 95% of all carbon fixation by C₃ plants (that includes all phytoplankton) occurs through RubisCO (Raven 1995b).

RubisCO is known to catalyze at least two reactions and both are shown schematically below (Fig. 1.5). Most importantly RubisCO catalyzes the reductive carboxylation of ribulose 1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglyceric acid (3-PGA). In addition RubisCO also catalyzes the oxygenation of RuBP to form one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG).
Figure 1.5 - Potential reaction pathways catalyzed by RubisCO. Both reactions occur at the same catalytic site and simultaneously and competitively.

The oxygenation of RuBP is referred to as photorespiration and has traditionally been seen as a wasteful process, in particular because the regeneration of RuBP in photorespiration leads to the evolution of CO$_2$ and requires free energy in the form of ATP. In addition RubisCO suffers from several other inefficiencies (Farquhar & Caemmerer 1982, Mizohata et al. 2002). Both reactions (carboxylation and oxygenation) occur in the same active site and compete, making the enzyme extremely sensitive to local partial pressures of CO$_2$ and O$_2$. RubisCO is also a slow catalyst with a very low turnover rate (<50 mol CO$_2$ / mol enzyme S$^{-1}$), which is lower then for any other carbon assimilation enzyme (Raven 1995b). Lastly, RubisCO rarely performs its function at a maximum rate ($K_{max}$), since the partial pressure of CO$_2$ in the vicinity of the enzyme is
often smaller than its Michaelis-Menten half-saturation constant ($K_m$). It is thus not surprising that some higher plants may have to invest as much as 50% of their total protein content by weight in RubisCO protein alone in order to satisfy their carbon fixation needs (Hartman & Harpel 1994).

**Molecular diversity of RubisCO**

RubisCO has been shown to occur in two distinct forms in nature termed form I and II respectively (Tabita 1988). Form I of the enzyme is an assemblage of eight 55-kDa large subunits ($rbcL$) and eight 15-kDa small subunits ($rbcS$). These subunits assemble into a 560-kDa hexadecameric protein-complex designated as $L_8S_8$ (Knight et al. 1990). Most photosynthetic prokaryotes that depend on the CBB-cycle for carbon assimilation and all eukaryotic algae express a form I type RubisCO. The exception to this rule are several marine dinoflagellates, which apparently contain a nuclear encoded form II of RubisCO (Morse et al. 1995, Whitney et al. 1995, Whitney & Yellowlees 1995, Rowan et al. 1996). Form II of RubisCO is a multimer of large subunits ($L_2$) (Tabita & McFadden 1974a, b) and is otherwise found in many photosynthetic and chemoautotrophic bacteria. More recently it has been recognized that some bacteria, such as *Rhodobacter sphaeroides* and many chemoautotrophs contain functional genes for both a form I and a form II of RubisCO (Yaguchi et al. 1994, Tabita 1995, Hernandez et al. 1996, Shively et al. 1998).
Figure 1.6 - Schematic representation of the phylogenetic diversity of known rbcL sequences. At least three forms of the gene are recognized based on sequence analysis. Form I includes all phytoplankton with the exception of certain dinoflagellates, which are known to contain a form II of the enzyme. Recently sequenced archaeal genomes indicate a novel grouping for RubisCOs from these organisms, form III.

Phylogenetic analysis of large number of form I rbcL DNA sequences revealed the division of form I into four major clades referred to as IA, IB, IC and ID (Tabita 1995). Form IA is commonly found in nitrifying and sulfur oxidizing chemosynthetic bacteria as well as some marine Synechococcus (marine type A) and all Prochlorococcus strains sequenced to date. All other cyanobacteria as well as all green algae possess a form IB type enzyme. Form IC of rbcL is expressed by some photosynthetic bacteria
such as hydrogen oxidizers. Form ID encompasses a diverse group of eukaryotic lineages including essentially all chromophytic, eucaryotic algae such as phaeophytes, rhodophytes, diatoms, prymnesiophytes, pelagophytes and several other phyla.

Surprisingly putative RubisCO like sequences were also found in the recently sequenced achaeal genomes of *Archaeoglobus fulgidus* and *Methanococcus jannaschii* (Bult et al. 1996, Klenk et al. 1997) even though these organisms are not thought to fix carbon via the CBB-cycle. RubisCO like sequences were also identified in *Archaeoglobis fulgidis* and *Pyrococcus sp.* The amino acid sequences of these putative archaeal RubisCOs however only slightly resemble those of the form I and II type enzymes (Tabita 1999) and their classification into at least one or more (form III and IV) novel forms has been proposed (Tabita 1999). The RubisCO of *M. jannaschii* (form III) has been shown to form a homodimer of two large subunits (L₂) and displays perhaps the lowest capacity to distinguish between CO₂ and O₂ of any RubisCO studied to date (Watson et al. 1999). The significance of these findings remains unknown.

The phylogeny of RubisCO displays several interesting incongruencies with phylogenies derived from ribosomal DNA sequences. This has lead to the speculation that over evolutionary history numerous lateral gene transfers may have occurred, disseminating RubisCO among divergent lineages (Delwiche & Palmer 1996). For example the dinoflagellates *Gonyaulax polyhedra* and *Symbodinium sp.* contain a nuclear encoded form II RubisCO most similar to sequences found in α-proteobacteria. Within the form I clade Delwiche and Palmer suggest as many as six lateral transfers to explain the unusual phylogeny observed among the cyanobacteria, proteobacteria and plastids. Some γ-, β- and α-bacteria may have acquired a green-like cyanobacterial gene, while
marine *Synechococcus* and *Prochlorococcus* almost certainly obtained their RubisCO genes from a purple bacterium (Watson & Tabita 1997).

*rbcL* is a very conserved gene. Even the most distantly related molecules are generally no less then 30% similar at their amino acid sequence level and similarity may be appreciably higher in portions of the gene responsible for activation of the catalytic domain (Hartman & Harpel 1994). For example the overall amino acid sequences of *Rhodospirillum rubrum* and spinach are more than 31% similar. In addition all RubisCOs also form a monophyletic clade in which functional consistency has been retained. Considering its size and the presence of multiple domains evolving at dissimilar rates the gene makes for a useful molecular marker. Over 16500 (as of May 2003) sequences for *rbcL* have been added to the database since the first phylogeny for RubisCO was published by Andersen and Canton (Andersen & Caton 1987). This large number of published reference sequences allows for reasonably accurate phylogenetic identification of sequences recovered from the environment.

**Catalytic structure of RubisCO**

In order to understand the function and structure of the RubisCO enzyme much attention has been given to X-ray Crystallographic studies of the enzyme and three-dimensional structures are now known for a number of species, including spinach (Knight et al. 1990, Andersson 1996), tobacco (Curmi et al. 1992), *Synechococcus* (Newman & Gutteridge 1990, 1993), *Rhodosparillum rubrum* (Schneider et al. 1986, Schneider et al. 1990), the archaeon *Thermococcus kodakaraensis* (Kitano et al. 2001)
and most recently the green alga *Chlamydomonas reinhardtii* (Mizohata et al. 2002). Based on these and other studies it is now believed that the primary catalytic structure of RubisCO is a dimer of two large subunits (L₂). In form I RubisCO, four L₂ dimers are combined to form L₈S₈ hexadecameric superstructure whereby the major contacts between the L₂ dimers are mediated by the small subunits. A Mg²⁺ cofactor as well as the carbamylation of Lys201 (in spinach; Lys 191 in *Rhodospirillum*) are required for the activity of the enzyme. A loop in the beta barrel and two other elements of the large subunit, one in the N- and one in the C-terminus of the protein form the active site in *Synechococcus*. Small subunits apparently do not contribute to the formation of the active site. Description of the crystal structure of RubisCO from the hyperthermophilic archaeon *Thermococcus kodakaraensis* recently revealed a novel assembly in which five L₂ dimers assemble to form an (L₂)₅ toroid-shaped decamer. This further supports the classification of these newly described archaeal RubisCOs into a novel type III grouping.

**Regulation of RubisCO transcription and activity**

Transcription of the cbb operon (the term ‘cbb’ is used for the rbcL operon in proteobacteria) in facultatively photoautotrophic and chemolithotrophic proteobacteria is highly regulated and several reviews have addressed the issue (Tabita 1995, Gibson et al. 1996, Kusian & Bowien 1997). Genes involved in the Calvin cycle in bacteria are often organized in operons regulated by a single promotor (Windhovel & Bowien 1991, Gibson et al. 1996), although the exact arrangement and composition of individual gene clusters is highly species specific. In addition to being under the control of a transcriptional
regulatory protein of the LysR family (CbbR), which binds in the intergenic region between the cbbR and cbbL genes (Windhovel & Bowien 1991, Kusian & Bowien 1995), a two component regulatory systems also appears to be involved in transcriptional activation of cbb gene clusters (Qian & Tabita 1996).

Regulation in cyanobacteria is somewhat different. rbcL and rbcS are usually co-transcribed and are, depending on the organisms, under the regulation of a light dependent or light independent regulatory mechanism (Gibson et al. 1996). In Anabaena sp. for example rbcL expression is regulated at the transcriptional level in response to the nitrogen and oxygen status of the cell, but remains constant during heterocyst formation (Haselkorn et al. 1983, Lang & Haselkorn 1989). Synechococcus RF1 has evolved a mechanism to temporally separate nitrogen and carbon fixation. Cells exhibit very strong circadian diel-regulation of nif-genes and nitrogen fixation (Huang et al. 1990) and as light is turned on there is an immediate and concomitant decrease in nif and increase in rbcL expression (Chow & Tabita 1994). The exact mechanism of this switch remains unknown.

Metabolic regulation of carbon fixation enzymes in prokaryotes appears to primarily be achieved at the transcriptional level, since transcriptional activity is usually reflected by the activity profiles of a transcript’s enzyme product (Kusian & Bowien 1997). Regulation is nonetheless subject to several post-transcriptional factors, particularly in eukaryotes. Many bacteria package RubisCO into small, polyhedral inclusion bodies called carboxysomes. The number of carboxysomes in turn appears to be proportional to the specific activity of RubisCO in Thiobacillus intermedius (Purohit et al. 1976). Activation of the large subunit by carbamylation is sterically inhibited in vivo
by several sugar-phosphates such as 2-carboxyarabinitol-1-phosphate (CAMP) and 2-carboxyarabinitol-1,5-bisphosphate (CABP) (Tabita 1988) allowing RubisCO activity to be under more strict control of cell physiology and energy status. Also potentially involved are molecular chaperones, which allow for the correct assembly and folding of RubisCO under various conditions, when its activity is needed.

*rbcL* gene expression in the environment

Microbial communities are often composed of a large number of morphologically uniform yet functionally diverse species. It is thus often difficult to link individual biochemical transformations to particular microbial species. The most promising approach to this problem is thought to be the measurement of gene expression. Attention has in particular been given to quantifying the transcript levels of genes involved in the fixation of carbon and nitrogen in both cultures and the environment. Studies usually assume that the levels of particular mRNAs are indicative of the assembly and activation of functional proteins and that through group specific transcription measurements the individual contribution of respective microbial species can be determined.

Methods for quantifying gene expression however traditionally relied on signal normalization to a constant cellular component such as 16S rDNA. Because universal probes do not provide species specific normalizers and because the relationship between a gene of interest and the amount of 16S rRNA in the environment is generally not known, Pichard and Paul (Pichard & Paul 1993) normalized the expression of catechol-2,3-dioxygenase (*xylE*) to target DNA providing a specific measure of gene expression in
aquatic microorganisms. Later this approach was applied to the quantification of $rbcL$ mRNA in Tampa Bay and the Gulf of Mexico (Pichard et al. 1993). Using a riboprobe derived from the $rbcL$ gene of *Synechococcus* sp. WH6301, $rbcL$ mRNA levels were shown to decrease 3 to 8-fold with a concomitant decrease in primary productivity and chlorophyll $a$ in a transect of Tampa Bay. Form IB $rbcL$ mRNA to DNA ratios in an offshore vertical profile were highest in the subsurface layer with the highest photosynthetic assimilation rates and exhibited dramatic diel variation in a deck-top incubated mesocosm experiment. Similarly, mesocosm experiments as well as a Lagrangian study of an oligotrophic site in the southeastern Gulf of Mexico showed that form IB $rbcL$ mRNA exhibited a strong diel signal in surface waters (Pichard et al. 1996). Both $rbcL$ mRNA and carbon fixation peaked in the early morning hours and were significantly correlated, suggesting that form IB $rbcL$ mRNA was a good proxy for carbon fixation in these waters. In cultures, diel regulation for $rbcL$ in cyanobacteria was confirmed in *Synechococcus* sp. strain RF-1 (Chow & Tabita 1994). The same study also demonstrated the temporal separation of expression and activity between $nif$ (nitrogenase) and $rbcL$. $nif$-transcription and nitrogenase activity occurred only during the dark, while $rbcL$ transcription occurred primarily in the light. In contrast *Trichodesmium thiebautii* was shown to produce the greatest amount of $nif$-operon mRNA during the early morning hours in samples from oligotrophic stations near the Bermuda Islands (Wyman et al. 1996). There was also a close coupling between $nif$-mRNA abundance and translation of the enzyme and greatest nitrogen fixation activity occurred simultaneously with greatest translational activity or as much as 2-3 hours thereafter.
Using multiple, group-specific gene probes, the temporal and spatial variation in \textit{rbcL} transcription was studied in several environments (Pichard et al. 1997a, Paul et al. 1999). In both cases form IB (cyano) and form ID (chromo) probes were used to quantify \textit{rbcL} transcript levels in size fractionated samples. In Tampa Bay \textit{rbcL} transcript levels and carbon fixation were predominantly associated with the >5 µm fraction, while both signals were stronger in the <1 µm fraction in the oligotrophic Gulf of Mexico. Both studies demonstrated highest ‘cyano’-\textit{rbcL} in vertical profiles at shallower depth dominated by picocyanobacteria (\textit{Synechococcus} and \textit{Prochlorococcus}), while ‘chromo’-\textit{rbcL} transcript was highest at the deep chlorophyll maximum (SCM), a depth where chromophytic picoeukaryotes dominated as determined by flow cytometry or epifluorescence microscopy. In a Lagrangian diel study a temporal separation in the peaks of form ID and form IB transcripts was observed. Form IB transcript was predominantly found during morning hours, while chromophitic \textit{rbcL} mRNA was most abundant in the late afternoon. The temporal separation in transcriptional activity between individual groups of phytoplankton was confirmed in cultures of the chromophytic alga \textit{Pavlova gyrans} and the cyanobacterium \textit{Synechococcus sp.} WH7002 (Paul et al. 2000c). Both species exhibited strong diel regulation in \textit{rbcL} gene transcription and carbon fixation while grown under 12:12 light-dark conditions. In \textit{Synechococcus} \textit{rbcL} levels peaked at noon and RubisCO enzyme activity was highest in the afternoon (4pm). \textit{rbcL} transcription in \textit{Pavlova} was highest at 4pm.

The transcription of \textit{cpeBA} (phycoerythrin biosynthesis), \textit{rbcL} and \textit{glnA} (glutamine synthetase) has been studied in diel experiments in a cyclonic eddy in the north Atlantic (Wyman 1999). While \textit{cpeBA} transcription varied little, there was strong
regulation in the transcription of rbcL and glnA with respect to the light-dark cycle. The mRNA data also suggested that there was a temporal separation in the assimilation of carbon and nitrogen in natural populations of marine Synechococcus. Most recently rbcL transcription has been measured in depth profile inside and outside of a coastal, low salinity plume in the Gulf of Mexico (Paul et al. 2000a). Outside of the plume from IA rbcL transcription was low and accounted for only 3% of the levels observed in plume surface waters. In addition to high form IA rbcL transcriptional levels the plume also contained a large population of Synechococcus as well as comparatively high levels of carbon fixation. Prochlorococcus was absent from plume surface waters indicating that Synechococcus may have been particularly important in the region of the plume that was sampled.

Coastal plumes

The Mississippi River is the sixth largest river in the world. As a consequence the river represents the dominant contributor of fresh water, nutrients, sediments and pollutants to the continental shelf of the northern Gulf of Mexico. The Mississippi receives water from the third largest drainage basin in the world, which covers over 41% of the continental United States and where over one quarter of the US population resides. By the time the river reaches the Gulf of Mexico its flow amounts in average to ca 1.84*10^4 m^3 s^-1 of nutrient rich fresh water, although flow varies seasonally and is highest in Spring. The majority of this water (approximately 70%) enters the northern Gulf through the delta region. The remainder is diverted through the Atchafalaya River to the
west of the delta (Walker 1996). On the shelf, river water forms a distinctive low salinity plume, which has received much attention due to its enormous implications to productivity in the northern Gulf of Mexico.

Based on ship-board surveys it was originally hypothesized that the Mississippi River plume generally moves westward and disperses over the continental shelf along the Louisiana and Texas coastlines and that dispersion of the plume during the late summer may sometimes be towards the southeast (Smith 1980, Cochrane & Kelly 1986, Dinnel & Wisemann 1986). Both hypotheses were later confirmed by studying the dispersion of the Mississippi River plume using monthly composite ocean color images (Müller-Karger et al. 1991) obtained from the Coastal Zone Color Scanner (CZCS). Between November 1978 and May 1980 the plume was observed to spread predominantly westward from the delta in a band along the coast reaching as far as Tampico Mexico. Sporadically small areas of discolored water were also observed to move eastward as thin bands near the coast. On occasion large parcels of discolored water are seen to move southeastward along the West-Florida shelf. Two such events were observed during the study period, one in March of 1979 and one in April of 1980. It thus appears that such episodes of eastward plume dispersal may be a yearly reoccurring event during spring, when river discharge is high and westerly winds combined with local ocean circulation are favorable. A rigorous analysis of the frequency of large eastward dispersal events into the oligotrophic GOM has not been published (Del Castillo et al. 2001).

Regardless of its dispersal vector, the importance of the plume to primary production on the GOM coastal margin has long been recognized (Riley 1937, Thomas & Simmons 1960, Sklar & Turner 1981). Initial observations however lacked the resolution
to adequately describe the controlling mechanisms of productivity in the plume (Lohrenz et al. 1990). Only recently has primary production at the plume/oceanic interface been surveyed more systematically (Lohrenz et al. 1990). In this study the immediate discharge area close to the delta of the Mississippi River was sampled. A rapid depletion of nutrients in surface waters was observed as salinity increased. Productivity was to a large extent explained by empirical models based solely on light and phytoplankton biomass, with highest production occurring at salinities between 25 and 30 ‰. Other factors such as grazing, strong salinity gradients and the ephemeral nature of the plume could not be excluded and were potentially significant factors in controlling phytoplankton productivity. At salinities >30 ‰, however, productivity appeared to be controlled by nutrient availability. In a later study (Lohrenz et al. 1997) the linkage between river-borne nutrient fluxes and productivity on the continental shelf in the northern Gulf of Mexico was investigated. Over the span of six years (1988-1994) it was found that productivity on the shelf was significantly correlated with the river-borne flux of dissolved inorganic nitrogen (DIN) in the form of nitrate and nitrite. Light limitation of primary production was more important during the winter. The supply of nitrite and nitrate was adequate to support >100% of productivity observed in the areas of the Gulf affected by the plume.

Conversely it has been hypothesized that nitrogen may become limiting in the plume (Sklar & Turner 1981, Lohrenz et al. 1990). To test this hypothesis the ratio of particulate amino acid to particulate protein (AA/Pr) has been used as an indicator of nitrogen deficiency in phytoplankton in the plume (Dortch & Whitledge 1992). It was hypothesized that phytoplankton growing under a sufficient supply of nitrogen will
accumulate high levels of intracellular amino acids. Neither the AA/Pr ratios nor ambient nutrient concentrations indicated that nitrogen deficiency was wide-spread in the plume. High rates of ammonia regeneration were invoked to explain the lack of nitrogen limitation. Dissolved nutrient concentrations however indicated that silicate was as likely or sometimes even more likely to be a limiting nutrient, which would profoundly affect the ambient diatom population. Rapid regeneration of nitrate has been found to occur in the plume by means of dark bottle incubations, suggesting a zone of intense nitrification at intermediate salinities (18-27 ‰) (Pakulski et al. 1995). Nitrate production coincided with highest \(^3\)H-leucine incorporation (bacterial production), highest community O\(_2\) consumption and inorganic carbon regeneration. A net accumulation of ammonium was observed at low salinities while there were low rates of ammonium consumption at higher salinities (27 ‰). The ratio of nutrients in river outflow however is not constant throughout the year. DIN (NO\(_3^-\) + NO\(_2^-\) + NH\(_4^+\)) to phosphate is highest in spring and lowest during the late summer and fall. This is reflected in plume surface nutrient ratios, which indicate a higher probability of nitrogen limitation during fall and phosphate limitation during spring (Lohrenz et al. 1999).

Using isotope dilution and enrichment experiments it has been shown that ammonium regeneration is highest near the surface in the plume, where bacterial production and primary productivity are also high (Gardner et al. 1993). Particularly during the summer bacterial production, ammonium regeneration and amino acid turnover are highest in the plume at intermediate salinities (Cotner & Gardner 1993, Amon & Benner 1997). In the winter it was found that ammonium regeneration rates did not vary in different areas of the plume. With increasing distance from the river delta
leucine incorporation rates have been found to decrease in surface plume waters, while there was a concomitant increase in this parameter below the pycnocline (Amon & Benner 1997). A variable proportion of primary production was consumed by bacteria and POM (Particulate Organic Matter) export was hypothesized to be depend on zooplankton grazing.

The examination of vertical profiles of some plume associated sites has revealed the existence of a subsurface biomass maximum and that there may be exchange between the subsurface and surface layers leading to the reduction of nutrient concentrations at intermediate salinities (Lohrenz et al. 1999). The vehicle for exchange is presumably the flux of particulate organic matter (POM) out of the plume to below the mixed layer. POM export in the plume has been studied using free floating trap systems (Redalje et al. 1994a). Rates of export in this study were variable, being highest in May and lowest in July and August. The proportion of primary productivity exported below the euphotic zone was highly variable and depended mainly on phytoplankton species composition and zooplankton grazing. Paradoxically highest export occurred in regions of the plume with lowest production. To further address the impact of species composition on sedimentation of POC, Fahnenstiel (Fahnenstiel et al. 1995) investigated the taxon specific loss rates of a number of diatoms, cryptomonads, dinoflagellates and haptophytes in the northern GOM. Growth and loss rates were determined during two cruises in the Mississippi River plume region. Growth rates were highest in the plume region and over half of growth rate variability was explained by soluble nitrogen. Sedimentation rates were <1% of growth rates for all taxa, but large colonial diatoms
exhibited highest sedimentation rates indicating that the downward flux of POC was greatly influenced by the silicification of the ambient phytoplankton population.

The downward flux of POC below the mixed layer is the driving force behind carbon sequestration in the oceans. Organic particles have two potential fates as they are moved into the abyss: They can be remineralized or buried and sequestered in sediments. In the northern Gulf of Mexico it has been shown that Mississippi River flow correlates well with the formation of oxygen depletion in bottom waters on the shelf (Justic et al. 1993). These observations imply that there is close linkage between river borne nutrients, productivity in the plume and the downward flux of particulate carbon and concomitant depletion of oxygen below the mixed layer due to bacterial respiration. Dark bottle experiments have shown that bacterial respiration in the Mississippi River plume area was highest during the summer in areas of highest primary production and intermediate salinities (Gardner et al. 1994) and that oxygen depletion below the mixed layer on the Louisiana shelf was caused by carbon flux derived from phytoplankton production (Sen Gupta et al. 1996). Stable carbon isotope records in sediment cores taken near the river delta indicated that buried carbon was primarily of marine and not terrestrial origin (Eadie et al. 1994), linking productivity in the plume directly to carbon burial in GOM coastal sediments.

The exact biological and chemical implications of the plume, when it is in the eastern oligotrophic GOM still remain unstudied. The plumes’ effect on the biological productivity of the oligotrophic Gulf also remains unknown. Almost nothing is known regarding the plumes’ impact on phytoplankton species composition and diversity in the oligotrophic GOM.
Nitrogen limitation

A key parameter limiting oceanic phytoplankton productivity is the bioavailability of nitrogenous nutrients. Depending on their source and availability DIN (Dissolved Inorganic Nitrogen; i.e. nitrate, nitrite and ammonia) can support or limit both new and recycled production. Although our view of the oceanic nitrogen cycle is becoming increasingly complex (for a recent review see (Zehr & Ward 2002) and although it is now recognized that not only the filamentous cyanobacterium *Trichodesmium* but also certain oceanic *Synechococcus* are also capable of fixing dinitrogen gas (Zehr et al. 2001, Zehr & Ward 2002), it is thought that most phytoplankton are solely dependent on the uptake of external DIN to satisfy their nitrogen needs and that nitrogen limitation frequently occurs.

Nitrogen limitation in marine phytoplankton populations has nonetheless proven difficult to demonstrate and has only been investigated in relatively few studies (Zehr et al. 2001). Traditionally nitrogen limitation has been invoked because oceanic DIN concentrations often become unmeasurable before those of phosphate (Nixon & Pilson 1983). However, many phytoplankton have affinities for DIN and P below detection limits of analytical methods making such measurements difficult to interpret. A more direct approach for measuring nitrogen limitation involves measuring the chemical composition of phytoplankton. Assuming that cellular stores of nutrients are indicative of nutrient uptake and growth rates, such data has been used to infer nutrient limitation in marine systems (Yentsch et al. 1977, Goldman et al. 1979, Sakshaug & Olson 1986). Also frequently used to measure nutrient limitation are nutrient enrichment studies. In
such experiments nutrients are added to a test system, which is monitored for the stimulation of phytoplankton growth. Unfortunately such enrichment experiments yield results highly dependent on experimental design (Hecky & Kilham 1988).

Recently, molecular markers have been introduced to investigate the nutritional status of phytoplankton. Molecular markers are proteins, whose synthesis is indicative of metabolic status. Using antibodies or nucleic acid probes specific for such proteins or their mRNA, cell populations may be interrogated and their nutritional environment assessed. Several potential targets have been identified in eukaryotic algae. For example, in the haptophyte Emiliania huxleyi a 82-kDa nitrogen regulated protein, Nrp1, has been identified using cell surface biotinylation (Palenik & Koke 1995). Molecular markers have more successfully been applied to prokaryotic phytoplankton, especially marine Synechococcus. In Synechococcus WH7803 a 32-kDa cell wall associated phosphate-binding protein (PstS) has been identified (Scanlan et al. 1993) which was shown to be expressed if ambient phosphate levels dropped below 50 nM, a concentration typical of the oligotrophic ocean. Using an immuno-fluorescence assay, expression of PstS was subsequently detected in single Synechococcus cells in culture and in the environment (Scanlan et al. 1997).

In order to determine the nitrogen status of marine Synechococcus, relative expression levels of the transcriptional activator NtcA have been used (Lindell & Post 2001). ntcA mRNA is made in Synechococcus grown on a variety of nitrogen sources, yet expression is repressed to a basal level in the presence of ammonia (Luque et al. 1994, Lindell et al. 1998). Highest levels of transcript are made in cells completely deprived of any nitrogen source (Lindell & Post 2001). Because the main mechanism for the
assimilation of inorganic nitrogen in cyanobacteria is the GS/GOGAT pathway (Wolk et al. 1976, Flores & Herrero 1994), it is possible to completely starve a cell population of all nitrogenous nutrients by the addition of the inhibitor L-methionine-D,L-sulfoxamine (MSX), which sterically inhibits the glutamine-synthetase reaction. Using treatments of 100 µM MSX and 100 µM NH$_4^+$ the nitrogen status of the cyanobacterial population in the Gulf of Aqaba (Red Sea) has been assessed by quantification of $ntcA$ mRNA indicating that the ambient population was growing under ammonia replete conditions (Lindell & Post 2001). It was my original intent to adapt this technique for the study of *Synechococcus* populations in the Mississippi River plume.

**Nitrogen assimilation in *Synechococcus***

Nutrient uptake in phytoplankton is of course intricately linked to carbon fixation. Overall cellular ratios of C,N and P are dictated by cellular requirement to synthesize components such as proteins, nucleic acids, carbohydrates and lipids. Ratios of these elements are maintained at relatively constant cellular proportions (Redfield ratios) and the expression of genes involved C, N and P assimilation are greatly intertwined.

As indicated, the uptake of inorganic nitrogen in *Synechococcus* is tightly regulated by the transcriptional activator NtcA which was initially recognized in a *Synechococcus* sp. PCC7942 mutant unable to assimilate nitrate (Vega-Palas et al. 1990). Transformations with a cosmid clone carrying the $ntcA$ gene were able to complement cells and fully restore the wildtype. A 25 kDa gene product was shown to be transcribed from the $ntcA$ gene, which belongs to a family of transcriptional activators that include
Crp and Fnr in *E. coli* (Vega-Palas et al. 1992). NtcA has also been shown to inhibit the uptake and reduction of alternate nitrogen sources such as nitrate, nitrite and urea in the presence of ammonium by repressing the expression of the *nir* operon (Guerrero & Lara 1987, Flores & Herrero 1994).

NtcA is now known to be a dimeric cyclic AMP binding protein, which affects the transcription of a large number of genes, which are primarily involved in the uptake and assimilation of alternate nitrogen sources (Luque et al. 1994), and has also been shown to affect the transcription of *rbcL*. Figure 7 shows a diagram of the mode of action of NtcA.

**Figure 1.7 - Schematic of the NtcA regulatory pathway.** NtcA through some unknown cell membrane associate mechanism senses ambient ammonium and CO2 concentrations. Phosphorylation leads to activation of NtcA dependent promoters. Phosphorylation of GnlB inhibits nitrate transport.
It is thought that through some yet unknown mechanism the cell senses ambient ammonia availability. The resultant signal leads to phosphorylations of GnlB, a cyanobacterial analog of the enterobacterial P\textsubscript{II} regulatory protein (Forchhammer & Tandeau de Marsac 1994) and of NtcA. Additionally to being sensitive to the ammonia concentrations it has been shown that NtcA phosphorylation is dependent on the inorganic carbon supply (Lee et al. 1999). In the presence of ammonia phosphorylated GnlB inhibits the uptake of nitrate and nitrite performed by the general transporter NrtP. In the absence of ammonia both GnlB and NtcA are phosphorylated and NtcA acts as a positive transcriptional activator of itself and a series of other genes mainly involved in carbon and nitrogen assimilation (\textit{nrt, nir, nar, rbc}) (Luque et al. 1994) by binding to the palindromic sequence GTAN\textsubscript{8}TAC (Luque et al. 1994). A good review discussing \textit{ntcA} has recently been published by Herrero et al (Herrero et al. 2001).
Specific Objectives

In the context of the reviewed literature my objectives were:

1. to develop a real-time PCR assay for detection Diatom rbcL mRNA and to apply this technology in cultures and in environmental samples.

2. to explore the diversity and vertical distribution of phytoplankton species at a site associated with the Mississippi River plume by means of culture independent RT-PCR and cloning of rbcL sequences.

3. to explore diversity and community change of phytoplankton species along a transect of the offshore Mississippi River plume using rbcL clone libraries and pigment analysis.

4. to analyze the nitrogen uptake rates measured along a transect of the offshore Mississippi River plume in order to determine if the plume contributes a significant amount of new production to the oligotrophic Gulf of Mexico.

5. to use a combination of image analysis and in-situ sampling to determine the impact of the Mississippi River plume on basin-wide oligotrophic primary production.

6. to investigate and analyze the phytoplankton species composition using rbcL cDNA as well as expression patterns of rbcL and primary production during the Geochemical Rate/RNA Intergrated Study (GRIST)

7. to investigate the transcriptional regulation of the transcriptional activator NtcA by ammonia dependent transcription of anti-sense RNA in the cyanobacterium Synechococcus WH7803.

8. to generate a genomic BAC library from a natural Prochlorococcus population in the Gulf of Mexico.
CHAPTER 2

Real-Time PCR quantification of \textit{rbcL} (Ribulose-1,5-bisphosphate Carboxylase/Oxygenase) mRNA in Diatoms and Pelagophytes.

The following chapter has been peer reviewed and published essentially in this form in the journal Applied and Environmental Microbiology (AEM 68(8) p.3771-3779)

CHAPTER SUMMARY

Transcriptional activity is often used as a surrogate for gene expression in environmental microbial communities. We have developed a Real-Time PCR assay using the ABI-Prism® (PE Applied Biosystems) detection system for the quantification of large subunit ribulose-1,5-bisphosphate caboxylase/oxygenase (\textit{rbcL}) mRNA in diatoms and pelagophytes both in cultures and from natural phytoplankton communities. Plasmid DNA containing \textit{rbcL} inserts as well as in-vitro transcribed mRNA of these plasmids were used to generate standard curves with a dynamic range of more than six orders of magnitude with high accuracy and precision ($R^2=0.998$). Expression levels in a cultured diatom, \textit{Phaeodactylum tricornutum}, were quantified through one light/dark cycle using traditional $^{35}$S labeled oligonucleotide hybridization and Real-Time PCR. Detected mRNA levels were of similar magnitude using both techniques and correlated well ($R^2=0.95$, slope=1.2). Quantities obtained by hybridization were slightly, yet significantly
larger ($t=5.29$, $p=0.0011$) than those obtained by real-time PCR. This was most likely because partially degraded transcripts were not detected by real-time PCR. \textit{rbcL} mRNA detection by real-time PCR was three orders of magnitude more sensitive than by hybridization. Diatom/Pelagophyte \textit{rbcL} mRNA was also quantified in a profile from an oligotrophic site in the Gulf of Mexico. We detected the least amount of diatom \textit{rbcL} expression in the surface water and a maximum expression coinciding with the depth of the subsurface chlorophyll maximum (SCM). These results indicate that real-time PCR may be utilized for the quantification of microbial gene expression in the environment.
INTRODUCTION

One of the continuing challenges in microbial ecology is to estimate microbial activity. Even small water or soil samples usually contain large numbers of diverse microbial species, yet it is difficult to determine their individual contributions to particular processes using bulk assay techniques. While tremendous advances have been made once quantitative methods for the analysis of nucleic acids via various hybridization techniques (Sharp et al. 1980, Thomas 1980, Suzuki et al. 2000) became available, applications were generally limited by the sensitivity of the procedures. Such methods for the quantification of gene-expression have traditionally involved the use of radio-labeled probes for the detection of a particular mRNA cross-linked to charged filters. These protocols are time-consuming, costly and involve the generation of radioactive waste. PCR technology has greatly increased the sensitivity of methods for gene detection, but it is inherently non-quantitative. Quantitative PCR combines the sensitivity of PCR with the real-time measurement of amplification allowing the quantification of the original target concentration.

A PCR based quantitative assay, first described by Holland et al. (Holland et al. 1991) and referred to as ‘real-time PCR’, has recently emerged (Gibson et al. 1996, Heid et al. 1996). This technique, while offering all the advantages of conventional PCR, such as high sensitivity and specificity, also allows for the quantification of PCR-product formation during the exponential phase of the reaction. PCR product formation is monitored by an increase in fluorescence, either due to the binding of amplicon to a fluorescent DNA stain such as SYBR green (Higuchi et al. 1992), or the release of a
fluorescent moiety from an oligonucleotide probe (i.e. TaqMan® probe) specific for the
amplicon. TaqMan® probes are short oligonucleotides, which are labeled with a
fluorescent chromophore and a quencher at the 5’ and 3’ ends, respectively. During
template elongation the probe is cleaved by the 5’ → 3’ exonuclease activity of Taq-
DNA polymerase, releasing the 5’ linked dye from the 3’ linked quencher, resulting in an
increase in fluorescence with product formation. Even though real-Time PCR was
originally developed for clinical applications, it has recently been applied to microbial
ecology; e.g., for the detection of small-subunit ribosomal RNA (Suzuki et al. 2000) and
nirS gene copy abundance (Gruntzig et al. 2001) in natural microbial communities. Both
of these applications of real-time PCR technology were directed at quantifying DNA
gene copy abundance in environmental microbial communities. The work presented here
is directed at the detection of mRNA as a surrogate for gene expression and functional
activity of phytoplankton in the marine environment.

Concerns about global warming have led to interest in processes that might
influence the potential removal and/or sequestration of carbon dioxide from the oceans
and the atmosphere. By far, the most important global carbon sink is photosynthetic
carbon dioxide fixation, almost half of which is performed by oceanic phytoplankton
(Chisholm 2000). Phytoplankton primary production is thought to lead to burial and
sequestration of significant amounts of organic carbon in marine sediments and
consequently to the permanent removal of carbon from the immediate carbon cycle (Karl
et al. 1998, Legendre & Michaud 1998). Indeed it is thought, that most of the carbon
sequestered by the oceans is buried in sediments along continental margins (Hedges &
Keil 1995), where high rates of nutrient input, as a result of continental runoff, lead to
correspondingly high rates of new production in the water column. Since coastal phytoplankton communities are dominated by the activity of eukaryotic algae such as diatoms and phytoflagellates (Marshall & Nesius 1996, Webber & Roff 1996), measuring the expression of carbon fixation genes in diatoms may be a useful indicator of new production. We have developed a method for the detection of \textit{rbcL} (gene encoding for the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)) transcript abundance in diatoms and pelagophytes using Real-Time PCR.

RubisCO is the key enzyme of the Calvin-Benson-Basham (CBB) cycle, catalyzing the first step where CO$_2$ is reductively assimilated into organic carbon. Several forms of RubisCO exist in nature. Virtually all oceanic picophytoplankton contain a form I RubisCO, which is a 550 kd holoenzyme composed of eight large and eight small subunits (Knight et al. 1990). The genes encoding for the small and large subunits are referred to as \textit{rbcS} and \textit{rbcL} respectively (in proteobacteria they are named \textit{cbbS} and \textit{cbbL}). Phylogenetic analysis revealed that there are four distinct evolutionary lineages within the form I clade of the \textit{rbcL} sequences (Tabita 1999, Paul et al. 2000b). Form IA is found in most marine picocyanobacteria within the genus \textit{Prochlorococcus} as well as some \textit{Synechococcus} species, while it appears that all green algae and other cyanobacteria contain a form IB \textit{rbcL}. Chromophytic (non-green) algae (prymnesiophytes, pelagophytes, diatoms, eustigmatophytes etc.) all contain form ID \textit{rbcL}, which are closely related to the proteobacterial form IC sequences. Understanding the factors influencing the transcription of \textit{rbcL} in these important phytoplankton groups may provide useful information about the factors controlling oceanic primary productivity and phytoplankton biocoenosis structure.
We have previously described PCR primers (Paul et al. 1999) that amplify a wide range of chromophytic algal rbcL genes. Combining these primers with a diatom/pelagophyte specific TaqMan® probe has provided a means to estimate rbcL gene expression by these organisms by real-time PCR. This was compared to results obtained with more traditional mRNA detection assays using hybridization and probing. Our results show great promise for the use of real-time PCR to estimate gene expression by transcript abundance as a measure of the functional activity of specific groups of phytoplankton both in the marine environment and in culture.
MATERIALS AND METHODS

Algal cultures and Diel experiments

*Phaeodactylum tricornutum* (CCMP 630) was obtained from the Provasoli – Guillard Center for culture of Marine Phytoplankton (West Boothbay Harbor, ME) and grown in F/2 media (Guillard 1975). Cultures were maintained at 25º C and illuminated using cool white fluorescent tubes (25-40 µE m$^{-2}$) during 12:12 light dark cycles. For diel experiments two liter cultures were grown in a four liter Erlenmeyer flask. Cultures were stirred using a magnetic stir-bar and constantly aerated with sterile filtered air. Growth of cultures was monitored spectrophotometrically at 480 nm. Once the culture had reached sufficient cell density (late log-phase) the culture was sampled every two hours for whole cell carbon fixation, DAPI cell counts, enzyme activity and *rbcL* gene expression.

Primers and probe design

Degenerate PCR-primers for the amplification of *rbcL* from form ID containing phytoplankton have been described previously (Paul et al. 2000b, Wawrik et al. 2003). These primers amplify a 554 bp fragment of *rbcL*, which includes the functional site of the enzyme (forward primer: 5’-GATGATGARAAYATTAACTC-3’, reverse primer 5’- ATTTGDCCACAGTGDATACCA-3’). We have found these primers to amplify a great diversity of chromophytic algae from environmental samples including diatoms, pelagophytes, prymnesiophytes and eustigmatophytes (Paul et al. 2000b, Wawrik et al.
The TaqMan® probe was designed by obtaining all available diatom rbcL sequences (as of July 2000) in GenBank (www.ncbi.nlm.nih.gov). A conserved site 15 bp downstream of the 5' form ID rbcL primer was located. One degeneracy was introduced by inserting inosine (I) at an ambiguous position (Probe sequence: 5’-TGGCGTTGGAGAGAICGTTTCTTA-3’). All primer and probe sequences given are stated using IUPAC degeneracies. The TaqMan® probe was 5’-labeled with the fluorescent dye FAM (6-carboxyfluorescein) and 3’-labeled with the quencher TAMRA (6-carboxytetramethyl-rhodamine). Oligonucleotides and probes were obtained from Operon Technologies (Alameda, CA).

**PCR**

For PCR five µl of sample was directly added to 45 µl RT-PCR reaction mixture prepared from 2X RT-PCR TaqMan® Master Mix (PE Applied Biosystems, Foster City, CA) containing 1 µM of each primer, 2 mM MgCl and 200 nM probe (diluted from 100 µM stock solution). Cycle parameters were as follows: 3 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 52°C and 1 min 30 sec at 72°C. Cycling was preceded by a 10 min 95°C incubation to activate Amplitaq Gold™. For reverse transcription all of these steps were preceded by a 30 min incubation at 48°C. Amplifications were analyzed using the ABI prism™ detection system.
RNA sampling and extraction

For RNA extraction 20 ml of culture was sampled in triplicate and filtered onto 25 mm, 0.45 µm HV polyvinylidene difluoride filters (Millipore Durapore). Filters were then placed into 2.0 ml screwcap tubes containing 0.5 g muffled glass beads (Biospec Products), 750 µl of RLT lysis buffer (Qiagen, Valencia, CA) and 7.5 µl β-mercaptoethanol. Tubes were frozen in liquid nitrogen and stored at -80° C until extraction. Glass beads were baked over night at 450˚ F in a muffle furnace. Cells were disrupted by bead-beating (Paul 2001) and 500 µl of lysate were extracted using RNeasy spun columns (Qiagen, Valencia CA) as recommended by the manufacturer. For real-time PCR from cultures, DNA was removed by a 15 min DNAse digestion on the RNeasy columns using RNAse free DNAse available from Qiagen. This removed small amounts of DNA contamination usually present in RNeasy extracts. To double-check that extracted RNA was not contaminated with DNA, a digestion control was performed. A crude lysate was split equally and individual aliquots were left undigested, DNAse, RNAse or RNAse+DNAse digested respectively. Extracts were then amplified by RT-PCR to show purity of extracted RNA.

Environmental sampling

Gulf of Mexico samples were taken on a cruise during July of 2001 on the R/V Walton-Smith (Lat 25º 18.781 N, Long 84º 13.213 W) on a calm day during the early morning hours. Eighthundred ml of seawater were filtered and filters were stored as
indicated for culture samples. Cells were disrupted by bead-beating (Paul 2001) and 500 µl of lysate were extracted using RNeasy spun columns (Qiagen, Valencia CA) as recommended by the manufacturer.

**Quantitative hybridization**

mRNA from cultures and the environment was extracted using RNeasy columns as indicated above. However, samples were not DNAse digested on the columns but divided into three equal aliquots. The first aliquot remained undigested on ice, the second was digested with DNAse and the third aliquot was digested with RNAse. Extractions were then dot-blotted onto Zeta-Probe charged nylon filters (Bio-Rad). These filters were dried under a heatlamp and the RNA was crosslinked to the filters with a UV crosslinker (Fisher Scientific) using the optimal crosslink setting on the equipment. The filter was then hybridized with a probe derived from *Cylindrotheca* sp. N1 (Paul et al. 1999). The $^{35}$S labeled riboprobe was generated by *in vitro* transcription of linearized plasmid DNA using $^{35}$S-UTP. Probing occurred at 55° as previously described (Paul 2001). Dotted RNA was quantified with a BioRad Model GS363 Molecular Imager. Standard curves were generated by in vitro transcription of the same *rbcL* clone employed to make the probe. Standards were dotted in duplicate in a range between 500 pg / dot and 0.5 pg /dot.
TaqMan® probe specificity

To screen for the specificity of our TaqMan® probe a representative subset of
$rbcL$ containing clones obtained from the Gulf of Mexico (Wawrik et al. 2003) was
screened by colony amplification. These clones are known to be amplifiable using the
described form ID $rbcL$ primers. Amplification was detected using the ABI prism™
detection system as described above. To verify amplification and correct amplicon size,
 aliquots from real-time PCR were run on a 1% agarose gel stained with ethidium-
bromide.

Standard curves for real-time PCR

Standard curves for $rbcL$ DNA were generated from plasmid DNA (PCR2.1,
Invitrogen, Carlsbad, CA) containing a diatom $rbcL$ insert cloned from a natural Gulf of
Mexico population (Wawrik et al. 2003). Highly purified plasmid was obtained by
extracting 500 ml of $E. \text{ coli}$ culture (clone P99FH13) using a Plasmid Maxi Kit (Qiagen,
Valencia, CA). DNA was quantified using Hoechst 33258 (Paul & Myers 1982). To
produce RNA standards, plasmid DNA was linearized using BamHI and in-vitro
transcriptions were performed using T7 RNA polymerase for 2.5 hours at 37 ° C. To
eliminate DNA contamination, RNA was digested for 15 min using RQ1-DNAse
(Promega). RNA was purified using a RNeasy column (Qiagen, Valencia CA) as
recommended by Qiagen and quantified using a Ribogreen RNA quantification kit
(Molecular Probes, Eugene OR) using the 16S rRNA standard included in the kit. Serial
dilutions of DNA and RNA were prepared using sterile RNAse free water. Five µl of
diluted nucleic acid were then added to 45 µl RT-PCR master mix and PCR was
performed as indicated above.

**Competitive PCR**

To determine the effect of chromophytic *rbcL* DNA from algae other than diatoms and pelagophytes on the quantification of diatom DNA by real-time PCR, an experiment was performed using a range of concentrations of plasmid DNA containing a *rbcL* sequence not detected by our probe. We were concerned that coamplification of such non-target nucleic acid in environmental samples might interfere with the quantification of diatom and pelagophyte *rbcL* target sequences. One pg of a diatom *rbcL* containing plasmid DNA (Clone P99FH13) was added to 0.4, 4, 40 and 400 pg of non-target plasmid DNA in triplicate. The non-target DNA clones chosen (P99BH7 and P99FH16) would amplify with PCR primers but not be detected by the probe. To a third series tubes we added 0.4, 4, 40 and 400 pg of a 50/50 mixture of both non target plasmid DNA. Amplification was performed as indicated above.

**Whole cell carbon fixation**

Photosynthetic carbon fixation was measured by a method described by Strickland and Parson (Strickland & Parson 1968) as modified by Carpenter and Lively (Carpenter & Lively 1980). Ten ml of culture were incubated in acid leached liquid
scintillation vials. Light vials were incubated under 80 μEm²s⁻¹ average light flux for 30 min, while dark vials were shielded from light using black electrical tape. Radioactive \(^{14}\)C-bicarbonate (Amersham Corp., Arlington Heights, IL) was added to both vials to a final concentration of 0.5 μCi ml⁻¹ (18.5 KBq ml⁻¹). Before incubation and after 30 min, two ml of culture were sampled in duplicate and filtered onto 25 mm, 0.22 μm GS filters (Millipore Corp.). Filters were then placed in 0.5 ml 0.5N hydrochloric acid to drive off residual bicarbonate and radioactive counts were obtained by liquid scintillation.

**RubisCO enzyme activity**

Twenty ml of culture were sampled in duplicate in sterile Oakridge tubes and centrifuged for 10 min at 10,000 g to collect cells. Harvested cells were washed in 50 mM Tris-HCl pH 8.0, containing 1 mM EDTA pH 8.0, 10 mM MgCl₂, 50 mM NaHCO₃ and 1mM DTT. Cells were then centrifuged as indicated above and cell pellets were stored in liquid nitrogen for storage at -80°C until assayed. For enzyme assays cells were thawed, resuspended in the above buffer (Tabita et al. 1978) and then assayed for RubisCO enzyme activity using a radiometric assay (Gibson et al. 1991).

**Cell counts**

Cell density was monitored by autofluorescent cell counts. One ml of culture was diluted in fresh media and preserved using 3.7 % formalin. Cells were then filtered onto black polycarbonate 25-mm, 0.2 μm Poretics filters (Osmonics, MN). Filters were placed
on glass microscope slides and cells counted under oil immersion (1000X) and blue excitation. To check for bacterial contamination of algal cultures, samples were DAPI stained and slides inspected using epifluorescence microscopy (Porter & Feig 1980).

**Flow cytometry**

Twenty µl of 10% para-formaldehyde was added to one ml of seawater. Samples were incubated at room temperature for 10 min and frozen in liquid nitrogen until analysis in the lab. For processing samples were shipped on dry ice to the Flow Cytometry Lab of Texas A&M University. A Becton Dickinson FACSCalibur (San Jose, CA) flow cytometer equipped with a 488 nm, 15 mW Argon laser was used to quantify *Prochlorococcus*, *Synechococcus* and pico-eucaryotic algal populations. The fluorescence parameters measured for each event were forward and right angle light scatter of green (530 ± 30 nm), orange (585± 30 nm) and red (650±30nm) excitation wavelength. For signal normalization of fluorescence signals, purple-yellow calibration beads (2.2 µm, Spherotech Inc., IL) were added to each sample. Data collection was performed using CellQuest™ software (V. 3, Becton Dickinson 1996) and analyzed using CYTOWIN software ((Vaulot et al. 1989), [http://www.sb-oscoff.fr/Phyto/cyto.html#cytowin](http://www.sb-oscoff.fr/Phyto/cyto.html#cytowin)). Event rates were recorded for each sample. Abundances were corrected for volume analyzed and enumeration efficiency factor. Event rates and counts obtained from a series of known concentrations of calibration beads were used to determine efficiency factors.
Table 2.1

Specificity of real-time PCR for diatoms and pelagophytes

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phylogenetic association</th>
<th>GenBank accession #</th>
<th>Amplification</th>
<th>Detection by Real-Time PCR</th>
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<tr>
<td><strong>Positive controls</strong></td>
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<tr>
<td>P994AH8</td>
<td>Diatom</td>
<td>AF381735</td>
<td>+</td>
<td>+</td>
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<tr>
<td>P994CH1</td>
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<td>+</td>
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<td>Diatom</td>
<td>AF381735</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Negative controls</strong></td>
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<td>Prochlorococcus</td>
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Figure 2.1

Standard curves generated from plasmid DNA (A) containing a diatom rbcL insert and in-vitro transcribed mRNA (B) of a diatom rbcL clone obtained from an ambient phytoplankton population in the Gulf of Mexico. Target nucleic acid concentration is plotted against threshold cycle (CT). All values are averages of triplicate measurements. Error-bars indicate one standard deviation.
Figure 2.2 (A-C)

(A) Cell counts

(B) Carbon fixation

(C) Rubisco enzyme activity

60
Figure 2.2 (D-E)

**rbcL by real-time PCR**

![Graph D](image)

**rbcL by hybridization**

![Graph E](image)
Figure 2.2

Growth parameters and rbcL mRNA of a *Phaeodactylum tricornutum* (CCMP630) culture during the course of a 16-hour sampling period. (A) Epifluorescence cell counts in $10^6$ cells ml$^{-1}$. (B) µg CO$_2$ fixed per hour per $10^6$ cells (C) RubisCO enzyme activity in nmol CO$_2$ fixed per cell. (D) rbcL mRNA expression in ng rbcL mRNA cell$^{-1}$ as measured using Real-Time PCR. (E) ng rbcL mRNA cell$^{-1}$ as measured by dot-blotting and hybridization with a $^{35}$S labeled probe. Error bars indicate one standard deviation calculated from triplicate samples. Black bars indicate dark periods.
(A) Relationship between rbcL mRNA quantification by hybridization and Real-Time PCR. (B) Percentage of rbcL mRNA detected by real-time PCR, where rbcL mRNA detected by hybridization equals to 100% is plotted vs. the quantities obtained by hybridization.
Figure 2.4

A) ug fucoxanthin l⁻¹

B) 10^3 Picoeucaryotes ml⁻¹

C) pg rbcL mRNA liter⁻¹ by hybridization

D) pg rbcL mRNA liter⁻¹ by real-time PCR
Figure 2.4

Biological parameters measured at station 8, an oligotrophic station in the Gulf of Mexico. (A) Pigments. Chlorophyll $a$ and fucoxanthin (indicative of diatoms). (B) Flow-cytometry and primary productivity. (C) $rbcL$ mRNA measured by real-time PCR and hybridization.
Change of threshold cycle in Real-Time PCR as a function of competitive PCR target not detected by our TaqMan® probe. X-axis values are the ratio of total PCR target DNA and the amount of DNA recognized by the diatom probe. Errors bars indicate one standard deviation calculated from triplicate samples. P99BH7 (▲), P99FH16 (■), P99BH7 + P99FH16 (♦).
RESULTS

TaqMan® probe specificity

The specificity of our probe was confirmed by amplification of a representative sample of \( rbcL \)-containing clones obtained from an ambient population in the Gulf of Mexico. Table 2.1 shows a list of the screened clones, their GenBank accession numbers as well as their phylogenetic affinities (Wawrik et al. 2003). Seven diatom and two pelagophyte sequences were positively identified by our probe. Prymnesiophyte and eustigmatophyte sequences were also amplified using our primer-set as detected by agarose gel-electrophoresis (data not shown), yet amplification was not detected by the ABI prism® detection system.

Standard curves

Standard curves were generated with good precision through a dynamic range of six orders of magnitude using either plasmid DNA (Fig. 2.1A) or in-vitro transcribed RNA (Fig. 2.1B) with our diatom TaqMan® probe and the ABI prism® 7700 detection system. Coefficients of determination (\( R^2 \)) for the standard curves were better than 0.99 for both types of standards. Larger quantities of DNA or RNA led to non-linear amplification due to overloading of the reaction with target. Smaller quantities than the low end of our standards (<1000 targets for plasmid DNA) were detected but reliable quantification was not possible.
Culture experiments

In order to quantify and compare of \textit{rbcL} mRNA using either real-time PCR or hybridization with radiolabeled riboprobes, a culture of \textit{Phaeodactylum tricornutum} was sampled throughout one light/dark cycle. Cell counts changed little throughout most of the sampling period but increased towards the end of the experiment (Figure 2.2A). Cellular carbon fixation rose steadily throughout the illumination period, peaking in the late afternoon and decreasing thereafter (Figure 2.2B). Cellular RubisCO enzyme activity also peaked during the late afternoon (Figure 2.2C). During these time periods, \textit{rbcL} mRNA levels were quantified by real-time PCR (Fig. 2.2D) and hybridization with $^{35}$S labeled riboprobes (Fig. 2.2E). Clear diel regulation of transcript abundance was indicated by both methods, reaching a maximum in the early morning hours and decreasing to a low around 4 pm. Quantities measured by both methods were, in general, within a similar range. However paired t-test analysis indicated that quantities obtained by hybridization were significantly higher than those obtained by real-time PCR at the 99% confidence interval ($t=5.29$, $p=0.0011$). Quantities measured by hybridization ranged between 1.75 and $3.56 \times 10^{-10}$ ng \textit{rbcL} mRNA cell$^{-1}$, while quantities obtained by real-time PCR varied between 1.09 and $3.30 \times 10^{-10}$ ng \textit{rbcL} mRNA cell$^{-1}$. Paired t-test analysis of standard deviations for both methods found no significant difference in their precision at the 99% confidence interval ($t=2.923$, $p=0.022$). Regression analysis revealed a good correlation between the two types of measurements (Fig. 2.3A, $R^2=0.96$, slope = 1.2). The increased slope was due to a relatively larger decrease in the amount of transcript detected by real-time PCR than that detected by hybridization as transcript
levels decreased and reached their minimum during the afternoon hours. Figure 2.3B demonstrates the relationship of the discrepancy between the two methods as a function of the total amount of rbcL mRNA detected by hybridization. During the peak in expression in the early morning hours real-time PCR accounted for more than 90% of the rbcL mRNA detected by hybridization. In the afternoon however, when mRNA levels decreased, quantities obtained by real-time PCR decreased to a greater degree and only accounted for 58% in the 4 pm sample. The discrepancy between the two methods declined again in early evening hours and real-time PCR accounted for ca 85% of the rbcL mRNA in the 8 pm sample.

Environmental Samples

Figure 2.4 shows the biological parameters measured at station 8 in the Gulf of Mexico. Pigment profiles are shown in Fig. 2.4A. Chlorophyll a and fucoxanthin (a diatom pigment) both peaked at ca. 80 m of depth, indicating the presence of the SCM (Subsurface Chlorophyll Maximum). Flow-cytometry (Fig. 2.4B) showed good correlation between the concentration of chlorophyll a and the abundance of picoeukaryotes ($R^2=0.98$). Picoeukaryotic abundance was about $10^3$ cells ml$^{-1}$ in the surface and increased more than threefold at the SCM around a depth of 80 m. Below the SCM, cell abundance rapidly declined. Primary production at station 8 followed a bimodal distribution in the water column peaking at 20 m depth and the SCM. Figure 2.4C shows the amount of rbcL mRNA detected by real-time PCR and hybridization. mRNA expression measured by real-time PCR was low in the surface water, increased
rapidly at mid-depth and reached its maximum at the SCM, below which it rapidly
dropped similar to the decline in the abundance of picoeukaryotes. Quantities obtained
by hybridization were highest in the surface, remained of similar magnitude to a depth
below the SCM and then declined.

**Competitive PCR experiments**

Our degenerate primer set was not only capable of amplifying the *rbcL* gene
sequences of diatoms and pelagophytes, but also sequences from a large array of other
chromophytic algae (Wawrik et al. 2003). We were concerned that the presence of
competitive PCR targets in environmental samples, which might not be detected by our
TaqMan® probe, could influence the detection of Diatom/Pelagophyte *rbcL* mRNA.

Figure 2.5 shows the behavior of the threshold cycle (CT value) as a function of
competitive PCR target. When the diatom *rbcL* DNA accounted for at least 2.5 % of the
total *rbcL* DNA in the reaction, detection was possible and the CT value changed little
with competitor concentration. At lower relative concentrations detection rapidly
degraded and the threshold cycle became erratic or was equal to the total number of PCR
cycles (the ABI prism® detection system returns a CT value equal to total number of
cycles if no target is detected).
A real-time PCR method has been developed to detect diatom/pelagophyte $rbcL$ gene expression with great precision and dynamic range. Using our diatom TaqMan® probe we were able to distinguish diatom/pelagophyte $rbcL$ sequences from non-target DNA. In culture experiments real-time PCR proved to be of similar precision as hybridization, yet displayed a three orders of magnitude greater dynamic range. Quantities obtained by real-time PCR were slightly, yet significantly lower than those observed by hybridization, a difference that can most likely be attributed to a potential bias of hybridization experiments towards partial or degraded target sequences. Alternatively there may have been partial internal homology of the Cylindrotheca derived riboprobe. Such homology can lead to the internal binding probe to itself before binding to target on the filter and lead to increased signal.

One of the most important benchmarks of an environmental detection method is specificity (i.e. the ability to distinguish between the target sequence and other closely related non-target organisms present in a sample). We screened twenty $rbcL$ clones obtained from an ambient phytoplankton community in the Gulf on Mexico, indicating the fidelity of this technique. It is important to note that our primer set amplifies $rbcL$ from a much broader range of phytoplankton than our TaqMan® probe is designed to detect. This approach allows for the development of multiple probes for different phytoplankton groups, while using only a limited number of primer sets.

Equally important are sensitivity and dynamic range. Real-time PCR detection of $rbcL$ target nucleic acid has proven to be both, extremely sensitive; in addition a large
dynamic range with a quantitative detection limit of approximately 1000 gene copies has been displayed. The assay is linear through at least six orders of magnitude covering the entire range of traditional probing assays, while being almost three orders of magnitude more sensitive. Similar amplification and detection characteristics have been reported by Grünzig et al. (Gruntzig et al. 2001) for the amplification of nirS in *Pseudomonas stutzeri* and Becker et al. (Becker et al. 2000) for the 16S rDNA ITS-1 from *Synechococcus sp*. These results suggest that the lower quantitative detection limit of real-time PCR based assays in the environment is approximately $10^2$ targets. This seems high compared to the theoretical limit of just one copy per reaction tube, but was not viewed as a problem, because of the abundance of *rbcL* mRNA in natural samples and our ability to increase sensitivity by a variety of concentration protocols (e.g. vacuum or tangential flow filtration). Real-time PCR detection of a particular gene sequence is therefore more likely to be limited by adequate nucleic acid extraction protocols, primer and probe design, and not the sensitivity of Real-Time PCR.

*rbcL* mRNA expression is known to exhibit strong diurnal regulation in both picoplankton cultures (Paul et al. 2000c) and in samples taken from the environment (Paul et al. 1999, Wyman 1999). For marine *Synechococcus* species, *rbcL* mRNA levels increased before sunrise, reaching a maximum at daybreak (Wyman 1999). In the form IB containing marine cyanobacterium *Synechococcus* PCC7002, cellular *rbcL* mRNA content was highest during mid afternoon, preceeding the maximum in cellular CO$_2$ fixation (Paul et al. 2000c). Ambient form IB containing phytoplankton populations near Cape Hatteras have been observed to exhibit a peak *rbcL* expression during early afternoon hours (Paul et al. 1999), while chromophytic, form ID containing, picoplankton
displayed the highest rbcL mRNA levels during the late afternoon/early night hours (Paul et al. 1999) similar to the patterns observed in the prymnesiophyte Pavilion gyrans (Paul et al. 2000c). For Phaeodactylum tricornutum rbcL expression increased just before the light period, resulting in the appearance of peak cellular mRNA concentrations during early morning (Fig. 2.2D&E) hours. These observations indicate an interesting succession in the appearance of rbcL transcript by different phytoplankton groups. Early morning to sunrise hours appear to be utilized by form IA containing cyanobacteria for peak rbcL mRNA synthesis. Shortly thereafter, diatoms (or at least Phaeodactylum) reach their expression maximum. Throughout early afternoon hours form IB containing phytoplankton (mainly green algae) are most active at transcribing rbcL, while prymnesiophytes are transcriptionally most active late in the day.

In Phaeodactylum rbcL transcription is not well correlated with carbon fixation and RubisCO enzyme activity (Fig. 2.2). Net cellular carbon fixation peaked during the late afternoon together with RubisCO enzyme activity. This suggests that some type of post-transcriptional regulatory mechanisms modulates the production of functional RubisCO. Such decoupling of rbcL mRNA expression from enzyme activity has also been observed in the chromophytic alga Pavilion gyrans, where peak RubisCO enzyme activity actually preceded mRNA expression (Paul et al. 2000c). Possible regulatory mechanisms, which could explain these observations are known to exist in higher plants (Portis 1992) and may involve regulation of the carbamylation step as well as binding of sugar phosphates to RubisCO (Hartman & Harpel 1994). The significance of this decoupling however remains poorly understood and is perhaps common in eukaryotes,
while regulation in cyanobacteria seems to be at the level of transcription (Watson & Tabita 1997, Paul et al. 2000c).

We observed a good correlation between standard hybridization and real-time PCR ($R^2=0.95$). The quantities obtained by these two methods were also of similar magnitude, but significantly different. Real-time PCR consistently produced lower estimates, ranging between 58 % and 94 % of those obtained by hybridization. The discrepancy between the two methods was correlated ($R^2 = 0.65$, slope $\approx 0.2\times10^{-10}$ ng cell$^{-1}$) to the amount of $rbcL$ transcript detected by hybridization (Fig. 2.3B). A strict correlation between the methods should yield a regression line with a slope of approximately zero in this Figure. A possible explanation for this deviation from direct correlation of the two detection methods is the formation of partial $rbcL$ transcripts as the result of normal cellular degradation of mRNA. Quantities obtained by hybridization measurements are thus necessarily higher, because no distinction is made between partial and complete mRNA transcripts. PCR on the other hand requires a continuous, uninterrupted nucleic acid molecule (at least for the sequence between the primer sites) for proper amplification of the target sequence. It should be noted, that this caveat represents a distinct advantage of real-time PCR methods over hybridization techniques, since partial targets are, of course, not translated into functional enzyme. Standard deviations of the measurements obtained by the two methods were not significantly different, indicating that both methods exhibit similar precision. This demonstrates that real-time PCR measurements represent a real alternative to conventional hybridization techniques and should be accepted with a similar degree of confidence.
It was also important to compare the *rbcL* mRNA levels using the two methods in a natural phytoplankton community. Such communities are dominated by picoplankton (0.2-2 µm) in offshore, nutrient deplete environments. Picoeukaryotes (including diatoms and pelagophytes) are found throughout the photic zone and numerically dominate the phytoplankton community at the subsurface chlorophyll maximum (SCM). The diversity of picoeukaryotes at any particular site in the ocean may be quite large, simultaneously including green algae, coccolithophorids and a large diversity of haptophytes such as prymnesiophytes, pelagophytes, eustigmatophytes and diatoms (Paul et al. 2000b, Moon-van der Staay et al. 2001, Wawrik et al. 2003). Little is known about the numerical contribution of these groups to oceanic picoplankton communities, nor is there appreciation of the relative contributions of these different organisms to primary productivity. When several biological parameters were measured at an oligotrophic station in the Gulf of Mexico (Fig. 2.4), we observed a strong chlorophyll maximum at 80 m depth coinciding with a maximum in fucoxanthin (a pigment indicative of diatoms). Both pigments correlated well with the abundance of picoeukaryotes throughout the water column. This is not necessarily expected for a natural population, since there is usually considerable photo-adaptation of cellular pigment content with depth. Primary productivity was bimodally distributed, peaking at the SCM and at 20 m of depth. While the peak in primary productivity at the SCM is most likely caused by the activity of picoeukaryotes, near surface productivity is likely due to *Prochlorococcus*, abundantly found in this region of the water column (data not shown). Real-time PCR demonstrated highest diatom/pelagophyte *rbcL* mRNA expression at the SCM. Expression levels above the SCM were slightly higher than could be expected from a direct correlation between
picoeucaryotic abundance or chlorophyll $a$ and $rbcL$ expression. This suggests that diatoms and pelagophytes might contribute more actively to primary productivity than other picoeukaryotes in the portion of the water column dominated by *Prochlorococcus*. Expression levels in the surface were very low, suggesting that diatom/pelagophyte success in the surface layer might have been limited. We have however in the past had difficulty amplifying $rbcL$ mRNA from Gulf of Mexico surface water, leading us to believe that the surface $rbcL$ expression value reported here might be underestimated. This interpretation is supported by the observation that highest hybridization levels were in fact observed in the surface sample. $rbcL$ hybridization levels were consistently high throughout most of the water-column and declined below the SCM. Direct correlation between the two methods was poor ($R^2 = 0.34$), however if the questionable surface value was removed from the data-set the two methods correlated reasonably well ($R^2 = 0.74$) and were not significantly different by paired t-test analysis ($p=0.21$, $t=1.42$). It is possible that DNA damage in the surface water, due perhaps to high ultra violet radiation or enhanced nuclease levels, could lead to poor, partial or erroneous transcript formation. Such damaged transcripts would be included in the hybridization signal, yet would not be amplifiable. Alternatively this discrepancy might be explained by the presence of other chromophytic algae, which cross-hybridized with the diatom-derived riboprobe, but were not detected by our diatom real-time PCR probe.

PCR based techniques used in the study of environmental communities are subject to many biases. PCR reactions are often inhibited by contaminants and clone libraries often include artifacts due to the chimera formation in PCR (Winzingerode et al. 1997). Related sequences are not always amplified with similar efficiency and not all
target molecules are equally accessible to PCR primers, perhaps because primers do not bind with equal efficiency or because extension efficiencies are not always equal for all templates (Suzuki & Giovannoni 1996). For example Suzuki and Giovannoni (Suzuki & Giovannoni 1996) have shown that the proportion of products formed in PCR may be biased towards a 1:1 ratio regardless of the initial ratio between templates. G+C content of the template DNA has also been suggested to influence PCR amplification (Reysenbach et al. 1992, Dutton et al. 1993).

Figure 2.5 demonstrates another type of PCR bias in mixed template experiments. It appears that amplification of minor components in mixed assemblages of targets may be completely repressed by the presence of a dominant template. This has large implications for the interpretation of clone libraries since sequences only present at low abundance may not be recovered. Also the inability to quantitatively detect minor targets in real-time PCR applications may limit the capacity to use non-specific primers for amplification.

Regardless of the shortcomings of PCR, real-time PCR presents an extremely sensitive, reliable and accurate technique for the estimation of microbial activity in the environment and offers great potential for use in the studying the diversity and contributions of genes encoding for important biogeochemical processes.
CHAPTER 3

Vertical Structure of the Phytoplankton Community associated with a Coastal Plume in the Gulf of Mexico

This chapter has been peer reviewed and published essentially in this form in the journal Marine Ecology Progress Series (MEPS 251-(4) p.87-101)

CHAPTER SUMMARY

Low salinity plumes of coastal origin are occasionally found far offshore, where they display a distinct color signature detectable by satellites. The impact of such plumes on carbon fixation and phytoplankton community structure in vertical profiles and on basin wide scales is poorly understood. On a research cruise in June 1999 ocean-color satellite-images (SeaWiFS) were used in locating a Mississippi River plume in the eastern Gulf of Mexico. Profiles sampled in and outside of the plume were analyzed using flow-cytometry, HPLC pigment analysis and primary production using $^{14}$C incorporation. Additionally RubisCO large subunit ($rbcL$) gene expression was measured by hybridization of extracted RNA using three full-length RNA gene probes specific for individual phytoplankton clades. We also used a combination of RT-PCR/PCR and TA-cloning in order to generate cDNA and DNA $rbcL$ clone libraries from samples taken in the plume. Primary productivity was greatest in the low salinity surface layer of the
plume. The plume was also associated with high *Synechococcus* counts and a strong peak in form IA *rbcL* expression. Form IB *rbcL* (green algal) mRNA was abundant at the SCM, whereas form ID *rbcL* (chromophytic) expression showed little vertical structure. Phylogenetic analysis of cDNA libraries demonstrated the presence of form IA *rbcL* *Synechococcus* phylotypes in the plume. Below the plume two spatially separated and genetically distinct *rbcL* clades of *Prochlorococcus* were observed. This indicated the presence of the high-light and low-light adapted clades of *Prochlorococcus*. A large and very diverse clade of Prymnesiophytes was distributed throughout the water column, whereas a clade of closely related prasinophytes, may have dominated at the subsurface chlorophyll maximum (SCM). These data indicate that the Mississippi River plume may dramatically alter the surface picoplankton composition of the Gulf of Mexico, with *Synechococcus* displacing *Prochlorococcus* in the surface waters.
INTRODUCTION

One of the key processes influencing the global carbon cycle and the balance between the organic and inorganic pools of carbon in the atmosphere and ocean is the rate of oceanic carbon sequestration. Carbon sequestration is the removal of carbon from the immediate carbon cycle resulting from transport and burial of organic and inorganic particulate carbon into sediments. Biotic and abiotic carbonate precipitation as well as photoautotrophic carbon fixation by phytoplankton have been implicated in this process (Karl et al. 1998, Legendre & Michaud 1998). Carbon burial is thought to be particularly important along ocean margins (Jahnke 1996). New primary production along coastlines, which is primarily the result of the availability of NO$_3^-$, accounts for as much as 27% to 57% of total oceanic new primary production (Wollast 1993, Chavez & Toggweiler 1995). Large phytoplankton species with high sinking rates, particularly diatoms, are thought to be the most significant new producers and to mediate the sequestration of carbon into coastal sediments. In contrast, production in the oligotrophic ocean is thought to be mostly recycled through microbial loop-like processes. Diatoms are less numerous and picocyanobacteria, such as *Synechococcus* and *Prochlorococcus*, dominate the phytoplankton community presumably because of their greater affinity for low concentrations of reduced and recycled forms of nitrogen (mainly ammonium).

The dominant source of nutrients to the coastal ocean is river discharge. The most important source of river-water in the Gulf of Mexico is the Mississippi River, which contributes as much as 90% of the total freshwater input in this environment. Near-shore nutrient laden plume waters are areas of intense new production, supporting large
populations of diatoms and are virtually devoid of *Prochlorococcus* (Liu et al. 1999). Little is known however about the autotrophic picoplankton community associated with these highly productive offshore plume waters. In this paper we have used molecular and traditional techniques to describe the diversity and activity of the photoautotrophic picoplankton community in the offshore Mississippi River plume, to gain insight into the biological processes at work in this environment.

The molecular techniques we have used focus on the diversity and expression of genes involved in photosynthetic carbon fixation (Pichard et al. 1993, Pichard et al. 1997a, Pichard et al. 1997b, Liu et al. 1999, Paul et al. 2000a, Paul et al. 2000b). The key enzyme in the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway of photosynthetic carbon fixation in phytoplankton is ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Raven 1995a, Tabita 1999). RubisCO is found in several forms distinguished by subunit assembly and biochemical properties (Tabita 1988, Watson & Tabita 1996, Watson et al. 1999). All eukaryotic algae (with the exception of Dinoflagellates), cyanobacteria and photosynthetic as well as chemoautotrophic proteobacteria have a form I type enzyme (Tabita 1999). Form II of RubisCO is expressed by certain photosynthetic bacteria and some dinoflagellates, while Forms III and IV are found in Archaea and certain bacteria (Tabita 1999). Phylogenetic analysis of sequence information for the large subunit of RubisCO (*rbcL*) from form I containing organisms has revealed that there are four distinct lineages referred to as form IA, IB, IC and ID (Tabita 1995) respectively. Form IA *rbcL* is commonly found in nitrifying and sulfur oxidizing chemoautotrophic bacteria as well as some *Synechococcus* and all *Prochlorococcus* strains. Form IB is carried by most cyanobacteria and all green
algae. Form IC is expressed by some photosynthetic bacteria as well as hydrogen oxidizers and form ID \( rbcL \) is produced by a diverse group of algae including essentially all chromophytic eukaryotes (phaeophytes, rhodophytes, diatoms, prymnesiophytes, pelagophytes and several other groups).

We have previously described a low salinity surface plume feature in the NE Gulf of Mexico in 1997 that we attributed to riverine input from coastal waters ie. the “Green River” (Paul et al. 2000a). This feature showed a steep vertical stratification in the distribution of form IA \( rbcL \) mRNA, but no other \( rbcL \) forms. To characterize a similar feature in detail, we used satellite imagery to locate a surface plume originating from the Mississippi River that had traversed far into the Gulf of Mexico. The purpose of this study was to compare the vertical structure of the phytoplankton community within and outside this plume using flow-cytometry and \( rbcL \) gene expression. A second goal was to describe the diversity transcriptionally active \( rbcL \) phylotypes present in a profile of the plume site to determine the identity of the photosynthetically active members of the phytoplankton community.
MATERIALS AND METHODS

Sampling

Sampling was performed aboard the R/V ‘Pelican’ in the SE Gulf of Mexico on June 14 and 15, 1999 (station 4 -Lat 27°, 7.92 min. N, Long 85°, 2.55 min W, station 5 - Lat 26°, 32.00 min. N, Long 85° 30 min W, see Fig. 3.1). The sampling site was chosen by following the movement of a high chlorophyll plume resulting from Mississippi River discharge between June 8 and June 13 using SeaWiFS satellite images. The low salinity feature associated with the surface absorbance (Fig. 3.1) was located using the onboard MIDAS continuous sampling system. Seawater was sampled during early morning hours using a rosette of Niskin bottles attached to a Seabird CTD. Samples from greater than 40 m depth were protected from light by wrapping carboys in black plastic bags during filling and sample processing. Sampling depths were as follows: 3, 10, 40, 60, 78, 86, 100 and 130 m below surface.

rbcL mRNA analysis

Bulk mRNA was extracted from seawater using RNeasy spin columns (Qiagen) as described by Paul (Paul 2001). Eight hundred ml seawater samples were treated with 0.1% v/v DEPC (Di-Ethyl-Pyrocarbonate; Sigma Chemical Corp.) and immediately filtered onto 25 mm, 0.45 μm HV polyvinylidene difluoride filters (Millipore Durapore). Filters were then transferred to 2.0 ml screwcap tubes containing 0.2 g muffled glass
beads (Biospec Products) and 750 µl of RLT lysis buffer (Qiagen, Valencia, CA). Tubes were then frozen and stored in liquid nitrogen for processing in the lab. Cells were lysed by bead-beating and 550 µl of lysate was then extracted following the RNeasy kit (Qiagen, Valencia, CA) protocol. One third of the extract remained undigested on ice, while the other two thirds were digested with DNAse free RNAse or RQ1-DNAse. Samples were then dot-blotted onto Zeta-Probe charged nylon filters (Bio-Rad). The filters were dried and RNA was immobilized by UV-crosslinking. Duplicate samples were probed with form IA, form IB and form ID probes. The form IA rbcL probe was derived from *Synechococcus* WH7803 (Watson & Tabita 1996). The form IB and ID rbcL probes were derived from *Synechococcus* PCC6301 and *Cylindrotheca* sp. N1 respectively (Paul et al. 1999). Radio-labeled probes were made by *in vitro* transcription of these genes using $^{35}$S-UTP. Dot blots were quantified with a BioRad Model GS363 Molecular Imager using standard curves made from in-vitro transcripts generated by transcription of the same rbcL clones used to make the ribo-probes.

**Flow cytometry**

One ml samples were fixed with 20 µl of 10% para-formaldehyde at room temperature for 10 min and frozen in liquid nitrogen shipboard until analyzed in the lab. *Prochlorococcus*, *Synechococcus* and pico-eucaryotic algal populations were then quantified using a Becton Dickinson FACSCalibur (San Jose, CA) flow cytometer equipped with a 488 nm, 15 mW Argon laser. Forward angle light scatter, right angle light scatter, green (530 ± 30 nm), orange (585± 30 nm) and red (650±30nm)
fluorescence parameters were collected for each event. Purple-Yellow calibration beads (2.2 µm, Spherotech Inc., IL) were added to each sample to permit normalization of all fluorescence signals. Data was collected using CellQuest™ software (V. 3, Becton Dickinson 1996), transferred to a personal computer and analyzed using CYTOWIN software (Vaulot et al. 1989), http://www.sb-oscoff.fr/Phyto/cyto.html#cytowin). Event rates were recorded for each sample and abundances were corrected for volume analyzed and enumeration efficiency factor. The efficiency factor was derived from event rate and counts for series of known concentrations of calibration beads. For a brief review on flow-cytometric methodology see Campbell, L. 2001 (Paul 2001).

**HPLC pigment analysis**

Four liters seawater samples were filtered in duplicate through 25 mm Whatman GF/F glass fiber filters. The filters were folded in half, wrapped in aluminum foil and then immediately frozen in liquid nitrogen and stored at -80°C. Extractions were performed using acetone and analyzed by B. Pedersen, Mote Marine Lab, using the method of Millie et al. (Millie et al. 1993).

**Chlorophyll a analysis**

Samples for chlorophyll a were filtered onto 25 mm Whatman GF/F glass fiber filters, frozen in liquid nitrogen and stored at -20°C in the dark until extraction. Triplicate
samples were extracted with methanol and the chlorophyll $a$ concentration was determined fluorometrically (Holm-Hanson & Rieman 1968).

$^{14}$C-carbon fixation

$^{14}$C-carbon fixation was measured as described by Carpenter and Liveley (Carpenter & Lively 1980) using sterile, acid cleaned 500 ml polycarbonate flasks and 325 ml water samples. Flasks were incubated under natural irradiance that was adjusted to resemble the intensity and spectral features of the underwater light field using neutral-density screening and colored acetate filters. Irradiance intensity as a function of depth was determined by use of a Li-Cor light meter equipped with Li-190SA and Li-192SA surface and underwater photosynthetically active radiation sensors. Water column productivity was integrated for one m$^2$ of surface ocean as follows. The water column was divided into segments bounded by the surface, the half-way points between each pair of adjacent sampling depth and the depth of the bottom sample (station 4 intervals were 0-5, 5-25, 25-50, 50-69, 69-82, 82-93, 93-115 and 115-130 meters, Station 5 intervals were 0-10, 10-30, 30-50, 50-67.5, 67.5-78.5, 78.5-91, 91-115 and 115-130 meters). Primary productivity measurements taken within each interval were then extrapolated to the volume of the segment and all estimates for the water column segments were then added. This procedure approximates and yields slightly lager numbers than trapezoidal integration, except for the uppermost and lowermost segments. I assumed this procedure to me more accurate than trapezoidal integration, because the plume surface layer was
likely to be well mixed and because no sample directly from the surface (shallower than a
depth of 5 m) was available.

**DNA sampling and extraction for PCR**

Eight hundred ml of seawater was filtered onto 25 mm 0.45 μm polyvinylidene
difluoride filters (Millipore Durapore) and stored in 2 ml screwcap tubes in 1 ml of STE
(0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) in liquid nitrogen. Extraction was
performed by boiling lysis in STE and 1% SDS (sodium dodecyl sulfate) as in Pichard et
al. (Pichard et al. 1993). *Prochlorococcus* cells were provided to us by Dr. Frédéric
Partensky and Ms. F. Le Gall (strains, NATL1, NATL2B, SS120, TAKA9803-2 and SB)
and Dr. Lisa Campbell (strain PAC1) as frozen cell pellets. Pellets were resuspended in 1
ml of STE and extracted as described above.

**RNA extraction for RT-PCR**

Samples of 800 ml of seawater were filtered onto 25 mm, 0.45 μm polyvinylidene
difluoride filters (Millipore Durapore) and extracted as indicated above. Samples were
not treated with DEPC (diethylpyrocarbonate) due to its interference with the RT-PCR
reaction. Samples were DNAse digested on the RNeasy columns for 15 min using the
RNAse-Free DNAse reagent set (Qiagen) according to the protocol provided by Qiagen
in order to remove contaminating DNA from RNA preparations. To show that DNAse
digested RNA was not contaminated with DNA a digestion control was run. A crude
lysate was split equally and individual aliquots were left undigested, DNAse, RNAse or RNAse+DNAse digested. Extracts were then RT-PCR amplified to show purity of extracted RNA.

**RT-PCR**

Reverse transcriptions were performed with freshly extracted RNA using random hexamers, 4.7 mM MgCl₂, and M-MLV Reverse Transcriptase (Promega, Madison, WI) for 30 min at 37°C. Five µl of the reaction was then added to a PCR reaction such that a final volume of 100 µl was attained. The two primer sets used to amplify the *rbcL* fragments were the form IA/B primer set (615 bp fragment; forward primer: TCIGCITGRAACTAYGGTCG, reverse primer: GGCATRTGCAIAACRTGRAT) and form ID set (554 bp fragment; forward primer: GATGATGARAAYATTAACTC, reverse primer ATTTGDCCACAGTGDATACCA). Primer sequences are shown using IUPAC conventions. The final primer concentration in all PCR reaction mixtures was 1 µM for both primers. The MgCl₂ concentration was 1.5 mM and all nucleotides were added to a final concentration of 2.5 mM each. Five Units of TAQ polymerase (Promega, Madison, WI) was used per reaction tube. Cycle parameters were as follows: 3 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 52°C and 1 min 30 sec at 72°C. Cycling was followed by a 15 min 72°C elongation step.
PCR of *Synechococcus* like clones

During the course of these experiments we discovered that our form IA/B primers (Paul et al. 2000b) would not amplify *Synechococcus rbcL* sequences. No *Synechococcus*-like clones were obtained in any of our cDNA libraries despite the high abundance of these organisms. Using recently deposited sequence information in GenBank a new form IA reverse primer was designed to detect *Synechococcus* like genotypes: CTGAGIGGIAARAACTACGG. These primers yield a 455 bp amplicon, which overlaps in its entirety with the amplicons from the other two previously described primer sets. DNA was extracted as described above and 5 µl of DNA extract was added to a 100 µl reaction mixture. PCR was performed as indicated above.

Cloning and screening of clone libraries

*rbcL* mRNA was RT-PCR amplified from eight different depths of station 4 using the form ID and form IA/B primer sets. Station 4 surface water DNA was later amplified with the modified cyanobacterial reverse *rbcL* primer. *Prochlorococcus* DNA (strains NATL1, NATL2B, SB, SS120, TAKA9803-2 and PAC1) was amplified with the Form IA/B primer set. Immediately after amplification the amplicons were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Amplicons were then ligated into pCR® 2.1 vector using a TA cloning kit (Invitrogen corp., Carlsbad, CA) according to the protocol provided by the manufacturer. All white colonies were then picked and streaked onto individual 2xYT plates containing 50 µg ml⁻¹ Kanamycin and Ampicilin. To screen
for the presence of clones with the correct insert size, clones were PCR amplified by touching a colony with a sterile loop and transferring this loop to a PCR reaction tube. PCR reaction mixture and parameters were as described above. Amplifications were then run on 1.5% agarose gels stained with ethidium-bromide. Amplifications with products of the correct size were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and taken up in 50 µl of DI water. Twenty µl of purified product were then digested with 5 units of each Sau3AI (digests at ↓GATC↑) and AluI (digests at AG↓↑CT) in Buffer B (Promega, Madison, WI) for 30 min. at 37°C. Digestions were run on 2.5% low melting agarose gels (Fisher, Fair Lawn, NJ) at 13.5 V cm⁻¹ for 2 hours and stained with ethidium bromide. Gels were photographed and clones with unique restriction patterns were later visually selected for sequencing. Individual colonies from plates of selected clones were then picked into five ml of 2xYT media containing 50 µg ml⁻¹ Kanamycin and Ampicillin and grown for 16 hours. Plasmid DNA was then extracted using a Wizard® SV Plasmid Mini kit (Promega) and sequenced in an Applied Biosystems model 373 sequencer by the University of Florida core sequencing laboratory (University of Florida, Gainesville, FL).

**Determination of PCR error**

Two clones (P99FH2 and P99HH25) were grown in 500 ml of 2xYT media for 16 hours and plasmid was extracted using a Qiagen (Qiagen) plasmid kit. One ng of plasmid DNA for both clones was then added to individual PCR reactions and the rbcL insert was amplified as indicated above. Amplified DNA was then purified using a QiaQuick
(Qiagen) PCR purification kit. Amplicons were then ligated into the pCR® 2.1 vector using a TA cloning kit (Invitrogen corp., Carlsbad, CA) according to the protocol provided by the manufacturer. From FH2 and HH25 plates respectively, 10 and 12 colonies were then picked, the plasmid was extracted and the inserts were sequenced. Sequences obtained were then aligned using Omiga 1.1 (Oxford Molecular Group, Oxford, UK) and PCR and sequencing errors were counted as deletions, insertions or differences in sequences from the original clones.

**Phylogenetic analysis**

Deduced amino acid sequences were aligned with a representative sample of *rbcL* sequences obtained from GenBank, using Omiga 1.1 (Oxford Molecular group, Oxford, UK), which uses Clustal W, a pairwise, weighted alignment method. Amino acid sequences were used, because third codon positions were saturated and in order to avoid potential biases introduced by codon usage and GC content. Alignments were then corrected manually for obvious misalignments, and exported to Mega 2.0 beta (Kumar et al. 1993). Phylogenetic analysis was then performed using parsimony and the Neighbor-Joining method using a gamma distribution (gamma parameter = 2.0) to correct for rate heterogeneity across sites. Accession numbers for the clones obtained here are as follows: AF381648-AF381753 and AY042086-AY042090.
Area estimates

In order to estimate the area of the plume a seven day composite of level 3 SeaWiFS images was used. The June 8 composite was imported into a graphics editor and the plume was first outlined by hand and then shaded in white. All non-plume pixels were eliminated by shading them black. All white pixels were then counted by importing the image into Matlab (Mathworks). Images used in this analysis were preprocessed by the USF remote sensing group and provided to me at a resolution of 2.2 by 2.2 km. An area estimate can be derived using these numbers. In a similar fashion an area estimate for the total oligotrophic Gulf of Mexico (oGOM) as well as the eastern portion of the oGOM was obtained. The Gulf of Mexico was bounded by a line from the tip of Yucatan to Key West and all pixels south of this line were excluded from area estimates. For the eastern portion of the oGOM a second line was drawn from Yucatan to the Mississippi delta and the portion of the Gulf to the west of this line was excluded. In both cases the desired area was then shaded in white excluding high-chlorophyll coastal regions and the total number of white pixels was counted in Matlab.
Table 3.1

Cellular pigment content at station 4. Bulk chlorophyll (µg L⁻¹) and cellular pigment content (fg cell⁻¹) of divinyl-chlorophyll a (div-chl a) in Prochlorococcus (Pro), fucoxanthin (fuco) plus 19-heanoyloxyfucoxanthin (hex) in picoeukaryotes (Picoeu), 19-butanoyloxyfucoxanthin (19-but) in picoeukaryotes and zeaxanthin in Synechococcus (Syn).

<table>
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<th>Depth in Meters</th>
<th>Bulk Chl a µg L⁻¹</th>
<th>fg div-chl a Pro⁻¹</th>
<th>fg fuco+hex Picoeu⁻¹</th>
<th>fg zeaxanthin Syn⁻¹</th>
<th>fg 19'-but Picoeu⁻¹</th>
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Table 3.2

The distribution of \textit{rbcL} phylotypes detected at individual depth in our \textit{rbcL}-cDNA libraries.

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<th>Number of unique clones obtained</th>
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<th>Prymnesiophytes</th>
<th>Eustigmatophytes</th>
<th>Deeply rooted haptophytes</th>
<th>Diatoms</th>
<th>Pro high light</th>
<th>Pro low light</th>
<th>Prasinophytes</th>
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Chlorophyll-a concentration patterns derived from the Sea-Viewing Wide Field-of-view Sensor (SeaWiFS) over the eastern Gulf of Mexico in June 1999. Blue colors indicate low concentrations and green, yellow, and red indicate increasing concentrations. Concentrations near the Mississippi River Delta and in the river plume, as well as in other coastal areas, are suspect because of known effects by high concentrations of colored dissolved organic matter, suspended sediments, or bottom reflectance, which can artificially raise chlorophyll-a estimates. Land and clouds are colored black. Station locations are indicated. (Image courtesy of Frank Müller-Karger, Douglas Myhre)
Figure 3.2A

Station 4 CTD trace. Shown are carbon fixation (▲), in-situ fluorescence and salinity.
Station 5 CTD trace. Shown are carbon fixation (▲), in-situ fluorescence and salinity.
Flow cytometry of station 4 (A) and station 5 (B). *Synechococcus* (■), *Prochlorococcus* (●), Picoeukaryotes (▲). *Synechococcus* is abundant in the plume surface feature, while *Prochlorococcus* is found below.
Figure 3.4
Fig. 3.4

Pigment data from station 4. (A) divinyl-chlorophyll $a$ (µg L$^{-1}$, ), chlorophyll $b$ (µg L$^{-1}$, — ), prasinophytes, chlorophytes, euglenophytes), zeaxanthin (µg L$^{-1}$, — ), Synechococcus, Prochlorococcus), chl $c_1$ & $c_2$ (µg L$^{-1}$, — ), diatoms, prymnesiophytes, dinoflagellates). (B) violaxanthin (ug L$^{-1}$, — , eustigmatophytes), fucoxanthin (µg L$^{-1}$, — ), diatoms and prymnesiophytes), 19-butanoyloxyfucoxanthin (µg L$^{-1}$, — , prymnesiophytes and chrysophytes), 19-hexanoylfucoxanthin (µg L$^{-1}$, — , prymnesiophytes only).
Figure 3.5

*Prochlorococcus* and *synechococcus*), form IB (ng L\(^{-1}\), ■, green algae), form ID (ng L\(^{-1}\), ●, chromophytes).
Fig. 3.6

Consensus tree obtained from Neighbor Joining analysis of a 133 amino acid long alignment of sequences deduced from cloned cDNA fragments cloned from station 4. This Figure shows the diversity of form IB rbcL phylotypes recovered, together with their closest relatives found in GenBank. 27 novel and 3 previously published OUTs (Paul et al. 2000b) are shown on this tree. Outgroup to the shown clade were several form ID and one form II (Gonyaulax polyedra) sequences. Numbers at internal nodes indicate bootstrap values obtained from 500 bootstrap replicates.
Figure 3.7

Prochlorococcus TAK9803

High Light

Prochlorococcus MED4

P994DY19F

P974CH9

P974AH3

P974CH11

P994DY11F

P994DY16F

P994DY10F

P994BY13

P994DY20F

Prochlorococcus SB

P994DY17F

Prochlorococcus GP2

P994BY2

P994BY1

P994BY7

P974CH12

P994CY1

68

Prochlorococcus MIT9313

P994Y8

64

Prochlorococcus SS120

P994GY23

P994GY20

P994GY7

Vent clone AB038642

Vent clone AB038643

P994GY17

74

P994GY27

P994GY1

52

Prochlorococcus PAC1

P994GY4

88

Prochlorococcus NATL1

P994GY14

81

Vent clone AB038644

Vent clone AB038645

P994FY23

61

Synechococcus WH7803

P99SY12

P99SY5

P99SY1

Synechococcus PCC8102

Vent clone AB038633

Thiobacillus ferrooxidans

0.02

Low Light

Form IA

Marine A–type 2

Synechococcus
Consensus tree obtained from Neighbor Joining analysis of a 133 amino acid long alignment of sequences deduced from cloned cDNA fragments cloned from station 4. This Figure shows the diversity of form IA \textit{rbcL} phylotypes recovered together with their closest relatives found in GenBank. Six previously published sequences (Paul et al. 2000b) and 25 novel OTUs are shown. Outgroup to the shown clade were several form IA, IB, ID and one form II (\textit{Gonyaulax polyedra}) sequences. Also included are inferred \textit{rbcL} sequences from the DOE microbial genome project for \textit{Synechococcus WH8102}, \textit{Prochlorococcus marinus} MED4 and MIT9313 (www.jgi.doe.gov).
Figure 3.8
Consensus tree obtained from Neighbor Joining analysis of a 178 amino acid long alignment of sequences deduced from cloned cDNA fragments cloned from station 4. This Figure shows the diversity of form ID $rbcL$ phylotypes recovered together with their closest relatives found in GenBank. 53 novel OTUs are shown. Outgroup to the shown clade were several form IA, IB and one form II ($Gonyaulax polyedra$) sequences.
RESULTS

Description and area estimate

A SeaWiFS ocean color satellite image of the northern Gulf of Mexico taken on the day of sampling is shown in Figure 3.1. The image shows a high chlorophyll plume originating at the Mississippi delta. This plume was estimated to extend over approximately 26800 km² covering ca. 2% of the total area of the oligotrophic Gulf of Mexico and ca. 6% of the eastern portion. Figure 2 shows salinity, carbon fixation and in-situ fluorescence traces of a station within (station 4, Fig. 3.2A) and outside the plume (station 5, Fig. 3.2B). The salinity at station 4 varied between 36 and 36.5 ppt. throughout most of the euphotic zone. In the uppermost 10 m however we observed a well defined low salinity feature (33.5 ppt.; Fig. 3.2A) resulting from the Mississippi River plume. The most prominent feature of the CTD trace at station 4 (Fig. 3.2A) is the bimodal distribution of in-situ fluorescence. The subsurface chlorophyll maximum (SCM) occurred between 80-100 m of depth and an equally prominent peak in fluorescence occurred in the low salinity surface layer. No such increased surface in-situ fluorescence was observed at station 5 (Fig. 3.2B).

Productivity estimates (station 4 and 5)

Photosynthetic carbon fixation at station 4 was also bimodal, with a surface maximum of 3.39 µg C L⁻¹ h⁻¹ and a second, much smaller peak at the SCM (86 m sample) of 0.59 µg C L⁻¹ h⁻¹ (Fig. 3.2A). There was no similar surface peak in primary
production at station 5 and highest primary productivity of 2.05 µg C L⁻¹ h⁻¹ occurred in the 82 m, the SCM sample (Fig. 3.2B). Integrated water column primary productivity was estimated at ca. 63 mg C h⁻¹ m² for station 4 and ca. 84 mg C h⁻¹ m² for station 5. Light intensity measurements indicated that the ratio of subsurface to surface light intensity (I₂/I₀) was greater at station 5 than station 4 below 10 m, the average difference ranging from 14 to 34 % (data not shown). Thus, the plume may have shaded the lower water column, decreasing its rate of carbon fixation.

Flow cytometry

Flow cytometry data for stations 4 and 5 are shown in Figures 3.3A and 3.3B respectively. *Prochlorococcus* was completely absent in the surface plume of station 4, but was more than tenfold more abundant than *Synechococcus* in surface samples from station 5. At station 4 *Prochlorococcus* was most abundant between 10 and 80 m of depth with a maximum cell count of 1.7x10⁵ cells ml⁻¹ in the sample from 40 m. *Prochlorococcus* counts below 20 m of depth (below the low-salinity plume) were of similar magnitude for both stations and their profiles correlated well to each other by paired t-test analysis (t = 0.20 and P = 0.85). *Synechococcus* counts in surface plume samples from station 4 were approximately fivefold higher than counts obtained from station 5 surface samples. In the lower water column *Synechococcus* counts reached a second maximum just above the SCM at both stations and then decreased. *Synechococcus* profiles of the two sites did not correlate well to each other using paired t-test analysis (t = 1.8 and P = 0.13). Picoeukaryotes were the least numerous picoplankton fraction and
reached their highest abundance at the SCM at both stations (4.9 x 10^3 cells ml\(^{-1}\) and 4.6 x 10^3 cells ml\(^{-1}\) at station 4 and 5 respectively).

**HPLC pigment data**

HPLC pigment data for station 4 is shown in Figure 4. Divinyl-chlorophyll \(a\), a pigment diagnostic of *Prochlorococcus*, was absent from the low salinity surface plume corroborating the observation made by flow cytometry that *Prochlorococcus* was absent there. Below the plume the divinyl-chlorophyll \(a\) concentration increased with depth and reached a maximum just above the SCM. Highest cell counts of *Prochlorococcus* were observed at slightly shallower depth (40 m) than the divinyl-chlorophyll \(a\) maximum (60 m). If the divinyl-chlorophyll \(a\) concentrations are divided by *Prochlorococcus* cell counts (Table 3.1, column 3), cells between 78 and 120 meters of depth are observed to contain significantly more (\(t = 3.9, P = 0.008\)) divinyl chl \(a\) than cells from shallower depth, explaining this discrepancy. While this observation could potentially be the consequence of photoacclimation by *Prochlorococcus*, *rbcL* sequence data (see below) indicate the presence of two ecotypes of *Prochlorococcus*, one adapted to high light intensities and one adapted to lower light flux.

Zeaxanthin is found in both *Synechococcus* and *Prochlorococcus* and was found in surface to mid depth samples, but decreased where divinyl-chl \(a\) reached its maximum. Zeaxanthin was almost absent at and below the SCM. Chlorophyll \(b\), an indicator of chlorophytes (particularly Prasinophytes), as well as chlorophylls c1 and c2, which are found in many chromophytic algae, were bimodally distributed, displaying one maximum
at the surface and the second at the SCM (Fig. 3.4A). Fucoxanthin, produced by diatoms and prymnesiophytes, as well as 19-hexanoyloxyfucoxanthin, produced by both prymnesiophytes and chrysophytes, exhibited a similar bimodal distribution (Fig. 3.4B). At station 5 (data not shown) no such bimodal distributions were observed. Chlorophyll $b$, chlorophylls $c1$ and $c2$, divinyl-chl $a$, 19-hexanoyloxyfucoxanthin, as well as 19-butanoyloxyfucoxanthin only displayed one peak at the SCM. Zeaxanthin was very similarly distributed at both stations.

*rbcL* mRNA

Profiles of form IA, B and D *rbcL* mRNA were quantified at stations 4 and 5 (Figures 3.5A & B respectively). Concentrations of form IA *rbcL* mRNA were bimodally distributed with depth at both stations. However, while the shallow and deep maxima at station 5 were of similar magnitude, surface form IA *rbcL* concentrations at station 4 were twice as high as concentrations observed at the deeper maximum and almost ten-fold higher than both form IB or form ID concentrations. At station 4 form ID *rbcL* mRNA was present at all depths (Fig. 3.5A) and its highest abundance was observed at the SCM. At station 5 form ID transcript could only be detected at and below the SCM (Fig. 3.5B). The form IB *rbcL* transcript profile at station 4 was bimodal and concentrations were highest in the surface and SCM samples. Form IB *rbcL* was not detected at 60 m, which coincided with the highest concentrations of *Prochlorococcus* observed. At station 5 form IB *rbcL* mRNA was detected at all depths with noTable minimum at the SCM.
**rbcL cDNA libraries**

Four hundred-eighty nine *rbcL*-containing clones were screened by the PCR-restriction analysis described in the methods. One hundred and five unique *rbcL* sequences were recovered from station 4. Clones were designated by cruise date (P99), Station (4), depth (A-H) and primers used for PCR amplification (‘Y’ for form IA/B, ‘H’ for Form D primers and ‘SY’ for the cyanobacterial reverse primer). A rich assembly of *rbcL* phylotypes related to green algae was recovered from our clone libraries (Fig. 3.6). Predominant among these was a group of sequences closely related to *Chlorella* and a group of OTUs forming a micro-diverse clade closely resembling *Bathycoccus prasinos*. Nucleotide similarity within the prasinophyte clade was between 96 to 98% identity. Several more deeply rooted green algal sequences formed three clades. These were not closely related to any *rbcL* sequence in the database, but were distantly related to *Sphagnum palustre*. Among the form IB sequences were also two sequences, which were almost identical to the *rbcL* of the freshwater *Synechococcus* PCC6301 (formerly *Anacystis nidulans*), indicating the presence of form IB cyanobacteria.

Figure 3.7 shows the diversity of form IA sequences recovered from station 4. Most notable is the presence of two clades of sequences closely resembling *Prochlorococcus*. One group of these sequences was exclusively isolated from samples between 10 to 60 m depth and shared between 89 and 98 % DNA sequence identity. Shallow depth *Prochlorococcus*-like sequences formed a tightly conjoined clade with *Prochlorococcus GP2* and the *rbcL* sequences amplified from DNA samples of
Prochlorococcus marinus strains MED4, TAKA9803-2 and SB. These cultured strains have been described as high-light adapted Prochlorococcus isolates (Moore et al. 1995, Shimada et al. 1995, Garczarek et al. 2000). The second group of Prochlorococcus-like sequences was recovered from water samples taken between 78 and 130 m of depth. These sequences shared between 82 to 94 % sequence identity and appeared closely related to Prochlorococcus strains MIT 9313, a low-light adapted Prochlorococcus isolate (Moore et al. 1998), and the rbcL sequences cloned from Prochlorococcus strains NATL1 and NATL2B, both of which are also low light adapted strains (Partensky et al. 1993). Interestingly it was found that several rbcL sequences recently recovered from deep-sea thermal vent bacteria (Elsaied & Naganuma 2001), GenBank Accession # AB038641, AB038642, AB038643) were closely associated with the clade of low-light adapted Prochlorococcus sequences. Between group comparison (high-light vs. low-light) of Prochlorococcus-like sequences revealed that the two clades were between 76 and 81 % identical at the DNA level, supporting the hypothesis that the two clades are phylogenetically distinct. To further test this assumption individual or several branches were moved from the high-light into the low-light clade or vice versa and the tree topology was analyzed using Templeton (Templeton 1983) statistical analysis. No significant (P < 0.05) alternate topology was found if the division between these two groups was not preserved.

A third clade of from IA sequences was amplified upon reexamination and redesign of our form IA/B reverse primer. These sequences were closely related to the marine cyanobacterium WH7803, indicating the presence of form IA Synechococcus in station 4 surface water. The richness of form ID rbcL phylotypes recovered from station
4 was very large (Fig. 3.8). Fifty-three novel form ID OTUs were obtained. Twenty-three of these were most closely related to Prymnesiophytes. Six pelagophyte-like, thirteen diatom-like, five eustigmatophyte-like, three haptophyte-like and three more deeply rooted, unidentified sequences were also obtained. Table 3.2 shows the distribution of clones we obtained and their phylogenetic affinity. Prymnesiophytes were observed throughout the water column, while other chromophytic algae were largely recovered from subsurface samples. No diatoms were observed in the surface clone samples, whereas deeply rooted haptophytes were found in those samples. All prasinophyte-like sequences were cloned from water sampled at the SCM. Prochlorococcus lowlight and highlight sequences are divided into shallower and deeper depth respectively.

**PCR error**

In order to determine PCR and sequencing error a total of 11264 base pairs were sequenced from 22 rbcL clones. Among these one gap and 10 PCR or sequencing errors were found, indicating accuracy of better than 99.9 % in the determination of sequence identity from PCR amplified rbcL sequences.
DISCUSSION

A low salinity, high chlorophyll Mississippi River plume was tracked in the central north-eastern Gulf of Mexico using SeaWiFS satellite images and sampled. Primary productivity was greatest in the low salinity surface layer, which contained large numbers of *Synechococcus* and an abundance of form IA rbcL mRNA. A previous study of a plume-like feature in the Gulf of Mexico yielded 23 unique rbcL clones derived from PCR amplification of extracted DNA (Paul et al. 2000b). In this previous study it was however not known, if sequences were derived from live or dead cells or even from extracellular DNA. In the present study, 105 unique sequences were derived from 489 clones screened by RFLP analysis, all of which were derived from mRNA. Phylogenetic analysis of cDNA clone-libraries from a profile at this station revealed a diverse group of rbcL phylotypes including *Synechococcus*, the high-light and low-light adapted clades of *Prochlorococcus* and several clades of eukaryotic algae. Among the eukaryotes prymnesiophytes were most prevalent and were distributed throughout the water column. Diatoms were found in the lower portion of the water column and prasinophytes may have dominated picoeukaryotic phytoplankton at the subsurface chlorophyll maximum (SCM).

Oceanic picoplankton community structure has been studied using flow cytometry and epifluorescence microscopy (Binder et al. 1996, Blanchot & Rodier 1996, Blanchot et al. 2001). Based on these and many other studies it is now believed that in tropical and subtropical environments *Prochlorococcus* numerically dominates the picoplankton and usually exceeds other groups by one to two orders of magnitude (Campbell et al. 1994,
Campbell et al. 1997, Blanchot et al. 2001). Picoeukaryotes are usually distributed independently of light intensity and often represent the majority of the picoplankton at the SCM. *Prochlorococcus* occasionally dominates over picoeukaryotes at the SCM if the SCM is located above the nitracline (Blanchot & Rodier 1996). Both *Prochlorococcus* and picoeukaryotes generally outnumber *Synechococcus* at low light intensities and *Synechococcus* is often most abundant where *Prochlorococcus* is least numerous (Olson et al. 1990a, Partensky et al. 1996).

These generalizations held true for the non-plume Gulf of Mexico station described here (station 5). In contrast, plume station 4 had a significantly altered picoplankton community structure. Picoeukaryotes were less abundant than at station 5 at all depths and *Prochlorococcus* was all but absent from the surface plume. The surface community at station 4 was instead dominated by *Synechococcus*, which was fivefold more numerous than at station 5. Similar observations for high chlorophyll, low salinity plume features have previously been reported (Paul et al. 2000a) suggesting that large numbers of *Synechococcus* are a dominant feature of such plumes in the Gulf of Mexico. Paul et al. (Paul et al. 2000a) also reported elevated form IA *rbcL* gene expression associated with the plume reminiscent of the surface peak in form IA *rbcL* described here.

The taxonomic group *Synechococcus* is an assembly of unicellular, coccoid cyanobacteria divided into six provisional genera. Two of these clusters are designated marine A & B (for example marine cluster A contains *Synechococcus* strains WH7805, WH8103 and WH7803, while marine cluster B contains *Synechococcus* strains WH8101 and WH8007) and express a form IA *rbcL*. We consequently hypothesized that the
elevated levels of form IA rbcL observed in the plume were due to the presence and activity of the proliferating population of *Synechococcus* observed by flow cytometry. In order to support this hypothesis we hoped to demonstrate the presence of *Synechococcus* marine clusters A & B like sequences by RT-PCR and cloning. Unfortunately no such sequences were recovered from our cDNA libraries. Only two form IB *Synechococcus*-like clones were obtained (Fig. 3.6) from the 60 m and 100 m (just above and at the SCM) samples, both of which were closely related to *Synechococcus* strain PCC6301. Upon reexamination of new rbcL sequence information for marine *Synechococcus* isolates found in GenBank we discovered that our cyanobacterial primer set did not match these newly submitted sequences and the lack of form IA *Synechococcus* rbcL OTUs in our libraries might consequently be expected. Using a newly designed 3’ primer, four unique OTUs most closely related to *Synechococcus* strain WH7803 were recovered by PCR amplification of DNA extracted from station 4 surface samples (all RNA samples had been exhausted and only DNA samples were available at this point in the investigation). Unfortunately sequence information obtained from DNA samples cannot be taken as evidence of transcriptionally active cells and can only demonstrate the presence of such genotypes in the plume.

Oligotrophic sites in the Gulf of Mexico typically exhibit a bimodal distribution of form IA rbcL mRNA similar to station 5 (Fig. 3.5B). This distribution is expected due to the presence of large numbers of *Prochlorococcus*. *Prochlorococcus* isolates belong to one of two distinct chlorophyll b/a₂-ratio phenotypes (Morel et al. 1993, Partensky et al. 1993, Moore et al. 1995). Cells with the low b/a₂ phenotype form a dimly fluorescing population in shallower, ‘high-light’ environments, while the deeper part of the euphotic
zone harbors a more brightly fluorescing population with the high $b/a_2$ (‘low-light’) phenotype (Campbell & Vaulot 1993, Veldhuis & Kraay 1993, Blanchot & Rodier 1996, Partensky et al. 1996, Zubkov et al. 1998, Blanchot et al. 2001). *Prochlorococcus* GP2, a high-light adapted strain first isolated from the western Pacific Ocean (Shimada & Miyachi 1996), was the first to be shown to encode a form IA $rbcL$ (Shimada et al. 1995).

With the exception of one isolate all $rbcL$ sequences for *Prochlorococcus* available in GenBank and all strains screened in this study are also of the form IA type. A bimodal distribution of form IA $rbcL$ mRNA is expected due to the presence of the two ecotypes of *Prochlorococcus*, which both express this form of $rbcL$. At station 4 elevated form IA $rbcL$ mRNA levels in the plume are superimposed on the expression levels typically found due to *Prochlorococcus* (Fig. 3.5A). Flow-cytometry in fact indicated that *Prochlorococcus* was absent from the surface plume and we recovered no *Prochlorococcus*-like sequences from our station 4 surface cDNA libraries. Why *Prochlorococcus* is absent from the plume remains unclear, but might be due to the lower salinity found there. *Prochlorococcus* is, for example, not found in low salinity estuarine waters (Campbell, L., personal communication).

A large diversity of *Prochlorococcus*-like sequences was recovered from samples taken below the plume, where *Prochlorococcus* was very abundant. Phylogenetic analysis of these sequences revealed that *Prochlorococcus*-like phylotypes were found in two distinct clades. One group of sequences was recovered from samples taken between the plume and mid-depth and is most closely related to the high-light adapted *Prochlorococcus* isolates MED4, GP2, SB and TAKA9803-2. The second group sequences was exclusively isolated from deeper water samples, which included the SCM.
These sequences were more closely related to the low-light Prochlorococcus isolates MIT9313, NATL1, NATL2B and SS120. The tight linkage in phylogenetic analysis to rbcL sequences from cultured isolates lends strong support to the hypothesis that the two observed clades of Prochlorococcus-like rbcL phylotypes described here are indeed the highlight and lowlight adapted Prochlorococcus ecotypes previously described. This surprising correlation between depth, pigment content and phylogenetic inference is remarkable, but has also been recognized for 16 S rDNA sequence information from Prochlorococcus isolates (Urbach & Chisholm 1998) as well as genotype specific gene probing in the North Atlantic Ocean (West & Scanlan 1999). It is also interesting to note that several recently isolated deep sea thermal vent bacterial form IA rbcL sequences (Elsaied & Naganuma 2001) were found to group within the low-light Prochlorococcus clade, suggesting a gene transfer event from possibly extremophilic, chemotrophic bacteria to Prochlorococcus may have occurred.

Even though form IA rbcL expressing phylotypes were apparently dominant in the plume they were not found to the exclusion of other phytoplankton. We also found both form IB and form ID rbcL mRNA in the plume. Form IB rbcL mRNA surface levels were in fact of similar magnitude as surface values recorded at station 5 and reached ca. 15% of form IA levels, suggesting that form IB bearing phytoplankton may not have been affected strongly by the presence of the plume. The form ID sequences found in the plume corroborate the pigment data indicating the presence of chromophytic algae, such as diatoms and prymnesiophytes. However, the co-occurrence of fucoxanthin and 19-hexanoyloxyfucxanthin, the presence of prymnesiophyte clones, and the absence of
diatoms in the surface clones, strongly argues for the presence of prymnesiophytes over diatoms in the plume at this location.

Phylogenetic analysis of our cDNA libraries revealed two groups of form IB clones. Several clones were most closely related to *Chlorella* and these were found both in surface and deeper samples (Table 3.2, Fig. 3.6). The other group of sequences was isolated from samples within the SCM and these sequences formed a micro-diverse clade ingroup to *Bathycoccus prasinos* (Fig. 3.6) suggesting that prasinophytes may have been particularly important there. This hypothesis is substantiated by the presence of high concentrations of chlorophyll *b*, a green algal pigment (prasinophytes, chlorophytes and euglenophytes), at the SCM, where this pigment reached a higher concentration than any other pigment except chl *a* (Fig. 3.4A). It is also interesting to note that at the SCM form IB *rbcL* mRNA levels reached ca. 50 % of form IA levels. Because *Prochlorococcus* was more than tenfold more abundant than picoeukaryotes at this depth (Fig. 3.3A) it appears that form IB carrying phytoplankton, particularly prasinophytes, may have exhibited a 5 fold higher transcriptional activity of *rbcL* than form IA containing picocyanobacteria.

Form ID *rbcL* mRNA was absent in the upper water-column at station 5 (Fig. 3.5B) and only found at the SCM, yet at station 4 chromophytic *rbcL* was found in the entire water-column (Fig. 3.5A). This suggests that the presence of the plume may have stimulated chromophytic *rbcL* expression above the SCM where other forms of *rbcL* (ie. form IB from picoeukaryotes and picocyanobacteria) may otherwise dominate. Depth integrated counts of picoeukaryotes at station 4 were not elevated over station 5 averaging ca. 87 %, if similar depths are compared between the two stations. No elevated counts of picoeukaryotes were observed in the surface plume. This may mean that
chromophytic picoeucaryotes (ie. Prymnesiophytes) replaced form IB-containing picoeucaryotes in the plume. A large diversity of form ID phylotypes was observed at station 4 (Fig. 3.8) encompassing four major phytoplankton groups. Prymnesiophytes were by far the most diverse group of organisms and were found at all depth except the center of the SCM, although pigment data (Fig. 3.4B) suggests their abundance at this depth. We also detected the rbcL sequences of eustigmatophytes and pelagophytes at station 4. Unfortunately there appears to be no clear pattern in the distribution of these clones. This lack in resolution is possibly the consequence of the insufficient scale of our clone libraries or potential drawbacks inherent in PCR based techniques. PCR suffers from many biases (for a review see (Winzingerode et al. 1997)) as is illustrated by our initial failure to detect form IA Synechococcus in the plume in 1997 and in the current sampling. Regardless of our ability to detect these organisms pigment signatures suggest the presence of all three groups in the entire water column.

The observed phytoplankton diversity at station 4 is not surprising. It has already been put forward by Hutchinson (Huchinson 1961) that species diversity is often far greater than could be expected as a result of niche-partitioning based only on a few limiting resources. Besides the sheer number of species, two additional facets of diversity were observed in our dataset. A very diverse and deeply rooted group of eukaryotic algae, including the chromophytes and Chlorella-like green alga was observed. These organisms were found to be only distantly related and covered almost the entire phylogenetic spectrum of the known pelagic eukaryotic phytoplankton. These phylotypes are likely representative of unique species. The greater genetic distances between these forms suggests that these have evolved some time ago, or are under some genetic constraint in
the evolution of the \textit{rbcL} gene. On the other hand prasinophytes, and the high/low light \textit{Prochlorococcus} clusters exhibited marked microdiversity reminiscent of microdiversity observed for \textit{nosZ} (Scala & Kerkhof 1999), \textit{nifH}-containing bacterial populations (Zehr et al. 1998), and \textit{Prochlorococcus} in the plume sampled in 1997. The simultaneous occurrence of a large numbers of organisms with minute differences in sequence identity (less than 10% DNA dissimilarity) we believe indicates a rapidly evolving or recently evolved clade of organisms. This is not limited to prokaryotes because a cluster of eukaryotes such as the group of prasinophytes showed a similar microdiversity. If microbial microdiversity is a general phenomenon however it would appear that most genetic diversity is found in small variations of functional genes, a fact most likely missed by recent microbial genome sequencing efforts.

It is interesting to speculate about the importance of the Mississippi River plume to primary productivity in the oligotrophic Gulf of Mexico. The Mississippi River discharges on average 1.7 x 10^{4} m^{3} s^{-1} fresh water onto the northern Gulf of Mexico shelf, most of which is carried westward along the Texas coastline (Müller-Karger et al. 1991). Eastward transport of the Mississippi River plume is apparently sporadic and short lived and occurs either as focused or diffuse dispersal along the coastline or as larger discolored water masses which are carried into the open Gulf. The plume described in this paper is an example of such a large discharge event. Müller-Karger (Müller-Karger et al. 1991) also investigated the frequency of occurrence of large offshore plumes by screening CZCS (Coastal Zone Color Scanner) images between November 1978 and May 1980. Two plume occurrences were observed in these images. This indicates that large eastward extending plumes are by no means frequent events, yet are also not rare. Image
analysis indicates that the plume described here covered ca. 2% of the total oligotrophic Gulf of Mexico and ca. 6% of the eastern portion typically influenced by such plumes. Surface primary productivity at station 5 was measured at 0.54 µg C liter⁻¹ h⁻¹, while surface productivity at station 4 was estimated at 3.39 µg C liter⁻¹ h⁻¹. If these rate measurements are used as averages for non-plume and plume respectively, it can be estimated that during the time of sampling the plume may have accounted for as much as 11% of total surface productivity in the oligotrophic Gulf of Mexico (upper 10 m of the water column). The plume may have also accounted for as much as 28% of oligotrophic surface productivity of the eastern portion of the Gulf. However, such estimates of basin wide production based upon two points should be viewed with caution. Despite the positive impact on surface productivity the reverse may have been true at the SCM. SCM primary productivity at station 4 was only 29% (0.59 µg C liter⁻¹ h⁻¹) of the rate observed outside of the plume (2.03 µg C liter⁻¹ h⁻¹). In fact, if primary productivity is integrated throughout the water column, it is found that station 4 only displayed ca. 75.4% of water column productivity observed at station 5. This is most likely due to the attenuated light intensity below the plume. These observations suggest that the plume may have significantly reduced productivity at a depth critical to the downward flux of carbon into the deep ocean via the biological pump and may have significantly impacted the rate of carbon sequestration in the Gulf of Mexico.

Collectively these data indicate that the presence of a low salinity, high-chlorophyll plume derived from Mississippi River discharge may have significantly impacted the phytoplankton community structure of the Gulf of Mexico by allowing *Synechococcus* to replace *Prochlorococcus* in the low salinity surface layer and
potentially stimulating the growth of larger, chromophytic phytoplankton in the upper
portion of the water column. Using culture independent techniques we detected two
spatially separated clades of *Prochlorococcus* reminiscent of the high-light and low-light
adapted *Prochlorococcus* ecotypes which numerically dominated below the plume. We
also detected a clade of prasinophytes, which may have been important at the SCM. In
combination with the altered picoplankton community of the plume we observed that
surface primary productivity of the oligotrophic Gulf of Mexico was enhanced by as
much as 9.5 %, while productivity may have been negatively impacted at the SCM by the
presence of the plume. Despite this enhancement of surface productivity in the plume
integrated water column productivity may have in fact been reduced by as much as 25%
by its presence.
CHAPTER 4

Phytoplankton Community Structure and Productivity
Along the axis of the Mississippi River plume

The following chapter has not been published, but is currently in review. The article has been submitted essentially in this form to the journal Aquatic Microbial Ecology.

CHAPTER SUMMARY

The Mississippi River is the largest freshwater input into the Gulf of Mexico (GOM) and contributes a large nutrient load to northern GOM waters. During the summer the Mississippi River plume is sometimes found to extend into the eastern oligotrophic GOM as far south as the Dry Tortugas. The objectives of this study were to determine the relative contribution of the Mississippi River Plume to the total surface water production in the oligotrophic GOM and the impact of this feature on the composition of phytoplankton found there. Using SeaWiFS satellite images we located and sampled the offshore Mississippi River plume along its axis. In-situ sampling in combination with remote sensing allowed us to estimate integrated plume primary productivity. Carbon fixation in the northern GOM averaged 0.53 µg C L⁻¹ h⁻¹ for non-plume stations and 9.3 µg C L⁻¹ h⁻¹ in plume stations. We estimated integrated productivity of the plume at ca. 3.28*10⁹ g C h⁻¹, which accounted for 41% and 13% of all surface and total water column productivity in the oligotrophic GOM respectively at
the time of sampling. Analysis of \textit{rbcL} cDNA clone libraries and HPLC pigment data indicated that our sampling transect traversed several regions with distinctly different phytoplankton assemblages. Non-plume communities were numerically dominated by \textit{Prochlorococcus}, and contained prymnesiophytes and eustigmatophytes. Diatoms dominated the most productive inshore station, while \textit{Synechococcus} dominated in the mid-plume just off the Louisiana shelf. The least productive and most offshore portion of the plume was also diatom dominated. Diatoms were the most diverse algal class observed accounting for over 42\% of all unique \textit{rbcL} genotypes detected in the plume. Collectively these results indicate that the Mississippi River Plume contributes significantly to oligotrophic productivity in the GOM resulting from localized blooms of both \textit{Synechococcus} and diatoms.
INTRODUCTION

The Mississippi River is the seventh largest freshwater input into the world’s oceans (Milliman & Meade 1983) and strongly effects the biological productivity of the northern Gulf of Mexico shelf (Walsh 1988, Lohrenz et al. 1994, Redalje et al. 1994b, Fahnenstiel et al. 1995, Rabelais et al. 2002). The region surrounding the Mississippi delta is in fact so productive that it accounts for 70-80% of Gulf of Mexico (GOM) fishery landings (Grimes 2001), the US portion of which alone was valued at ca. $994 million during the year 2000 (National Marine Fisheries Service, personal communication). The forces controlling biological productivity and phytoplankton community composition in this region are thus of great ecological and economical interest.

On a basin wide scale phytoplankton distribution in the GOM has been studied using ocean color satellite images (Müller-Karger et al. 1991). Surface pigment concentrations on the Gulf of Mexico shelf are generally higher than those observed for the oligotrophic Gulf and peak during winter. Pigments also undergo a marked seasonal cycle strongly influenced by the depth of the mixed layer. In contrast, surface pigment concentrations in the oligotrophic portion of the Gulf are generally low and display little seasonality. The Mississippi River plume, which usually extends westward along the Louisiana and Texas coastline is thought to be responsible for the high productivity of these waters. Particularly during the summer months however, low salinity, high chlorophyll and highly productive water can be carried south-eastward into the oligotrophic GOM. Under these circumstances the plume appears as a conspicuous, high-
chlorophyll surface feature in SeaWiFS images stretching from the Mississippi delta across the Gulf as far as the Dry Tortugas (Müller-Karger et al. 1991, Perez et al. 1999, Wawrik et al. 2003). Often a second plume containing elevated surface chlorophyll originating from the Yucatan upwelling, joins the Mississippi River plume tracing and completely outlining the loop current. The Mississippi outflow westward of the Mississippi delta has been studied intensely, in part, because the plume has been implicated in the formation of extensive areas of hypoxic bottom water (Justic et al. 1996, Rabalais et al. 1996). Far less is known about the far-field effect of the Mississippi River plume in the oligotrophic GOM where it can on occasion be a prominent feature. Little is known in particular about its impact on basin-wide primary production and phytoplankton community composition.

We have previously described the vertical structure of the picoplankton community at a site within the offshore Mississippi River plume (Wawrik et al. 2003). Using a combination of flow cytometry, mRNA hybridization, pigment analysis and rbcL cDNA clone libraries we demonstrated the vertical distribution of phytoplankton species in the water column. The plume harbored a large bloom of *Synechococcus*, which replaced *Prochlorococcus* in the upper water column as determined by comparison with a nearby non-plume site. Our observations concerning a single site in the offshore Mississippi River plume led us to the current investigation of the phytoplankton dynamics along a transect of the plume from the Louisiana Shelf far into the oligotrophic Gulf of Mexico. Specifically we were interested in determining if the phytoplankton community composition was dynamic and changed along the axis of the plume. Secondly we wanted to determine the relative importance of the plume to surface water production
of the Gulf of Mexico basin. Our results indicate that phytoplankton shifts occur as the plume matures and the plume, at times, can account for as much as 41% of the surface production in the Gulf.
MATERIALS AND METHODS

Sampling

Sampling was performed aboard the R/V ‘F. G. Walton-Smith’ in the NE Gulf of Mexico between July 15 and 23, 2001. The station locations are indicated in Figure 1. Using SeaWiFS satellite images downloaded daily to the vessel, we determined the approximate location of the plume. The onboard auto-sampling system (which recorded near surface in-situ fluorescence and salinity) was then used we located the frontal boundary between the plume and the non-plume GOM. Stations were chosen some distance away from the frontal boundary, inside the plume, so that our stations formed a series, which followed the length of the plume towards the Mississippi shelf. Stations 1 & 8 were located outside of the plume, while stations 2-7 were located inside. Station locations were as follows: station 1 - Lat 26º, 00 min. N, Long 83º, 35.6 min W, station 2 - Lat 27º, 17 min. N, Long 87º, 23 min W, station 3 - Lat 27º, 30 min. N, Long 88º, 24 min W, station 4 - Lat 27º, 58 min. N, Long 87º, 35 min W, station 5 - Lat 28º, 33 min. N, Long 87º, 24.9 min W, station 6 - Lat 28º, 59.9 min. N, Long 86º, 38.6 min W, station 7 - Lat 26º, 28.7 min. N, Long 86º, 38.6 min W, station 8 - Lat 25º, 15.78 min. N, Long 84º, 13.21 min W. Before sampling the CTD (conductivity temperature depth recorder) was cast to a depth of 200 m in order to determine the fluorescence profile in the water column and determine the depth of the subsurface chlorophyll maximum (SCM). For profiles seawater was sampled from a total of eight depths. Sampling depths were chosen so that one sample was taken immediately above, inside and immediately below the
SCM. The near surface samples were taken at a depth of 3 m using a small electric impeller pump. The remaining samples were taken at depth spaced evenly throughout the euphotic zone. Sampling was performed during morning hours using 20 L Niskin bottles attached to rosette sampler equipped with a Seabird CTD. Samples from greater than 40 m of depth were protected from light by wrapping carboys in black plastic bags during filling and sample processing.

**14C carbon fixation**

14C-carbon fixation was measured by a technique adopted from Carpenter and Liveley (Carpenter & Lively 1980) in sterile, acid cleaned 500 ml polycarbonate flasks assaying 325 ml water samples. Flasks were incubated under irradiance adjusted to resemble the intensity and spectral features of the underwater light field using neutral-density screening and colored acetate filters. Depth profiles of irradiance intensity were determined using a Li-Cor light meter equipped with Li-190SA and Li-192SA radiation sensors. Surface plume productivity was determined assuming a plume surface layer of 10 m. Water column productivity was integrated for one m$^2$ of surface ocean as follows. The water column was divided into segments bounded by the surface, the half-way points between each pair of adjacent sampling depth and the depth of the bottom sample. Primary productivity measurements taken within each interval were then extrapolated to the volume of the segment and all estimates for the water column segments were then added.
**RNA extraction for RT-PCR**

Between 200 and 800 ml of seawater were filtered onto 25 mm, 0.45 µm HV polyvinylidene difluoride filters (Millipore Durapore). Filters were then stored under liquid nitrogen in 2 ml screw-cap tubes containing 750 µl RLT buffer (Qiagen), 7.5 µl β-mercapto ethanol and 0.2 g muffled, baked glass beads. Samples were not treated with DEPC (diethylpyrocarbonate), as is commonly done for probing experiments, since DEPC inhibits RT-PCR reactions by chemically modifying RNA. For extraction samples were thawed in warm water for two minutes and cell lysis was achieved by bead-beating as described previously (Wawrik et al. 2003). Lysate was then extracted using a Qiagen RNeasy RNA extraction kit (Qiagen). Samples were DNAse digested on the RNeasy columns for 15 min using the RNAse-Free DNAse reagent set (Qiagen) according to the protocol provided by Qiagen to remove DNA contamination.

**PCR**

Reverse transcriptions were run using random hexamers, 4.7 mM MgCl, and M-MLV Reverse Transcriptase (Promega, Madison, WI) for 30 min at 37° C and freshly extracted RNA. Five µl were then added to a PCR reaction. Two primer sets were used to amplify the *rbcL* fragments. The form IA/B primer set produced a 615 bp fragment (forward primer: TCIGCITGRAACTAYGGTCG, reverse primer: CTGAGIGGIAARAAACTACGG) and the form ID set produced a 554 bp fragment (forward primer: GATGATGARAAYATTAACTC, reverse primer...
ATTTGDCCACAGTGDATAACCA). All sequences are stated using IUPAC
degeneracies. PCR conditions were as follows: 1 µM final concentration for both
primers, 1.5 mM MgCl₂, 2.5 mM of each nucleotide and five units of Taq-polymerase
(Promega, Madison, WI). Cycle parameters were: 3 min at 95° C followed by 40 cycles
of 1 min at 95° C, 1 min at 52 °C and 1 min 30 sec at 72° C. Cycling was followed by a
15 min 72° C elongation step.

Clone libraries

*rbcL* mRNA was RT-PCR amplified from the surface samples of all eight
stations using both primer sets. Immediately after amplification products were purified
using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Amplicons were then
ligated into PCR® 2.1 vector using a TA cloning kit (Invitrogen corp., Carlsbad, CA). All
white colonies were then picked onto individual 2xYT plates containing 50 µg ml⁻¹
Kanamycin and Ampicilin. The number of clones picked for stations 1-8 were 40, 37, 43,
39, 41, 43, 42 and 45 respectively for the form ID primer set and 39, 40, 40, 39, 40, 30,
38 and 39 respectively for the form IA/B primers. Clones were screened for the correct
insert size using PCR by touching a colony with a sterile loop and transferring this loop
directly to a PCR reaction tube. The number of colonies with correct insert sizes from
station 1-8 for the form ID primers set were 30, 13, 33, 38, 35, 36, 28 and 37 respectively
and 21, 32, 30, 17, 17, 10, 17, and 16 for the form IA/B primers. Amplifications with
products of the correct size were purified using a QIAquick PCR purification kit (Qiagen,
Valencia, CA). Twenty µl of purified product were then digested with 5 units of each
Sau3IA (digests at $\mathrm{\text{GATC}}$) and AluI (digests at $\mathrm{AG\text{CT}}$) in Buffer B (Promega, Madison, WI) for 1 hour at 37° C. Digestions were run on 2.5 % low melting agarose gels (Fisher, Fair Lawn, NJ) at 13.5 V cm$^{-1}$ for 2 hours and stained with ethidium bromide. Clones with unique restriction patterns were visually selected for sequencing. Sequencing we performed in an Applied Biosystems model 373 sequencer by the University of Florida core sequencing laboratory (University of Florida, Gainesville, FL).

**Phylogenetic analysis**

cDNA sequences were translated into amino acids and these were aligned with a representative sample of $rbcL$ sequences obtained from GenBank as well all sequences we previously recovered from similar environments (Paul et al. 2000b, Wawrik et al. 2003). Alignment was performed using Omiga 1.1 (Oxford Molecular group, Oxford, UK) using a Clustal W pairwise weighted alignment method. Amino acid sequences were used, because third codon positions were saturated and in order to avoid potential biases introduced by codon usage and GC content. Alignments were then eyeballed for obvious misalignments, and exported to Mega 2.0 beta (Kumar et al. 1993). Trees were built using both parsimony and the Neighbor-Joining method using a gamma distribution (gamma parameter =2.0) to correct for rate heterogeneity across sites. Both methods generally agree if $rbcL$ alignments are analyzed. Accession numbers for the clones obtained here are as follows: AY157365-AY157486.
**Plume area and productivity estimates**

A seven day composite of level 3 SeaWiFS images was used. The image was imported into a graphics editor. The plume was then first outlined by hand and then shaded in white. All non-plume pixels were shaded black. All white pixels were then counted by importing the image into Matlab (Mathworks). Level 3 SeaWiFS images provided to me by the USF remote sensing lab (curtesy of Frank Müller-Karger and Doug Myhre) were mapped to a resolution of 2.2 by 2.2 km per pixel and area estimates were obtained by using this resolution (4.4 km$^2$ per pixel). In a similar fashion an area estimate for the total oligotrophic Gulf of Mexico (oGOM) as well as the eastern portion of the oGOM, bounded by the tip of Yucatan, Key West and the Mississippi delta, was obtained. For productivity estimates we divided the plume longitudinally into sections bounded by the longitudinal midpoints between our stations. The area for each section was then estimated individually and productivity was extrapolated from the individual stations located with the sections assuming a plume thickness of 10 m. Station 8 was a blue water station and served as the productivity baseline. Water column productivity was integrated for one m$^2$ of surface ocean as follows: The water column was divided into segments bounded by the surface, the half-way points between each pair of adjacent sampling depth and the depth of the bottom sample. Primary productivity measurements taken within each interval were then extrapolated to the volume of the segment and all estimates for the water column segments were then added.
Statistical analysis of the community data

The community data shown in Table 3 was imported into the PRIMER software package (Primer-E Ltd., Plymouth Marine Laboratory, UK) and normalized so that relative numbers (percentages) of total abundance were obtained. A square root transformation was applied to the normalized community data matrix and a dissimilarity matrix was calculated using Bray-Curtis dissimilarity coefficients. The resulting triangular dissimilarity matrix was used as the basis for clustering and Multi-Dimensional Scaling (MDS) analysis. In order to test if there was significant genetic overlap between the libraries from individual sampling sites pair-wise F-tests (Martin 2002) were performed. For F-test analysis all sequences were first aligned and alignments for individual stations were imported into Arlequin (http://anthro.unige.ch/arlequin) as individual populations assuming a frequency of one for each unique OUT and setting alpha to 0.05. To test if the communities we sampled harbored distinct phylogenetic lineages we performed pair-wise P-tests (Martin 2002). For P-test analysis a best possible tree is generated from an alignment of all clones sampled from two communities. A single character representing sampling site is then mapped to this tree and its parsimony length is calculated. This length is then compared to a histogram of 1000 randomly rearranged trees. This analysis can be performed in PAUP (Sinauer Associates, Sunderland MA).
Contour plots

CTD profiles were first exported from Seasave v.5.21f (Seabird Electronics) at a density of 1 data point per second. This produced profiles with a data density slightly higher than 1 data-point per m depth. The data series were then trapezoidally splined in Matlab (Mathworks) to a density of 1 datapoint per m in order to obtain the same spacing for all profiles. Contour plots were then generated using the Kriging method in Sigmaplot (SPSS inc.).

Pigment analysis

Four liters of seawater were filtered in duplicate through 2.5 cm Whatman GF/F glass fiber filters. Filters were wrapped in aluminum foil, frozen in liquid nitrogen and stored at -80°C until processing. Samples were extracted with acetone and analyzed using the method of Millie et al. (Millie et al. 1993). The contribution of diatoms to bulk fucoxanthin and chlorophyll $a$ was estimated using previously published pigment ratios. Pelagophytes and haptophytes fucoxanthin was subtracted from bulk fucoxanthin by using a fucoxanthin-to-19-hexanoloxyfucoxanthin ratio of 0.02 and a fucoxanthin to 19-butanoyloxyfucoxanthin ratio of 0.14 (Letelier et al. 1993). For diatom chlorophyll $a$ estimates we used a chl $a$-to-fucoxanthin ratio of 0.8 (Claustre et al. 1994). Estimate of the contribution of *Synechococcus* to total chl $a$ were obtained as described by Morel (Morel 1997). A concentration of 2 fg chl $a$ per *Synechococcus* cell counted by flow
cytometry was assumed as this value represents a typical concentration under growth-saturating irradiance (Kana & Glibert 1987).
Table 4.1

Salinity and productivity in surface samples

<table>
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<tr>
<th>Station</th>
<th>Sampling date</th>
<th>Salinity</th>
<th>Carbon fixation</th>
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<td>07/19/01</td>
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<td>36.05</td>
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Table 4.2

Pigment data

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<th>Station</th>
<th>Chl a</th>
<th>Fucoxanthin</th>
<th>Chl b</th>
<th>19'-hex</th>
<th>19'-but</th>
<th>Zeaxanthin</th>
<th>Fucoxanthin due to Diatoms</th>
<th>Chl a due to Synecococcus</th>
<th>Chl a due to other phytoplankton</th>
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<td>88.2%</td>
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Table 4.3

*rbcL* cDNA library composition.

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<th>Synechococcus</th>
<th>Trichodesmium</th>
<th>Prymnesiophytes</th>
<th>Eustigmatophytes</th>
<th>Deeply rooted haplophytes</th>
<th>Diatoms</th>
<th>Prochlorococcus</th>
<th>Bolidophytes</th>
<th>Chlorophytes</th>
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Table 4.4

Pariwise F and P test results

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P- test $\alpha=0.05$

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</table>
Surface chlorophyll \( a \) concentrations in the NE Gulf of Mexico derived using the Sea-Viewing Wide Field-of-View Sensor (SeaWiFS). Lighter colors indicated higher amounts of colored material in surface water. Concentrations near the Mississippi River Delta and in the river plume, as well as in other coastal areas, are subject to known effects by high concentrations of colored dissolved organic matter, suspended sediments, or bottom reflectance, which can artificially raise chlorophyll \( a \) estimates. Land and clouds are colored black. Station locations are indicated. (Image courtesy of Frank Müller-Karger, Douglas Myhre)
Figure 4.2

A

distance from station 6 in km

B
Figure 4.2

(A) In-situ fluorescence in the upper 50 m of the water column along the plume transect as measured during the CTD casts. Darker colors indicate higher fluorescence. Units are FU (fluorescence units) indicated by the CTD instrument. (B) Salinity in the upper 50 m of the water column along the plume transect as measured during the CTD casts. Lighter colors indicate higher salinity.
Figure 4.3

(A) % chl a due to diatoms
(B) % chl a due to Syn

Fucoxanthin
chl b
Figure 4.3

Pigment data. (A) Estimation of contribution to bulk chlorophyll $a$ by diatoms ($●$) and *Synechococcus* ($▲$). (B) Bulk chl $b$ ($▲$, this pigment is diagnostic for chlorophytes) and fucoxanthin ($■$, indicative of diatoms, pelagophytes and haptophytes).
Figure 4.4

Diatoms
- Cylindrotheca sp
- Detonula confervacea
- Odontella sinensis
- Skeletonema costatum
- Phaeodactylum tricornutum
- Bollidomonas pacifica
- Bollidomonas mediterranea
- Bollidophytes
- Pseudopedinella elastica
- Dictyochophyceae
- Heterococcus caespitosus
- Nanoaloropsis CCMP533

Eustigmatophytes
- Chrysochromulina hirta
- Emiliania huxleyi
- Calcidiscus leptoporus

Dictyochophyceae
- Xanthophyceae
- Detonula confervacea
- Odontella sinensis

Prymnesiophytes
- Prymnesium parvum
- Chrysochromulina parva
- Cylindrotheca sp
- Skeletonema costatum

Unknown deeply rooted chlorophytes
Figure 4.4

Neighbor Joining bootstrap consensus tree obtained from an analysis of the derived amino acid sequences of all form ID sequences obtained here and their closest match in GenBank. Outgroup to the shown clade were several form IB and one form II (Gonyaulax polyedra) sequences. Numbers at internal nodes indicate bootstrap values obtained from 1000 bootstrap replicates.
Figure 4.5

Neighbor Joining bootstrap consensus tree obtained from an analysis of the derived amino acid sequences of all form IA and form IB sequences obtained here and their closest match in GenBank. Outgroup to the shown clade were several form ID and one form II (*Gonyaulax polyedra*) sequences. Numbers at internal nodes indicate bootstrap values obtained from 1000 bootstrap replicates.
Tree resulting from cluster analysis of the community data. The tree was generated from a Bray-Curtis dissimilarity matrix. The individual clades were labeled non-plume, young plume and declining plume respectively based on their geographic location.
Figure 4.7

Time versus lineage plots for all eight stations generated from neighbor joining consensus trees assuming a molecular clock. Time intervals were of equal length and the time unit was arbitrarily defined as 0.1.
RESULTS

Plume description, size and sampling sites

During July of 2001 the Mississippi River plume was carried into eastern oligotrophic Gulf of Mexico (Fig. 4.1) causing a substantial phytoplankton bloom detected by SeaWiFS ocean color imagery. Using these remote sensing images the plume was estimated to cover an area of 33470 km$^2$, accounting for ca 9.16 % and 2.75% of the NE and total oligotrophic GOM respectively. Our sampling transect of this feature began on the West-Florida shelf and proceeded along the approximate plume axis towards the Mississippi delta. A total of 8 profiles were sampled at stations shown in Figure 4.1. Station 1 was on the Florida shelf and was not associated with the Mississippi River plume, but nonetheless displayed slightly lower salinity (34.8 ppt, Table 4.1) than is typical for the offshore Gulf of Mexico (ca. 36.1 ppt, see station 8, Table 4.1). Station 1 also exhibited slightly elevated surface chlorophyll as seen in SeaWiFS images (data not shown). Station 8 was a station typical of the oligotrophic conditions found in the SE GOM. Stations 7, 2, 3, 4, 5 and 6 were a series of stations located along the approximate axis of the plume and progressively closer to the Mississippi delta. Station 3 was our most westward station and may have been located in a small eddy seen in sea-surface color images at the western margin of the plume.
Plume productivity

Carbon fixation at non-plume stations averaged 0.53 µg C L\(^{-1}\) h\(^{-1}\) and 9.3 µg C L\(^{-1}\) h\(^{-1}\) in the plume stations (Table 4.1). Using these *in-situ* measurements as a basis for extrapolation we estimated that integrated productivity of the plume was ca. 3.28*10\(^9\) g C h\(^{-1}\). Given our measurements of surface productivity at station 8, our oligotrophic non-plume reference site, we calculated that the plume accounted for as much as 41% of all carbon fixation occurring in the upper 10 m of the oligotrophic GOM. The same calculations also indicate that the plume may have accounted for as much as 71% of all surface production occurring in its NE portion. Our sampling strategy allowed the estimation of integrated water column productivity at four of the eight sites sampled (not enough data was available at the remaining sites). Integrated productivity ranged between 0.04 g C h\(^{-1}\) m\(^{-2}\) and 0.27 g C h\(^{-1}\) m\(^{-2}\). Production was lowest at station 8, our most oligotrophic station and highest at station 6, the most inshore plume profile.

The plume also influenced productivity in the lower water column below the plume. Stations 4 and 6 had no discernable SCM and productivity below the plume was minimal at these stations (data not shown). The SCM at station 3 was similar to a typical oligotrophic profile, but SCM productivity (0.33 µg C L\(^{-1}\) h\(^{-1}\)) was only ca. 15% of the rate measured at the SCM of station 1 (2.1 µg C L\(^{-1}\) h\(^{-1}\)) and only ca. 67% of the rate in our sample from station 8 (0.50 µg C L\(^{-1}\) h\(^{-1}\)). No data is available for the SCM of station 5. Station 2 displayed a broad, poorly defined SCM between 50 and 100 m depth. Productivity in this feature was similar to values measured at station 8 (0.37 µg C L\(^{-1}\) h\(^{-1}\) at 55 m and 0.59 µg C L\(^{-1}\) h\(^{-1}\) at 100 m depth) but only 17-27% of the rate at station 1. It
should be noted that the SCM sample obtained at station 8 was taken ca. 8 meters above the actual peak in in-situ fluorescence (data not shown). The carbon fixation rate measured in this sample may consequently be a considerable underestimate of productivity at the true SCM. This assumption is supported by data previously collected in our sampling area (Wawrik et al. 2003). Productivity at the SCM of station 5 (2.03 µg C L\(^{-1}\) h\(^{-1}\)) during our cruise on the R/V Pelican in 1999 was considerably higher than our measurements at station 8.

**Surface fluorescence and salinity**

Elevated surface pigment concentrations observed by SeaWiFS were corroborated by in-situ observations. Surface fluorescence as measured by the onboard auto-sampling system increased eleven-fold from 0.13 fluorescence units (fu) at station 8 to 1.45 fu at station 4. Figure 4.2A shows a composite contour plot of the fluorescence values measured during our CTD casts as a function of depth and distance from the most inshore station (station 6). Salinity in this same transect ranged from 36.1 ppt in the non-plume station (station 8) to 30.9 ppt at the most inshore site (station 6). A composite contour plot of the in-situ salinity is shown in Figure 4.2B. A low-salinity surface lens containing elevated in-situ fluorescence extended more than 500 km into the oligotrophic Gulf of Mexico.
Pigment concentrations were determined by HPLC analysis (Table 4.2) and the increase in in-situ fluorescence detected along the axis of the plume was reflected in chlorophyll \(a\) measurements. Chl \(a\) was found to steadily increase from 0.25 \(\mu g\) L\(^{-1}\) at station 8 to 1.99 \(\mu g\) L\(^{-1}\) at our most inshore plume site (station 6). Chlorophyll \(b\) (a diagnostic pigment for green algae such as chlorophyceae, prasinophyceae and euglenophyceae) was present at measurable quantities at all stations increasing from 0.027 \(\mu g\) L\(^{-1}\) at station 6 to a maximum of 0.094 \(\mu g\) L\(^{-1}\) at station 4. Chl \(b\) concentrations were low in the off-shore portions of the plume (stations 2, 3 and 7) and were not significantly elevated over non-plume surface concentrations (Fig. 4.3B). The distribution of zeaxanthin was bimodal exhibiting peaks at stations 5 and 3 corresponding to the respective peaks in the abundance of Synechococcus and Prochlorococcus as measured by flow cytometry (data not shown). 19’-hexanoyl-oxyfucoxanthin (diagnostic for prymnesiophytes) levels were highest at station 5 (0.148 \(\mu g\) L\(^{-1}\)) and were elevated in most plume samples.

Fucoxanthin concentrations ranged between 0.004 and 1.14 \(\mu g\) L\(^{-1}\) displaying a bimodal distribution with peaks at the most coastal plume site and the distal plume stations 2 and 7 (Table 4.2, Fig 4.3B). Concentrations were lowest at station 4 and in non-plume samples. Since fucoxanthin is not only found in diatoms but also present prymnesiophytes, chryophytes and raphidophytes a pigment algorithm was used to estimate the diatom derived fraction of this pigment. Diatoms clearly dominated the fucoxanthin pool accounting for more than 80% of this pigment at every station sampled.
An analogous pigment algorithm was used to calculate the contribution of diatoms and *Synechococcus* to total chl *a* (Table 4.2, Fig. 4.3A). *Synechococcus* was most prominent at stations 4 and 5 while diatoms accounted for a larger percentage of total chl *a* at plume stations 6, 2 and 7. Except at station 3, where both diatoms and *Synechococcus* were only minor contributors to chl *a* (<15% combined), diatoms appeared most dominant where *Synechococcus* was least important.

**Clone libraries**

Using molecular cloning of RT-PCR amplified *rbcL* mRNA we recovered one hundred and sixteen unique *rbcL* sequences from the eight surface samples and their phylogenetic affiliations are shown in Figures 4.4 and 4.5. Most importantly we observed, that the bulk of the eukaryotic, autotrophic picoplankton in our sampling region was composed of diatoms (36 clones), prymnesiophytes (24 clones) and an array of OTUs closely related to a diverse group of green algae (25 clones). Together these three algal groups accounted for 73% of the species richness observed. The remaining sequences included six eustigmatophytes, nine prochlorophytes, five silicoflagellates, two sequences related to *Trichodesmium thiebautii*, three deeply rooted haptophytes and one sequences most closely related to the recently described bollidophytes. With the exception of the two *Trichodesmium*-like sequences all form IB clones were related to chlorophytes and no other form IB cyanobacteria were found in our libraries. Form IA containing cyanobacteria (hereafter referred to as alpha-cyanobacteria) belonged either to the marine A cluster of *Synechococcus* or the high-light adapted clade of
Prochlorococcus. A breakdown of our libraries by station and algal group is shown in Table 4.3.

Clustering analysis

Using the data shown in Table 4.3 a clustering analysis was performed to investigate the spatial patterns of community change in our sampling region. In such an analysis unique communities form ‘clades’ of sites with similar community composition. We hypothesized that the plume contained a distinct and unique species assemblage dissimilar from the non-plume GOM. A second objective was to determine if a succession of communities occurred along the axis of the plume. Figure 4.6 shows the resulting tree, which was obtained regardless if the community data matrix remained untransformed, was root or double root transformed. Three clusters of sites were obtained in this analysis. Based on results of our pigment analysis and our sampling strategy these were labeled ‘Non-Plume’ (1,3 & 8), ‘Diatom/Distal Plume’ (2,6 & 7) and ‘Synechococcus/Central Plume’ (4 & 5) respectively. To test the statistical significance of this division several non-parametric analyses were performed. The ANOISM function of Primer (the software package used for the cluster analysis) indicated that the plume (diatom and/or Synechococcus) contained a community significantly different from Non-plume sites (R=0.81, significance level=1.8%, global R=1). The same analysis also indicated that the ‘Synechococcus plume’ contained a community significantly different from the ‘Diatom plume’ at the 10% confidence interval (R=0.92, global R=1). Analogous results were obtained by direct analysis of sequence information contained in
our libraries by F and P-test analysis (Martin 2002). A significant F-test indicates less genetic diversity within individual communities (here sampling sites) than for both communities combined. A significant P-test indicates that two communities harbor distinct phylogenetic lineages not found at the site it is being compared to. F- and P-tests were significant comparing ‘Synechococcus’ and ‘Diatom plume’ both individually or combined to ‘Non-plume’ sites at the 95% confidence interval. The F-test comparing ‘Synechococcus’ and ‘Diatom’ plume samples were also significant at the 10% confidence interval. It should also be noted that ‘Synechococcus plume’ stations were characterized by a high abundance of Synechococcus, as determined by flow cytometry (data not shown), high nitrate levels and a high N to P ratio (Wawrik et al. in review) in comparison with the distal plume. Pair-wise F and P tests were also performed for all individual stations and the resulting matrix is shown in Table 4.4.

As suggested by Martin (Martin 2002) we also generated ‘Time-Versus-Lineage’ plots for all eight stations (Fig. 4.6). NoTable is the right-shift of stations 4 and 5 in this plot indicating an excess of closely related OTUs in the libraries from stations 4 and 5 as compared to our other samples (Martin 2002).
DISCUSSION

The Mississippi River drains approximately 40% of the continental United States (Moody 1967) and is the dominant source of new nutrients to the northern GOM. These nutrients lead to enhancement of productivity in the delta region. Although the importance of the river to productivity of the northern GOM has long been recognized (Riley 1937, Fox et al. 1987, Lohrenz et al. 1990, Chin-Leo & Benner 1992, Pakulski et al. 1995, Rabalais et al. 1996, Lohrenz et al. 1997), many details of the biological processes linked to it are still not well understood. Remote sensing in combination with targeted sampling of the offshore Mississippi River plume allowed us to sample this feature without a basin-wide shipboard sampling program. Using a combination of in situ data and image analysis of remote sensing data we estimated integrated plume primary productivity. We also surveyed the ambient phytoplankton populations at several stations within and outside the plume using molecular techniques and pigment analysis.

Our estimates of plume surface layer productivity dramatically illustrate the importance of the Mississippi River plume to productivity in the GOM. Integrated estimates revealed that as much as 41% and 71% of all surface productivity in the oligotrophic GOM (total and north-eastern portion respectively) were directly linked to productivity in the Mississippi River plume, at least during July of 2001. These estimates confirm and upwardly modify similar estimates previously reported by our group (Wawrik et al. 2003; see chapter 2). This previous study however relied on just one station with characteristics most similar to those of station 7, our plume station farthest from the delta (salinity = 33.5 ppt, primary productivity = 3.39 µg C L⁻¹ h⁻¹, integrated
productivity $= 63.1 \text{ mg } \text{ C h}^{-1} \text{ m}^2$). The more productive regions of the plume were not sampled during the 1999 cruise and we believe that estimates presented here are more representative and accurate.

In the prior study we had hypothesized that productivity at the SCM of some plume-associated sites may in fact be reduced and that total water column productivity can in some cases be lowered due to light attenuation by the plume (Wawrik et al. 2003). Data presented here partially confirms this hypothesis. Productivity as well as its ratio to chl $a$ (data not shown) in SCM plume samples was clearly influenced and reduced by the presence of the plume. A depression in total water column productivity due to presence of the plume however was not supported by our data. Stations 2, 6 and 7 all displayed substantially greater integrated productivity than station 8 (not enough data were available to integrate other stations). Productivity at these stations was also higher than at both stations sampled during our 1999 cruise (Wawrik et al. 2003), non-plume $= 0.083 \text{ g C h}^{-1} \text{ m}^2$, plume $= 0.063 \text{ g C h}^{-1} \text{ m}^2$). Thus, if plume associated reduction in total water column productivity occurs, it is likely to be limited to a small and remote portion of the plume and of minor significance to the bulk processes and productivity of the plume.

Several authors have previously reported daily productivity data for the GOM. For non-plume coastal regions these estimates range between 0.3 and 0.5 g C day$^{-1}$ m$^2$ (Vargo et al. 1987). Coastal waters impacted by the plume have been reported to exhibit productivity between 0.68 and 5 g C day$^{-1}$ m$^2$ (Lohrenz 1999, Chen et al. 2000). Estimates for the offshore GOM are in the range of 0.14-0.48 g C day$^{-1}$ m$^2$ (Lohrenz 1999). More recently Chen (Chen et al. 2000) estimated productivity at 0.24 g C day$^{-1}$ m$^2$ and 0.52 g C day$^{-1}$ m$^2$ for the open and coastal Gulf waters respectively. Based on our
remote sensing analysis roughly 15% of the GOM exhibits an elevated chlorophyll \( a \) signature due to coastal proximity (data not shown), while the remaining 85% can be characterized as offshore and oligotrophic. If 0.5 and 0.25 g C day\(^{-1}\) m\(^{-2}\) respectively are used as averages for integrated coastal and open ocean carbon fixation, then total GOM productivity can be estimated at ca. 4.1*10\(^{11}\) g C day\(^{-1}\). Assuming a rate of carbon fixation in the plume of 3.28*10\(^{9}\) g C h\(^{-1}\) for a 12 hours per day occurring in the upper 10 m of the water column, it can be estimated that the plume accounted for as much as 13% of all primary production in the oligotrophic GOM during July of 2001, while covering less than 2.8% of its area.

Critical to the quality and quantity of POC export to the deep ocean is the makeup of the oceanic food-web. Picoplankton dominated recycled production is not thought to lead to significant export of surface production to the deep ocean. Larger eukaryotic phytoplankton such as diatoms and coccolithophorids on the other hand may display appreciable sinking rates and are also important food-sources for higher trophic levels. In the northern GOM in particular these assumptions have been investigated for several relevant eukaryotic phytoplankton taxa (Fahnenstiel et al. 1995). Growth rates were highest inside the plume region and were also greater during summer. Sinking rates were apparently controlled by the degree of silicification. Sedimentation was important in several diatom species, in particular large colonial types such as *Skeletonema costatum* and *Thalassiosira rotula*. Hence, if significant downward flux of carbon occurs in the offshore plume, it is likely coupled to productivity and growth of a proliferating diatom population in the more remote and less productive portions of the plume.
Our data indicates that diatoms were important in portions of the plume. Cluster and pigment analysis suggest the division of the plume into coastal and offshore diatom dominated regions (stations 2, 6 and 7) and a *Synechococcus* dominated mid-plume (stations 4 and 5). At stations 4 and 5 *Synechococcus* accounted for 29 and 56 % of total chl *a* respectively, while diatoms only accounted for 4.6 and 9.6% respectively. The reverse was observed for stations 2,6 and 7, where diatoms accounted on average for a fourfold greater proportion of the chl *a* pool than *Synechococcus*, a difference that was significant at the 98% interval (P=0.02). At stations 2 and 7 the contribution of *Synechococcus* to total chl *a* was also significantly lower than at ‘non-plume’ stations (T=2.2, P=0.092).

A total of 35 unique diatom *rbcL* sequences were recovered from the plume accounting for a remarkable 42% of all phytoplankton species detected there. Considering this diversity, the diatom pigment signature and the observation that chromophytic *rbcL* mRNA increased almost 24-fold between station 8 and its peak at station 4 (see chapter 5) it seems that diatoms played an important role in the phytoplankton dynamics and productivity of the plume. Interestingly the companion study (see chapter 5) indicated that productivity in the plume was driven mainly by rapid recycling of ammonium. Diatoms are mostly thought to be new producers and to depend on nitrate for growth. Based on our data it is feasible to hypothesize that diatom production in the plume was driven by ammonium (recycled production). If diatom driven recycled production in occurs in the plume remains to be demonstrated.

Nitrate input into the Gulf of Mexico by the Mississippi River has at least doubled over the last half century, while silicate concentrations in the river have simultaneously...
decreased by 50% (Rabalais et al. 2002). The size and type of diatoms in the plume may as a consequence have been limited, reducing the amount of large, heavily silicified forms in the phytoplankton (Turner et al. 1998, Dortch et al. 2001). The impact of these changes on carbon sequestration is not known, but plume productivity may have shifted from diatoms to picocyanobacteria and consequently reduced total carbon flux below the euphotic zone. On the other hand the increase in nutrients in general has no doubt increased overall production in the plume potentially leading to greater carbon export.

Nowhere in the plume did diatoms and Synechococcus combined account for more than 61% of total chl $a$ and in most cases their combined contributions was less than 50%. Other eukaryotic algal groups must therefore be inferred to account for the remaining pigment. Since pigment data suggested that prymnesiophytes were only of minor importance in the plume is it appears that much of the remainder may have been contributed by chlorophytes. Fucoxanthin, found in both diatoms and prymnesiophytes, was almost entirely accounted for by diatoms, even in non-plume samples and over 58% of all prymnesiophytes-like clones were recovered from only two, non-plume samples (stations 1 and 8), emphasizing this group’s preference for offshore and oligotrophic environments. Chl $b$ was abundant in the plume and particularly concentrated in the in-shore and Synechococcus dominated portion of the plume, where the greatest number of unique green algal clones were detected in our libraries.

Sequence data analysis also indicated that ‘Synechococcus plume’ samples (stations 4 & 5) potentially contained an excess of closely related sequences (Fig. 4.7). An excess of recently diverged lineages is thought to suggest a highly variable environment where extreme conditions drive rapid microbial evolution. Stations 4 and 5
are located in the transition zone between the coastal, shelf associated and the offshore plume. It is possible that highly variable chemical and physical characteristics in the region foster rapid evolutionary processes characterized by frequent invasion and reinvansion of both coastal and oligotrophic species. Alternatively it is possible that the observed shift was due to primer biases resulting in the absence of the dominant *Synechococcus* from our libraries. There was a conspicuous lack of *Synechococcus*-like *rbcL* sequences in the libraries obtained from stations 4 and 5. It is possible that the large population of *Synechococcus* observed at these sites was transcriptionally inactive or dying and was as a result not recovered by our RNA based sampling strategy. More likely however this population was not detected because of small genetic differences between the naturally occurring strains of *Synechococcus* in the upper plume and the cultured representatives used in the design of our PCR primer set. Supporting this hypothesis is the observation that the *rbcL* sequences of several green *Synechococcus* strains isolated from the plume could not be amplified using our PCR primer set (data not shown).

Noteworthy is also the presence of several uncommon sequences in our libraries. One clone (WS01ST7CH3) appeared most closely related to *rbcL* sequences of the recently described algal class *Bolidophycheae* (Guillou et al. 1999a), yielding the first evidence for the presence of this algal group in the GOM. Bolidophytes have however been demonstrated to constitute only a minor component of the eukaryotic phytoplankton in the equatorial Pacific and Mediterranean Sea (Guillou et al. 1999b) and are likely of minor importance in the plume. Also we observed several *rbcL* sequences which formed a small clade clustering basally to the remaining heterokont algae, yet had no apparent close match in GenBank (as of September 2002). We have recovered representatives of
this clade during two separate cruises (1999 and 2001) both within and outside of the plume (Fig. 4.4) suggesting the abundance of this unknown taxonomic algal group.

In summary, it appears that our sampling transect traversed three oceanographic regimes containing distinct phytoplankton assemblages. Non-plume communities were numerically dominated by *Prochlorococcus* as determined by flow cytometry and were the preferred environment of prynesiophytes, eustigmatophytes and haptophytes. The plume was divided into two regimes, one *Synechococcus* dominated, the other diatom dominated. Our data suggest that least productive portions of the plume were dominated by a diverse group of diatoms, indicating the possibility of significant carbon export to below the mixed layer in the oligotrophic NE GOM. High productivity in the Mississippi River plume significantly enhanced basin-wide production accounting for as much as 43% of total oligotrophic surface productivity in the GOM, at least during July of 2001, while covering less than 3% of its surface area.
CHAPTER 5

High Rates of Ammonium Recycling Drive Phytoplankton Productivity in the Offshore Mississippi River Plume

The following chapter has been submitted essentially in this form to the journal Aquatic Microbial Ecology for review.

CHAPTER SUMMARY

As part of an integrated study of the regulation of carbon fixation in the offshore Mississippi River plume, we measured the rates of $^{15}$N-labeled ammonium and nitrate uptake in the surface plume waters from offshore to nearshore along the plume axis towards the Mississippi delta. Concentrations of nitrate in the plume ranged from 0.19 to 2.5 µM with the highest concentrations primarily in the shoreward stations, while ammonium ranged from 0.17 to 0.44 µM, showing little spatial variability. Rates of ammonium uptake ranged from 16.5 to 260 nM h$^{-1}$, and showed a strong trend of increasing values from offshore towards the Mississippi Delta. In contrast nitrate uptake rates ranged from 3.2 to 25 nM h$^{-1}$. The high rates of ammonium uptake in the presence of low ammonium concentrations and elevated nitrate was made possible by elevated rates of ammonium regeneration that exceeded ammonium uptake by 1.7- to 5.7-fold in the plume. The plume exhibited relatively low f-ratios and also contained elevated levels of *Synechococcus* as determined by flow cytometry and high levels of form IA (α-cyanobacterial) *rbcL* transcript levels. These data suggests that a major portion of the
carbon fixation observed in the offshore Mississippi River plume represents recycled production supported by high rates of ammonium regeneration.
INTRODUCTION

High chlorophyll surface plumes originating from the world’s major river deltas are often seen as prominent features in ocean color satellite images. Such plumes can extend far into the open ocean. For example, the Amazon and the Orinoco River plumes have been observed to extend as much as 1000 km into the western oligotrophic Atlantic Ocean and Caribbean Sea respectively (Müller-Karger et al. 1989, Longhurst 1993, Müller-Karger et al. 1995).

The prominent river plume in the Gulf of Mexico (GOM) originates from the Mississippi River. The Mississippi discharges ca. 536±130 km$^3$ of nutrient-rich water onto the northern GOM shelf each year, making it the sixth largest river worldwide (Dai & Trenberth 2002). Exacerbated by heavy fertilization of large areas within the Mississippi watershed (>2 million tons of nitrogen fertilizer per year) the river carries a very high nitrate (111±4.3 µM) and phosphate (7±0.4 µM) load as it reaches the delta (Amon & Benner 1997). During most of the year this leads to massive phytoplankton blooms along the Louisiana and Texas coastlines, which receives most of the Mississippi River plume freshwater input. In some instances, however, particularly during the summer months when local wind forcing and surface circulation are favorable, the Mississippi River plume instead reaches hundreds of kilometers into the eastern oligotrophic Gulf of Mexico. Under these circumstances ocean color images have shown the Mississippi River plume to extend along the Florida shelf break as far as the Dry Tortugas or even the Florida Straits (Müller-Karger et al. 1991, Wawrik et al. 2003).
No targeted survey of the plume in this environment has thus far been conducted to study the plume’s effect on oligotrophic ocean ecology, phytoplankton composition and nutrient cycling. Previous studies of the plume have primarily been concerned with the plume’s impact on coastal ecosystems in the northern GOM shelf region. The plume has however been shown to greatly enhance oligotrophic surface productivity and phytoplankton species composition in the oligotrophic GOM (Wawrik et al. 2003).

On the shelf, nutrients, irradiance and primary production of the Mississippi River plume have been studied extensively (Lohrenz et al. 1990, Dortch & Whittedge 1992, Lohrenz et al. 1994, Pakulski et al. 1995, Lohrenz et al. 1999, Pakulski et al. 2000). Productivity in the most coastal region of the plume is initially limited by turbidity and highest productivity occurs at intermediate salinities as the plume matures. Nutrients display distinct non-conservative mixing behavior along the salinity gradient of the plume and both silicate as well as nitrogen have been reported to limit productivity in the higher salinity portions of the plume. High productivity in the plume has been implicated in the formation of extensive regions of hypoxia in bottom waters along the Louisiana and Texas shelf during summer stratification (Dortch et al. 1994, Eadie et al. 1994, Rabalais et al. 1994, Justic et al. 1996, Rabalais et al. 1996).

Nitrogen mineralization rates in the Mississippi River plume area have been measured using HPLC (High Performance Liquid Chromatography) in isotope dilution and enrichment experiments (Gardner et al. 1993). It was found that highest ammonium regeneration rates occurred in samples from shallow depths where primary and bacterial production was high. In the Mississippi River plume region, ammonium regeneration
rates, bacterial production, and amino acid turnover have been observed to be greatest at intermediate salinities during the summer (Cotner & Gardner 1993).

In order to determine the effect of the offshore Mississippi River plume on nitrogen cycling in the oligotrophic Gulf of Mexico we measured the concentrations and relative uptake rates of dissolved inorganic nitrogen (DIN) species along a transect of the plume from offshore to onshore. Our results indicate that ammonium concentrations averaged over nine-fold higher than nitrate concentrations in the plume, yet uptake rates of ammonium were almost seven-fold greater than nitrate uptake rates. Recycled production thus dominated within the plume, an observation that was corroborated by the presence of a numerically dominant population of *Synechococcus*. In a companion study we explore the importance of the offshore plume to oligotrophic productivity in the Gulf and describe phytoplankton species dynamics using molecular techniques and pigment analysis (Wawrik & Paul in review).
METHODS

Gulf of Mexico Sampling

Surface samples were obtained aboard the R/V ‘F.G. Walton-Smith’ between July 16 and 26, 2002 along the approximate axis of the offshore Mississippi River plume. For a more detailed description of station locations as well as a SeaWiFS satellite image of the sampling region and our sampling strategy please refer to chapter 4.

Ambient nutrient concentrations

Water from each depth of the vertical profile was filtered through precombusted (450°C for 2 hr) Whatman GF/F filters. Concentrations of nitrate, phosphate and silicate were measured with a Technicon AutoAnalyzer. Concentrations of NH$_4^+$ were measured manually with the phenol/hypochlorite technique (Grasshoff et al. 1999). No unfiltered samples were analyzed.

Uptake and regeneration of inorganic nitrogen

Rates of ammonium and nitrate uptake were measured with $^{15}$N tracer techniques using 0.05 µM tracer additions as previously described (Bronk et al. 1998). All $^{15}$N tracer incubations were done in on-deck flow-through incubators under simulated in situ light and temperature conditions. Light was attenuated with blue Plexiglas shields and
neutral density screens. Experiments were done in 1.0 L PETG bottles, and samples were incubated for ~3 hr. At the end of each incubation, samples were filtered through precombusted GF/F filters. Filters were subsequently dried and analyzed on a Europa GEO20/20 mass spectrometer with an ANCA sample processing unit. The filtrate from the ammonium incubations were collected and frozen for later determination of the atom % enrichment of the NH$_4^+$ pool. The ammonium pool was isolated using the solid phase extraction technique (Dudek et al. 1986, Brzezinski 1987). All NH$_4^+$ uptake rates were corrected for isotope dilution (Glibert et al. 1982). For this the atom % enrichment of the ammonium pool (i.e. the ratio of $^{15}$N:$^{14}$N in the ammonium pool after you add the label) is determined at the beginning and the end of the experiment. During the course of the incubations there are a number of processes, such as zooplankton and bacterial remineralization, which will release $^{14}$N ammonium back into solution. This release will dilute the ratio of $^{15}$N:$^{14}$N in the ammonium pool. The starting $^{15}$N isotopic enrichment can then be used to estimate the rate of regeneration.

*rbcL* mRNA analysis

mRNA was extracted from seawater using RNeasy spin columns (Qiagen) as previously described (Paul 2001). Briefly, between two hundred and eight hundred ml seawater samples were treated with 0.1% v/v DEPC (Di-Ethyl-Pyrocarbonate; Sigma Chemical Corp.) and filtered onto 25 mm, 0.45 µm HV polyvinylidene difluoride filters (Millipore Durapore). Filters were stored in liquid nitrogen in 750 µl of RLT lysis buffer (Qiagen, Valencia, CA) together with 0.2 g of glass beads (Biospec Products). Cell lysis
was achieved by bead-beating. The lysate was then extracted following the RNeasy kit (Qiagen, Valencia, CA) protocol. Samples were split three ways. One third was digested with DNAse free RNAse and one third was digested with RQ1-DNAse. RNA was then immobilized onto Zeta-Probe charged nylon filters (Bio-Rad) by dot-blotting and UV-crosslinking. Duplicate samples were probed with form IA, form IB and form ID \( rbcL \) probes previously described (Watson & Tabita 1996, Paul et al. 1999). Riboprobes labeled with \(^{35}\text{S-UTP}\) were prepared by in vitro transcription. Dot blots were analyzed using a BioRad Model GS363 Molecular Imager. Standard curves were made from opposite orientation in-vitro transcripts generated from the same \( rbcL \) clones used to make the ribo-probes.

**Flow cytometry**

Samples of one ml were fixed with 20 \( \mu \text{l} \) of 10 % para-formaldehyde and frozen in liquid nitrogen. *Prochlorococcus*, *Synechococcus* and pico-eucaryotic algal populations were then quantified using a Becton Dickinson FACSCalibur (San Jose, CA) flow cytometer outfitted with a 488 nm, 15 mW Argon laser. Forward angle light scatter, right angle light scatter, green (530 ± 30 nm), orange (585± 30 nm) and red (650±30nm) fluorescence parameters were collected for each event. Purple-yellow calibration beads (2.2 \( \mu \text{m} \), Spherotech Inc., IL) were added to each sample to permit normalization of all fluorescence signals. Data was collected using CellQuest™ software (V. 3, Becton Dickinson 1996) and analyzed using CYTOWIN software (Vaulot et al. 1989);http://www.sb-oscoff.fr/Phyto/cyto.html#cytowin). Event rates were recorded for
each sample and abundances were corrected for volume analyzed and enumeration efficiency factor. The efficiency factor was calculated from event rate and counts for series of known concentrations of calibration beads.

**Assessment of nutrient limitation**

To investigate potential nutrient limitation ratios of DIN, phosphate and silicate were calculated and compared to typical ratios. N:P ratios $\geq 30$ were taken to indicate phosphate limitation and ratios $\leq 10$ nitrogen limitation (Healey & Hendzel 1979, Healey 1985, Suttle & Harrison 1988, Dortch & Whitledge 1992). Si:N ratios much greater than 1 are thought to indicate nitrogen limitation, where as ratios much smaller than 1 indicate silicate limitation (Brzezinski 1985, Levasseur & Therriault 1987, Dortch & Whitledge 1992). For the purpose of this study Si:N ratios of 0.8 and 1.2 were taken to indicate silicate and nitrogen limitation respectively. Silicate limitation is indicated by Si:P ratios $\geq 3$ (Harrison et al. 1977, Dortch & Whitledge 1992).
Table 5.1

N, P and Si limitation in the plume. N:P, Si:N and Si:P ratios are shown. Based on these ratios limitation by N, P and Si is determined using published ratios. “+” indicates limitation by a particular nutrient. “-“ indicated that no evidence for limitation was observed based on nutrient ratios.

<table>
<thead>
<tr>
<th>Station</th>
<th>N:P</th>
<th>Si:N</th>
<th>Si:P</th>
<th>N:P</th>
<th>Si:N</th>
<th>Si:P</th>
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<td>P</td>
<td>N</td>
<td>N</td>
<td>Si</td>
<td>N</td>
<td>Si</td>
</tr>
<tr>
<td>#6</td>
<td>4.09</td>
<td>0.87</td>
<td>3.55</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>#5</td>
<td>29.6</td>
<td>0.36</td>
<td>10.6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#4</td>
<td>31.1</td>
<td>0.06</td>
<td>1.84</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#3</td>
<td>27.4</td>
<td>0.56</td>
<td>15.5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#2</td>
<td>21.6</td>
<td>0.33</td>
<td>7.16</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>#7</td>
<td>6.07</td>
<td>0.42</td>
<td>2.54</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>#8</td>
<td>2.04</td>
<td>5.00</td>
<td>10.2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>#1</td>
<td>3.83</td>
<td>1.34</td>
<td>5.12</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5.1

(A) Nitrogen concentration in uM as a function of station. 

(B) Nitrogen rates in nM h⁻¹ as a function of station.
Ambient nutrient concentrations and DIN uptake rates along our sampling transect. Station numbers are indicated on the x-axis. (A) Ammonium (▲) and nitrate (■) concentrations in the plume in µM. (B) Uptake rates of ammonium (▲) and nitrate (■) in nM h⁻¹ as measured by ¹⁵N tracer technique. Also shown is the rate of ammonium regeneration(●). (C) Ambient concentrations of ortho-phosphate (●) in µM and N:P ratio (▲).
Figure 5.2

A

B

F-ratio

C-fixation

ug C fixed L$^{-1}$ h$^{-1}$

Silicate uM

% cfix due to NH$_4$ uptake

6A 5A 4A 3A 2A 7A 8A 1A

6A 5A 4A 3A 2A 7A 8A 1A
(A) Carbon fixation (▼) in µg C fixed L$^{-1}$ h$^{-1}$ and f-ratio (■) along our transect. The f-ratio is calculated as the ratio between nitrate and nitrate+ammonium uptake. (B) Ambient silicate concentrations (■) in µg L$^{-1}$ and the percent carbon-fixation accounted for by ammonium uptake (▲) assuming a Redfield ratio of 6.6 for C:N uptake.
Figure 5.3
Figure 5.3

(A) Results from flow cytometry analysis. Shown are Prochlorococcus (▲), Synechococcus (●) and the number of picoeukaryotes (♦) per ml. (B) Detected levels of form IA (●), form IB (▲) and form ID (■) rbcL mRNA along our sampling transect.
RESULTS

Sampling was initiated on the West Florida Shelf and proceeded from offshore to onshore along the plume axis (Fig. 4.1). On our return trip we sampled the most distal plume station (station 7) and an offshore/oligotrophic reference station (station 8). Stations 1 and 8 were outside the plume while stations 2-7 were located within. A discussion of salinity, productivity and composition of phytoplankton along this transect together with a more detailed summary of our sampling strategy is found in chapter 4.

Nitrate and ammonium concentrations

Ammonium concentrations varied 2.5-fold and ranged between 0.18 µM and 0.44 µM, being highest at stations 2 and 4 (Fig. 5.1A). No discernable pattern in the variability of ammonium was observed. The three stations closest to the delta did not contain a significantly different concentration of ammonium than the more offshore located plume sites, or the stations outside the plume. Nitrate concentrations varied much more dramatically and increased 69-fold from 0.036 µM to 2.5 µM between station 8 and their peak at station 4 (Fig. 5.1A). Nitrate concentrations were significantly greater in plume samples than in non-plume samples (t=1.9, p=0.1). Nitrate concentrations were significantly greater in samples from station 2 through 5 than in the remaining plume samples (t=5.9, p=0.004).
Ammonium and nitrate uptake

Despite elevated nitrate concentrations in the plume, ammonium uptake was much greater than nitrate uptake. Ammonium uptake ranged between 16.5 nM h\(^{-1}\) at our most oceanic plume station (station 7) and 260 nM h\(^{-1}\) at station 3 and displayed a strong trend of increasing values toward the Mississippi Delta (Fig. 5.1B). Ammonium uptake in non-plume samples averaged 18.7 nM h\(^{-1}\). Nitrate uptake rates were on average almost 7-fold lower within the plume and almost 24-fold lower outside. Nitrate uptake rates in the plume ranged between 3.1 nM h\(^{-1}\) and 25 nM h\(^{-1}\). Uptake averaged 1.03 nM h\(^{-1}\) in non-plume samples. Data analysis also revealed that nitrate uptake was significantly correlated to the ratio of dissolved nitrate to dissolved ammonium (R\(^2\)=0.79, p=0.003, Fig. 5.1). The exception was station 6, where an intermediate nitrate uptake rate of 15.7 µM h\(^{-1}\) coincided with the lowest nitrate concentration measured anywhere in the plume. Nitrate uptake rates were significantly correlated to ammonium uptake rates along our transect (R\(^2\)=0.78, p=0.004).

Phosphate and Silicate

Phosphate concentrations along the transect ranged within the narrow range between 85 nM and 104 nM (Fig. 5.1C). Concentrations were significantly higher in non-plume samples relative to plume samples (t=3.4, p=0.01). The N:P ratio was calculated by adding dissolved nitrate and ammonium concentrations and dividing this number by dissolved phosphate (Fig. 5.1C). Silicate varied more than 8-fold within the plume...
ranging between 0.17 µM at station 4 and 1.38 µM at station 3. There was no obvious pattern of silicate concentrations along the plume and concentrations at non-plume stations were not significantly different from those within the plume.

Analysis of nutrient ratios (Table 5.1) indicated that phosphate limitation of phytoplankton growth may have occurred at stations 4 and 5. Based on N:P and Si:N ratios nitrogen was limiting at non-plume stations 1 and 8. N:P ratios also indicated potential nitrogen limitation at stations 6 and 7, while there was no evidence for nitrogen limitation in the remaining plume, which contained elevated nitrate. Based on Si:N and Si:P ratios silicate appeared limiting in the central portion of the plume surveyed here. Non-plume stations were not silica limited.

**Carbon fixation and f-ratio**

Carbon fixation in non-plume samples averaged 0.53 µg C L\(^{-1}\) h\(^{-1}\) and steadily increased almost 25-fold along the plume to a rate of 16.2 µg L\(^{-1}\) h\(^{-1}\) at station 6 (Fig 5.2A). Variability in carbon fixation along the transect was not correlated with ammonium or nitrate uptake, but rather displayed a negative correlation with salinity (R\(^2\)=0.74, p=0.006). The f-ratio is calculated to be the fraction of ‘new’ to total production by dividing nitrate uptake by nitrate plus ammonia uptake (Eppley 1981). The f-ratios were lowest in non-plume samples, where they averaged 0.053, and were significantly higher in the plume (t=2.8, p=0.029). Close examination revealed no significant correlation of the f-ratio to any other parameter described here. Assuming that all ammonium uptake leads to primary production in the plume and assuming a Redfield
ratio of 6.6 for C:N uptake we calculated the percent carbon fixation due to ammonium assimilation (Fig. 5.2B). This percentage was significantly correlated to ambient silicate concentrations in our sampling region (p=0.034, R²=0.75).

**Flow cytometry**

*Synechococcus* was more abundant at the more coastal stations, reaching a maximum of 2.3*10⁵ cells ml⁻¹ at station 5, but declining in the central portion of the plume (Fig. 5.3A). At stations 2 and 7 *Synechococcus* was no longer significantly elevated over surface abundance at non-plume sites. With the exception of station 3, *Prochlorococcus* was not abundant in plume surface samples. Counts in the plume were < 10⁴ cells ml⁻¹ at stations 2, 6 and 7 and <2*10⁴ cells ml⁻¹ at stations 4 and 5. At station 3 *Prochlorococcus* was present at an abundance of 1.68*10⁵ cells per ml. This cell density was even 2.6-fold greater than concentrations observed at the most oligotrophic site (station 8). The concentration of picoeukaryotes increased steadily from offshore to onshore ranging between 9.5*10² cells ml⁻¹ and 4.5*10³ cells ml⁻¹. The abundance of picoeukaryotes was significantly positively correlated with rates of carbon fixation (R²=0.86, p=0.001) and negatively correlated with salinity (R²=0.80, p=0.003).

**rbcL mRNA**

The dominant *rbcL* transcript observed at all stations sampled was the alpha-cyanobacterial form IA of this gene. Form IA *rbcL* mRNA concentrations ranged
between 0.01 ng L\(^{-1}\) at station 1 and 0.081 ng L\(^{-1}\) at station 2 and in average exceeded form IB and ID transcript levels by 8.8- and 8.0-fold respectively (Table 5.1). Form IA and IB \(rbcL\) mRNA concentrations were well correlated (\(R^2=0.84\), \(p=0.008\)) and increased significantly between West Florida Shelf and the Mississippi Delta (Fig. 5.3B). Form IB expression, which ranged between 0.0011 ng L\(^{-1}\) at station 1 and 0.0090 ng L\(^{-1}\) at station 5, was also significantly correlated to carbon fixation (\(R^2=0.70\), \(p=0.05\)), while form IA and form ID were not. The greatest range in expression values was observed for form ID transcript, which varied almost 24-fold between lowest values at station 1 (0.0014 ng L\(^{-1}\)) and their high at station 4 (0.033 ng L\(^{-1}\)). Form ID expression was significantly correlated to in-situ concentrations of ammonium (\(R^2=0.74\), \(p=0.037\)) and the ratio of nitrate to nitrate uptake (\(R^2=0.84\), \(p=0.008\)). We observed no significant correlations between the forms of \(rbcL\) quantified here and our flow cytometry counts for picoeukaryotes, \textit{Synechococcus} and \textit{Prochlorococcus}. 


DISCUSSION

The Mississippi River plume, extending westward on the continental shelf, has been studied intensely. Data presented here represents the first targeted survey and transect of the relatively high-salinity and offshore portion of the Mississippi River plume that periodically wanders into the oligotrophic NE GOM.

Ammonium concentrations ranging between 0.29 and 2.5 \( \mu M \) have been reported along a salinity gradient for the plume (Pakulski et al. 1995). In transects extending offshore from the Southwest Pass and the Atchafalaya River ammonium concentrations ranging between 0 and 2.6 \( \mu M \) were measured, with concentrations peaking at mid-salinities (Gardner et al. 1997). Similar results (with concentrations up to 3.58 \( \mu M \) ammonium at mid salinities) were obtained in a similar survey of the Southwest Pass discharge region (Bode & Dortch 1996). Ammonium concentrations reported here, for the offshore plume were in the lower range, of these previously reported values. Potential ammonium uptake rates in the coastal Mississippi River plume have been reported in the rage between 0.4 and 1.8 \( \mu M \) h\(^{-1}\) (Gardner et al. 1997) and up to 4.4 \( \mu M \) h\(^{-1}\) (Bode & Dortch 1996), while ammonium regeneration rates ranged between 0.08 and 0.75 \( \mu M \) h\(^{-1}\) (Gardner et al. 1997) and 0.03 and 1.09 \( \mu M \) h\(^{-1}\) (Bode & Dortch 1996). Both studies observed peak uptake and regeneration rates at intermediate salinities. Ammonium uptake and regeneration rates reported here are consistent with these previous observations and support the hypothesis that most intense recycled production occurs at intermediate salinities. In the offshore plume, nitrate exceeded ammonium at all but our most inshore plume station, while ammonium exceeded nitrate concentrations at our non-plume oligotrophic stations. Despite higher nitrate concentrations, sub-micromolar levels
of ammonium were the preferred source of nitrogen and production was fueled by high levels of ammonium regeneration (Fig. 5.1C). The dominance of regenerated production in the offshore plume is in contrast to the coastal plume, which exhibits high rates of nitrate driven new production.

Not all nitrate uptake represents new production, however. Evidence has been reported for intense nitrification in the plume, particularly at intermediate salinities (Pakulski et al. 1995). Nitrate found in offshore plume waters may thus be, at least in part recycled, blurring the distinction between new and recycled production commonly used. It should be noted that, if this were the case, that our nitrate uptake measurements may be an underestimate of actual rates due to isotope dilution of the nitrate pool during incubations. One possible indication for nitrification is the strong non-conservative mixing behavior of nitrate concentrations along our sampling transect (with peak values at stations 4 & 6). It is possible that this increase in nitrate was due to the activity of nitrifying bacteria, although Mississippi outflow heterogeneity, eddy induced mixing and upwelling in this region should not be discounted.

Ammonium is thought to be the preferred source of nitrogen for phytoplankton growth (McCarthy et al. 1977, Dortch 1990). This preference is mediated by specific cell-surface associated transporters, which follow substrate dependent Michaelis-Menten enzyme kinetics. It is further thought that oceanic species are adapted to their environment by possessing high substrate affinities (Dugdale & Goering 1967) and that nitrate uptake may be dramatically reduced by the presence of even low concentrations of ammonium (Wheeler & Kokkinakis 1990). In the field, uptake kinetics of natural phytoplankton assemblages have been studied by the addition of 10-1000 nM $^{15}$NH$_4^+$ and
Almost without exception the Michaelis-Menten equation was an appropriate descriptor of uptake kinetics in samples from a wide range of physical, chemical and biological conditions. Ammonium was preferred over nitrate across a large spectrum of nitrogen concentrations and inhibited nitrate uptake with an inhibition half-saturation parameter ($K_i$) of 40-50 nM. Significant inhibition of nitrate uptake by ammonium has also been reported by other authors (Wheeler & Kokkinakis 1990). Ammonium only accounted for <1% of total DIN, yet accounted for 44-89% of total N assimilation and nitrate assimilation was negatively correlated with ambient ammonium concentrations (Wheeler & Kokkinakis 1990). These observations held only partially true for the offshore Mississippi River plume stations of this study, where ammonium uptake was clearly dominant, despite low concentrations. Nitrate uptake in the plume, however, was both positively and significantly correlated with ammonium uptake and the nitrate to ammonium ratio. One possible explanation for this observation may be that nitrate transport and reduction pathways are expressed only when sufficient nitrate is present and when ammonium concentrations are insufficient to repress their expression. As a result, a high ratio of nitrate to ammonium combined with more favorable uptake enzyme kinetics and cellular demand could result in conditions more favorable to the utilization of nitrate.

Nutrient discharge by the Mississippi River has been implicated in sustaining high levels of primary productivity in the northern GOM (Riley 1937, Sklar & Turner 1981, Lohrenz et al. 1990, Redalje et al. 1994a, Wawrik et al. 2003). As Mississippi River water enters the northern GOM it carries a high load of nitrate (between 20 and 200 µM, depending on season (Lohrenz 1999), but only low concentrations of orthophosphate
(between 0.3 and 5 μM; (Lohrenz 1999)). Ammonium as well as nitrite are also not present in significant quantities (Antweiler et al. 1995). Productivity in the discharge area is initially limited by the availability of light due to high turbidity of the river water. As a result of the interplay between the availability of nutrients and turbidity, the highest productivity in the plume is most often found in regions of intermediate salinities between 10 and 30‰ (Lohrenz et al. 1990, Dagg & Whitledge 1991, Dortch & Whitledge 1992, Hitchcock & Whitledge 1992, Lohrenz 1999), where high nutrient water is no longer limited by light availability. Additionally, factors other than light have also been implicated in constraining biomass and productivity even in the most turbid portions of the plume (Lohrenz et al. 1990). Both phosphate and silicate have been found to limit phytoplankton productivity in the Mississippi Delta region (Smith & Hitchcock 1994, Nelson & Dortch 1996). As river water mixes into the oceanic end-member, nitrate and silicate are rapidly depleted from the plume, while supporting intense new production. Nitrate concentrations usually approach the limits of detection at salinities greater than 30-33‰ (Lohrenz et al. 1990).

It has been hypothesized that at least one or more nutrients (in particular nitrogen) will eventually become limiting in the plume (Sklar & Turner 1981, Lohrenz et al. 1990) as has been observed for silicate in the Hudson River plume (Malone et al. 1980). Dortch and Whitledge (Dortch & Whitledge 1992) specifically addressed the hypothesis that nitrogen or silica may become limiting to productivity in the Mississippi River plume and nearby regions. Using ratios of cellular free amino acids to protein (AA/Pr) as well as ambient nutrient concentrations they concluded that nitrogen limitation was not wide
spread in the plume and was most likely to occur during summer month at high salinities. Nutrient ratios indicated that silicate was at least as likely to be limiting.

Although nutrient ratios should be interpreted with caution since they only possess limited use as a predictor of nutrient limitation, several observations can be made based on our measurements (Table 5.1). Nitrogen appeared not to be limiting phytoplankton biomass in the plume, at least at stations with ≥ 1µM nitrate. Ammonium regeneration rates were on average 2.5-fold greater than ammonium uptake in plume samples, supporting the notion that the rate of DIN supply was more than sufficient to support the observed rates of primary production. The nutrient most likely limiting in the offshore plume, based on nutrient ratios, was silicate, followed by or in combination with phosphate. Phosphate was relatively depleted and near the detection limits at all our stations indicating that it may have been a limiting nutrient throughout. N:P ratios however indicated that phosphate may have been particularly scarce at stations where *Synechococcus* was dominating over diatoms (station 3-5; see chapter 4). During the past century nitrate loading of the Mississippi River has at least doubled, while silica concentrations have been reduced by half, reducing the Si:N ratio in river discharge from 4:1 to 1:1 (Turner & Rabalais 1991, Rabalais et al. 2002). These changes potentially influenced the size and type of diatoms found in the Mississippi watershed, favoring small, less heavily silicified forms or even non-silicified phytoplankton. Silica limitation, however, is not thought to ultimately limit phytoplankton biomass and may only result in the adjustment of the ambient species composition, which could have large implications for food web dynamics, nutrient cycling and the rate of carbon sequestration. Also, less
silicified populations of diatoms may exhibit lower cellular Si:N ratios, potentially alleviating silicate limitation in the plume.

Analysis of pigment data and the composition of $rbcL$ cDNA clone libraries obtained from our plume samples indicated that the offshore plume was divided into a more coastal diatom dominated, a central *Synechococcus* dominated and a more oceanic diatom dominated region (Wawrik & Paul in review). Flow cytometry supports these observations (Fig. 5.4A). Numerically *Synechococcus* was the principle phytoplankter at all plume stations and was particularly abundant at intermediate salinity stations (30.8-31.5 ‰), where pigments indicated their dominance (see chapter 4). Similar observations for *Synechococcus* have been reported for the dilution zone of the Yangtze River, China, where abundance ranged between $10^2$ and $10^5$ cells ml$^{-1}$ in the summer (Vaulot & Xiuren 1988) and increased in the offshore direction. Highest abundance was observed at salinities between 25 and 30 ‰. Further offshore plume stations were dominated by diatoms as indicated by pigment ratios (see chapter 4) despite indications of silica limitation in this region. This suggests that factors other than silica or nitrogen limitation may be controlling phytoplankton composition and dynamics in the most offshore portion of the plume. This point is particularly well illustrated by the observation of a large population of *Prochlorococcus* at station 3, which coincided with the highest ammonium uptake rates measured in the plume. Station 3, which was located on the edge of the plume, contained a phytoplankton community most similar to our non-plume stations (see chapter 4), but otherwise exhibited characteristics (salinity, nutrient concentrations, ammonium uptake rate and productivity) very similar to adjacent and more centrally located stations 2 and 4. The large numbers of *Prochlorococcus* at station 3 is a
somewhat surprising finding, since this organism is typically not abundant in plume surface waters (Wawrik et al. 2003).

It is also interesting to note that despite the numerical dominance of *Synechococcus* in the plume, carbon fixation was most tightly correlated with flow cytometry counts for picoeukaryotes. Unfortunately we performed no size-fractionation experiments and it was thus difficult to assign either productivity or nitrogen uptake measurements to individual components of the phytoplankton using our data. Clone library data indicated the presence of a large number of diatom species, which may have dominated some portions of the plume based on pigment information (see chapter 4). Since diatoms are capable of supporting their growth using ammonium, it is possible that the high rates of ammonium uptake and primary productivity we observed were at least in part due to the presence of a diverse group of these organisms. *Phaeodactylum tricornutum*, for example, has been shown to assimilate both L-arginine and ammonium simultaneously and individually at a rate sufficient for growth (Flynn & Wright 1986). Alternatively a diverse group of green algae was also present in the plume and was actively transcribing their carbon fixation genes. Form IB *rbcL* mRNA concentrations were significantly correlated with carbon fixation \((R^2=0.5, p=0.05, N=8)\), while form ID (diatom/chromophytes) *rbcL* was not.

The most abundant form of *rbcL* transcript found at all stations was nonetheless the form IA (α-cyanobacterial) type, corroborating the observation that *Synechococcus* numerically dominated that plume. Both the cyanobacterial and chromophytic algal *rbcL* forms (form IA and ID respectively) were not significantly correlated with carbon fixation, exemplifying the highly variable abundance and contribution to total chlorophyll
of these organisms in our sampling region (see chapter 4). In addition it is possible that
chemolithotrophic, nitrifying bacteria (which also contain a form IA or the RubisCO
gene) may influenced our form IA signal and obscured its correlation with productivity.
Considering evidence of nitrification in the plume (Pakulski et al. 1995) it is feasible that
high levels of form IA \( rbcL \) were in part due to the presence of such organisms. Form ID
\( rbcL \) expression was the most variable, increasing 24-fold between non-plume and plume
stations. Expression was significantly greater in the plume, but most tightly correlated to
the ratio between dissolved nitrate and nitrate uptake, indicating that chromophytic algae
were particularly successful where other nitrate using phytoplankton may have been less
abundant or less active.

Together these observations suggest that an intense bloom of diatoms in the near-
shore plume (based upon coastal sampling by others), where nitrate levels are high and
silica is not limiting, is replaced by smaller, ammonium preferring cells, in particular
\textit{Synechococcus} as plume water moves into the oligotrophic GOM. New production
becomes negligible as recycling of sub-micromolar ammonium ensues in the offshore
portion of the plume.
CHAPTER 6
The Geochemical Rate/RNA Integration Study (GRIST): I. RubisCO Transcription and Photosynthetic Capacity of Photoautotrophic Plankton

Data in this chapter has been submitted for review to the journal Applied and Environmental Microbiology

CHAPTER SUMMARY

The Geochemical Rate/mRNA Integration Study (GRIST) was a pilot field experiment aimed to assess the relationship between traditional biogeochemical rate measurements and transcriptional activity of microbial populations. We participated in GRIST in order to quantify the transcriptional activity of the large subunit gene (rbcL) of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). Group specific gene probes and quantitative PCR were used to determine form IA, IB, ID and diatom rbcL mRNA abundance over two diel cycles in surface an bottom water (15 m). In addition we described the phytoplankton species makeup at LEO15 based on rbcL cDNA library composition. rbcL data is compared to measurements of photosynthetic capacity (Pmax) provided by Jorge Corredor at the University of Puerto Rico. Strong diel signals in rbcL gene transcription and photosynthetic capacity were observed in three of four time series, with maxima occurring between 10 AM and 1 PM. Lowest P_{max} and rbcL levels were detected between 6 PM and 10:30 PM. Significant correlations were observed between
$P_{\text{max}}$ and all forms of $rbcL$ mRNA. The best correlation was observed between the additive hybridization $rbcL$ signal (form IA+IB+ID, $R^2=0.58$). Diatom $rbcL$ and $P_{\text{max}}$ mRNA was modestly correlated ($R^2 = 0.49; n=15$), but correlation improved dramatically upon removal of two outliers ($R^2 = 0.88; n=13$), which originated from afternoon samples with high $P_{\text{max}}$ but negligible mRNA levels as determined by quantitative PCR. Pigment information and total mRNA yield data indicated that diel signals observed in $rbcL$ mRNA were not caused by changing abundance of phytoplankton, but were an inherent property of the ambient population. $rbcL$ cDNA library data indicated the dominance of chromophytic algae, particularly diatoms, and eukaryotic green flagellates. Picocyanobacteria were not important during GRIST.
INTRODUCTION

The Geochemical Rate/RNA Integrated Study (GRIST) was designed as a pilot study to investigate the linkages and feedback mechanisms between solar irradiance, microbial activity and the carbon and nitrogen cycles. In order to study the relationships between individual rate processes, emphasis was placed on the application of molecular biological techniques during two one day diel experiments. It was hypothesized that a better understanding of patterns regulating the expression (transcription, translation and gene activity) of biogeochemically important genes in the marine environment will provide significant insight and more detailed understanding of otherwise difficult to study natural rate processes. Several research groups focused on quantifying transformations of carbon and nitrogen, including primary production, bacterial production, dissolved organic matter production/uptake, inorganic and organic N assimilation as well as N redox cycling in water column and sediment samples. These measurements were to be compared to quantities of specific mRNAs of several biogeochemically important genes. Our group contributed to this experiment by quantifying transcription of the major $rbcL$ forms (form IA, IB and ID). We also determined diatom $rbcL$ mRNA quantities using methods developed in chapter 2. In addition we determined water column total mRNA quantities and generated an $rbcL$ cDNA library to describe the phytoplankton species composition at LEO-15. In particular we were interested in comparing this data with measurements of carbon fixation and photosynthetic capacity of autotrophic plankton (measured by Jorge Corredor at the University of Puerto Rico) in order to assess if
significant correlations existed between these parameters and if \textit{rbcL} mRNA measurements may be used as proxies for carbon fixation processes.

The Long-term Ecosystem Observatory (LEO) is operated by Rutgers University and was originally designed as a prototype for future coastal observatory systems. It provides permanent and real-time communication with equipment on the seafloor in 15 m depth offshore near Tuckerton NJ (Grassle et al. 1998, Glenn et al. 2000). Archived data is available on the web through the Rutgers based Ocean Data Access Network (RODAN) (Zhang et al. 2001). LEO-15 is located on the highly productive New Jersey shelf, which is subject to recurrent coastal upwelling during summer month (Glenn et al. 1996). In addition this ecosystem receives large quantities of nutrients from New Jersey watersheds and has traditionally been characterized as a diatom driven eutrophic environment.
METHODS

The GRIST experiment was carried out at the Rutgers University Marine Field Station (RUMFS) at Tuckerton, NJ (19-25 July 2002). Sampling was performed at the Long-Term Ecosystem Observatory (LEO-15). The site is centered on a sand ridge in 15 m of water offshore from RUMFS. Diel experiments were performed by sampling at 4-hour intervals over two daily cycles (20 July 2002 and 22 July 2002). Filtration for biogeochemical and molecular analyses occurred at sea aboard the R/V Arabella.

Chl \( a \) and particulate carbon

Note: these data were provided to us by Jorge Corredor at the University of Puerto Rico.

Samples were flash-frozen on-deck in liquid \( N_2 \). Bulk chlorophyll \( a \) (Chl \( a \)) concentrations were determined by filtering sea water onto GF/F-filters and freezing filters for extraction in the lab. After 90% acetone extraction (Welschmeyer 1994) chl \( a \) concentrations were determined flurometrically. Particulate organic carbon and nitrogen content was determined using a CE Elantech CHN elemental analyzer.

Photosynthetic parameters

Note: These assays were performed by Jorge Corredor at the University of Puerto Rico. Methods provided herein are stated for the purpose of completeness.
Surface and bottom water samples for photosynthetic capacity measurements were immediately transported to the dock-side laboratory at the RUMFS after sampling (approximately a 30 minute trip). Water was sampled in 20 L polycarbonate carboys. Bottom samples were protected from light by wrapping carboys in black plastic bags. Sub-samples of 650 ml were transferred to 1 L light shielded, acid washed polyethylene bottles. These samples were then spiked with 0.108 mCi $^{14}$C-bicarbonate (Amersham Pharmacia Biotech) for a 0.167 µCi initial activity. Aliquots (40 ml) of the spiked water were transferred to 40 ml borosilicate EPA vials and incubated for 2 h in a photosynthetron apparatus (CHPT Mfg Co.) at constant temperature (17°C) and irradiances ranging from 0 (dark sample) to 614 uE m$^{-2}$ s$^{-1}$. A zero time sample blank was immediately filtered prior to commencement of incubation. Following incubation, samples were filtered onto 25 cm GF/F glass fiber filters and treated with 250 µl 10% HCl to drive off unfixed $^{14}$C-bicarbonate. After 24 h, 10 ml of BCS scintillation fluid (Amersham Pharmacia Biotech) were added and sample radioactivity was determined on a Beckman L6000 liquid scintillation counter using the channels ratio mode. The resulting data was plotted in $P$ vs. $E$ (irradiance) curves and the photosynthetic parameters $\alpha$ (response to low light), $P_{\text{max}}$ (photosynthetic capacity) and $\beta$ (susceptibility to photoinhibition) were computed using an exponential formulation (Platt et al. 1990).
**rbcL mRNA analysis**

mRNA extraction methods are essentially the same as described in chapters 3 and 5 and are described elsewhere (Paul 2001). Between 100 and 200 ml (depending if the filter clogged quickly) seawater were treated with 0.1% v/v DEPC (Di-Ethyl-Pyrocarbonate; Sigma Chemical Corp.) and filtered. For methods see above. Duplicate samples were probed with form IA, form IB and form ID rbcL probes previously described (Watson & Tabita 1996, Paul et al. 1999, Wawrik et al. 2003). For quantitative PCR of diatom rbcL mRNA DEPC treatment omitted during sampling. Samples were DNAse digested on the RNeasy columns for 15 min using the RNAse-Free DNAse reagent set (Qiagen) according to the protocol provided by Qiagen. Amplification and quantification was achieved as described in chapter 2 (Wawrik et al. 2002). RNA yields for all RNA extractions were determined using Ribogreen RNA quantification reagents (Molecular Probes Inc., Eugene, OR).

**PCR**

Reverse transcriptions were run using random hexamers, 4.7 mM MgCl, and M-MLV Reverse Transcriptase (Promega, Madison, WI) for 30 min at 37° C and freshly extracted RNA. Five µl were then added to a PCR reaction. Two primer sets were used to amplify the rbcL fragments. The form IA/B primer set produced a 615 bp fragment (forward primer: TCIGCITGRAACTAYGGTCG, reverse primer: CTGAGIGGIAARAACCTACGG) and the form ID set produced a 554 bp fragment
(forward primer: GATGATGARAAYATTAACCT, reverse primer
ATTTGDCCACAGTGDATACCA). All sequences are stated using IUPAC
degeneracies. PCR conditions were as follows: 1 µM final concentration for both
primers, 1.5 mM MgCl₂, 2.5 mM of each nucleotide and five units of TAQ polymerase
(Promega, Madison, WI). Cycle parameters were: 3 min at 95°C followed by 40 cycles
of 1 min at 95°C, 1 min at 52°C and 1 min 30 sec at 72°C. Cycling was followed by a
15 min 72°C elongation step.

Clone libraries

Immediately after amplification products were purified using the Quiaquick PCR
purification kit (Qiagen, Valencia, CA). Amplicons were then ligated into PCR® 2.1
vector using a TA cloning kit (Invitrogen corp., Carlsbad, CA). All white colonies were
then picked onto individual 2xYI plates containing 50 µg ml⁻¹ Kanamycin and
Ampicilin. Clones were screened for the correct insert size using PCR by touching a
colony with a sterile loop and transferring this loop directly to a PCR reaction tube.
Amplifications with products of the correct size were purified up using a QIAquick PCR
purification kit (Qiagen, Valencia, CA). Twenty µl of purified product were then digested
with 5 units of each Sau3AI (digests at ↓GATC↑) and AluI (digests at AG↓↑CT) in Buffer
B (Promega, Madison, WI) for 1 hour at 37°C. Digestions were run on 2.5 % low
melting agarose gels (Fisher, Fair Lawn, NJ) at 13.5 V/cm for 2 hours and stained with
ethidium bromide. Clones with unique restriction patterns were selected for sequencing.
Sequencing was performed in an Applied Biosystems model 373 sequencer by the University of Florida core sequencing laboratory (University of Florida, Gainesville, FL).

**Phylogenetic analysis**

cDNA sequences were translated into amino acids and these were aligned with their closest match recovered from a BLAST search of GenBank. Alignments were generated using Omiga 1.1 (Oxford Molecular group, Oxford, UK) and inspected manually for obvious misalignments. Alignments were then exported to Mega 2.0 beta (Kumar et al. 1993). Trees were generated using both parsimony and the Neighbor-Joining analysis. Both methods usually agree for rbcL sequences. Accession numbers for the clones obtained here are as follows: AY356325-AY356346.
## Tables

Table 6.1

Photosynthetic parameters of phytoplankton assemblages during the GRIST experiment.

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Table 6.2

Concentration of photosynthetic pigments at LEO-15 during the GRIST experiment.

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Table 6.3

Closest Relative in GenBank of *rbcL* clones obtained during the GRIST experiment

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Figure 6.1

Satellite thermal image showing sea surface temperature at LEO-15 on July 22, 2002.

(Image courtesy of Lee Kerkhoff at Rutgers University)
Figure 6.2

Vertical profiles of water temperature and *in situ* fluorescence versus time during the GRIST study. Boxes indicate times of the first and second time series. (A) temperature (B) *in situ* fluorescence. (Data courtesy of Lee Kerkhoff at Rutgers University)
Figure 6.3

Chl $a$, POC and PON at the LEO-15 Node A during the GRIST experiment. Cicles indicate bottom samples. Squares indicate surface samples. Day one samples are shown in left panels, while day 2 samples are shown in the right panel. (Data curtesy of Lee Kerkhoff at Rutgers University)
Day 1: Time course for \( rbcL \) mRNA abundance and light-saturated photosynthetic rate

(A) Near-surface samples (B) Bottom samples. (\( P_{\text{max}} \) data provided by Jorge Corredor)
Day 2: Time course for $rbcL$ mRNA abundance and light-saturated photosynthetic rate

(A) Near-surface samples (B) Bottom samples. ($P_b^{\text{max}}$ data provided by Jorge Corredor)
Figure 6.6

Neighbor-joining tree of $rbcL$ sequences obtained from the GRIST site (labeled GRIST and clone specific postfix). Numbers at internal nodes indicate bootstrap consensus values obtained from 1000 bootstrap replicates. Clone labeled P994DY5 is from the Gulf of Mexico (Wawrik et al. 2003).
mRNA yields achieved by means of bead-beating and extraction on an RNeasy column (Qiagen). Yields are shown in ng L$^{-1}$ sea water, while only 100-200 ml were filtered during the experiment.
Figure 6.8

(A) Form I A rbcL mRNA hybridization vs. Pmax (B) form IB rbcL mRNA hybridization vs. Pmax (C) form ID rbcL mRNA hybridization vs. Pmax (D) All forms or rbcL (A+B+D) vs. Pmax
Diatom \textit{rbcL} real-time PCR vs. Pmax. (A) all time points (B) two afternoon samples with high carbon fixation but low \textit{rbcL} mRNA removed.
RESULTS

Field site conditions, chl $a$, PON and POC

Sea surface temperature (SST) varied appreciably at LEO15 during the course of the three day GRIST study (Fig. 6.1). SSTs ranged from 17° C at the beginning of the experiment to 24° C at the end of day 2 as the result of warm water intrusion. Stratification of the water column was observed on the first day of sampling and water temperatures varied between 15° C in bottom samples and 22° C at the surface (Fig. 6.2A). Stratification was disrupted during day 2 of the experiment as the result of wind induced mixing caused by an approaching continental weather front. Chlorophyll $a$ (chl $a$) concentrations were lower in bottom than in surface samples on both days. On day one surface chl $a$ concentrations were constant in the morning and early afternoon hours, but doubled in late afternoon samples reaching a maximum of ca. 16 µg L$^{-1}$. Low values in bottom samples (ca. 3 µg L$^{-1}$) prevailed most of the day, except for the last sampling, when concentrations increased to near surface values. During the second sampling period bottom chl $a$ concentrations were consistently low (<5 µg L$^{-1}$), while surface concentrations peaked in the 6:00 pm sample (34.7 µg L$^{-1}$). Particulate organic nitrogen (PON) and particulate organic carbon (POC) concentrations followed a pattern similar to chl $a$ and were significantly correlated to this parameter in both surface and bottom samples. chl $a$, PON and POC were consistently greater in surface samples than in bottom samples on both sampling days (Fig. 6.3).
Light saturated photosynthetic rate normalized by chl \(a\) (\(P_{\text{B}}^{\text{max}}\))

Note: This data was kindly provided by Jorge Corredor at the University of Puerto Rico.

Only three measurements for \(P_{\text{B}}^{\text{max}}\) were available for surface waters of day one (Table 6.1). chl \(a\) normalized rates in the surface increased from 6.65 mg C chl \(a^{-1}\) \(\mu\text{E}^{-1}\) h\(^{-1}\) in the morning sample to 7.95 in the late afternoon, when chl \(a\) concentrations doubled. \(P_{\text{B}}^{\text{max}}\) in bottom samples first decreased from 6.62 mg C chl \(a^{-1}\) \(\mu\text{E}^{-1}\) h\(^{-1}\) in the early morning sample to 4.86 mg C chl \(a^{-1}\) \(\mu\text{E}^{-1}\) h\(^{-1}\) in the noon samples and peaked at 9.50 mg C chl \(a^{-1}\) \(\mu\text{E}^{-1}\) h\(^{-1}\) in the late afternoon. Variability of \(P_{\text{B}}^{\text{max}}\) was much greater during the second diel experiment, when a strong diel signal was observed in both bottom and surface samples. In the surface \(P_{\text{B}}^{\text{max}}\) varied 9.6-fold, peaking in the noon sample at 7.01 mg C chl \(a^{-1}\) \(\mu\text{E}^{-1}\) h\(^{-1}\). The lowest rate of 0.72 mg C chl \(a^{-1}\) \(\mu\text{E}^{-1}\) h\(^{-1}\) was observed in the early evening. \(P_{\text{B}}^{\text{max}}\) variability in bottom samples mirrored the diel signal observed in the surface. Rates varied 2.9-fold and peaked at 11.6 mg C chl \(a^{-1}\) \(\mu\text{E}^{-1}\) h\(^{-1}\) in the noon sample. This sample contained the highest rate for \(P_{\text{B}}^{\text{max}}\) observed during the GRIST experiment. \(P_{\text{B}}^{\text{max}}\) rates were significantly greater near the surface than at the bottom (\(p=0.01\)), while there was no significant difference between the two depth if non-normalized rates are compared (\(p=0.08\)).

**Pigment concentrations (HPLC)**

HPLC pigment data is shown in Table 6.2. Patterns of chlorophyll \(a\) as determined by HPLC were in good agreement with fluorometrically determined quantities, although HPLC
quantities were consistently lower and accounted in average for only 65% of those obtained by fluorometry. Chlorophyll \( b \) is indicatitive of green algae, including Chlorophytes, Pasinophytes and Euglenophytes. Concentrations of chl \( b \) were significantly greater in surface samples than near the bottom on both days (day one: \( p=0.03 \), day two: \( p=0.002 \)). Concentrations averaged 0.98 \( \mu g \) \( L^{-1} \) and 2.82 \( \mu g \) \( L^{-1} \) in surface samples of day one and two respectively. Chl \( b \) was also significantly higher during the second diel than during the first (\( p=0.02 \)). Near the bottom chl \( b \) was not prevalent averaging only 0.32 \( \mu g \) \( L^{-1} \) and 0.11 \( \mu g \) \( L^{-1} \) during day one and two respectively. Noticeable however was a >14-fold increase in bottom chl \( b \) between the 7:30 pm and 11:30 pm samples of the first diel experiment. Chlorophyll \( c1 \) found in diatoms, prymnesiophytes and raphidophytes was absent from all bottom samples except the 11 pm sample of day one. Surface concentrations averaged 0.19 \( \mu g \) \( L^{-1} \) during day one and 0.10 \( \mu g \) \( L^{-1} \) during day two but were not significantly different (\( p=0.23 \)). Chlorophyll \( c2 \) is found in the majority of chromophytic algae, including dinoflagellates and cryptophyceae. Concentrations were significantly greater in surface than in bottom samples during both diels (\( p=0.02 \) and \( p<0.001 \)) and averaged 0.48 and 0.95 \( \mu g \) \( L^{-1} \) in the surface for day one and two respectively. Peridinin is a diagnostic marker for dinoflagellates and was largely absent from day one surface and all bottom samples. Concentrations however were appreciable in near surface samples during the second diel experiment, averaging 0.40 \( \mu g \) \( L^{-1} \). Zeaxanthin, mainly found in cyanobacteria and rhodophytes, was absent from bottom samples except the 11:00 sampling of day one. Surface concentrations averaged 0.47 \( \mu g \) \( L^{-1} \) during day one and 0.37 \( \mu g \) \( L^{-1} \) during day two and noticeably declined to zero during the evening hours of the first day of sampling. Fucoxanthin, mainly found in diatoms, was the dominant accessory pigment observed during GRIST and was significantly correlated with
chl a concentrations at LEO 15 ($R^2=0.52$, $p<0.01$). Concentrations were significantly lower in bottom samples than in surface samples (day 1: $p=0.03$; day 2: $p=0.01$). Highest concentrations were observed in day two 9:40 am surface samples (5.90 µg L$^{-1}$).

*rbcL* mRNA

*rbcL* signals observed during the first diel experiment are shown in Figure 6.4. In surface samples (Fig. 6.4A) *rbcL* mRNA levels decreased in the morning hours for all major forms we quantified (form IA, IB and ID). While form IB and form ID *rbcL* mRNA remained invariable for the remainder of the day, form IA increased during the afternoon and remained elevated into the evening hours. Diatom *rbcL* mRNA levels showed little variability over the course of the day, but were lowest in the afternoon. In bottom samples (Fig. 6.4B) we observed a strong diel signal for form ID and diatom *rbcL* mRNA. Both peaked at 1:00 pm and then decreased to their lowest quantities in the 7:00 pm samples. Form IA and IB signals remained relatively invariant throughout most the day but more than doubled between the 7:30 and 11:00 pm samples. On the second day of sampling all forms of *rbcL* were observed to exhibit strong diel variation in both surface (Fig. 6.5A) and near bottom (Fig. 6.5B). Peak values were observed during morning hours, between 9:00 and 11:00 am. Analogous to the first sampling day, form IA, IB, ID and diatom *rbcL* mRNA increased 3-, 2.5-, 3.9- and 6.1-fold respectively between 7:00 and 11:00 pm in bottom samples. All three forms of *rbcL* mRNA quantified by hybridization were significantly correlated to each other at the 1% confidence interval (A to B: $p=0.001$, A to D: $p=0.01$, B to D: $p=0.001$). Form IB and form ID *rbcL* mRNA were also significantly correlated with
diatom \textit{rbcL} mRNA quantified by real-time PCR. Form IA was not (p=0.12). Pmax was significantly correlated with all forms of \textit{rbcL} we quantified (A: p=0.01, B: p=0.005, D: p=0.001, diatom: p=0.007). Pmax was least well correlated to form IA \textit{rbcL} (R^2=0.41, Fig. 6.8A). Pmax exhibited progressively tighter correlation to form IB (R^2=0.47, Fig. 6.8B) and to form ID \textit{rbcL} mRNA (R^2=0.56, Fig. 6.8C). The best predictor of Pmax was the cumulative \textit{rbcL} mRNA signal obtained by adding form IA, IB and ID hybridization numbers (R^2=0.58, Fig. 6.8D). Diatom \textit{rbcL} mRNA was significantly, but not as well correlated to Pmax (R^2=0.44, Fig. 6.9A). Upon removal of two afternoon samples, which contained a near zero signal, however this correlation was substantially more predictive (R^2=0.88, Fig. 6.9B).

\textit{rbcL} clone libraries

Cloning and sequencing of transcriptionally active \textit{rbcL} genes from this environment yielded 22 unique \textit{rbcL} clones (Fig. 6.6). Table 6.3 shows the closest match in GenBank obtained by BlastP analysis (Altschul et al. 1997) and their percent identity to the query at the amino acid level. Fourteen of the recovered clones were most closely related to sequences from chromophytic algae. Sequences likely originated from four diatoms, four prymnesiophytes, four chrysophytes, a rhodophyte, and a deeply rooted sequence, which was most closely related to \textit{Olithodiscus luteus}. All remaining eight clones were of the form IB type. Among them were two sequences most closely related to flagellated chlorophytes such as \textit{Pyramimonas}, one sequence closely related to \textit{Bathycoccus} and a small clade of deeply rooted forms. No picocyanobacterial sequences (ie. \textit{Synechococcus} or \textit{Prochlorococcus}) were detected.
Total mRNA yield

Average mRNA yields (n=6) during GRIST ranged between 2.6 µg mRNA L\(^{-1}\) and 36.3 µg mRNA L\(^{-1}\) (Fig. 6.7). The peak average value was observed in the 11:30 pm surface sample of the first diel experiment, while lowest mRNA yields were observed in the 6:30 pm bottom sample of the second diel experiment. Overall mRNA yields were significantly greater in surface samples than in bottom samples (p<0.001, N=11). mRNA yields were highly variable in surface samples during the first diel and increased from average levels of 13.0 µg mRNA L\(^{-1}\) at 5:30 am to 36.3 µg mRNA L\(^{-1}\) in the last sample of the day. The opposite trend was observed during the second diel, where average surface mRNA levels decreased from 31.5 µg L\(^{-1}\) in the early sample to 15.4 µg L\(^{-1}\) in the latest sampling. Levels in bottoms samples were low throughout most of the day. Levels significantly increased however between 7:00 pm and 11:00 pm during both days (N=6, p<10\(^{-5}\) during both diel experiments).
DISCUSSION

Molecular approaches to measuring microbial community composition and activity are thought to circumvent many shortcomings of culture dependent techniques (MacGregor 1999). The central dogma of molecular biology implies that RNA transcription leads to translation and production of functional enzymes. These enzymes in turn are responsible for all observed biogeochemical transformations. Each stage in the process of gene expression however may be heavily regulated and individual techniques focusing on DNA, RNA, protein or enzyme activity measures a different aspect of microbial activity (MacGregor 1999). The GRIST experiment was designed to investigate the relationship between mRNA transcription of carbon and nitrogen assimilation genes and biogeochemical rate processes in order to describe the correlations between these different aspects of gene expression.

While \( rbcL \) mRNA levels were depressed in daylight surface samples of the first day of sampling and form IA and form IB were invariable in bottom samples, strong diel pattern were observed for form ID and diatom \( rbcL \) mRNA in bottom samples. During the second diel we observed strong diel signals for all forms of \( rbcL \) in surface and bottom samples peaking between 10:00 and 11:00 am, which coincided with a similar diel pattern in Pmax.

Diel variations of phytoplankton carbon fixation in the environment have long been recognized (Doty & Oguri 1957) and photosynthetic parameters have been described for natural assemblages, which were used to propose working models for the evident diel variation (MacCaull & Platt 1977). Variation in Pmax and photosynthetic efficiency (\( \alpha^B \)) have been studied in the Gulf of Maine (Legendre et al. 1988), where it
was found that both parameters varied in phase. Variations were not related to total chl \(a\) concentrations in seawater and based on these observations it was concluded that oscillations in carbon fixation are caused by endogenous circadian rhythms and not environmental factors.

During the GRIST study there was some indication that diel variations observed in mRNA signals and Pmax were reflected by pigment levels as determined by HPLC (Table 6.2) bringing into question if the observed diel signals were merely the result of varying cell populations. This scenario however seems unlikely based on the data at hand. During day one surface concentrations of the major pigments (Table 6.2) reached their maximum values in the near midnight sample and chl \(a\) concentrations approximately doubled in the afternoon. This pattern was only reflected by the form IA signal but not the other forms of \(rbcL\). In day 1 bottom samples and at both depth during day 2, highest \(rbcL\) levels were found to occur between 11:00 am and noon, while the major pigment peaks (with the exception of fucoxanthin) occurred either in the first or last sample of the day or later in the afternoon (between 3pm and 5pm) indicating that high \(rbcL\) levels either preceded high pigment levels or were independent of their levels. High carbon fixation rates are expected to lead to de novo synthesis of pigments. More importantly, while mRNA yields (Fig. 6.7) exhibited good correlations with pigment levels, particularly to chl \(c_1\), fucoxanthin and zeaxanthin (\(R^2=0.68, 0.57\) and 0.77 respectively; \(p<0.001\) for all three), yields were not significantly correlated with the individual mRNA signals (form IA: \(p=0.38\); IB: \(p=0.17\); ID: \(p=0.29\); diatom \(p=0.627\)). This indicates that variations in \(rbcL\) mRNA levels were not causes by population level
dependent variations of total mRNA yield, but were caused by transcriptional regulation of phytoplankton $rbcL$ mRNA synthesis.

Virtually all carbon fixation in autotrophic plankton is mediated by RubisCO and it follows that RubisCO gene expression and regulation may in large part modulate photosynthetic diel variations. The principal factor controlling RubisCO expression in phytoplankton is light (MacCaull & Platt 1977, Tabita 1988, Hartman & Harpel 1994) and at least in prokaryotes regulation is mostly mediated at the transcriptional level. $rbcL$ transcript levels have been used as an indicator for RubisCO gene expression in the environment in several studies. Mesocosm experiments and a langrarian study in the oligotrophic SE Gulf of Mexico revealed strong diel variation in form IB $rbcL$ mRNA levels in surface waters reminiscent of photosynthetic diel variations (Pichard et al. 1996). Form IB $rbcL$ mRNA was significantly correlated with carbon fixation and both were high during morning hours. This correlation was confirmed in cultures of $Synechococcus$ sp. strain RF-1 (Chow & Tabita 1994). In two later studies form IB and form ID $rbcL$ mRNA were quantified in size fractionated samples taken in Tampa Bay and the oligotrophic Gulf of Mexico (Pichard et al. 1997a, Paul et al. 1999). $rbcL$ transcript and carbon fixation were predominantly associated with the $>5 \mu m$ fraction in Tampa Bay and the $<1 \mu m$ fraction in the oligotrophic Gulf of Mexico. A lagrangian study also revealed the temporal separation between the peaks in form ID and form IB transcript. The maximum in form IB $rbcL$ mRNA occurred in the morning hours, while peak form ID transcript levels were observed during the afternoon. Observations in cultures confirmed these observations in the form ID containing chromophytic alga $Pavlova$ gyrans and the form IB containing $Synechococcus$ WH7002 (Paul et al. 2000c).
The diatom *Phaeodactylum tricornutum* conversely was shown to exhibit highest *rbcL* transcription during morning hours (Wawrik et al. 2002) indicating that the afternoon peak in *rbcL* mRNA is a feature of prymnesiophytes. Highest levels of form ID and diatom *rbcL* during GRIST were observed between 11:00 am and noon, which is consistent with the assumption that diatoms dominated the phytoplankton community during GRIST.

Although gene transcription has been used as a surrogate for gene expression, a large degree of uncertainty remains regarding the predictability of biogeochemical activity based upon transcript abundance. Data presented here further illustrates this by demonstrating that *rbcL* mRNA may not always be an accurate predictor of carbon fixation. While Pmax was overall significantly correlated with all types of *rbcL*, correlations were not significant for the first diel experiment alone. This indicates that factors other than *rbcL* transcription may have been dictating primary production in the water column during the first diel.

Enzymes can be subject to a host of post-transcriptional controls and particularly RubisCO appears to be tightly regulated at the post-translational level in eukaryotes (Hartman & Harpel 1994). Mechanisms for the metabolic regulation of RubisCO activity include reversible activation of the active site by CO$_2$, binding of non-competitive inhibitors to the active site and binding of effectors at sites other than the active site. These controls appear to respond to the relative abundance of RubisCO substrates and to cellular energy and reductive states (ADP/ATP and NADP/NADPH ratios). *rbcL* transcription and RubisCO enzyme activity have also been shown to be temporally uncoupled from whole cell carbon fixation in the chromophytic alga *Pavlova gyrans*, while transcription, carbon fixation and enzyme activity were tightly correlated in *Synechococcus* WH7002 (Paul et al. 2000c). These
data indicated that \textit{rbcL} mRNA may be a better surrogate for gene expression in prokaryotic algae than in eukaryotes.

Despite these shortcomings our results suggest that transcriptional regulation may have been a good indicator of carbon fixation during the second diel of the GRIST experiment. We observed strong diel variations in all forms of \textit{rbcL} mRNA, which were in phase with a similar diel pattern observed for \( P_{\text{max}} \). \( P_{\text{max}} \) variability was greater than one order of magnitude (13 – 160 mg C L\(^{-1}\) h\(^{-1}\)) and coefficients of determination (R\(^2\)) between the various forms of \textit{rbcL} mRNA and \( P_{\text{max}} \) ranged between 0.4 for form IA to 0.6 for form ID respectively.

Diatoms were the dominant phytoplankton during both diel experiments (see pigment discussion below). Real-time diatom \textit{rbcL} mRNA however correlated only moderately with \( P_{\text{max}} \) (\( R^2 = 0.44; n = 15 \)). If two afternoon (3:40 PM day 1 and 4:20 PM day 2) surface samples, which exhibited barely detectable levels of diatom \textit{rbcL} mRNA despite the high photosynthetic capacity, were removed from the analysis the overall correlation between diatom \textit{rbcL} mRNA and \( P_{\text{max}} \) was the best observed for any form of \textit{rbcL} (Fig. 6.8, \( R^2 = 0.88; n = 13 \)). Low levels of diatom \textit{rbcL} mRNA detected in the two outlying samples may be attributed to mRNA degradation in the presence of high levels of functional RubisCO protein. More likely RT-PCR reactions were inhibited, potentially by UV damage to nucleic acids or contaminants, as has been observed for Gulf of Mexico surface samples (Wawrik et al. 2002).

RT-PCR and cloning of \textit{rbcL} from the transcriptionally active phytoplankton population indicated the presence of an array of chromophytic algae including diatoms, prymnesiophytes, and chrysophytes (Fig. 6.6). In addition we observed several deeply rooted haptophytes and several chlorophytic flagellates. These observations are in good agreement
with pigment data collected during GRIST (Table 6.2) and indicate that diatoms and chlorophytes were the dominant phytoplankton during the first diel experiment, while several other chromophytic flagellates formed minor components of the community. It should be noted that only one \textit{rbcL} library was generated from morning samples of the first diel and that this clone library should not be taken to indicate conditions found during later times in the experiment. In fact, it appears that community composition may have shifted considerable between the two sampling days. We observed a substantial and significant increase in \textit{chl b} levels in surface waters of the second diel (Table 6.2), indicating that chlorophytes may have become proportionally more important. Also Surface waters on the second day of sampling contained appreciable amounts of peridinin, indicating the advection of a population of dinoflagellates not present during the first diel cycle. Pigment data also revealed significant differences between surface and bottom communities, suggesting a stratified system with independent phytoplankton communities in surface and bottom waters. Overall pigment abundance was a poor predictor of individual forms of \textit{rbcL} mRNA and no significant correlations were observed.

Cyanobacteria, such as \textit{Synechococcus} and \textit{Prochlorococcus}, were only minor components of the community, as was evidenced by the absence of zeaxanthin from all but one bottom sample and low levels of this pigment in surface waters. We also recovered no cyanobacterial \textit{rbcL} sequences from our cDNA library. It seems also likely that the low levels of zeaxanthin we observed were entirely accounted for by \textit{Synechococcus} since Tuckerton is located at ca. 40 deg. N, the approximate northern limit to the occurrence of \textit{Prochlorococcus} (although no flow cytometry was available to confirm this assumption).
CHAPTER 7

Transcription of a Short Cis-encoded Antisense mRNA Homologous to the Global Nitrogen Regulatory Hene ntcA in Synechococcus WH 7803

A manuscript containing data from the following chapter is currently in preparation to be submitted for review soon

CHAPTER SUMMARY

In cyanobacteria NtcA transcriptionally regulates many genes involved in the uptake and assimilation of DIN (dissolved inorganic nitrogen). Here we report that in the marine cyanobacterium Synechococcus WH7803 and three other marine isolates down-regulation of ntcA transcription concurs with the formation of a short cis-encoded antisense ntcA mRNA transcript. A significant decrease in cellular ntcA mRNA levels was observed in Synechococcus WH7803 one hour after the addition of 100µM ammonium, whether cells were grown on 2 mM ammonium, 8 mM nitrate or were nitrogen starved. Concomitantly we observed a 3 and 4.8-fold, significant increase in cellular levels of antisense ntcA mRNA as the result of ammonium addition in ammonia and nitrate grown cultures. These observations were confirmed in three phycoerythrin containing marine Synechococcus isolates. Southern blotting indicated that ntcA is encoded by a single locus in WH7803. Northern blotting demonstrated that the sense ntcA transcript in WH7803 is ca. 1.2 kb in length. The antisense transcript of ntcA was found to be ca. 380 nucleotides long. These data suggest that NtcA gene expression may be regulated by the formation of a cis-encoded antisense mRNA.
INTRODUCTION

Biomass and productivity in the oligotrophic and mesotrophic ocean are dominated by autotrophic picoplankton (Guillou et al. 1999a). Picoplankton is composed of *Synechococcus*, *Prochlorococcus* and a diverse array of small eukaryotes (Campbell et al. 1994, Li 1994, Guillou et al. 1999a). The picocyanobacterial component of the picoplankton often represents the dominant fraction of photosynthetic biomass in the oligotrophic, tropical oceans (Burkhill et al. 1995, Campbell et al. 1997, Partensky et al. 1999a, Partensky et al. 1999b), where productivity is frequently limited by the bio-availability of dissolved inorganic nitrogen (DIN) (Vitousek & Howarth 1991, Bronk et al. 1994, Tyrrell & Law 1997). How picocyanobacteria, in particular *Synechococcus*, mediate and regulate DIN uptake is thus of interest.

Ammonium is the preferred source of inorganic nitrogen in cyanobacteria, since other forms of nitrogen such as nitrate, nitrite and dinitrogen gas must first be reduced in order to be assimilated into organic matter via the GS/GOGAT pathway (Guerrero & Lara 1987, Flores & Herrero 1994, Lindell et al. 1998). Genes involved in the transport and reduction of nitrate and nitrite in *Synechococcus* PCC 7942 have been shown to be organized in an operon (*nirA-nrtABCD-narB*) and their expression to be inhibited by the presence of ammonium (Guerrero & Lara 1987, Luque et al. 1992, Suzuki et al. 1993, Flores & Herrero 1994). Ammonium also reversibly inhibits the transport of nitrate and nitrite transport across the cell membrane after incorporation through glutamine synthetase (Lara et al. 1987, Flores & Herrero 1994, Lindell et al. 1998). Complementation of pleiotropic mutants of *Synechococcus* PCC 7942 unable to
assimilate nitrogen sources other than ammonium revealed a 669 base pair long gene, designated \( ntcA \), that could restore the wild-type (Vega-Palas et al. 1990, Vega-Palas et al. 1992).

NtcA was later shown to be a transcriptional activator of the cyclic AMP receptor protein (CRP) family (Vega-Palas et al. 1992). Under ammonium deplete conditions NtcA binds to promoters of an array of genes involved in nitrogen assimilation and positively influences their transcription. Genes activated by NtcA share an upstream NtcA binding site with the consensus sequence of GTAN₈TAC (Luque et al. 1994). Among the activated genes identified in several strains of cyanobacteria are the \( nir \)-operon, \( glnA&B \), \( amt1 \), the \( urt \)-operon, \( ntcB \), \( hetC \), \( devBCA \), \( nrtP \) and \( ntcA \) itself (Herrero et al. 2001). NtcA has also been reported to have inhibitory effects on several genes. Most notably NtcA has been shown to bind to the promoter region of \( rbcL \) (large subunit gene of ribulose-1,5-bisphosphate Carboxylase/Oxygenase) (Chastain et al. 1990, Ramasubramanian et al. 1994) linking the expression of genes involved in both carbon and nitrogen assimilation. NtcA has been found to bind to the promoters of \( gifA&B \) (glutamine synthetase inactivating factors IF7 & IF17) and full expression of these genes only occurs in the presence of ammonium (Garcia-Dominguez et al. 2000). NtcA is known to be auto-regulatory and expression is repressed in the presence of ammonium in \textit{Synechococcus} WH7803 and PCC7942 (Luque et al. 1994, Lindell et al. 1998). \( ntcA \) was shown to exist as a single copy in WH7803 with a transcriptional start-point just downstream of the NtcA consensus binding sequence (Lindell et al. 1998). mRNA levels were shown to drop quickly (2 min half-lives) upon ammonium rifampin addition. Nitrate assimilating cells were shown to contain higher levels of \( ntcA \) mRNA than ammonium
grown cells. Highest levels of \textit{ntcA} were observed in nitrogen starved cells, although nitrate uptake capacity did not develop as the result of N starvation. \textit{ntcA} mRNA accumulates in \textit{Synechococcus} WH7803 grown on a variety of nitrogen sources, but not if cells are grown on ammonium (Lindell & Post 2001). mRNA levels rapidly increase as ammonium levels in media drop below 1µM but decline again upon the addition of >1µM ammonium to media.

The exact mechanisms by which NtcA senses the presence of ammonium in the environment and integrates this signal to balance cellular C to N demand still remains unknown. Here we report evidence that the regulation of \textit{ntcA} transcription may involve the transcription of a short cis-encoded antisense mRNA.
FIGURES

Figure 7.1

A

B

ASW + 2mM ammonium

ASW + 8 mM nitrate
ntcA sense transcript regulation. (A) Cells were grown in ASW with 2mM ammonium and either remained untreated (black bars) or received a treatment of 100 uM ammonium final concentration (gray bars) for 60 minutes. (B) Cells were grown in ASW + 8 mM nitrate.
Figure 7.2

(A) ASW + 2 mM ammonium

(B) ASW + 8 mM nitrate
Figure 7.2

*ntcA* antisense transcript regulation. (A) Cells were grown in ASW with 2mM ammonium and either remained untreated (black bars) or received a treatment of 100 uM ammonium final concentration (gray bars) for 60 minutes. (B) Cells were grown in ASW + 8 mM nitrate.
Figure 7.3

A

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<td>3e-5</td>
</tr>
<tr>
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<td>2e-5</td>
</tr>
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B

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ntcA sense and antisense transcript regulation in nitrogen starved cultures. (A) ntcA sense mRNA. Cells remained untreated (black bars) or received a treatment of 100 µM ammonium final concentration (gray bars) for 60 minutes. (B) ntcA antisense ntcA mRNA
Neighbor joining analysis of partial \textit{rbcL} sequences cloned from \textit{Synechococcus} isolates used in this study. Bootstrap values $>60\%$ out of 1000 replicates are shown. Poisson correction was applied to correct for rate heterogeneity. G5A, GG3L, B13, J15, N5D, A13 and S13 are Gulf of Mexico phycoerythrin containing isolates grown in our lab. (*) isolates used in this study.
Southern blot genomic *Synechococcus* WH7803 undigested and restriction digested DNA. (L) Lambda HindIII markers (P) 1 ng plasmid containing ntcA insert + 100 bp ladder (U) undigested (1) HindIII digested (2) EcoRI digested (3) EcoRV digested (4) Sall digested (5) XohI digested. Molecular weights are indicated in the left margin in kb. The blot was probed using $^{35}$S labeled sense *ntcA* mRNA *in-vitro* transcript (Riboprobe)
Northern blot of bulk *Synechococcus* WH7803 mRNA probed with an antisense *ntcA* riboprobe. (1&5); RNA molecular weight markers (Promega); sizes are indicated in kb in the left margin. (2&6) 1 ng sense *ntcA* in-vitro transcript; (3&7) 1 ng antisense *ntcA* in-vitro transcript; (4&8) bulk DNAse digested mRNA extracted from 30 ml of WH7803 culture. The size of the sense *ntcA* message is indicated in the right margin.
Northern blot of bulk *Synechococcus* WH7803 mRNA probed with a sense *ntcA* riboprobe. (1) Molecular weight markers (Ambion); (2&6) Molecular weight markers (Promega); (3&7) 1 ng AS *ntcA* *in-vitro* transcript; (4&8) total DNase digested RNA extracted from 20 ml of *Synechococcus* WH7803 culture; (5&9) total DNase digested RNA extracted from 20 ml of WH7803 culture after 1 hour incubation following 100 µM ammonium addition.
METHODS

Cultures and culture conditions

*Synechococcus* WH7803 was obtained from the Provasoli – Guillard Center for culture of Marine Phytoplankton (West Boothbay Harbor, ME). Cultures are maintained in our lab in SN media prepared from aged seawater at 16°C under 12:12 light dark cycle illumination. The strains A13, B13 and N5D are non-axenic, phycoerythrin containing marine *Synechococcus* we obtained from Gulf of Mexico oligotrophic waters by serial dilution of seawater samples with SN in 15 ml plastic tubes followed by growth in SN media and clonal isolation on SN solid media. Attempts to produce axenic cultures by multiple transfers from solid to liquid media, antibiotic and lysozyme treatments were not successful.

For mRNA experiments cultures were transferred out of SN into nitrogen free artificial seawater medium (ASW) described previously (Wyman et al. 1985, Lindell et al. 1998) and grown through at least two transfers before experiments were performed. This was necessary, since ntcA transcriptional regulation and responses to ammonium additions were not consistent in SN media in our hands. Media was supplemented only with vitamin B12 and not the full Va vitamin mix (Waterbury & Willey 1988). In order to keep media nitrogen free the trace metal mix was modified by replacing ferric ammonium citrate with ferric chloride and cobalt nitrate with cobalt chloride. Nitrate containing media (ASW$_{NO3}$) was supplemented with 8 mM nitrate and ammonium containing media (ASW$_{NH4}$) was prepared by adding ammonium chloride to a final
concentration of 2 mM. Nutrients and trace metals mix were added using sterile technique after the media was pH adjusted to pH 8.0 with a few drops of concentrated hydrochloric acid and autoclaved. Nutrient and trace metal stocks were autoclaved separately. Vitamin B12 stock was filter sterilized though a 0.22 Acrodisc filter (Life Sciences Groups, Ann Arbor, MI). All cultures were grown in acid leached 500 ml Erlenmeyer flasks on a shaking Table at 145 rpm. Cultures were maintained at room temperature and illuminated using cool white fluorescent tubes during 12:12 light dark cycles. Cultures were grown through at least two transfers in the media used during experiments in order to acclimate cells (either ASW$_{\text{NO}_3}$ or ASW$_{\text{NH}_4}$). For mRNA experiments 40 ml of dense culture were inoculated in duplicate during the late afternoon into 500 ml flasks containing 160 ml ASW$_{\text{NO}_3}$ or ASW$_{\text{NH}_4}$. Cultures were then grown for 36 hours and sampled during early morning hours after the light had turned on in our incubator. One flask remained untreated, while the other received an addition of 100 µM final concentration freshly prepared, sterile filtered ammonium chloride. For nitrogen starvation experiments 40 ml of nitrate grown culture was spun and washed twice with nitrogen free ASW media. The pellet was then suspended in 200 ml of nitrogen free ASW and maintained for 48 hours before sampling.

**Cloning of the NtcA gene**

To generate a probe, *ntcA* was cloned from *Synechococcus* WH7803 by PCR amplification and TA cloning. 25 ml of dense culture were spun down and the pellet was resuspended in 1 ml STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 8.0).
DNA was extracted using SDS boiling lysis described elsewhere (Pichard et al. 1993). Several dilutions of extracted DNA were then PCR amplified using 2.5 U Taq-polymerase (Promega, Madison, WI), 1.5 mM MgCl and the following primers:

**GCACCCGCTCCCACCAGCAGAACC (NTCA1)** and **CACGAGCCCTGATTGCAGAA** (NTCA2). Cycling conditions were: 92° C for 50 sec, 56° C for 40 sec, 72° C for 1 min 30 sec followed by a 15 min elongation step at 72° C. This produced a 597 bp amplicon, which was cloned into the PRCII vector (Invitrogen, Carlsbad, CA) using an Invitrogen dual promotor TA-cloning kit according to the manufacturers instructions. Several clones were then screened for the correct insert size by PCR amplification. One clone containing the correct insert size was selected and the insert sequenced to confirm its identity (the plasmid was named pNTCA).

**Genetic identification of Isolates**

To determine the identity of the environmental isolates used here we PCR amplified and sequenced their **rbcL** (large subunit of ribulose-1,5-bisphosphate caboxylase/oxygenase) gene sequences. DNA was extracted from 20 ml of dense cultures using the DNeasy Tissue Kit (Qiagen, Valencia, CA) as indicated by the manufacturer for gram negative bacteria. Extracted DNA for the isolates G5A, GG3L, B13, J15, N5D, A13 and S13 was amplified using the following primers: **TCIGCITGAACTAYGGGCTG** and **CTGAGIGGGIAARAACTACGG** (all degeneracies are given in IUPAC code). Final reaction mixtures contained 2.5 U Taq-DNA polymerase (Promega, Madison, WI) and 1.5 mM MgCl. Cycling conditions were 95° C for 60 sec, 52° C for 60 sec, 72° C for 1
min 30 sec followed by a 15 min elongation step at 72°C. PCR amplicons were TA-cloned as indicated above and inserts sequenced. DNA sequences were then imported into the Omiga (Oxford Molecular) and translated into amino acid sequences. These were then aligned with a representative set of \(\text{rbcL}\) sequences obtained from GenBank. Alignments were exported to MEGA 2.0 beta (Kumar et al. 1993) and phylogenetic analysis was performed using the Neighbor Joining method.

**RNA extraction, dot-blotting and mRNA quantification**

After ammonium additions, cultures were incubated for sixty minutes. Both treatment and control cultures were sampled by filtering 30 ml samples in triplicate onto 0.45 µm polyvinylidene difluoride filters (Millipore Durapore, Billerica, MA). Filters were then placed into 2 ml screwcap tubes containing 750 µl RLT lysis buffer (Qiagen), 7.5 µl \(\beta\)-mercaptoethanol and several scoops of baked muffled glass beads. Tubes were frozen in liquid nitrogen and stored at -80°C until extraction. For extraction, tubes were thawed for two minutes in warm water and cell lysis was achieved by bead-beating. RNA was then extracted from 500 µl lysate using RNeasy spun columns (Qiagen) as recommended by the manufacturer. Each extract was split three ways and one third remained undigested on ice, while one third was DNAsa digested and one third was RNAsa digested. Samples were then dot-blotted onto Zeta-Probe charged nylon filters (Bio-Rad, Hercules, CA) and RNA was immobilized on filters by UV-crosslinking. Filters were then hybridized to sense and antisense \(^{35}\)S labeled mRNA ribo-probes generated by *in vitro* transcription of restriction digested pNTCA. Standards for
quantification were generated by opposite strand *in vitro* transcription of pNTCA. A dilution series *ntcA* sense and antisense mRNA was dot-blotted onto Zetaprobe and immobilized by UV-crosslinking. Duplicate standard curves were incubated with filters containing sample RNA. *ntcA* sense and antisense mRNA was then quantified using a Bio-Rad GS363 molecular imager. All RNA signals were normalized to cell densities. For cell counts 1 ml of culture was fixed by dilution into 3.7% formaldehyde. Triplicate sub-samples were filtered onto black-stained 0.22µm polycarbonate Poretics filters (Osmonics) and average cell counts were obtained by averaging ten fields counted by epifluorescence microscopy.

**Southern and Northern blot analysis**

For Southern blots 200 ml of dense *Synechococcus* WH7803 culture was spun and the pellet was extracted using a DNeasy tissue kit (Qiagen) according to the manufacturers instruction for gram negative bacteria. After inspection of the *ntcA* sequence of WH7803 found in GenBank, extracted DNA was digested with HindIII, EcoRI, EcoRV, Sall and Xohl because the gene contained no digestion-sites for these enzymes. Digested and undigested DNA was run on a 1.5 % agarose gel and transferred to Zetaprobe charged nylon membrane by standard southern blotting technique (Sambrook & Russel 2001). To determine the size of the sense *ntcA* transcript in WH7803 a northern blot was generated. Fifty ml of culture were spun and the pellet was suspended in 100 µl STET (0.1 M NaCl, 10 mM Tris·HCl, 1 mM EDTA pH 8.0, 5% Triton X-100) containing 500,000 U lysozyme ml⁻¹. The suspension was incubated at 37°
C for 15 minutes and RNA was extracted using an RNeasy spun column (Qiagen) according to the manufacturers protocol. RNA was DNase digested on the RNeasy column by using an RNase free DNase reagent kit (Qiagen). Samples were then run on a 1.5 % denaturing agarose gel and RNA was transferred to Zetaprobe (Bio-Rad) by standard northern blotting technique (Sambrook & Russel 2001). RNA was immobilized on filters by UV-crosslinking. The filter was then probed with an antisense $^{35}$S-UTP labeled riboprobe generated by in-vitro transcription from pNTCA. Determining the size of the antisense ntCA transcript abundantly detected in dot-blots was exceedingly difficult and repeated northern hybridization using agarose denaturing gel electrophoresis was unsuccessful. To determine its size RNA had to be run on a 10 % denaturing acrylamide gel containing 40% formamide and 7M urea. RNA was transferred to zetaprobe by electroblotting at 5V for 5 hours and immobilized by UV crosslinking. Filters were probed using a sense ntCA $^{35}$S labeled RNA riboprobe. All probed filters were analyzed using a Bio-Rad GS363 molecular imager and exported to Photoshop for digital cleanup and Figures generation.
RESULTS

ntcA sense and antisense transcriptional regulation was investigated in *Synechococcus* WH7803 and three environmental *Synechococcus* isolates in nitrate containing, ammonium containing and nitrogen deplete media. mRNA levels were determined by dot-blot hybridization of RNA extracted from control cultures and cultures, which had received a treatment of 100 µM ammonium 60 min prior to extraction. Figure 7.1 shows ntcA sense transcript quantities determined in treatments and control cultures. In ammonium grown cells (Fig. 7.1A) a significant decrease in ntcA levels was observed two out of four strains as the result of ammonium addition. Average transcript levels decreased 22% (A13), 40% (B13), 69% (N5D) and 70 % (WH7803) respectively. This decrease was not significant in strains A13 (p=0.12) and B13 (p=0.28), but was highly significant in N5D (p=0.005) and WH7803 (p=0.0025). A similar decrease in ntcA mRNA levels was observed in nitrate grown cells (Fig. 7.1B). Average transcript levels decreased 61% (A13), 80% (B13), 34% (N5D) and 55 % (WH7803) respectively. These differences were significant in A13 (p=0.089), B13 (p=0.013) and WH7803 (p=0.1) but not in N5D (p=0.35).

Concurrent with the decrease in ntcA mRNA levels as the result of the addition of 100 µM ammonium, a substantial and significant increase in levels of ntcA antisense hybridization was observed in all four strains. Average antisense ntcA mRNA levels in ammonia grown cultures increased 3.7, 3.1, 4.2 and 3.0-fold in A13, B13, N5D and WH7803 respectively (Fig. 7.2A). These increases were all significant (p=0.02, 0.1, 0.006 and 0.0005 respectively) at the 90% confidence interval. In nitrate grown cells
average antisense \textit{ntcA} mRNA signals increased 2.3, 3.2, 4.0 and 4.8 fold for A13, B13, N5D and WH7803 respectively (Fig. 7.2B). This increase was significant in strains B15 (p=0.002), N5D (p=0.006) and WH7803 (p=0.09), but not in A13 (p=0.28).

Changes in transcript levels found in nitrogen starved cultures were modest compared to nitrate and ammonia grown cultures (Fig. 7.3). One hour after the addition of 100 \(\mu\)M ammonium \textit{ntcA} sense transcript levels had decreased by 17, 19, 40 and 14\% in A13, B13, N5D and 7803 respectively. This decrease was significant in A13 (p=0.061) and WH7803 (p=0.067), but not in B13 (p=0.16) and N5D (p=0.51). Simultaneously antisense \textit{ntcA} mRNA levels remained unchanged in B13 as the result of ammonium addition and increased 59, 56 and 57 \% in A13, N5D and WH7803 respectively. These increases were significant in all three strains at the 90\% confidence interval (p=0.06, 0.008 and 0.018 in A13, N5D and WH7803 respectively).

To determine the phylogenetic affiliation of isolates used in this study we PCR amplified and sequenced a portion of their \textit{rbcL} (large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) genes. Phylogenetic analysis (Fig. 7.4) revealed that their \textit{rbcL} sequences were virtually identical to \textit{Synechococcus} WH7803 (94-100 \% identical at the nucleotide level and 100\% identical at the AA level for all strains).

Southern blot analysis was performed by digestion of chromosomal \textit{Synechococcus} WH7803 DNA (Fig. 7.5). While a single band was observed in lanes containing EcoRI, EcoRV, SalI and XohI digested DNA, two bands were observed in lane containing HindIII DNA. Digestion and blotting was repeated and to confirm this banding pattern.
Northern analysis of WH7803 mRNA revealed a single sense \textit{ntcA} transcript of approximately 1.2 kb in length (Fig. 7.6). The antisense transcript was detected as a single band and determined to be ca. 380 nucleotides in length (Fig. 7.7).
DISCUSSION

During a series of culture experiments on the marine cyanobacterium *Synechococcus* WH7803 we made the accidental discovery that dot-blots probed for the antisense transcript of *ntcA* produced ample hybridization signal. Antisense hybridization was shown to significantly increase upon the addition of 100 µM ammonium with a concomitant decrease in sense *ntcA* mRNA hybridization. These results were reproducible in other phycoerythrin containing *Synechococcus* isolates grown in our lab. Southern hybridization indicated that only one locus homologous to *ntcA* is found in the *Synechococcus* WH7803 genome. Northern hybridization demonstrated that the sense *ntcA* transcript was ca. 1.2 kb in length, while the antisense transcript is ca. 380 nucleotides long. These results suggest that regulation of *ntcA* transcription may involve the transcription of a small antisense mRNA.

Regulation of *ntcA* sense transcript accumulation observed in our lab (Fig. 7.1) is consistent with observations previously described for *Synechococcus* WH7803 (Lindell et al. 1998). In this study it was shown that *ntcA* expression was negatively regulated by the presence of ammonium and that *ntcA* transcript levels declined with half-lives of ca. 2 minutes upon ammonium or rifampin addition. Analogous down-regulation of *ntcA* transcription as the result of ammonium addition can also be found in other phycoerythrin containing marine *Synechococcus*, at least marine A-type strains closely related to WH7803 as indicated by their *rbcL* sequences (Fig. 7.1).

The sense *ntcA* mRNA transcript in *Synechococcus* WH7803 was found to be ca. 1.2 kb in length, which is consistent with lengths for transcripts found in other strains of
Synechococcus. *Synechococcus* PCC 7942 for example contains a single transcript of 1.3 kb (Luque et al. 1994) while *Anabaena sp.* PCC 7120 has been found to contain two transcripts of 1.4 and 1.0 kb length (Wei et al. 1994, Ramasubramanian et al. 1996).

*Cyanothece* sp. ATCC51142-BH68K produces two *ntcA* transcripts of 1.3 and 1.0 kb. Because the ORF (open reading frame) of *ntcA* is ca. 700 bp in length and because the transcriptional start point is located approximately 200 bp upstream from its start codon, it is believed that the transcript does not accommodate any genes located downstream from *ntcA* (Herrero et al. 2001).

Ammonium readily permeates biological membranes and uptake in *Synechococcus* is mediated by membrane associated permeases, if ambient concentrations are low (Herrero et al. 2001). Intracellularly ammonium has been hypothesized to mediate a signal to which NtcA responds (Herrero et al. 2001). This signal is thought to depend on ammonium incorporation into the cellular carbon skeleton.

In *Anacystis nidulans* nitrate reductase expression was repressed by the presence of ammonium, even in the presence of nitrate or under nitrogen starved conditions. Inactivation of glutamine synthetase by addition of the irreversible inhibitor L-methionine-D,L-sulfoxamine (MSX) restored nitrate reductase activity (Herrero et al. 1981). In *Synechococcus* PCC 7942 transcription of the *nirA-nrtABCD-narB* gene cluster, which codes for genes involved in nitrate uptake and reduction, was negligible if cells were utilizing ammonium. MSX however induced expression of this operon (Suzuki et al. 1993).

In *Synechococcus* the coordination of cellular carbon and nitrogen metabolism involves the signal transduction protein P$_{II}$ (encoded by *gnlB* in *Synechococcus*)
This trimeric protein carries varying numbers of phosphorylated serine residues depending on cellular nitrogen supply (Forchhammer & Tandeau de Marsac 1994). P_{II} is phosphorylated under high CO_{2} in the presence of nitrate or under nitrogen deprivation. The dephosphorylated form dominates if cells are grown on ammonium (Forchhammer & Tandeau de Marsac 1994, 1995b). P_{II} also binds to the metabolites 2-oxoglutarate and ATP thus allowing for the integration of cellular carbon and nitrogen demand (Forchhammer & Hedler 1997). 2-oxoglutarate and ATP are needed for modification of the protein (Forchhammer & Tandeau de Marsac 1995a, Forchhammer & Hedler 1997). In addition P_{II} has been shown to be transcriptionally regulated by NtcA in *Synechococcus* PCC 7942 (Lee et al. 1999). In this strain *gnlB* is transcribed constitutively to form a 680 nucleotide transcript. A shorter transcript of 620 nucleotides is transcribed from an NtcA depended promotor under nitrogen deplete conditions and P_{II} protein levels are in good correlation with levels of the two transcripts. In this context it should be noted that the response of *ntcA* transcription to ammonia addition appears to be moderated by prolonged nitrogen starvation (Fig. 7.3). Cellular *ntcA* mRNA levels decreased in average 50 and 58 % in ammonia and nitrate grown cells respectively as the result of the addition of 100 µM ammonium (average of all four strains used). *ntcA* mRNA levels in nitrogen starved cells however had only declined in average by 18 % one hour after 100 µM ammonium had been added. This suggests that cellular demand for nitrogen may have been able to curb the inhibitory effect of ammonium on *ntcA* transcription. It is enticing to speculate if this modulating effect was mediated by the antisense *ntcA* mRNA we detected by hybridization in all four strains. After ammonium addition cellular antisense *ntcA* mRNA levels increased in
average by 252 and 255% in ammonia and nitrate grown cells. Levels only increased by ca. 95% in cells maintained in nitrogen free media for 48 hours.

Small untranslated RNA molecules (sRNAs) other than tRNAs and rRNAs are now recognized to play important roles in a large number of cellular processes (Eddy 2001, Wassarman et al. 2001, Wassarman 2002). In E. coli an abundance of sRNAs has now been described (Eddy 2001). Although their functions are often not well understood, it has become evident that sRNAs can play critical roles in regulating cellular responses to environmental growth conditions (Wassarman 2002). sRNAs appear to fall into three functional classes, depending on their mode of action (Wassarman et al. 1999): Their functions can be mediated by RNA:Protein interaction, as is the case with the CsrB and 6S RNAs. Alternatively the molecule can have intrinsic activity such as RNase P RNA or, as is the case with the MicF and DicF transcripts, the sRNA can be directly involved in RNA:RNA interaction.

CsrA is a small RNA-binding protein, which has been shown to repress the expression of a suite of genes involved in glycogen metabolism, glycolysis and gluconeogenesis, motility and adherence in E. coli (Romeo 1998). Activity of CsrA is regulated by binding to csrB mRNA preventing target recognition (Romeo 1998, Wassarman et al. 1999). Multiple copies of CsrA bind to a single csrB RNA molecule and the ratio of CsrA to csrB transcript has been hypothesized to control CsrA activity. The 6S RNA in E. coli has has been shown to interact with the RNA polymerase/sigma70 complex repressing expression of genes from sigma70 dependent promoters during stationary phase (Wassarman & Storz 2000). The RNase P RNA is a 377 nucleotide long transcript encoded by rnpB which forms a critical component of RNase P enzyme
RNase P is found in all three kingdoms (archaea, eubacteria and eukaryotes) and it processes precursor tRNA molecules by removing 5’ leader elements. Enzymatic activity resides with the mRNA component of the complex, although substrate recognition is thought to occur through structural features and not base-pairing (Wassarman et al. 1999). The 93 nucleotide long micF transcript has been shown to inhibit the expression of the membrane porin OmpF (Wagner & Simons 1994, Delihas 1995, Pratt et al. 1996). The micF RNA binds to the Shine-Delgardo sequence of the ompF transcript by forming a stable duplex. The ompF mRNA is then destabilized via mechanisms involving an 80kd protein that is not yet well understood. In an analogous fashion DicF, a 53 nucleotide long regulatory mRNA, binds to the Shine-Delgardo sequence of the cell division gene ftiz and regulates its expression by RNA-hybrid formation and interference with ribosomal binding (Tetart & Bouche 1992, Wassarman et al. 1999).

sRNAs typically are small transcripts of less than 200 nt in length, which do not code for protein (Delihas 1995). Northern blot analysis of Synechococcus WH7803 mRNA revealed a band of ca. 380 nt in length which hybridized to labeled sense ntcA mRNA (Fig. 7.7) indicating that the antisense ntcA transcript we detected is likely to fall into this category of small RNA molecules. In addition sRNAs can either be transcribed from the same genetic locus as the genes they regulate (referred to as cis-encoded) or they are transcribed from partially homologous intergenic regions elsewhere on the genome (trans-encoded) that do not code for a complete ORF (open reading frame) (Delihas 1995, Wassarman et al. 2001). Southern blot results indicated that only a single locus encoding for ntcA is found in the genome of WH7803 (Fig. 7.5). Although we
observed two bands (at ca. 6.5 kb and 9.5kb) in the lane containing HindIII digested DNA, the remaining four digestions produced only a single band. Also, WH7803 has been previously described as containing only one copy of ntcA (Lindell et al. 1998) and only a single HindIII fragment (>6 kb) was found to hybridize in this study. The presence of the second, larger HindIII fragment suggest that a portion of the WH7803 population used for DNA extraction in our lab may have accumulated a mutation adjoining ntcA, deleting a HindIII site. In order to confirm that only a single locus homolgous to ntcA is found in *Synechococcus* we imported the complete genome of *Synechococcus* PCC8102 into Kodon (Applied Math). A homology search produced no significant matches (>100bp at 65% identity) to ntcA elsewhere in the genome.

Based on these results we hypothesize that the regulation of ntcA in marine A-type *Synechococcus* involves the transcription of a short, cis-encoded antisense mRNA. If this transcript is involved in the down-regulation NtcA gene expression remains to be shown. It also remains to be demonstrated how ammonium addition is sensed in *Synechococcus* and how this signal is integrated leading to the observed regulatory responses of sense and antisense ntcA transcription.
CHAPTER 8

Methods for the Generation and Screening of Bacterial Artificial Chromosome (BAC) libraries generated from cultures and the environment

CHAPTER SUMMARY

Less than 1% of marine bacteria are thought to be isolatable by current culturing techniques and much of our understanding of the uncultured component of microbial communities is based on PCR amplification of 16S rDNA genes. PCR based approaches are also limited by the phylogenetic range of PCR primers and can sometimes be heavily biased by inherent limitations of PCR chemistry. The generation of large insert BAC libraries generated from bulk environmental DNA has enabled the cloning of genome fragments from natural populations circumventing the selectivity of PCR. In order to study the diversity of \textit{rbcL} phylotypes in uncultured phytoplankton communities, techniques were adapted to generate BAC libraries from environmental DNA. In this process a genomic BAC library with 2.9-fold genome coverage was generated from the marine cyanobacterium \textit{Synechococcus} WH7803 and screened for \textit{rbcL} and \textit{ntcA} by colony lifting. Eight \textit{rbcL} and two \textit{ntcA} containing BAC clones were identified. A 5 kb region surrounding the \textit{ntcA} gene in a 25 kb \textit{ntcA} containing BAC clone was sequenced by primer walking and genomic comparisons were made with sequences from other \textit{Synechococcus} strains. This analysis revealed that gene organization surrounding \textit{ntcA} is
not conserved. *Synechococcus* WH8102 and *Prochlorococcus* MI9313 shared similar gene organization in the region surrounding *ntcA*, while *Nostoc* PCC7120 and *Prochlorococcus* MED4 only share similar gene content downstream of *ntcA*. The remaining *Synechococcus* strains all contained unique and different genes around *ntcA*. BAC libraries were also generated from New Jersey coastal waters during the Geochemical Rate/RNA Integrated Study (GRIST) and from a natural community in the Gulf of Mexico. The GRIST library was provided to Lee Kerkhoff at Rutgers University, while the Gulf of Mexico library is currently being filter arrayed at the Benaroya Research Institute.
INTRODUCTION

Less than 1% of bacterial species found in the environment have been cultured. As a consequence it is thought that a large proportion of the biochemical diversity of uncultured microbes remains unknown. This realization stems mainly from advances in microscopy and molecular techniques for the use in environmental studies. Electron microscopy (Schmidt et al. 1991), epifluorescence microscopy (Sieracki & Viles 1992) and flow cytometry (Robertson et al. 1998) were all instrumental to the realization that numbers of bacteria found in seawater greatly exceed the number of colony forming units (CFUs) detected by traditional plating techniques and that most marine bacteria remain to be cultured (Button et al. 2001). The analysis of sequence information for ribosomal genes (most importantly 16S) extracted from the environment has since become the tool of choice for culture-independent analysis of microbes in the environment (Olsen et al. 1986, Pace et al. 1986) and hundreds of studies have since described microbial communities from all types of environments using similar techniques. Several studies have however been particularly important to our current understanding of the composition of uncultured microbes. For example, bacterioplankton communities in the Atlantic and Pacific oceans were described using a 16S rDNA PCR/cloning approach (Giovannoni et al. 1990) and by detection of 16S in fragmented DNA cloned into bacteriophage lambda (Schmidt et al. 1991). Many unknown lineages with sequences similar to known bacterial divisions were revealed. In addition, several previously unknown bacterial lineages, most importantly the SAR11 cluster, which was later shown to be a significant component of the oligotrophic bacterioplankton (Giovannoni et al. 1990).
In a similar fashion the archaeoplankton was discovered (DeLong 1992, DeLong et al. 1994) and shown to be a major component of marine microbial communities, particularly in cold upwelling and deep waters. Archaeoplankton can account for >30% of bacterioplankton communities in North Sea surface waters (Pernthaler et al. 2002) and was completely absent from culture collections.

Despite these advances, PCR based approaches suffer from many drawbacks, biases and limitation. Primer design is often limited by the availability of sequence information from cultured microorganisms and PCR chemistry can dramatically favor or disfavor particular sequences in mixed target reactions (for a discussion of PCR biases see chapter 2). More importantly, the 16S based PCR approach fails to afford any relevant information regarding the biochemical roles and physiology of individual species detected by their ribosomal sequences. Community metabolism and dynamics remain a black box, because biochemistry, genetic inventory and gene expression of microbial communities are highly intertwined. Individual bacterial genomes are also thought of as mosaics of genes of diverse origin, which do not contain all genes necessary to perform the diverse biochemical transformations observed in typical microbial communities (Cary & Chisholm 2000).

Genomic analysis of natural communities is seen as the most promising avenue to circumventing these limitations. This approach relies on unspecific cloning of total community DNA and assumes that a sufficiently large library can capture the genomic community composition (referred to as the community ‘Metaganome’). This approach has already produced several interesting findings. Marine picoplankton communities in the North Pacific have been studied by generation of a fosmid DNA library and a 38.5 kb
A clone containing an archaeal rDNA sequence closely related to planktonic Crenarchaeota was identified (Stein et al. 1996). Several other arachaeal genes were identified, including elongation factor 2 (EF2) and two genes not previously observed in archaea (RNA helicase and glutamate semialdehyde-aminotransferase). Beja et al. (Beja et al. 2000b) constructed large insert BAC (bacterial artificial chromosome) libraries from marine plankton. The library was screened and its phylogenetic composition found to be in good agreement with previous 16S/PCR based studies. A significant proportion of clones originated from uncultured microbes and one euryarchaeotal clone was sequenced in its entirety, revealing several previously unknown archaeal genes. In the same BAC-library one clone was observed, which produced a type of bacteriorhodopsin (Beja et al. 2000a). Bacteriorhodopsins typically function as light-driven proton pumps in certain halophilic bacteria and had not previously been described for marine pelagic bacterioplankton. The pigment was shown to be associated with an uncultivated gamma-proteobacterium and shared sequence similarity with archaeal rhodopsins. Expression of the gene in *E. coli* produced a functional proton pump with typical photochemical properties. These results demonstrated the presence of a previously unknown marine bacterial population capable of light driven energy generation. BAC libraries have also been generated from soil (Rondon et al. 2000). A 1 Gbp library contained a wide diversity of 16S rDNA genes originating from low-GC bacteria, gram positives, acidobacterium, cytophagales and proteobacteria. In addition several clones were observed to express cloned genes in *E. coli* producing phenotypes, which included anti-microbial, lipase, amylase, nuclease and hemolysis activity. These finding indicated that metagenomic libraries can be a powerful tool for the exploration of biochemical properties and capabilities of uncultivated
microbes. A DNA polymerase and rDNA operon containing large genomic DNA clone obtained from the marine sponge associated symbiotic archaeon *Cenarcheum symbiosum* has been investigated in detail (Schleper et al. 1997). The derived amino acid sequence of the *Cenarcheum* DNA polymerase gene was most similar to sequences from the extreme thermophilic bacteria *Sulfolobus acidocaldarius* and *Pyrodictium occultum*. Protein expressed in *E. coli* was less thermosTable than polymerases from its thermophilic relatives and displayed both 3’-5’ and 5’-3’ exonuclease activity. These findings confirmed observations of ecological distributions, which indicated that *Cenarcheum*, although closely related to thermophilic archaea, is a non-thermophilic species. In a later study of the same organism was studied using a genomic approach (Schleper et al. 1998). Several large clones containing the ribosomal operon were identified which were shown to belong two closely related strains of *Cenarcheum* coexisting in their host sponge. Strains were >99% identical in their large subunit rRNA gene sequences and exhibited identical gene organization over a 28 kb strech of DNA, highlighting the inherent widespread genomic microheterogeneity observed inherent in natural microbial communities.

The purpose of this chapter was to adapt these techniques to generate large insert genomic libraries from naturally occurring phytoplankton communities and to apply this technology to a natural *Prochlorococcus* population in the Gulf of Mexico.

**METHODS**

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Preparation of DNA containing agar plugs

*Synechococcus* WH7803 was obtained from the ATCC culture collection (Manassas, VA) and maintained in SN (Waterbury et al. 1986a) media in 12:12 light:dark cycles at 18°C. Seawater was obtained from a series of stations in the NE Gulf of Mexico during a research cruise on the R/V Walton-Smith between July 14-22, 2002 and from LEO-15 during the Geochemical Rate/RNA Integrated Study (GRIST).

To generate agar plugs from WH7803 cultures, 500 ml of dense culture was centrifuged at 14,000 rpm for 10 min. The cell pellet was then resuspended into 2-4 ml of molten 1% SeaPlaque Low Melting Point agar (BME, Rockland, ME). For pellets from seawater between 200 and 400 L seawater was concentrated by vortex flow filtration as described elsewhere (Paul et al. 1991). Retentate was spun at 12,000 rpm for 15 min and pellets were suspended in 1% molten SeaPlaque LMP agar. Cell suspensions (both for cultures and natural samples) were then pipetted into 75 µl plug molds (BIO-RAD, Hercules, CA) and placed at 4°C for 30 minutes until the agar had solidified. One plug per ml was then extruded into lysis buffer (10 mM Tris·HCl pH 8.0, 50 mM NaCl, 0.1 M EDTA pH 8.0, 1% Sarcosyl, 0.2% sodium deoxycholate, 1 mg ml⁻¹ lysozyme). Plugs were incubated at 37°C for two hours and the lysis buffer was replaced with ESP buffer (1% sarcosyl, 1 mg ml⁻¹ proteinase K, 0.5 M EDTA pH 8.0). Plugs were then incubated over night at 55°C, the ESP buffer was replaced with fresh ESP (no proteinase K) and incubated for an additional 60 minutes. Plugs were then stored in 0.5 M EDTA pH 8.0.
For long term storage it is recommended to store plugs in 20% NDS buffer (0.5 M EDTA pH 8, 0.01 M Tris pH 8, 1% N-lauroyl-sarcosine).

**Restriction digestion of DNA in agar plugs**

Five plugs each were placed into 50 ml of 5% sarcosyl and incubated at 37°C for two hours. Plugs were then placed into 25 ml 20% ethanol and equilibrated for two hours at 4°C while shaking and subsequently placed into TE buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0). After one hour the buffer was replaced and equilibration was continued for 60 min. Plugs were then placed into 1X freshly prepared digestion buffer (Promega digestion buffer E is required for Promega HindIII – 6mM Tris · HCl, 100 mM NaCl, 1 mM DTT) and equilibrated for 1 hour. Equilibration was repeated twice with fresh buffer E. No more then five plugs were then placed into five ml of digestion mix (1X buffer E, 4mM spermidine, 50 ul BSA ml⁻¹ (Promega, Madison, WI), 200 U ml⁻¹ HindIII enzyme). The restriction enzyme was then diffused into plugs over night and 30 µl of 1M MgCl (6 mM final concentration MgCl) were added to the digestion mix after heating to 37°C. Reactions were terminated by placing plugs into 0.5 M EDTA at appropriate time intervals.

**DNA size fractionations**

Before size fractionation of partially digested DNA, plugs were twice equilibrated in 1X TE buffer for 1 hour. For analytical gels ½ plug from each digestion time-point was placed into individual wells of a 1% SeaKem LMP agarose (BME) gel. DNA was the
separated in 1XTBE (8.9 mM Tris, 8.9 mM boric acid, 2 mM EDTA pH 8.0) in a Bio-
Rad pulse field gel Electrophoresis Cell CHEF-DRII setup. Pulsing conditions were: 6 V
\( \text{cm}^{-1} \), 18 hours, 10-60 second switch times. The gel was post stained for 1 hour at 37˚ C in
1X TBE containing ethidium bromide. After the appropriate digestion time was
determined ten DNA containing plugs were digested accordingly. After equilibration in
1XTE plugs were cut in small pieces using a sterile razor blade. The homogenized DNA
containing agar was then placed in a long well (generated by using masking tape to join
adjacent wells on the gel comb). In order to determine migration distance of the desired
size fraction ½ digested plug was placed in an individual well and the gel was run as
indicated for the analytical gel. Wells containing the size marker and the ½ un-
homogenized plug were then cut from the gel, ethidium bromide stained. The portion of
the gel containing DNA desired for cloning was not stained and photographed because
UV light is extremely detrimental to cloning efficiency. Individual size fraction were then
cut using a sterile razor blade and stored in 0.5 M EDTA pH 8.0.

**Electroelution**

Spectrapor dialysis tubing (2.1 cm diameter) with a molecular weight cut off of
12-14 KDa (Allied, Fisher Scientific, Pittsburgh, PA) was cut in 15 cm long strips and
placed in 500 ml buffer (1 mM EDTA pH 8.0 + 2 % NaCO\(_3\)). Tubing was heated to 90˚ C
for 15 minutes and placed into boiling DI (deionized) water for 10 minutes. Tubing was
then rinsed in DI water and stored in 50% ethanol at 4˚ C until usage. Tubing was
thoroughly rinsed with DI just before usage. Appropriate molecular weight fractions were
equilibrated in 1X TE twice for 1 hour at 4˚C and sliced into thin sections using a sterile
razor blade. Slices were placed evenly into the dialysis tubing together with 300 µl of sterile 1X TE and the DNA was electro-eluted at 3V cm^-1 for 3 hours. After 3 hours the current was reversed for 30 seconds to facilitate DNA removal from the dialysis tubing. The dialysis bag was then dialyzed twice for one hour in DI at 4°C.

**Ligation and Electroporation**

Electroeluted DNA was quantified using Hoechst33258 DNA stain. Fifty ng DNA were then ligated into 50 ng HindIII digested pIndigoBAC (Epicentre, Madison, WI) according to the manufacturers recommendations using T4 DNA ligase. Ligations were incubated over night at 14° C and ligase was inactivated by heating to 65° C for 10 minutes. Ligations were drop dialyzed for two hours using Millipore 0.025µm VSWP filters on 0.5 x TE buffer. Between 5 and 25 µl of ligations were then electroporated into 25 µl electrocompetent cells (Epicentre) using a Bio-Rad MicroPulser. Disposable transformation cuvettes (1 mm, Bio-Rad) were used at 1.45 kV. Transformations were then immediately taken up in 0.5 ml of iced SOC media and cells were allowed to recover at 37° C for one hour before plating. For long-term storage cells were allowed to recover for three hours and were then frozen in 50% glycerol at -80° C.

**Minipreparation procedure and restriction analysis**

Transformants were plated onto 2XYT plates containing 12.5 µg ml^-1 chloramphinecol. X-GAL and IPTG were spread onto plates before plating to allow for blue/white screening. While colonies were picked using sterile tooth-picks and
transferred into 5 ml of 2XYT + 12.5 µg ml$^{-1}$ chloramphenicol. Cells were grown over night and extracted using either a SV Miniprep Kit (Promega) or a Qiagen Plasmid Maxi Kit (Qiagen) according to the manufacturers recommendations. Plasmid DNA was restriction digested using 10 U of NotI (Promega) for 2½ hours at 37$^\circ$ C. To determine clone sizes digestions were loaded onto a 1% SeaKem LMP agarose gel and run using the following pulsed field settings: 18 hours, 6 V cm$^{-1}$, 10-60 second switch times.

**Screening of libraries by colony-lifting**

Clones were either screened by direct colony lifting of plated transformations or by colony lifting of arrayed previously frozen clones. For direct lifting 250 µl of recovered transformed *E. coli* was spread onto 14 cm diameter 2XYT + 12.5 µg ml$^{-1}$ chloramphinecol plates. Cells were grown over night and colonies lifted onto NitroPure microcellulose filters (Osmonics). For arraying, individual colonies were first picked into invidual wells of 96-well microtiter plates. Clones were frozen in these plates after 25% final concentration sterile glycerol was added to each well. Individual wells were then picked using a sterile toothpicks and spotted in an array onto fresh plates. After over-night incubation the array of colonies was lifted as indicated above. To fix DNA to filters cells were lysed on gel blot paper wetted with 5% SDS (sodium dodecyl sulfate) in 2XSSC (0.15 M NaCl, 0.015 M NaCitrate) for two minutes. Filters were then microwaved for 2 ½ minutes and placed onto paper wetted with 1.5 M NaCl + 0,5 M NaOH for 15 minutes in order to denature the DNA. Filters were neutralized twice using 1.5 M NaCl + 0.5 M Tris·HCl. PH 8.0, dried and the DNA was crosslinked to filters by
UV cross-linking. To identify colonies containing genes in question \((rbcL\) and \(ntcA\)) $^{35}$S labeled RNA riboprobes were generated by in-vitro transcription from plasmid containing partial gene sequences. Probed filters were analyzed in a Bio-Rad GS363 molecular imager.

Partial sequencing of an \(ntcA\) containing clone by primer walking

Two \(ntcA\) containing BAC clones were detected in the library generated from *Synechococcus* WH7803. Plasmid was extracted from 500 ml of culture grown from individual colonies of both clones using a Qiagen Maxiprep kit. Clone identity was confirmed by restriction digestion of 2.5 µg plasmid DNA using 10 U BamHI and southern blotting of digested DNA. Four successive outward primer walk sequencing rounds were performed using the following primers in order to obtain the sequence of a 5kb section surrounding \(ntcA\): (round 1) forward primer: tagcaacgcacacggttcca reverse primer: ggacaggtgaagatagcggc (round 2) forward primer: gcttgagcctgccagttgga reverse primer: caaacacccgacaagcagat (round 3) forward primer:ggtcacggaattgcgcagag reverse primer: cgaacgcggcaccaccccttg. Sequences were imported into Sequencher 4.1.4 (Gene Codes) and assembled into a contig, which was exported to Codon (Applied Math) for sequence comparison.
RESULTS AND DISCUSSION

A large quantity of seawater was concentrated from station 3 (240 L), 4 (200 L), 7 (240 L) and station 8 (240 L) surface samples during a research cruise in July 2001 on the R/V Walton-Smith. In addition cells were concentrated from station 8 subsurface samples (240 L), which likely contained the highest abundance of *Prochlorococcus* (based on previous experience with similar oceanographic conditions, since no flow cytometer was available on board). Embedding in agar and cell lysis yielded 60, 50, 20 and 40 DNA containing plugs respectively from station 3, 4, 7 and 8. A large number of plugs were also generated from a culture of *Synechococcus* WH7803 so that the cell mass equivalent of ca. 10 ml of culture was embedded into one 75µl agar plug. DNA quantities per plug ranged from 3.8 µg plug\(^{-1}\) in station 3 plugs to 6.1 µg plug\(^{-1}\) in plugs from station 4. Pulsed field agarose gel electrophoresis demonstrated that both, plugs from cultures of *Synechococcus* and plugs generated from environmental samples, contained primarily high molecular weight DNA in the rage between 50 and 1000 kb (Fig. 8.1). These data indicated that Membrex concentration and embedding in agar plugs followed by lysozyme and proteinase K treatment is adequate for the generation of large quantities of high molecular weight DNA embedded in agar.

Partial digestion of DNA embedded in agar plugs was more difficult than initially anticipated. Two hundred units of HindIII enzyme per ml of digestion buffer produced no noticeable shift in migration distance of the agar embedded DNA originating from *Synechococcus* WH7803 on a pulsed field gel (Fig. 8.2A). Even five hundred units of HindIII per ml only partially digested the embedded DNA after 20 hours and we found
that a minimum of 1000 units of restriction enzyme per ml of digestion mix was needed to digest DNA embedded in agar to completion over night (Fig. 8.2B lanes 4-6). Despite high enzyme concentrations and repeated attempts under different conditions DNA from cruise surface stations remained recalcitrant to restriction digestion (Fig. 8.2B lanes 1-3). Station 3 DNA remained undigested even if another restriction enzyme (BamHI) was used. Similar observations were later made during the generation of a BAC library from surface sample taken at LEO-15 during the Geochemical Rate/RNA Integrated Study (GRIST). DNA concentrated during this study remained recalcitrant to digestions. However digestion was successful in a series of plugs generated from a Membrex concentrate obtained with a malfunctioning Membrex machine. The lower yield obtained from this machine led to the production of plugs containing a smaller amount of DNA. DNA in these plugs digested with similar kinetics as plugs generated from station 7 and 8 subsurface samples leading us to believe that our digestion difficulties may not have been related to the depth of sampling, but may have resulted from final yield and concentration of DNA and interfering compounds in the plugs. A lower concentration in the range of 2-4 µg plug\(^{-1}\) is likely to be most adequate.

Partially digested DNA was separated into individual size fractions (Fig. 8.3) and adequate fractions were chosen for electroelution and ligation. Unfortunately repeated attempts at producing libraries with very large insert sizes from cultures and natural environments were unsuccessful. Regardless which fraction of DNA shown in Figure 8.3 was used for cloning insert sizes always were in the range between 10 to 30 kb as is shown for the GRIST BAC library clones shown in Figure 8.4.
A library consisting of 1230 BAC clones in the range between 7-30 kb was generated from DNA of *Synechococcus* WH7803. Of these clones 475 were picked into individual wells of 96-well microtiter plates containing 2XYT media +15µg ml\(^{-1}\) chloramphinecol. After the addition of 25% final concentration glycerol the plates were stored at -80°C. Assuming an average insert size of 15 kb and a genome size of 2.5 Mb for WH7803 the library should provide a resource with slightly less than threefold coverage of the WH7803 genome. The remainder of the clones were plated, colony-lifted and probed for form IA *rbcL* (Fig. 8.5). A total of 14 colonies were identified which produced elevated hybridization (Fig. 8.5). These colonies were isolated on fresh plates and reprobed after colony lifting. Ten of these clones produced no significant hybridization upon reprobing (such as shown in Fig. 8.6A). Four clones were identified which produced high levels of hybridization. Two additional isolation streaks and confirmation by probing were performed to ensure clonality. An additional four clones containing *rbcL* were identified in individual wells of the frozen microtiter plates. All clones were hand-spottet onto fresh plates in an array of colonies. Colonies were lifted, probed and colonies producing hybridization were identified (Fig. 8.7A). Colonies were then re-picked out of the microtitier plates and hybridization was confirmed by re-probing (Fig. 8.7B). In addition two wells were identified which contained clones that hybridized to a probe generated from the *ntcA* gene of *Synechococcus* WH7803.

Three successive rounds of primer walking were performed on the *ntcA* containing BAC-clones in order to identify the genes surrounding *ntcA* in WH7803 (Fig. 8.8) and a 5445 bp contig was generated. Sequence analysis revealed the presence of two ORFs immediately upstream of *ntcA* with significant matches to ribonuclease PH and
cob(I)almine adenyltransferase in a BLAST search of GenBank. These genes were also observed in the upstream region of \textit{ntcA} in the fully sequenced \textit{Prochlorococcus} MIT9313 and \textit{Synechococcus} WH8102. This gene organization however was not conserved in any of the other cyanobacteria for which sufficient sequence information was available including the MED4 strain of \textit{Prochlorococcus}. \textit{Nostoc} PCC7120 contains two ORFs just upstream of \textit{ntcA} identified as \textit{escR} (enoyl-[acyl-carrier-protein] reductase) and \textit{hisB} (imidazoleglycerol-phosphate dehydrase) in GenBank. The two ORFs found upstream of \textit{ntcA} in \textit{Synechocystis} PCC6803, \textit{Synechococcus} PCC7002 and \textit{Thermosynechococcus elongatus} BP-1 have not been identified (and produced no significant matches to genes with known function in GenBank) are in each case unique to the organisms. The two genes upstream of \textit{ntcA} in MED4 are ATP:correnoid adnosyltransferase and deoxycytosine deaminase. The downstream region of \textit{ntcA} is somewhat more conserved than the gene content upstream of \textit{ntcA}. One large ORF was identified just downstream of \textit{ntcA} in WH7803, which produced significant matches to the hypothetical proteins predicted just downstream of \textit{ntcA} in \textit{Prochlorococcus} MIT9313, \textit{Nostoc} PCC7120, \textit{Synechococcus} WH8102, \textit{Prochlorococcus} MED4 and \textit{Synechocystis} PCC6803. In the annotated genome of \textit{Prochlorococcus} MED4 this gene is identified as a putative membrane protein. This gene however was not found at this location in \textit{Synechococcus} PCC7002 and \textit{Thermosynechococcus elongatus} BP-1. Both these species contained a unique gene organizations in this region of their genome. \textit{Synechocystis} PCC6803 is unique in that it contains the hypothetical membrane protein coding ORF just downstream of \textit{ntcA}, but does not contain the other putative protein further downstream shared by the other marine strains. These data indicate that \textit{ntcA} is
not part of a larger nitrogen assimilation operon. Genes encoded in the region
surrounding ntcA do not encode for known nitrogen metabolism genes nor is gene
organization conserved among different cyanobacteria. Assuming that the amount of
genomic rearrangement between two bacterial genomes is a measure of time since the
two species diverged, gene organization shown in Figure 8.9 can be used to predict a
particular phylogeny for these cyanobacteria. The marine *Synechococcus* WH7803 and
WH8102 as well as *Prochlorococcus* MIT9313 are most closely related as they share
similar gene content and arrangement in the immediate region surrounding ntcA. Gene
arrangement would also predict that *Nostoc* PCC7120 and *Prochlorococcus* MED4 form
a clade with these marine cyanobacteria to the exclusion of the remaining taxa shown in
Figure 8.9. *Synechococcus* PCC7002 and *Thermosynechococcus* are most distantly
related to *Synechococcus* WH7803. This phylogeny is not consistent with the known
phylogeny of these species, especially with respect to the two *Prochlorococcus* strains,
which are known to form a monophyletic clade among the cyanobacteria.

In addition to the BAC library generated from *Synechococcus* WH7803 we also
produced BAC libraries using DNA collected from station 8 during our cruise on the FG
Walton-Smith in July 2001 and during the GRIST experiment. Twelve transformations
were performed using ligated partially digested GRIST DNA producing a total of ca.
25,000 transformants. The majority of these transformants were stored in 50% glycerol
and provided to Lee Kerkhof at Rutgers University. Nine hundred and sixty clones were
picked into 96 well micro-titer plates and archived as a resource in 25% glycerol at -80°C
as a resource for future research. Clones were determined to range in size between 10
and 30 kb. Two libraries containing 5500 and 5200 transformants with similar insert sizes
were generated from station 8 plugs. Cells were frozen in 50% glycerol and sent to Benaroya Research Institute for filter arraying.
Figure 8.1

Pulsed field electrophoresis gel showing several plugs generated from cultures of *Synechococcus* WH7803 (A) and from Membrex concentrates of station 4 surface water (B). Six lanes each containing ½ of individual plugs chosen randomly in order determine if the amount of DNA obtained in individual plugs was homogenous. Promega poly-lambda markers were loaded as molecular weight markers (L).
Figure 8.2

A

B
Figure 8.2

(A) Agar embedded DNA of *Synechococcus* WH7803; unsuccessful digestion series using 200 and 500 units of HindIII ml\(^{-1}\) of restriction buffer. (L) Promega poly-lambda molecular weight marker (1) T=0 (2) 30 minutes (3) 60 minutes (4) 2 hours (5) 4 hours (6) 20 hours (B) Digestion of DNA in agar plugs generated from station 3 surface and station 7 subsurface water using 1000 units of HindIII ml\(^{-1}\). (L) Promega poly-lambda molecular weight markers (1) T=0 (2) Station (ST) 3 T=0 (3) ST 3, 8 hours (4) ST 4, 16 hours (5) ST 7 T=0 (6) ST 8, 8 hours (7) ST 7, 16 hours
Analytical gel of individual size fractions (1-17) cut from chef gel used to separate the partially digested DNA. Each lane contained a small portion of the ca. 1 mm thick slice that was cut from the gel that contained HindIII digested DNA sampled from Station 8 subsurface water. Increasingly larger fractions were loaded from left to right. Bounding lanes contained Promega poly lambda size markers (L).
Sizes of BAC clones generated from DNA concentrated during the GRIST experiment (A) Nineteen clones were randomly picked from plated transformations to estimate average insert size. Extracted plasmid was NotI digested and run on a chef-gel for analysis. P= lambda HindIII size marker; L=poly lambda size marker; 1-19= NotI digested plasmid DNA. (B) Eight \textit{rbcL} containing clones were identified and extracted plasmid was HindIII digested to estimate insert sizes. Digested DNA was run on a chef-gel. L=poly lambda size marker; 1-8= HindIII digested \textit{rbcL} containing BAC clones; 9= undigested WH7803 DNA embedded in agar.
Figure 8.5

Colony lift of plated transformation generated from partially digested *Synechococcus* WH7803 DNA ligated into pIndigoBAC. The filter was probed for *rbcL* containing clones using a $^{35}$S labeled riboprobe generated from an *rbcL* containing plasmid by *in vitro* transcription. Circles indicated colonies that were collected and replated onto fresh 2XYT chloramphenicol containing plates.
Re-plated colonies picked from colony lifted transformations in Figure 8.4. Plates were colony lifted and probed for $rbcL$ using a $^{35}$S labeled riboprobe generated from an $rbcL$ containing plasmid by *in vitro* transcription. (A) No hybridization found indicating that the BAC plasmid carried by cells does not contain $rbcL$. (B) Hybridization indicating the presence of $rbcL$ in the BAC plasmid carried by cells.
Colony lift hybridizations from arrayed WH7803 BAC clones probed for \textit{ntcA} using a 35S labeled riboprobed generated by \textit{in vitro} transcription from a plasmid containing a partial segment of the WH7803 \textit{ntcA} gene. (A) Initial screening. Clones in circles were picked and replated for re-screening. (B) hybridization by colony lift of clones picked for re-screening. One \textit{ntcA} containing clone was observed in the array of BAC clones screened in A.
Two ntcA containing clones were identified. Clone 1: plate 4 well C8. Clone 2: plate 5 well B1. Both clones were sequenced outward from the known portion of the ntcA gene in *Synechococcus* WH7803 by successive primer walking steps. The obtained sequences were aligned and converted into a contig using the Sequencher software package (Gene Codes). GenBank Accession number : AY 356324
Figure 8.9

*Synechococcus* WH7803

*Prochlorococcus marinus* MIT9313

*Synechococcus* WH8102

*Nostoc* PCC7120

*Prochlorococcus marinus* MED4

*Synechocystis* PCC6803

*Synechococcus* PCC7002

*Thermosynechococcus elongatus* BP-1
Figure 8.9

Shown is a comparison between the genomic loci containing ntcA in several cyanobacteria. Open reading frames (ORFs) with no close match in GenBank are indicated as hypothetical proteins. The ntcA ORF is shown in red. Light blue ORFs are genes of known function located upstream of ntcA shared by *Synechococcus* WH7803, WH8102 and *Prochlorococcus* MIT9313. Dark blue ORFs are genes of unknown function not found in the region surrounding ntcA in WH7803. Green ORFs are genes of known function not found in the region surrounding ntcA in WH7803. Pink ORFs are genes located downstream of ntcA in *Synechococcus* WH7803 and are shared by *Synechococcus* WH8102, *Prochlorococcus* MIT9313 and MED4, *Nostoc* PCC7120 and *Synechocystis* PCC7120.
REFERENCES AND BIBLIOGRAPHY


Amon RMW, Benner R (1997) Seasonal Patterns of Bacterial Abundance and Production in the Mississippi River Plume and Their Importance for the Fate of Enhanced Primary Production. Microbial Ecology 35:289-300


290


291


Dinnel SP, Wisemann WJ (1986) Fresh water on the Louisiana and Texas shelf. Cont. Shelf Res. 6:765-784


Ellis RJ (1979) Trends Biochem. Sci. 4:241-244

293


Forchhammer K, Tandeau de Marsac N (1994) The PII protein in the cyanobacterium *Synechococcus* sp. strain PCC 7942 is modified by serine phosphorylation and signals the cellular N-status. J Bacteriol 176:84-91


Goericke R (1990) Pigments as ecological tracers for the study of the abundance of growth of marine phytoplankton. Ph. D., Harward University


Healey FP, Hendzel LL (1979) Indicators of phosphate and nitrate deficiency in five algae in culture. Journal of the Fisheries Research Board of Canada 36:1364-1369


Holm-Hanson O, Rieman B (1968) Chlorophyll a Determination: Improvements in Methodology. Oikos 30:438-447


Kumar S, Tamura K, Nei M (1993) MEGA: Molecular Evolutionary Genetic Analysis. The Pennsylvania State University, University Park, PA


assessment, sustainability, and management. Blackwell Science, Malden, Mass., USA


Moestrup O (1991) Further studies of presumably primitive green algae, including the
description of Pedinophyceae clas. nov. and Reultor gen. nov. J Phycol 27:119-133
51:179-199
Moon-van der Staay SY, De Wachter R, Vaulot D (2001) Oceanic 18S rDNA sequences
Moon-van der Staay SY, Staay GWMvd, Guillou L, Vaulot D (2000) Abundance and
Diversity of Prymnesiophytes in the Picoplankton Community from the equatorial
Pacific Ocean inferred from 18S rDNA Sequences. Limnol. Oceanogr. 45:98-109
Moore LR, Goericke R, Chisholm SW (1995) Comparative Physiology of Synechococcus
and Prochlorococcus: Influence of Light and Temperature on Growth, Pigments,
Moore LR, Rocap G, Chisholm SW (1998) Physiology and molecular phylogeny of
Morel A (1997) Consequences of a Synechococcus bloom on the optical properties of
oceanic (case 1) waters. Limnol. Oceanogr. 42:1776-1754
Synechococcus: a comparative study of their size, pigmentation and related
in dinoflagellates. Science 268:1622-1624
distribution in the Caribbean Sea: Observations from Space. Progress in
Oceanography 23:23-69
Müller-Karger FE, Richardson PL, McGillicuddy D (1995) On the offshore dispersal of
the Amazon's Plume in the North Atlantic: Comments on the paper by A.
Longhurst, "Seasonal cooling and blooming in the tropical oceans". Deep-Sea
Research I 42:2127-2137
Phytoplankton Concentrations and Sea Surface Temperature Cycles of the Gulf of
Mexico as Determined by Satellites. Journal of Geophysical Research 96:12645-
12665
Nelson DM, Dortch Q (1996) Silicic acid depletion and silicon limitation in the plume of
the Mississippi River: evidence from kinetic studies in the spring and summer.
studies of recombinant Synechococcus ribulose-1,5-bisphosphate
carboxylase/oxygenase from Escherichia coli. J. Biol. Chem. 265:15154-15159
Newman J, Gutteridge S (1993) The X-ray structure of Synechococcus ribulose-
bisphosphate carboxylase/oxygenase-activated quaternary complex at 2.2-A
resolution. J Biol Chem 268:25876-25886
Carpenter EJ, Capone DG (eds) Nitrogen in the Marine Environment. Academic
Press, p 565-648


Rabalais NN, Wiseman W, Turner RE (1994) Comparison of continuous records of near-bottom dissolved oxygen from the hypoxia zone along the Louisiana coast. Estuaries 17:850-861


Riley GA (1937) The significance of the Mississippi River drainage for biological conditions in the northern Gulf of Mexico. Journal of Marine Research 1:6074


Tabita FR, McFadden BA (1974b) D-Ribulose 1,5-diphosphate carboxylase from Rhodosporillum rubrum. II. Quaternary structure, composition, catalytic and immunological properties. J. Biol. Chem. 249:3459-3464


Tetart F, Bouche JP (1992) Regulation of the expression of the cell-cycle gene ftsZ by DicF antisense RNA. Division does not require a fixed number of FtsZ molecules. Mol Microbiol 6:615-620


306


Vega-Palas MA, Flores E, Herrero A (1992) NtcA, a global nitrogen regulator from the cyanobacterium Synechococcus that belongs to the Crp family of bacterial regulators. Mol. Microbiol. 6:1853-1859


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