AQUATIC NITROGEN FIXATION: PATTERNS, RATES AND CONTROLS IN A SHALLOW, SUBTROPICAL LAKE

By

MOSHE DORON

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To my parents with all my love

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By

Moshe Doron

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Shallow lakes can act as sinks or sources of nutrients such as nitrogen (N) which is often a key nutrient limiting downstream coastal systems. Biological N_2 fixation is one of the key processes affecting lake N, but more research is needed to evaluate this process in relation to algal blooms, particularly in subtropical systems. To evaluate these changes, a project was undertaken to investigate the spatial and temporal patterns of nitrogenase activity and diazotrophic community composition in a large, shallow, subtropical lake (Lake George, Florida, USA). This lake may be limited by N rather than phosphorus (P), making it a good candidate for the study of diazotrophs in algal bloom formation. The objectives of this study were threefold: (1) to characterize and quantify N_2 fixation in Lake George water, (2) to identify environmental parameters related to N_2 fixation, (3) to characterize the diazotrophic community composition in Lake George. It was hypothesized that environmental conditions affecting carbon, N and P would impact the diazotrophic community (abundance, composition, and activity rate) and, thus, directly affect lake N budgets. We measured, diazotrophic community size (microscopy), composition (using *nifD*), and function (nitrogenase activity via acetylene reduction) from April to September, 2008. Nitrogenase activity ranged from 3 to 95 nmol l^{-1} h^{-1} during the study and

had a significant importance to the lake's N budget. Nitrogenase activity correlated with N limitation (estimated by dissolved inorganic N: total P ratio (DIN:TP ratio)), heterocystous cyanobacterial blooms, and was positively influenced by light. The chemical variation between sites was significant also showing a seasonal change. Analysis of *nifD* clone libraries revealed a diazotrophic community with low diversity that was dominated by filamentous heterocystous cyanobacteria (order *Nostocales*). Microscopy and nutrient patterns showed these blooms were episodic, possibly due to availability of P relative to N, and that each bloom was influenced by different parameters, with the later blooms depending on the outcome of those prior. The results of this study documented the potential for N_2 fixation to transform the lake into a N source to downstream systems, but further study is needed to determine the role of diazotrophs in the formation of algal bloom events.

CHAPTER 1
INTRODUCTION

As a vital component of amino and nucleic acids, the building blocks of proteins and DNA/RNA, nitrogen (N) is a key element required for all living organisms. Although the majority of the planet's N is found in the lithosphere in the form of nitrate, it is not accessible or bioavailable. The second largest N pool, dinitrogen gas (N_2) in the atmosphere, is also unavailable for direct use or uptake by organisms. This inaccessibility of N is the primary reason why many ecosystems are found to be limited by N, and only a limited number of prokaryotes and archea can convert atmospheric N_2 into a bioavailable form (ammonium, NH_4^+) through the process of biological N_2 fixation.

In N-limited ecosystems, N_2 fixation has the potential to supply N and increase primary production (Flett, 1980; Smith, 1983). As a result, microorganisms with the ability to fix N_2 (diazotrophs) are somewhat relieved of N limitation, and are expected to demonstrate increased growth under N-limiting conditions due to this competitive advantage (Karl, 1997). In turn, a community shift towards diazotrophs is expected and also explained by their ability to outcompete other organisms (Schindler, 1977; Tilman, 1982). This concept holds for aquatic systems, where diazotrophs proliferate under N limited conditions. For example, in many N-limited Lakes, the diazotrophic density (total cells counts per ml) can typically represent about 20-30% of the total phytoplankton community during the growth season with a maximum recorded up to 60% (Gao, 2005). Higher abundance of diazotrophs usually results in higher rates of N_2 fixation (Vitousek and Howarth, 1991). In eutrophic lakes for example, N_2 fixation constituted for up to 82% of total N loading (Howarth et al., 1988), while in shallow wetlands like the Everglades, watercolumn N_2 fixation was 3 times higher in nutrient impacted sites (Inglett et al., 2009).

Presumably, the relative proportions of nutrients in the water column are a key factor shaping the composition, growth and activity rates (per unit of the limiting nutrient) of the phytoplankton community. Nonetheless, the identification of nutrient limitation in natural environments by coupling nutrient concentrations to community growth is difficult due to the complexity and dynamics of natural systems (Hecky and Kilham, 1988) and internal storage of both N and P by phytoplankton (Lohman et al., 1991). In aquatic systems, more emphasis is put on N or phosphorus (P) because under normal conditions, carbon supply is generally found to be adequate. In most marine ecosystems, primary production is most frequently limited by N (Tilman et al., 1982), while most fresh water systems are generally limited by P (Schindler, 1977). For this reason, P additions can also increase the demand for N in freshwater systems, and thus lead to increasing N limitation (Paerl, 1987; Mills, 2004).

Availability of P can come through a variety of factors. Although anthropogenic loading is a major source of P that shifts fresh water systems into N limitation, some ground and surface water can deliver natural high concentrations of P (Sims et al., 1998) leading some lakes and rivers to be naturally rich in P (Smith, 1983; Havens et al., 2007). Temporal changes in precipitation, temperature, wind, salinity, pH, residence time, and light intensity can also have an impact on nutrient abundance patterns and distribution (Thayer, 1971). Due to the overall abundance of P contained in sediments, a summer shift in environmental conditions (e.g., salinity) that converts P into labile forms may increase the demand for N and its fixation. Such patterns may be related to phytoplankton proliferation and N_2 fixation rate (Paerl et al., 1996). In either case, the importance of P for N_2 fixation regulation should be reflected by an N:P ratio that evaluates which of the two elements limits primary production and affects diazotrophic activity.

Shallow lakes are more abundant than deep lakes and are receiving increasing attention because of their eutrophication around the globe (Dokulil and Teubner, 2003). In general, CO_2 limitation is rare in shallow lakes because of its high rate of introduction from the atmosphere and sediment (Jensen et al., 1994). The depth of a lake is an important parameter that has many implications for lake processes, including the amount of stratification and wind induced sediment resuspension (Bachmann et al., 2000). Thus, it must be considered when examining the nutrient cycle in such systems (e.g., resuspension of P rich sediment may shift system to N limitation). Unlike deep lakes where P concentration in the water column represents an internal pool, in shallow lakes the mobile sedimentary P may be directly available for primary producer's growth (Istvánovics et al., 2000).

Frequently, aquatic systems are hydrologically linked in the landscape, and therefore, processes occurring in systems higher in the watershed are likely to influence other downstream systems. For this reason, upstream eutrophic systems that contain many nutrients (including N) that move through the landscape (mostly in dissolved forms) can act as pollution sources to downstream systems. In this manner, N that was fixed in such systems may be transported to downstream systems; this is particularly true for shallow lakes (especially with P rich sediments) that are ideal environment for diazotrophic cyanobacteria.

Several factors affect the degree to which eutrophic systems act as pollution sources to downstream systems. In general, lakes and rivers show greater N exports in periods of high discharge, probably due to stored N in the landscape during dry periods and flushing during wet periods (Howarth et al., 2006). This export can range from 10 to 15% of the N inputs in drier watersheds to over 35% in the wetter watersheds (Howarth et al., 2006). As this exported N reaches downstream systems, it changes nutrient equilibrium and can result in eutrophication.

This is especially true when downstream systems are coastal/marine because N inputs drive eutrophication in N limited systems (Howarth, 1988). In fact, eutrophication of coastal systems is considered to be the biggest pollution problem in the coastal waters of the U.S. (Howarth et al., 2000; NRC, 2000). For example, about 4000 estuaries in U.S. are severely degraded from eutrophication driven mostly by N, and 67% is degraded to some extent (Bricker et al., 1999; EPA, 2001). By shifting nutrient limitation from P to N, P inputs promote N_2 fixation that leads to increased levels of N. Thus, P inputs to inland waters may lead to increased levels of N in downstream coastal systems indicating that control on both nutrients is needed to control eutrophication in these systems (Paerl, 2009; Schindler et al., 2008). In order to prevent such advert environmental consequences, it is imperative to study the dynamics of N_2 fixation in these upstream lakes.

From an ecological point of view, N_2 fixation has been measured in wide range of environments and conditions (Henson, 2003; Zehr, 1998). Thus, in order to simulate the processes that take place in the environment, an understanding of the environment and the community structure and function are essential. The first milestone in the path to understanding N_2 fixation was placed in 1895, when Winogradsky isolated the first free-living N_2 fixing bacterium using a pure culture.

Most of the diazotrophs in the phylum *Proteobacteria* are facultative or obligate anaerobic, chemoautotrophs, or heterotrophic and the few phototrophs that exist do not produce O_2. An example is the species *Rhodospirillum centenum* (also known as *Rhodocista centenaria*), a heterotrophic bacterium that can fix N_2 under aerobic growth conditions and may be common and broadly distributed in many environments including lakes. Additional diazotrophs are members of other phylum, including cyanobacteria and *Actinobacteria*; for example, *Frankia* are

filamentous bacteria, which form root nodules, live in symbiosis with plants, and were found in aquatic systems.

The phylum cyanobacteria,which its domination in fresh water systems has become synonymous with eutrophication, contains many diazotrophic members. Among diazotrophs, cyanobacteria are exceptional oxygenic photoautotrophs that inhabit almost every illuminated environment on Earth. Like other primary producers, they are essential for the foundation of the food web, but their ability to use atmospheric N_2 and their developed mechanisms for P management (by vertical movement to the benthic layer and internal storage of P) make them an important subgroup (Scheffer et al., 1997). Moreover, they are the only known organisms that can form heterocysts, which are specialized cells for N_2 fixation with several mechanisms of protection from O_2 that may enter from the environment or from nearby vegetative cells that produce O_2. Hence, it is not surprising that diazotrophic communities of subtropical inland water systems are usually dominated by cyanobacteria. As mentioned above, diazotrophs may be very distinctive from each other by many aspects including their activity rates, regulation mechanisms and preferred niches; thus, it is important to identity the diazotrophic community components and understand their activities and dynamics.

A common feature to all diazotrophs is the production of the nitrogenase enzyme which is the only known enzyme that fixes (reduces) N_2 (Henson, 2003; Zehr, 1998). Due to the extremely high energy demands of N_2 fixation (Witz et al., 1966), diazotrophs use a cascade of regulatory mechanisms for nitrogenase gene expression. The nitrogenase enzyme is composed of two separate protein components: 1) dinitrogenase reductase which donates two high potential electrons to dinitrogenase, and 2) dinitrogenase which actually catalyzes N_2 reduction. The nitrogenase complex is irreversibly inhibited by O_2 and its manufacturing usually does not take

place in the presence of O_2 and reduced N (Wang et al., 1985). The nitrogenase gene encodes the nitrogenase enzyme and is divided into several subunits called *nif* (e.g., *nifH* and *nifD*).

In general, there are challenges in cultivating many microbes because some cannot be grown in vitro (some would die, become non-viable, or form spores) while others require specific media or co-culture with other species. Even the microbial organisms that can be cultured might not be identified or adequately reflect their corresponding environmental conditions. The vast majority of the in vitro analysis is done on pure cultures of organisms and cannot supply an authentic picture of the processes as they occur in the environment. Moreover, even direct techniques for measurement of N_2 fixation is accomplished by measuring process rate and enzyme activities; thus even though it is not dependent on culturing, it gives only the fixation rate and ignores the composition of the diazotrophic community. Knowledge about the diazotrophic community composition is valuable for its characterization and can be used to understand their dynamics and potential activities, as well as the environmental conditions leading to observed patterns.

Before molecular biology advanced and PCR (polymerase chain reaction) was invented, it was difficult to identify the N_2-fixing organisms. Most methods that were used to identify diazotrophs in phytoplankton populations were culture based and included microscopy (morphology) and biomass estimates. Other relied on biochemical analysis or on the composition of cell membranes (fatty-acids). Other studies used biomarker pigments as means for monitoring large shifts in species composition of phytoplankton, but although freshwater cyanobacteria can be discriminated by the presence of specific biomarker photosynthetic pigment, it cannot separate diazotrophs (Rowan, 1989). In addition, monitoring the distribution of biomarker pigments in spatially complex and temporally transient waters remains a significant obstacle. In

many cases, these parameters were correlated to chlorophyll-*a* (*chl-a*) that by itself does not offer information about community composition or the presence of diazotrophs.

In fact, even the initial attempts to use PCR with 16S ribosome did not yield enough information to identify specific diazotrophs (Zehr et al., 1989). This was due to the fact that many of the diazotrophs do not share similar qualities apart from the ability to fix N_2 (Stracke et al., 2002). Only later, when specific primers were designed to target the subunits of the nitrogenase gene did quality information about the specific organisms, their roles, and interactions start to accumulate (Zehr et al., 1989). In other studies, only a single gene (usually *nifH* which encodes for the dinitrogenase reductase subunit) was investigated, and some of its regulators were identified, although not all members of the diazotrophic community were identified (Moisander et al., 2006). Some drawbacks of the molecular approach may arise when working with diazotrophs possessing more than one set of nitrogenase genes (for example, alternative or additional nitrogenase genes (Ludden et al., 1989)). In addition, results from phylogenetic analysis must be treated with caution because of the possibility that nitrogenase was transferred by lateral gene transfer (in contrasts to the Darwinian model of vertical descent, where genes are inherited from the preceding generation) (Young, 1992). E.g., *nifH* suggested that nitrogenase gene had been horizontally transferred from a *Proteobacterium* to cyanobacteria.

nifH and *nifD*

At the present, the *nifH* database contains one of the largest non-ribosomal gene datasets, yet it includes a relatively high number of uncultivated organisms (Zehr, 2003). Recent studies used *nifD* gene (encodes for the dinitrogenase subunit which is the actual site of N_2 reduction) sequences due to their ability to supply additional information that can be used to construct the phylotypes of the N_2-fixing community. Due to the fact that *nifD* was used less extensively, its

gene database contains a relatively low number of gene sequences (especially when compared to the popular *nifH* database).

Although the small database of sequences hampers the use of *nifD* for phylogenetic analysis, when used as a phylogenetic marker, it usually grants a higher resolution between closely related diazotrophs (especially cyanobacteria). In fact, Roselers et al., (2007) demonstrated that *nifD* better distinguishes between members of the *nif* gene family even in cases that organisms use one of the alternative nitrogenase enzyme (like nitrogenase-vanadium). When Roselers et al., (2007) were trying to develop a molecular technique that would detect N_2-fixing cyanobacteria in environmental samples, they realized that the gene sequences of *nifD* had conserved regions that permit the design of PCR primers specific for cyanobacterial *nifD*. The use of *nifD* primers and the construction of a genetic library are essential before real time quantative PCR (Q-PCR) techniques can be used to specifically quantify and identify spatial and temporal variation in nitrogenase transcription in complex microbial communities.

Need for Research

The latest assessment of many eutrophic lakes around the globe concluded that P-only reduction strategies would not be effective to control phytoplankton bloom (Conley et al., 2009). In these lakes, P fluxes between sediment and water, could potentially supply the phytoplankton community, which was frequently dominated by cyanobacteria. Using their ability to vertically migrate and consume excess P at the sediment-water interface, and then rise to the water surface enabled them to form blooms in periods of presumed P limitation.

Constant and simultaneous measurements and control of both P and N are necessary in these lakes in order to successfully control algal blooms and diazotrophic activity (Schindler et al., 2008). Fresh water lake/reservoir systems are ideal sites for phytoplankton proliferation dominated by diazotrophs that introduce new N into their and downstream systems. In order to

evaluate the importance of N_2 fixation to the N budget of upstream systems and its influence on downstream systems, it is impetrative to identify its major regulators in the system. By measuring enzyme activities under various environmental conditions that the system is exposed to, it may be possible to identify pattern of N_2 fixation and quantify the amount of N that is fixed in the entire lake

Lake George as a Model System

Lake George is located in subtropical region and is the second largest lake in Florida. Its estimated size is about 19.4 km long and 9.7 km wide, covering about 18,600 ha (Figure 1-1). It is a part of the SJR ecosystem in which it may serve as a source and a sink for various nutrients. As the river moves slowly (mean residence time of the lake is 84 days) northward, it flows through Lake George and continues 200 km on a low gradient (compared to its upstream 300 km) path to the Atlantic Ocean.

Lake George is a shallow (mean depth 2.5 m) lake that lies in four counties with different environmental characteristics such as P deposits, natural springs and a nearby forest. Lake George, like many systems in the SJR and Florida, has high natural concentrations of dissolved P due to large deposits of phosphate-rich sediments (Odu, 1952). In addition, Lake George has received large amounts of P and N pollution from point source pollution (among them septic tanks, fertilizers runoff and effluent from wastewater treatment plant) and continues to receive nutrients from nonpoint sources (agricultural runoff, particularly from dairy and livestock production facilities) (Stewart et al., 2006).

Like other shallow lakes, Lake George does not stratify completely and its water is relatively homogeneous vertically. Due to different inflow sources, its water chemistry can vary spatially, and like other eutrophic lakes, P levels in Lake George are relatively high and shifts nutrient limitation from to N. In such systems, N_2 fixation is a key pathway that can supply the

demand for labile N. As a result, the lake has become dominated by cyanobacteria (both diazotrophic and non-diazotrophic). The growth of these diazotrophs could act as a source of N to downstream river systems and result in an accelerated rate of eutrophication, in particular to marine systems. Recently, this area received attention due to its apparent connection with adverse phenomenon seen in the Jacksonville coastline and in the Atlantic Ocean (nuisance algal blooms and formation of hypoxic zones) (SJRWMD report, 2008). Similar processes are occurring elsewhere and have similar connections with inland eutrophication and sources of nutrients.

Lake George is an ideal site for this analysis for several reasons. It is the largest waterbody on the St. Johns River which is the major input of freshwater to the nutrient-sensitive estuary near the city of Jacksonville, Florida (NRC, 2000). Because its low residence time is ideal for cyanobacterial proliferation and its relative high P promotes diazotrophic dominance and higher rates of N_2 fixation, Lake George is also suitable for the study of cyanobacterial bloom dynamics and N_2 fixation,. In addition, the historical data on the lake and its regular monitoring by the SJR Water Management District (SJRWMD), including their experiments and reports, supply important information about the system. For example, data obtained by the SJRWMD has estimated the amount of N being added to Lake George water (calculated by difference between the inflow and outflow) at approximately 600 metric tons (MT) per year (Figure 1-2). This N may originate from various sources, including springs, leaching and biological N_2 fixation.

Research Objectives

Under typical summer conditions, downstream N limitation is a fairly consistent feature of many aquatic systems and may be satisfied by N derived from N_2 fixation in upstream water bodies. The morphology of such upstream systems (mostly size and depth) and their trophic status play an important role on the degree of nutrient export; in general, big eutrophic water

bodies have greater potential of exporting while shallow lakes are better environments to diazotrophic cyanobacteria. Thus, a eutrophic lake that is both large and shallow may export higher amounts of N to downstream systems.

The economic and environmental impacts of exported N has prioritized the need to determine nutrient thresholds and establish nutrient criteria such as Total Maximum Daily Loads (TMDLs). Before it is possible to control eutrophication and pollution, however, the complexity of the system and its variables must be understood. The need to evaluate the portion of N being added to the system through biological processes is essential to understanding the N budget in lakes and its significance to exported N. The examination of different parameters that affect N_2 fixation can be used to understand the process under natural conditions and in different time periods. Therefore, the specific objectives for this study were to:

1. Characterize and quantify N_2 fixation in Lake George water, by evaluating the influences of location, date and light and by estimating its annual contribution to the N budget of the lake. It is hypothesized that due to this lake's shallow water eutrophic characteristics, N_2 fixation is a significant source of N
2. Identify environmental parameters related to N_2 fixation. It is hypothesized that rates are affected by light and seasonality (through changes in N, P and their ratio).
3. Characterize the diazotrophic community composition in Lake George as it relates to lake location, date and N_2 fixation rate. It is hypothesized that lake conditions will affect the overall composition of the diazotrophic community

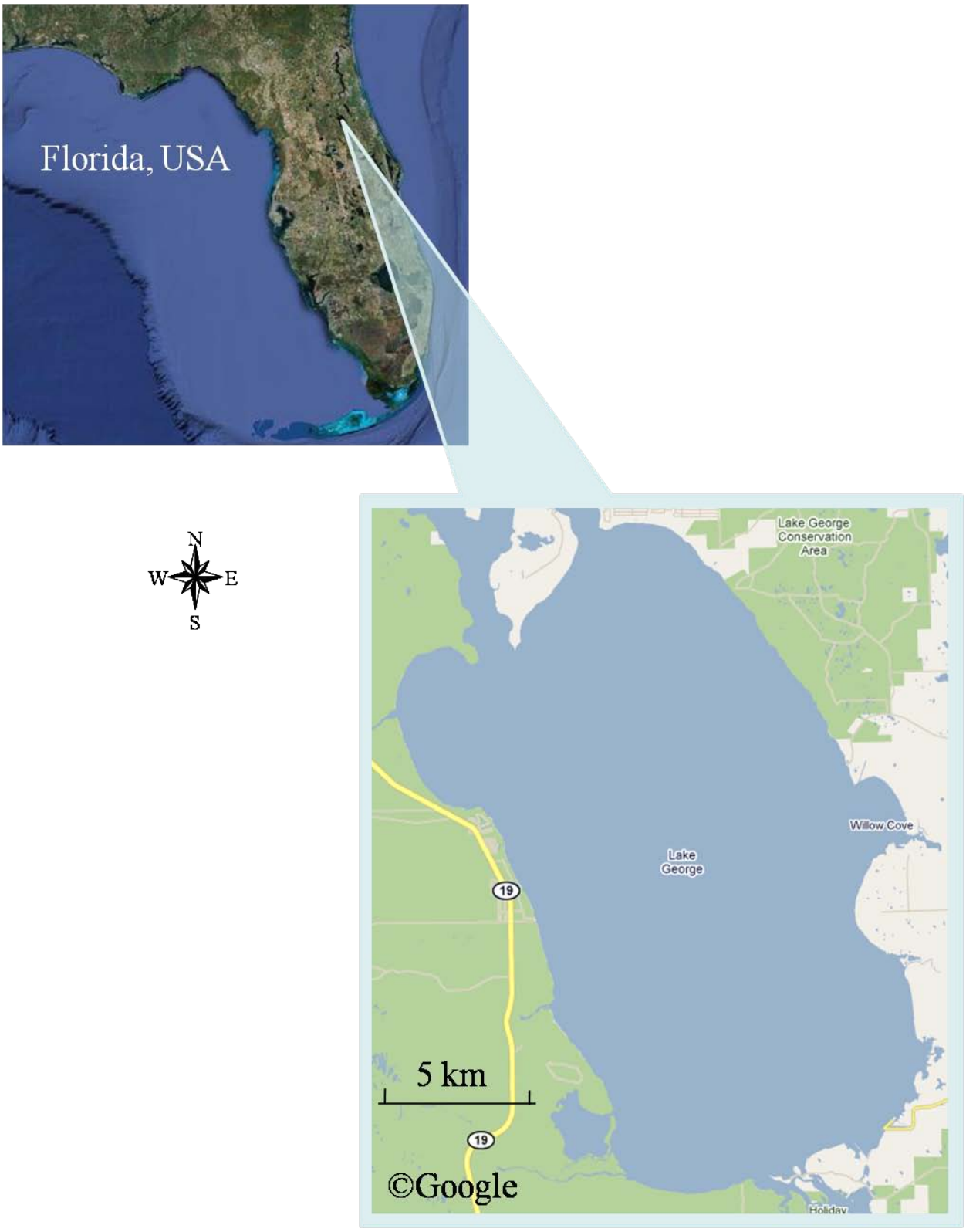


Figure 1-1. Map showing the location of Lake George in Florida including its main water source and output (SJR).

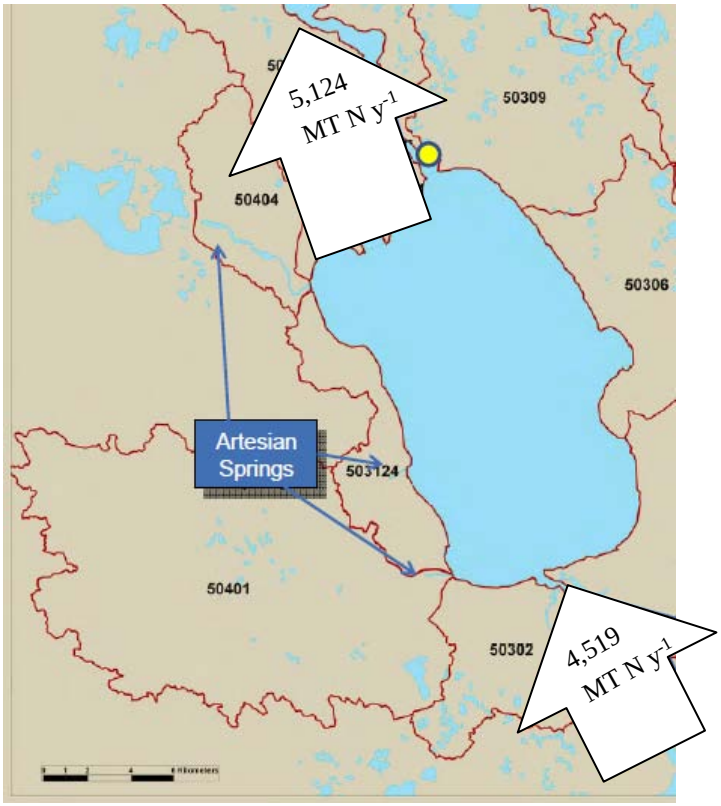


Figure 1-2. Autochthonous N loading and export in Lake George. A comparison between N inputs from SJR and Artesian Springs (west) to exported N through the SJR (adapted from Hendrickson, SJRWMD, 2008).

CHAPTER 2
BIOGEOCHEMICAL ANALYSIS

Due to its importance as a building block of biological systems and its role in countless biochemical pathways, N availability is often coupled to biogeochemical processes like photosynthesis and mineralization, thus shaping aquatic ecosystem ecology. As discussed in Chapter 1, as a system becomes more limited by N, higher rates of N_2 fixation are generally observed due to a competitive advantage diazotrophs have a over other organisms that do not possess this ability (Vitousek and Howarth, 1991; Karl et al., 1997). Nitrogen fixation by diazotrophs is the only biological activity (non-anthropogenic) that has the potential to introduce new N into the system and alleviate limitation of aquatic primary production (Flett et al., 1980; Smith, 1983).

Nutrient Limitation by N and P

Conventional views of nutrient limitation state that P limits primary production in most freshwater ecosystems, while N is limiting in coastal and marine waters (e.g., Hecky and Kilham, 1988). Limitation by P is common in many inland fresh water systems, but once P enters the system, the limiting nutrient often shifts to N resulting in a competitive advantage to diazotrophs (Scheffer et al., 1997; Havens, 2003). When observing eutrophic systems, a direct relationship between nutrients and N_2 fixation would demonstrate its regulators and importance to N export budgets and proliferation of diazotrophs. Although high in nutrients, eutrophic aquatic systems can experience both N and P limitation from shifts in nutrient loading ratios and biological controls on nutrient cycling. Thus, in eutrophic systems, N_2 fixation may be an important component of the N budget, especially in periods of N limitation (Keirn and Brezonik, 1971; Ashton and Hoare, 1981).

Algal N_2 Fixation

Algal blooms have caused much economic and ecological damage globally during the last decades (Riegman, 1998; Cloern, 2001). Nitrogen fixation by algae has been documented in natural waters for some time now (Hutchinson, 1941), and this primarily by cyanobacteria which are the dominant diazotrophs in most aquatic environments (Scheffer et al., 1997; Havens, 2003). Cyanobacteria are superior competitors under eutrophic conditions and frequently thrive in nutrient-enriched waters (Vincent, 1987). These bacteria can achieve optimal growth under low light conditions, are buoyant (Reynolds, 1987; Reynolds and Walsby, 1975), have a relatively slow growth rate (Paerl, 1998), and some members can differentiate their cells into specialized structures called heterocysts, where N_2 fixation is protected from O_2. For the above reasons, cyanobacteria are considered to be the most important aquatic diazotrophs (Keirn and Brezonik, 1971).

Eutrophication of Downstream Systems

Evidence that upstream eutrophic systems may accelerate eutrophication in downstream N-limited and marine systems (Howarth, 1988) indicates that a control on both N and P may be the best approach for long-term management of eutrophication in these systems (Paerl, 2009). In general, watersheds show greater N exports in periods of high discharge, probably by releasing N that was sequestered during dry periods. This export can range from 10 to 15% of the N inputs in dry watersheds to over 35% in wet watersheds. As exported N reaches downstream systems, it changes nutrient equilibrium and can accelerate eutrophication. In fact, eutrophication of coastal systems is considered to be the biggest pollution problem in the coastal waters of the U.S. (Howarth et al., 2000; NRC, 2000). For example, about 4000 estuaries in U.S. are severely degraded from eutrophication, and 67% are degraded to some extent (Bricker et al., 1999; EPA, 2001). Internal sources may also supply the required P for the phytoplankton community, and

thus, sequestered P in these systems may be released seasonally (e.g., salinity and light levels) thereby increasing the demand for N and N_2 fixation.

In many shallow subtropical lakes, P does not limit primary producers and as a result these lakes became inoculums for pest cyanobacteria (both diazotrophic and non-diazotrophic). Cyanobacterial growth and activity can act as a nutrient source (especially N) to downstream systems and result in an accelerated rate of eutrophication. Recently, such systems have received attention due to their apparent connection to adverse phenomena in many coastal and marine systems around the globe (hypoxic zones and toxic algal blooms). In fact, it is believed that restoration of such systems is crucial for the reduction of these global events.

In Florida, the St. Johns River Water Management District (SJRWMD) attempts to reduce the frequency and magnitude of these events in the Jacksonville coastline (a nutrient-sensitive estuary) by evaluating N cycle processes and loads within the watershed of the St. Johns River (SJR; Hendrickson, 2008). One key focus of this study is waterbodies capable of N_2 fixation through blooms of cyanobacteria. Several shallow lakes exist in this watershed and meet this criterion for study, among them is Lake George. Lake George is a good model for the investigation of N_2 fixation for several reasons including: 1) it is the largest watershed on the SJR (NRC, 2000), 2) this lake has a relative high P level promoting diazotrophic dominance, 3) it has a relatively low residence time, which is ideal for cyanobacterial proliferation, and 4) there is a large amount of historical data on Lake George including numerous experiments, reports and regular monitoring by the SJRWMD with over 70 years of data on water quality and over a decade of data on phytoplankton composition.

Research Objectives

Under typical summer conditions, downstream N limitation is a fairly consistent feature of the lower SJR system and may be alleviated by N derived from N_2 fixation in Lake George

(SJRWMD report, 2002). For many years, the area downstream from Lake George, including the Jacksonville region, has been experiencing algal blooms and zones of hypoxia. The economic and environmental impacts of exported N prioritize the need to determine nutrient thresholds and establish nutrient criteria such as Total Maximum Daily Load (TMDLs) in this region. The fact that Lake George is the biggest water body along the SJR, and its relative proximity to Jacksonville makes it one of the most important systems in this regard.

The SJRWMD estimated the amount of annual N being added to Lake George water between 1996- 2006 (calculated by difference in the inflow and outflow total N) as about 600 metric tons (MT) (Figure 1-2). This additional N may originate from various sources, including surface discharge, groundwater springs, biological N_2 fixation, etc. One of the most important variables needed for understanding the N budget of Lake George is the portion of N being added to the system through biological N_2 fixation. In turn, the examination of different parameters that affect N_2 fixation can be used to better understand the process under natural conditions and in different time periods.

Rough estimation of the amount of fixed N in Lake George done by the SJRWMD indicates that it may be a significant source of N to downstream systems (Figure 1-2), but because it was calculated indirectly, it may not represent actual values (Hedrickson; SJRWMD, 2008); thus, the overall goal of this study was to evaluate temporal patterns and quantify the amount of N that enters the system through N_2 fixation. Lake George is an ideal site for this analysis for the reasons mentioned above and its N_2 fixation rates were within the ranges observed in other shallow subtropical eutrophic lakes measured between the years 2000- 2002 (Gao, 2005). The specific objectives for this section were to:

1. Characterize and quantify N_2 fixation in Lake George water, by evaluating the influences of location, date and light and by estimating its annual contribution to the N budget of the

lake. It is hypothesized that due to this lake's shallow water eutrophic characteristics, N_2 fixation is a significant source of N

2. Identify environmental parameters related to N_2 fixation. It is hypothesized that rates are affected by light and seasonality (through changes in N, P and their ratio).

Materials and Methods

Site Description

Lake George is a large (186,000 ha), shallow (average depth 2.5 m), subtropical lake located in Florida, USA (Figure 2-1). Its estimated size is about 19.4 km long, 9.7 km wide, and it is a part of the SJR ecosystem in which it is the biggest water body. Its eutrophication has been accelerated by its geological characteristics, nearby human activity, and inflow from the SJR (SJRWMD report, 2008). Lake George does not stratify completely, and subsequently it is categorized as Class III by the state (this class supports healthy fish and wildlife populations).

Sample Collection

Integrated water samples were collected weekly during the summer of 2008 (22 weeks from April 8 to September 2) from nine locations in Lake George (three sites in each of the east, center, and west portions of lake, Figure 2-1). The sites were chosen to represent a range of water chemistry and environmental conditions in Lake George during the study period. The nine water samples were transported to the laboratory in 1-l carboys where they were composited into three transect samples based on lake location corresponding to East, West and Center transects (Figure 2-1). The three transect samples were mixed in 4-l carboys by constant stirring, and processed within two hours from sampling except on two dates (5/20 and 7/29). Phytoplankton biomass was concentrated from between 150 to 450 ml (depending on water turbidity) of site water by vacuum filtration through 0.7-µm glass fiber prefilters (Millipore CAT No. APFF04700). Nitrogenase activity was assayed on triplicate filters immediately following filtration.

Nitrogenase Activity

Measurements of nitrogenase activity (NA) were performed using a modified version of the Acetylene (C_2H_2) Reduction Assay (ARA) by Stal (1988). The principle behind this method is based on the fact that the nitrogenase enzyme also reduces C_2H_2 gas into C_2H_4 gas. Because both C_2H_2 and C_2H_4 are trace elements, it is more accurate measuring low concentration of them compared to the abundant N_2. A molar ratio of 3:1 between C_2H_4 to N_2 can be used to estimate the amount of N_2 that was fixed (Howarth et al., 1988b).

Filters containing phytoplankton biomass were placed in 50-ml Erlenmeyer flask (60 ml total volume), covered with filtered site water (10-ml), and capped with a rubber turnover stopper. Two water controls without filters were used; similar to the samples, they contained a volume of 10 ml water: blank controls flasks were filled with 10-ml D.I. water, while site water controls flasks were filled with 10-ml filtered site water. Using a syringe, a volume of 5 ml of headspace was removed from each flask and followed by injection of 6 ml acetylene gas. Triplicates of each sample were incubated at 23-26^{o}C inside a temperature-controlled shaker (100 rpm) and under three different light treatments. Dark incubation (0 µM photons m^{-2} s^{-1} PAR) flasks were placed inside an aluminum foil covered box, low light (66 µM photons m^{-2} s^{-1} PAR) flasks were covered by a screen mesh material (1-mm square mesh) and high light flasks were exposed to direct light (115 µM photons m^{-2} s^{-1} PAR) from a fluorescent light source.

Following incubation (2-3 hours), flasks were shaken to equilibrate the gas phases and sampled for headspace gas samples (4 ml) which were stored in evacuated, 3.5-ml serum bottles with gray, butyl-rubber stoppers and aluminum crimp seals. All gas samples were analyzed for ethylene within one week of incubation using a Shimadzu GC-8A gas chromatograph equipped with flame ionization detector (110^{o}C). Gas separation was done at 80^{o}C using a six foot,

Poropak-N column (Supelco, Bellefonte, PA). Ethylene concentrations were verified using a premixed standard gas (10 ppm) (Scott Specialty Gases, Inc., Plumsteadville, PA).

For the calculation of total AR per flask, ethylene production was derived from both headspace and aqueous phase volumes. The volume of ethylene in the aqueous phase was calculated by multiplying the headspace ethylene concentration by a tabulated solubility constant (Henry's Law under standard temperture and pressure) which was multiplied by the water volume of the sample. Using the Universal Gas law (under standard temperture and pressure of 1.1 atmosphere) the volume of ethylene was converted to moles of ethylene produce which was then based per volume of water filtered and length of incubation.

P= absolute pressure of ethylene (1)

V= volume of ethylene generated

n = number of moles ethylene generated

R= 0.08205784 L atm K^{-1} mol^{-1}

T= absolute temperature in Kelvin (K)

The result yielded the number of moles of ethylene produced in one liter of lake water per hour. Finally, the theoretical conversion was used to estimate the actual rate of N_2 fixation (Howarth et al., 1988b). The result was thus expressed as nmol N fixed per liter per hour of incubation.

Physicochemical and Algal Taxonomic Analysis

Samples of integrated lake water were taken and analyzed for physicochemical and nutrient parameters by SJRWMD according to their protocols (SJRWMD 2002). Total organic C (TOC) was calculated indirectly as the difference between total C (TC) and total inorganic C (TIC); particulate organic carbon (POC) was calculated as the difference between TOC and dissolved organic carbon (DOC); nitrate/nitrite (NO_x) was calculated as the difference between total N (TN) and total Kjeldahl N (TKN); dissolved organic N was calculated as the difference

between dissolved Kjeldahl N (TKN-D) and ammonium (NH_4^+); particulate organic N (PON) was calculated as the difference between TKN and TKN-D; dissolved inorganic N (DIN) was calculated as the sum of NH_4^+ and NO_x; dissolved organic P was calculated as the difference between total dissolved P (TP-D) and dissolved reactive P (DRP). Microscopic analysis of densities and biovolume of cyanobacteria, heterocystous cyanobacteria and heterocysts were performed by M. Cichra at the School of Forest Resources and Conservation (Fisheries and Aquatic Science), UF, Gainesville.

Statistical Analysis

For statistical analysis of seasonal patterns, the study period was divided into two sub-periods that each included two AR peaks (4/08 - 6/17 and 6/24 - 9/02). Statistical analysis was done using SAS® and JMP®, Version 6 (SAS Institute Inc., Cary, NC). Type III tests of fixed effects ANOVA model based on AR rate with season, transect location and light treatment as main effects. Multiple comparison of means were conducted for significant light treatment ANOVA result (P-value <0.05) using Duncan's Multiple Range procedure. Canonical analysis was used to separate the transect sites on the basis of their nutrient composition and environmental parameters. Log transformations were used to improve normality based on standard skewness and kurtosis values. Variables included in the data matrices were DOC, log NH_4^+, log DRP, DON, log TN:TP, and conductivity. To evaluate the order of influence of water chemistry on AR rate, both forward and backward, stepwise multiple regression were done after screening auto-correlated limnological variables (among them *chl-a* and biological oxygen demand (BOD)). Several analyses including 3-way ANOVA, stepwise analysis, and canonical correlation were done after interchanging different variables such as water temperature, pH, biovolume of heterocystous cyanobacteria, and concentration ratios of particulate and dissolved C, N and P (POC:PON, PON:PP, PON:PP, DOC:DON, DIN:DRP, and DOC:DOP).

Results and Discussion

The mean ARA at all sites demonstrated the high variability of nitrogenase activity (NA) during the study period, with a range of 0-165 nmol C_2H_4 l^{-1} h^{-1} (depending on conditions). The estimated rate of N_2 fixation was expressed on the basis of the lake's water volume (daily average of 550 µg N m^{-3} h^{-1}, or 20 nmol N_2 l^{-1} h^{-1}). The conversion of AR rates to the lake's surface area was done by dividing the AR rate per m^3 by the lake's average depth (2.5) resulting in a daily average of 220 µg N m^{-2} h^{-1}. These measured N_2 fixation rates were within the ranges observed in other subtropical eutrophic lakes measured (Table 2-3). Nitrogenase activity showed a pattern consisting of two sub-periods of high AR rates separated by low activity (Figure 2-2). Rates during the first sub-period (5/20 - 6/17) were higher (138 and 165 nmol C_2H_4 l^{-1} h^{-1}, respectively) than those of the second sub-period (8/6 - 9/2).

Like any method, ARA has some disadvantages. It measures the potential rate of N_2 fixation but it might introduce some biases such as exposure to O_2 during filtration, variation in bioenergetics and reducing cofactor levels, presence of other non-N_2 fixing organisms that can reduce acetylene (e.g. methanogens (Flett et al., 1975)) or oxidize ethylene (e.g., methane oxidizing bacteria), and grazing of diazotrophs. After considering these disadvantages, ARA still remains a reliable method, and its results in this study were consistent with literature. Thus, it was possible to use AR rates recorded during this study (2008) to roughly quantify the total amount of N that was fixed in Lake George water during the study period and examine its contribution to yearly N output.

Acetylene reduction rate measured in each transect was scaled up using a model of Lake George prepared by SJRWMD which separates the lake into 320 sections with associated depths and volumes. This model was divided into three lake portions to correspond to the three transects used for sampling. The volume of each transect was multiplied by its corresponding AR rate at

each of the 22 weeks of study and multiplied by the number of hours per week (the number of hours used is explained next). Among the assumptions used in this calculated estimate were: a) AR rate under high light approximates fixation rate during the day; b) AR rate under dark incubation represents fixation rate during the night; c) light penetrated through the entire water column; d) both light and dark periods were 12 hours per day. Results from calculation of the yearly estimate were expressed as metric tones (MT, or 10^6 g) of N.

Results suggested that during the study from late Spring through early Fall, N_2 fixation rate in the dark amounted to 135 MT of N, while daytime N_2 fixation accounted for 462 MT N to the lake. For a rough estimate of the amount of N fixed during the rest of the year (outside the study period) an additional 131 MT N was estimated (based on an assumed constant rate of 28 ng N $l^{-1}h^{-1}$). Thus, the total N fixed during the study period is estimated to be between 600 - 730 MT during the entire year of 2008. This quantity was of the same magnitude, but slightly larger than the estimated yearly amount of exported N (600 MT, SJRWMD). On an annual basis this equates to approximately 3 - 4 g N m^2 added to Lake George.

Although a similar pattern of AR was observed at all the sites, both the west and center sites appeared to be more closely related (except on 5/20) compared to the east site (Figure 2-3). The highest AR rate was measured on 5/20 at the west site. During the period of 6/07 to 7/15, AR remained consistently below 30 nmol C_2H_4 l^{-1} h^{-1}. When looking at effectors on AR rate, results of three way ANOVA were almost identical in both sub-periods (Table 2-2) and showed significant ($P< 0.005$) effects by date, site and their interaction (except to interaction between sites and light that was not significant during the first sub-period). These results supported our hypothesis that light level, spatial distribution, and date/season are potential regulators of N_2 fixation.

Depending on light level, AR also varied greatly, and trends of AR in both sub-periods were similar (Figures 2-2 and 2-4). Using selected light treatments enabled us to estimate both the amount of N_2 fixation in a 24 h cycle and the level of PAR required to achieve light saturation. Before comparing the effect of light on AR, rates were normalized by dividing each AR rate by the highest rate measured on that day. Normalized AR rates under the different light incubations showed similar temporal pattern yet varied by their activity rate (Figure 2-4). These results under different light incubations showed a clear difference between N_2 fixation in the dark (with a mean of 20 nmol C_2H_4 l^{-1} h^{-1} or normalized mean of 0.14 nmol C_2H_4 l^{-1} h^{-1}) versus light (with a mean of 54 nmol C_2H_4 l^{-1} h^{-1} or normalized mean of 0.48 nmol C_2H_4 l^{-1} h^{-1}) (Figure 2-4). This result reinforces the assumption that the dominant diazotrophs in Lake George use energy from photosynthesis to fix N_2 and are able to protect their nitrogenase from photosynthetically generated O_2.

The lack of significant difference between AR under low (with a mean of 54 nmol C_2H_4 l^{-1} h^{-1} or normalized mean of 0.48 nmol C_2H_4 l^{-1} h^{-1}) and high (with a mean of 58 nmol C_2H_4 l^{-1} h^{-1} or normalized mean of 0.6 nmol C_2H_4 l^{-1} h^{-1}) light incubations (Figure 2-4) indicates that light saturation of this diazotrophic community was likely achieved within the light levels of this incubation (up to 115 μmol m^{-2} s^{-1} PAR). Saturation at such low light levels is generally indicative of cyanobacterial dominance in the phytoplankton (Grimshaw et al., 1993; Havens, 2003). Moreover, low light levels might be ideal for organisms that dwell in the deeper areas or in the sediment and are more sensitive to radiation (Grimshaw et al., 1997) and indirect damage from high temperatures reduces AR by inhibition of the O_2 protective mechanisms surrounding nitrogenase (Gallon et al., 1993).

Limnological Parameters

The proximity and dependence of phytoplankton on their environment suggest the importance of limnological parameters in the study of N_2 fixation (Havens, 2003). Thus, the concentration and relative proportions of nutrients in the water column shape phytoplankton community composition, growth and N_2 fixation rate per unit of limiting nutrient. Nutrients that affect N_2 fixation are most generally associated with N limitation, thus promoting a community shift toward diazotrophs and increased rates of N_2 fixation. Among these nutrients are P (DRP and TP), N (DIN, NO_x, NH_4^+,TN) and C (DOC, TC) (Hecky and Kilham, 1988; Howarth et al., 1988(a)). Important limnological parameters also showed variation between sites (Table 2-1), and many of their mean concentrations in the water showed temporal patterns similar to AR pattern. The bimodal pattern was predominant among several parameters including *chl-a*, dissolved oxygen (DO), POC:PON ratio, pH, and conductivity, while DRP had an inverse pattern (Figure 2-5). Salinity and silica (SiO_2) showed same trend and together with water temperature had lowest values during the first weeks of study but reached their highest points in early June and remained high until end of August (Figure 2-5). In contrast, total dissolved Kjeldahl N (TDKN), dissolved organic carbon (DOC), and the DIN:DRP ratio showed no clear patterns. Among possible explanations to observed seasonal trend are higher precipitation rate observed during the second sub-period (data not included). Dissolved organic N (DON), which like other organic forms is usually connected to non-point sources, was almost constant during the entire study period with an average of 0.7 mg l^{-1} (ranged from 0.6 to 0.83 mg^{-1}).

Interestingly, during the low AR activity measured around the middle of study period (start of June to mid July), nitrate/nitrite (NO_x) levels peaked and showed a bimodal peak (Figure 2-7). Ammonia (NH_4^+) remained low, yet at the first week of July it peaked to its second highest level (0.016 mg l^{-1}) (Figure 2-7). Unlike the first and last peaks, the second peak of NO_x appeared

during low AR activity. Among possible reasons that may explain the trends of NH_4^+ and NO_x are point sources, flux from sediment, inflow of seawater (Morris, 2000; Malecki et al., 2004) and nearby environmental sources (e.g. agricultural land). Ammonium concentrations were low in the aerobic surface waters probably due to biotic uptake or oxidation except for a peak on 07/01 that coincided with a decrease in NO_x (decreased by 0.007 mg l^{-1} followed by an increase of 0.006 mg l^{-1}) (Figure 2-7). In addition, it may be a typical mid-summer peak in NH_4^+, and DRP concentrations in surface waters, from imbalances between assimilation and mineralization, that has been described in other systems (Kemp, 1989).

DOP and DRP did not show a clear trend during our study but increased afterwards (after September) in agreement with observation from previous years (SJRWMD report, 2002). Levels of DRP were consistently low and did not show significant correlation with ARA, yet high levels of *chl-a* might suggest that P was taken by phytoplankton and thus was not as limiting as N. In their work, Tilman et al., (1982) suggested using the resource competition theory as an explanation to species dominance by looking at the type and levels of resources required by each species. Thus, a focus on seasonal population dynamics and succession might be an approach that explains the competition for nutrients (e.g., diatoms that compete better for P given that N and silicon are not limiting). Measured nutrient flux (DRP and NH_4^+) from sediment to water column at LSJR showed a strong negative relationship with O_2 availability in the water column and suggested that phytoplankton blooms will increase nutrient flux from the sediment (Malecki et al., 2004). For the above reasons, it is more likely that P was released during bloom events and dark periods when DO levels were lower. As a result of released P, nutrient limitation shifts to N and N_2 fixation increases.

Evaluation of Nutrient Limitation

Probably the most common method to evaluate nutrient limitation was through examination of the nutrient levels and their ratio in the water column and phytoplankton biomass. In his paper, Redfield (1934) identified a constant relationship between nutrient levels in the marine environment and their phytoplankton biomass. He identified the stoichiometric ratio of C:N:P to be 106:16:1. Since its publication, the Redfield ratio has been extensively used to estimate phytoplankton nutrient limitation in both marine and fresh water systems. The Redfield ratio of molar TN:TP suggested that Lake George was limited mostly by P with several periods of N limitation (Figure 2-6). Particulate C:N can largely be looked upon as the C:N ratio of algal biomass, and thus, can also be used to identify relative patterns of C and N availability. In this study, the weight ratio of POC:PON ranged from 1.2 to 10.3 with an average of 4.4 (Figure 2-5). In this approach, values lower than 7 can be used to infer N limitation (Redfield, 1934), indicating that Lake George was mostly N limited during the period of this study.

In contrast to the Redfield ratio, an alternative metric using DIN:TP was found by Morris and Lewis (1988) to more accurately predict nutrient limitation of phytoplankton. This metric classified lakes with a DIN:TP ratio (by weight) of <0.6 as N-limited and lakes with a ratio > 4 as P-limited, while those with ratios between 0.6 and 4 were considered to be under both N and P limitation. When using this approach in Lake George, the averaged DIN:DRP molar ratio was lower than 0.6 during most of the study period indicating a N-limited system (Figure 2-6). Thus, the two metrics suggested different nutrient limitation: While according to the Redfield ratio, N limitation was short and began the first week of June until the end of July (7/22), according to the DIN:TP weight ratio of Morris and Lewis (1988) N was limited most of the study period (lower than 0.6) except for one month around the beginning of July and during the last date of sampling (Figure 2-6). The high N_2 fixation rates measured in the lake's water indicated a N

limited system, suggesting that the metric of Morris and Lewis (DIN:TP weight ratio) was more accurate than Redfield ratio in this system. Additional support for this conclusion is found in, the fact that close association of ARA with the DIN:TP weight ratio (Figure 2-6).

In natural environments, identification of nutrient limitation by coupling concentrations to primary producers' growth is difficult due to system complexity and dynamics (Hecky, 1988). In order to identify and rank the parameters most closely associated to N_2 fixation, a stepwise multiple regression models were developed for both sub-periods (Table 2-4). In the first sub-period, the stepwise model ranked DO as the most correlated parameter to ARA followed by NO_x (negatively correlated to ARA) ($R^2 = 0.48$ ($P \geq 0.0005$)). On the other hand, the model of the second sub-period ranked POC:PON ratio (negatively correlated with ARA) as the most correlated parameter to ARA (P=0.001) followed by DON (negatively correlated), TDKN, NH_4^+ and water temperature (negatively correlated) ($R^2 = 0.98$); the negative correlations implied that these parameters were either inhibitory to or resulted from the outcome of N_2 fixation, while N (TKN and NH_4^+ during second sub-period) was the most strongly related parameter to N_2 fixation. The positive correlation of DO with ARA is likely the result of the importance of heterocystous cyanobacteria in the diazotrophic community.

The reason P limitation was less apparent in the water column might be due to internal storing of P by cyanobacteria (SJRWMD, 2002) and P deposits in sediment that can be used when N enters the system. In addition, readily available forms of P (mostly as DRP) are not expected to remain in the water column for long periods of time (ca. days) due to fast uptake by cyanobacteria. The result from predictors of nutrient limitation supported previous studies in Lake George (SJRWMD, 2002 Annual Project Report) which demonstrated a community that was limited mostly by N but also co-limited by both N and P. It was also noticed that although

diazotrophic activity constantly introduced new N into the water column, N was generally deficient relative to P, and therefore P was not the sole limiting nutrient.

The ability of N_2 fixation to shift limitation from N to P may vary on different locations or time periods but may be reflected by different community during bloom events. Possible explanations for the variation between nutrient limitation and diazotrophic activity in Lake George could include differences in the stoichiometric requirements of the phytoplankton (Arrigo, 2005), possible N storage mechanisms in periods of high N availability, nutrient distribution/sources, and seasonal changes (e.g., formation of thermocline followed by depletion of the initial levels of N as seen in other eutrophic lakes and estuaries (Schindler et al., 2008).

The importance of distribution of environmental parameters can be seen in the resource competition theory and may explain species dominance and the spatial/temporal factors that play an important role. Due to heterogeneity of aquatic systems, three factors relating algal communities must be considered: a) all phytoplankton are motile (the non-motile are affected by water currents and Brownian motion); b) nutrient uptake is not directly coupled to reproduction; and that c) the water column is heterogeneous (Tilman et al., 1982). According to this theory, various niches and individual algal blooms can be limited by a single nutrient (e.g., diazotrophs by P), yet preventing the lake from being completely described as limited by a single nutrient. Thus, as mentioned earlier, an approach that focuses on seasonal population dynamics and their succession offer a better explanation for the competition for nutrients inside the system or between trophic levels (like diatoms).

The spatial distribution of environmental factors can be used to asses their importance on the distribution of N_2 fixation. Using measured limnological parameters, canonical analysis was performed to identify the distributions of environmental parameters that distinguished between

the three transects in order to identify pattern seen by ARA (Figure 2-10). The analysis showed that DOC, log NH_4^+, log dissolved reactive P (DRP), DON, log TN:TP, and conductivity were the most different between the east and west transects during the entire study period; on the other hand, during the first sub-period the central transect was similar to the east while on the second sub-period it shifted to be more like the west.

The fact that there were differences in the distribution of water chemistry parameters between the two sub-periods suggested that an environmental change took place. Among possible explanations to observed seasonal trend are higher precipitation rate observed during the second sub-period (data not included), wave action and spring inputs that can form a gradient of decreasing conductivity out from the west shoreline depending on flow rate (Stewart et al., 2006). Although the similarity in nutrient distribution was not identical to ARA distribution, it demonstrated their association and supported the assumption that nutrient distribution could be affecting N_2 fixation rates. Our results indicated that water by the shorelines (east and west) of the lake had different composition. Several of these parameters (mostly N and P) are known to affect AR and their distribution is expected to influence N_2 fixation rate and phytoplankton abundance. During the first sub-period, the similarity in water composition of the west and center sites compared to the east was also observed in AR rates, however, while the AR trends were kept during the second sub-period unlike nutrient distribution that shifted and could not be related to N_2 fixing.

Microscopic Analysis

Microscopy identified biovolume and density of *Cylindrospermopsis raciborskii* as the dominant genus in Lake George during our study period followed by *Anabaena* (Figure 2-9). While the first bloom was formed by both *Anabaena* and *Cylindrospermopsis*, the remaining blooms were formed mostly by *Cylindrospermopsis.*

Both *Anabaena* and *Cylindrospermopsis* are considered to be opportunists and are fitted for such systems for several reasons including: a) they exhibit N_2 fixation under N limited conditions, b) they form heterocysts, c) they have the ability to store P under saturated conditions and efficient buoyancy regulation, d) they have a relatively low light requirement, e) they have the ability to uptake DIN, and f) they have a high temperature and salinity resistance. *Cylindrospermopsis* was more fitted than *Anabaena* in this environment probably due to its ability to scavenge P rapidly under low concentration, smaller cells and higher number of heterocysts. Thus, it is possible that after the first bloom P levels were very low selecting for the scavenger *Cylindrospermopsis.*

Due to their ability to uptake DIN and to fix N_2, diazotrophs were expected to be mostly P limited; thus, P probably permits their blooms and higher N_2 fixation rate in Lake George. These factors increase the difficulty in understanding and predicting phytoplankton blooms (namely cyanobacteria and diazotrophs abundance) in subtropical aquatic systems by looking at environmental conditions, mostly in the water column, and their influence on N_2 fixation.

In addition, microscopic analysis revealed that cyanobacteria dominated the phytoplankton community (E.J. Phlips, personal communication). During the period of study, bloom events measured by microscopy (densities and biovolumes) showed temporal trends which seemed to follow AR rates and nutrient availability (DON, DIN, NO_x, NH_4^+, POC:PON ratio) (Figures 2-8 and 2-9). The lowest concentration of cyanobacterial biovolume was measured at the first and last days of study (4/08/08 and 9/02/08) at all except for the East sites where biovolumes increased at the end of study, and the highest biovolume was between 5/20/08 - 5/27/08.

In agreement with AR peaks, biovolumes of heterocystous cyanobacteria yielded four peaks (the first by *Anabaena* and *Cylindrospermopsis* and the others by *Cylindrospermopsis*)

supporting the assumption that most of the N_2 fixation is done by heterocystous cyanobacteria. Total cyanobacteria biovolume followed similar trend however with a lag compared to heterocystous cyanobacteria. The lag and fact that total cyanobacteria biovolume were low between the second and third blooms when heterocystous cyanobacteria biovolume were very low may be due to their dependence on fixed N (Figure 2-8). The result supported the assumption that heterocystous cyanobacteria were closely associated with the entire cyanobacterial community and supplied them with fixed N (Schindler et al., 2008).

The heterocystous *Anabaena* and *Cylindrospermopsis* were the dominant diazotrophs and their association with AR rate demonstrated their important role in biological N input into the lake (Figure 2-9). The period around the middle of study (start of June to mid July), was between blooms of heterocystous cyanobacteria (their biovolume levels were at their lowest) and as expected showed low rates of ARA (Figures 2-3, 2-8 and 2-9). In order to avoid the comparison of AR rates that were attributable to differences in heterocystous cyanobacteria biovolume, AR rates were normalized[1] by dividing each AR rate by the heterocystous cyanobacteria biovolume at that day. Normalized AR rates were expressed as AR rates in terms of moles of C_2H_4 generated per unit of heterocystous cyanobacteria biovolume (l) and time (h), a comparison was done between samples. In addition to removing variability due to difference in numbers of heterocystous cyanobacteria, normalized rates had the potential to identify the importance of individual heterocystous cyanobacteria within the cyanobacterial community (because different genera of heterocystous cyanobacteria have different sizes and numbers of heterocysts per biovolume, thus resulting in different AR rates).

[1] Also normalized to cyanobacteria biovolume expressed as N fixed per μ^3 cyanobacteria, the center on 4/22 was low (1.4) and ranged between 10^{-17} mol to 10^{-21} mol N per μ^3 cyanobacteria.

According to results, the highest AR rates were at center of lake and the lowest were at the west sites. Normalized AR rates ranged from 2.6×10^{-10} to 3.9×10^{-8} N μm^{-3} heterocystous cyanobacteria h^{-1}. In addition, the central transect had one N_2 peak between the second and third blooms (6/24 to 7/15), when biovolumes of heterocystous cyanobacteria were at their lowest. This pattern of central stations correlated with NO_x peaks, suggesting that N_2 fixation in heterocystous cyanobacteria may not have been inhibited by NO_x or that they released fixed N. The increased efficiency of fixation per biovolume may be due to low biomass or environmental conditions (e.g., water temperature) during that period.

Conclusions

Based on the seasonal measurements of AR, we can conclude that N_2 fixation was an important driver of cyanobacterial bloom formation (perhaps through supplied N) and resulted in a significant contribution to the lake's N budget (730 MT). This amount was within the range measured in other systems (e.g., Clear Lake (oak arm), CA (Table 2-3)) and could explain a significant portion of the ~600 MT N increase observed in the outflow of Lake George. In addition to this N, there are many other N sources affecting the amount of N export from Lake George, including other sources of N_2 fixation (e.g., from sediments), atmospheric deposition, groundwater discharges, and surface runoff from the areas surrounding the lake. The combination of these N sources with the amount of watercolumn N_2 fixation estimated in this study is potentially much larger than the total amount of measured N export from the lake. However, it is important to remember that other N cycle processes such as sediment accumulation and denitrification can also remove N from the lake. Inclusion of these processes could balance much of the N inputs, and therefore, it is quite possible that the amount of N derived from watercolumn N_2 fixation in this study is reasonable.

Light had a significant effect on AR rates. While ARA was used to measure nitrogenase activity, incubation under different light levels demonstrated the degree to which N_2 fixation was stimulated by light and that N_2 was fixed mostly by phototrophs requiring low light levels. This conclusion was supported by the measured abundance of heterocystous cyanobacteria, and as well, by the positive correlation of AR with DO. These heterocystous cyanobacteria formed the first bloom and appeared to promote proliferation of the entire cyanobacterial community as noticed by similar patterns of the two biovolumes. The lag seen in biovolume of total cyanobacteria compared to heterocystous cyanobacteria supported the idea of nutrient exchange within this community and the importance of diazotrophs to bloom formations.

The overall patterns of nitrogenase activity indicated the importance of location and seasonality as regulators of N_2 fixation and diazotrophic growth in Lake George. Patterns of algal blooms and selected nutrients varied seasonally and spatially as well, showing similar patterns to AR and indicating their association with observed rates of N_2 fixation (Figure 2-8). Moreover, 3-way ANOVA supported this conclusion with significant effects of both site and date on AR rates (Table 2-2). In particular, forms of N and P (DIN, NH_4^+ and DRP) somewhat explain the differences observed between the measurements of the East, West, and Central portions of the lake. Heterogeneity may be explained by different water sources, comprising mostly of the main river water source (from the St. Johns) and other inputs from lake shorelines.

The difference in parameters (conductivity, pH, DIN, DRP and NH_4^+) seen in the canonical analysis between the first sub-period and the second sub-period potentially showed the importance of external nutrient inputs which could include water inflow from upstream (SJR) and the west shoreline (by Artesian springs), natural events (such as rain events and water mixing by wind). The apparent change in the distribution of water parameters (especially,

conductivity and pH) agreed with higher precipitation rate recorded during the second sub-period (data not included). Nutrient distribution or nutrient levels are known to control N_2 fixation and algal bloom formation, and the effect of environmental conditions on the diazotrophic community clearly indicated a shift from *Anabaena* to *Cylindrospermopsis*. The ability of *Anabaena* to uptake P under moderate levels faster than *Cylindrospermopsis*, can explain its abundance in the first bloom, while the domination of *Cylindrospermopsis* during the rest of the year is probably due to its ability to scavenge P rapidly under low concentration. Never the less, it was not precisely clear how N and P, or which mechanisms control diazotrophic composition and domination of single species.

Due to low DRP concentration in the water, this parameter did not seem to correlate to N_2 fixation, yet the N:P ratio suggested a N limited system that agreed with higher AR rates. Higher P, and lower N:P ratio (DIN:DRP or TN:TP ratio) were expected to increase demand for fixed N. The indicator DIN:DRP seemed to better reflect nutrient limitation in Lake George more accurately than Redfield ratio when compared with AR rates. Acetylene Reduction in late summer was lower than early summer probably due to the higher ratio of non-heterocystous species of cyanobacteria that were seen by microscopy and can be explained by higher ratio of N:P (DIN:TP). It is also possible that P was deposited in the sediment or biomass of non-diazotrophic phytoplankton several months before bloom formation, or that there was a relatively constant supply of P obtained from the sediments. Apart from P, heterocystous cyanobacteria are also known to be affected by factors such as salt, high temperatures and intense sunlight that have adverse effects on many biochemical processes and might decrease N_2 fixation rate.

As mentioned above, various nutrient sources and anthropogenic activities likely have changed the balance of both N and P, in Lake George and affected phytoplankton community

structure (composition and biomass) and function (primary productivity and N_2 fixation) during the study period. These alterations in the biogeochemical cycles in Lake George supported conditions of N limitation (as indicated by N:P ratios) and diazotrophic proliferation. Fixed N acts to alleviate the demand for N in Lake George, and also turned the lake into a source of N to downstream systems. These results clearly demonstrated that role which inland waterbodies like Lake George and processes such as N_2 fixation can play in the health and function of entire watersheds.

Some of the limitations of this work include failure to correlate AR with nutrient distribution, use of few measurements to capture spatial and temporal patterns, understanding of bloom dynamics (shift from *Anabaena* to *Cylindrospermopsis*) and interaction within the phytoplankton community. For this reason, additional work should be focused on increasing the ability to predict, explain, and control nutrients/environmental conditions that lead to algal blooms in Lake George and similar systems. Some improvements for future studies should potentially include the identification of specific nutrients that may be transient or pulsed and act to initiate bloom formation. Tracing N sources and fixed N will help to identify external N supply as well as potential N transfer between cyanobacteria and other phytoplankton groups. Moreover, several ARA measurements during fall and winter months and better understanding of light intensity or diurnal patterns would help improve the calculation of system N budget.

According to this study, the restoration and control of the phytoplankton community is complex tasks that cannot be achieved solely by reducing single nutrient inputs. In fact, such a reduction has been done in the last years without much success, and even leading to accelerated eutrophication (Conley et al., 2009; Howarth and Marino, 2006). For this reason, other remediation approaches (e.g., introduction of submerged macrophytes to reduce sediment

resuspension and wave action thus limiting internal nutrient sources) may also be cautiously combined with nutrient reduction to reduce growth of cyanobacteria and other diazotrophs in the Lake.

Table 2-1. Characteristic values of selected chemical parameters in Lake George water. Values represent the means of all samples collected for each transect during the study period (Analyzed by SJRWMD).

Site	DO (mg l-1)	TN (mg l-1)	TP (mg l-1)	pH	Conduct. (µS cm-1)	Chl-a (mg l-1)	DIN (mg l-1)	DON (mg l-1)	DRP (mg l-1)	DOP (mg l-1)
East	6.97	1.29	0.06	8.4	1561	32.5	0.03	0.6	0.003	0.008
West	7.15	1.20	0.05	8.5	1605	32.3	0.03	0.6	0.003	0.009
Center	7.02	1.25	0.06	8.5	1565	35.2	0.02	0.6	0.003	0.008

Dissolved oxygen (DO); total nitrogen (TN); total phosphorus (TP); chlorophyll-*a* (*chl-a*); dissolved inorganic nitrogen (DIN); dissolved organic nitrogen (DON); dissolved reactive phosphorus (DRP); dissolved organic phosphorus (DOP).

Table 2-2. Type 3 test of fixed effects based on nitrogenase activity rate (ARA) for the two sub-periods (n=99).

Effect	Time Period	
	----------*P*----------	
	April- June	July- September
Site	0.01*	<.0001*
Date	<.0001*	<.0001*
Light	<.0001*	<.0001*
Site × date	<.0001*	<.0001*
Date × light	<0.002*	<.0001*
Site × light	0.49	<.0004*

* indicates a statistically significant difference ($P \leq 0.05$)

Table 2-3. Nitrogenase activity (measured using ARA) at various types of aquatic ecosystems.

System	Unit					Reference
	$\mu g\ N\ m^3\ h^{-1}$	nmol N $l^{-1}\ h^{-1}$	g N $m^{-2}\ y^{-1}$	nmole C_2H_4 $l^{-1}\ h^{-1}$	$\mu g\ N\ l^{-1}\ h^{-1}$	
Lake George (potential)	8760	52	4	156	1.5	This Study
Lake George	78					Paerl et al., 2000.
Eutrophic lake Valencia, Venezuela			1.3			Levine and Lewis, 1987.
Eutrophic lake Lyngby, Denmark				80		Ahmad, 1981.
Eutrophic Clear lake (oak arm) CA				310 (total 500 MT N y^{-1})		Horne and goldman, 1972.
Eutrophic Reservoir Waco, TX					0.6	Scott et al., 2009.
Mesotrophic Lake Washington, WA.			0.013			Tison et al., 1977.
Temperate Amazon flood-plain, Lake Calado		30				Doyle and Fisher, 1994.
North Sea in Europe			1450			Howarth et al. 1996.

Table 2-4. Model parameters for stepwise multiple regression analysis of log transformed AR rates of sub-period 1 and 2. Models are presented for combined transect samplings.

Sampling Date	Model R^2 (n)	Variables Included	Estimate	SE	Pr> F
Sub-period 1	0.617 (23)	Constant	-238.49	51.4991	<0.0001*
4/08- 6/17/08		DO	38.7184	6.65975	
		NO_x	-95.291	43.1937	
Sub-period 2	0.9786 (29)	Constant	21.6709	1.06814	<0.0001*
6/24- 9/02/08		POC:PON ratio	-338.53	0.99719	
		DON	-436.35	21.0301	
		TKN-T	19.9688	1.13567	
		NH_4^+	67.3339	15.0517	
		Water temperature	-110.49	41.8737	

***** indicates a statistically significant difference ($P \leq 0.05$)

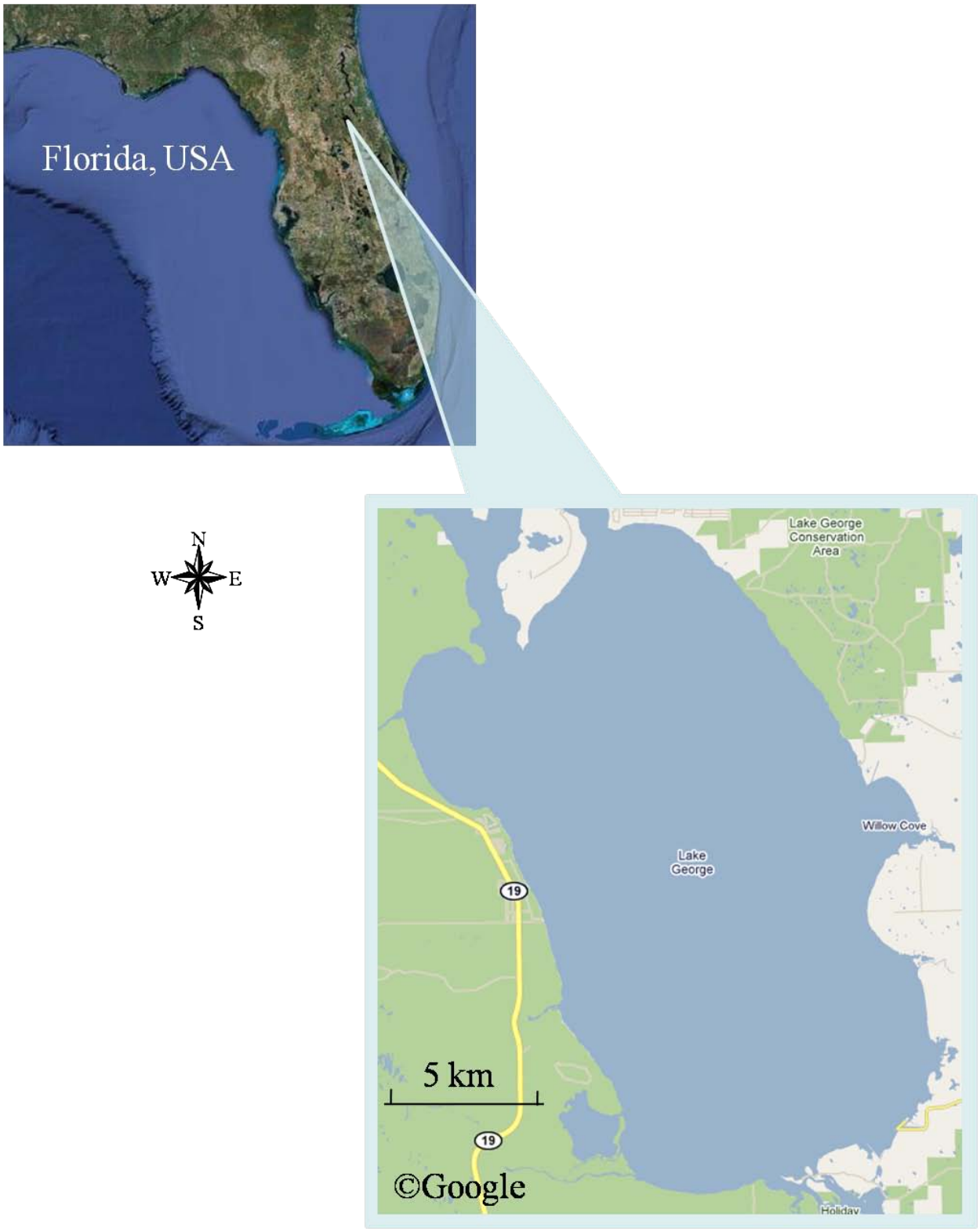


Figure 2-1. Map showing the location of Lake George in Florida including its main water source and output (SJR).

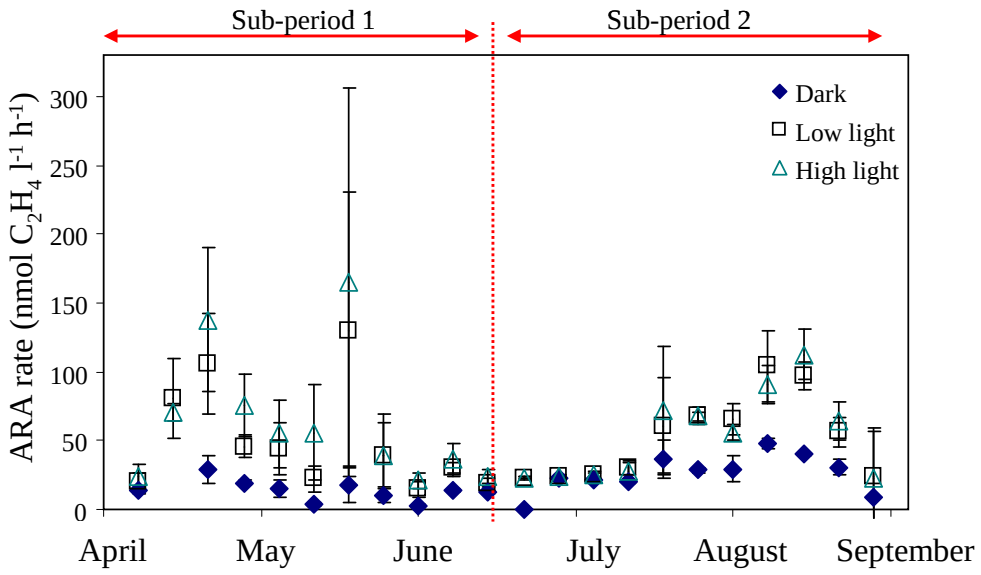


Figure 2-2. Temporal distribution of nitrogenase activity (measured by acetylene reduction assay) under three light levels (115, 66 and 0 µM m^{-2} S^{-1} PAR) for Lake George water. Dotted line separates between the two sub-periods. Values represent the mean of nine replicates (± 1 SE) obtained for each treatment during study period in 2008.

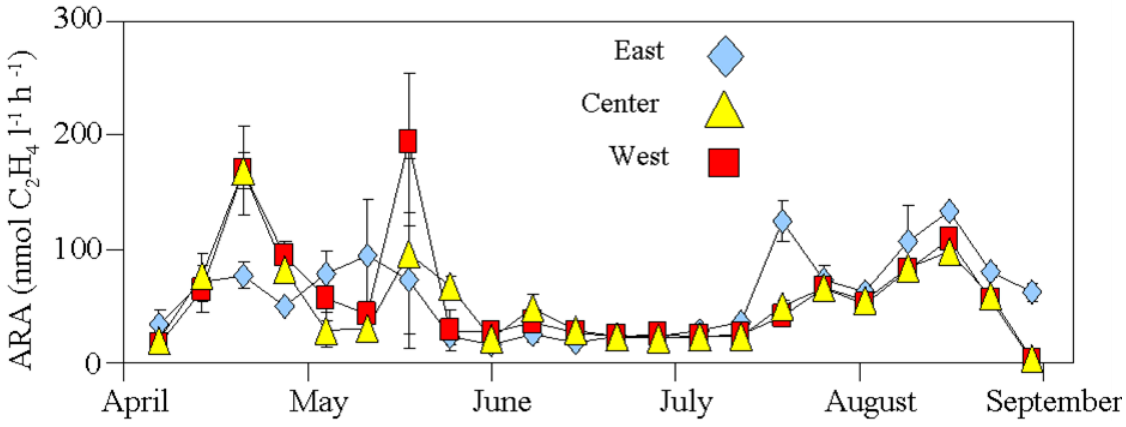


Figure 2-3. Spatial and temporal distribution of nitrogenase activity (measured using acetylene reduction assay) under high light incubation (115 µM m^{-2} S^{-1} PAR) for Lake George water during study period (2008). Values represent the mean of three replicates (± 1 SE) obtained from each transect.

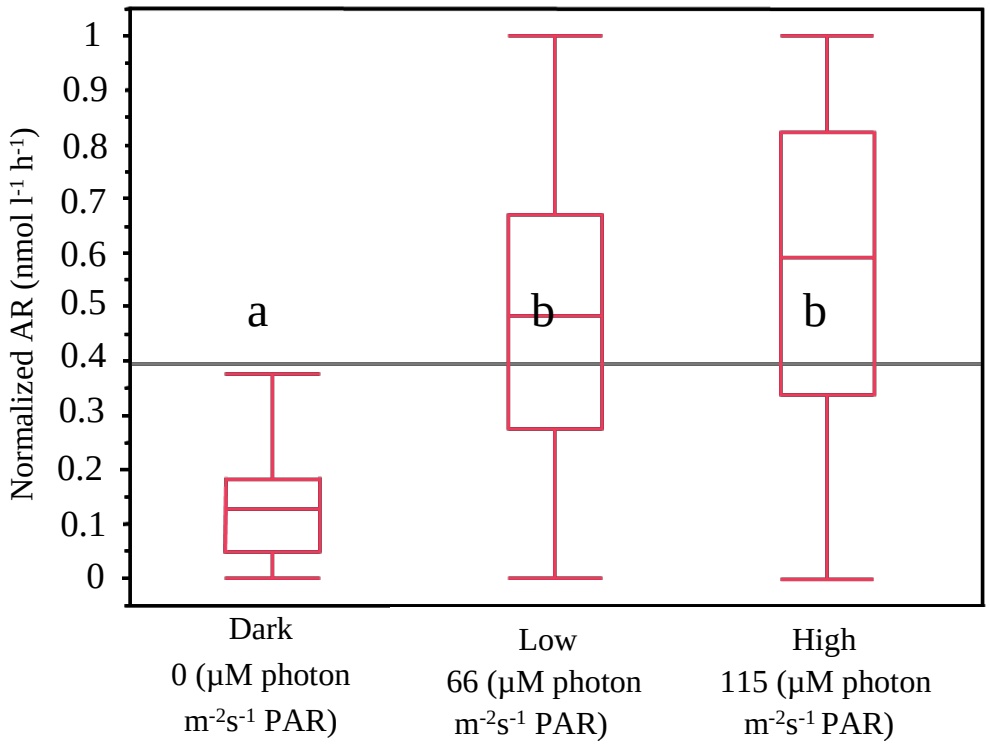


Figure 2-4. The effect of light intensity on acetylene reduction assay rates in Lake George water during the study period. Each rate was normalized as the percent of maximum AR value for a given date (see text for description).

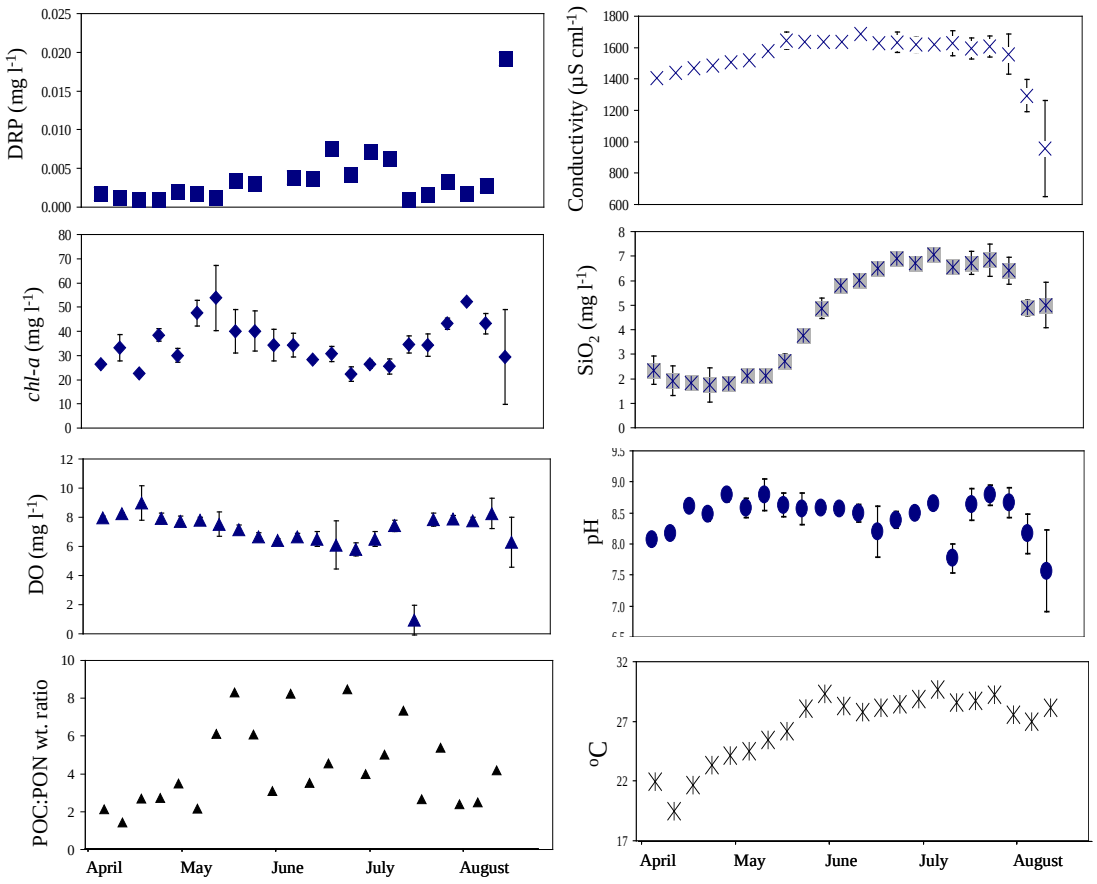


Figure 2-5. Temporal patterns of environmental parameters. Points represent the means of nine replicates (± 1 SE) obtained from Lake George water-column. A) DRP (dissolved reactive P). B) chlorophyll *a*- (*chl-a*). C) dissolved oxygen (DO). D) particulate organic carbon: particulate organic nitrogen ratio (POC:PON). E) conductivity. F) dissolved silica (SiO_2). G) pH. H) water temperature (Celsius). All analyses were done by the SJRWMD.

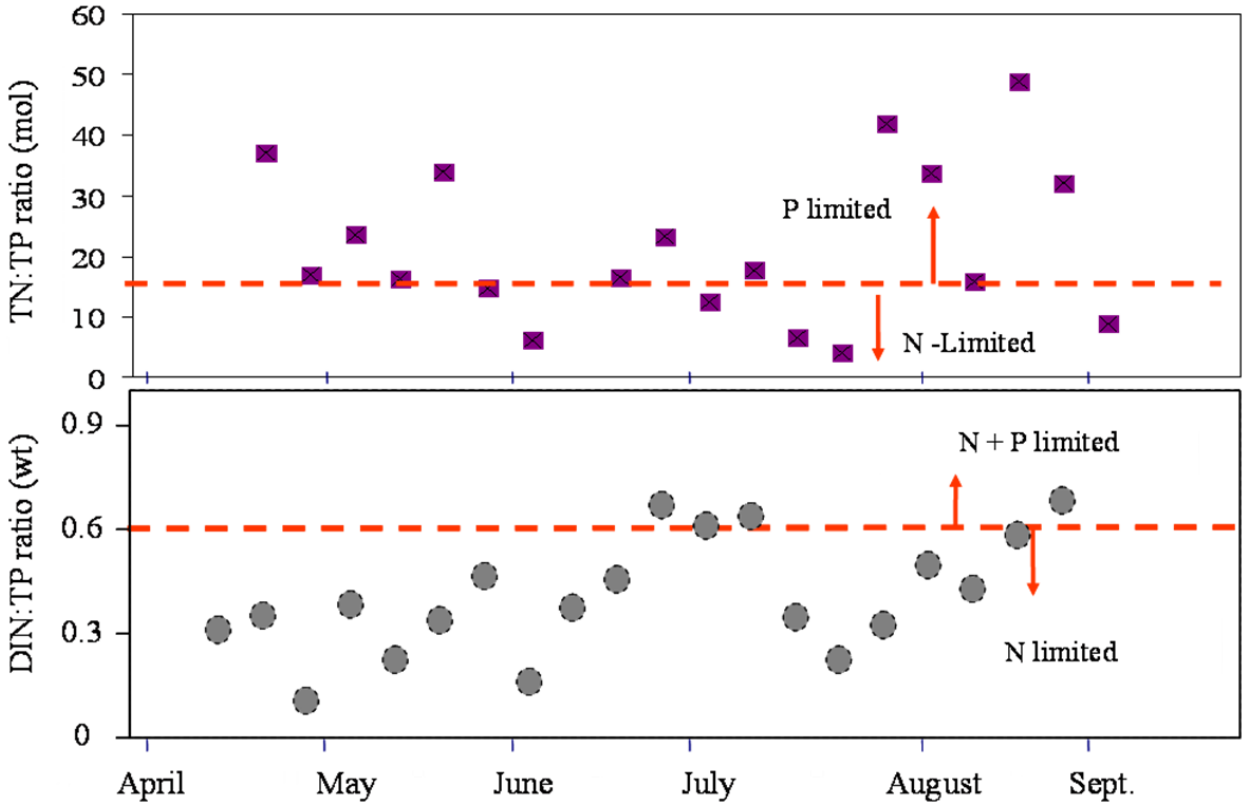


Figure 2-6. Temporal patterns of N:P ratio in Lake George water-column. Points represent means of nine replicates. A) molar total N to total P ratio (TN:TP). B) dissolved inorganic N to dissolved reactive P ratio by weight (DIN:DRP). Redfield ratio and Morris and Lewis ratio are marked by dotted red line.

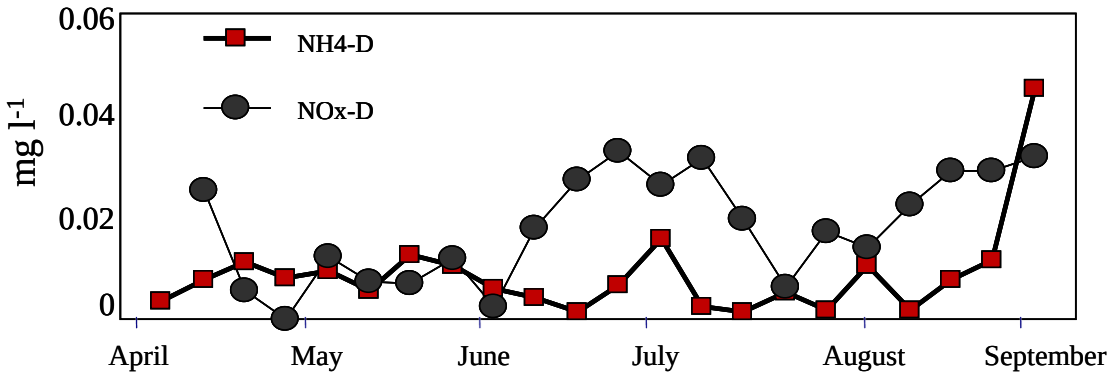


Figure 2-7. Temporal patterns of labile N: ammonium (NH_4^+) and nitrite/nitrate (NO_x) in Lake George water-column during study period. Points represent means of nine replicates.

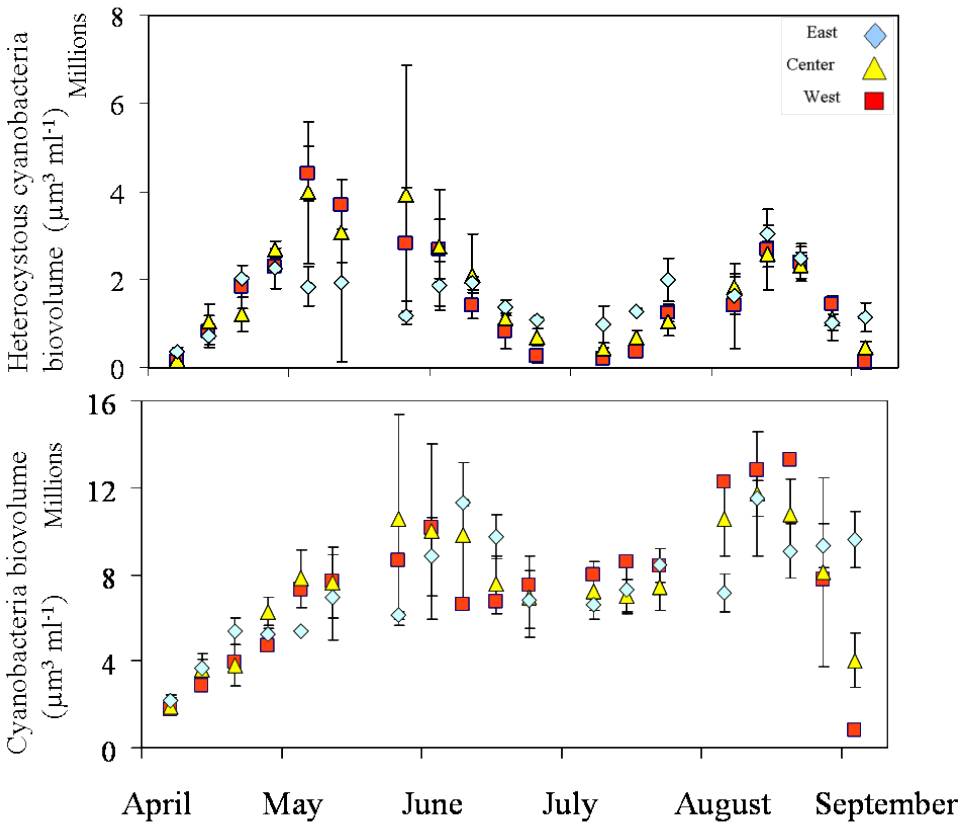


Figure 2-8. Spatial and temporal patterns of cyanobacteria biovolume (μm^{-3} ml^{-1}). Points represent the means of three replicates (± 1 SE) obtained from Lake George water-column. A) heterocystous cyanobacteria. B) total cyanobacteria. Microscopic analysis by Cichra and Phlips.

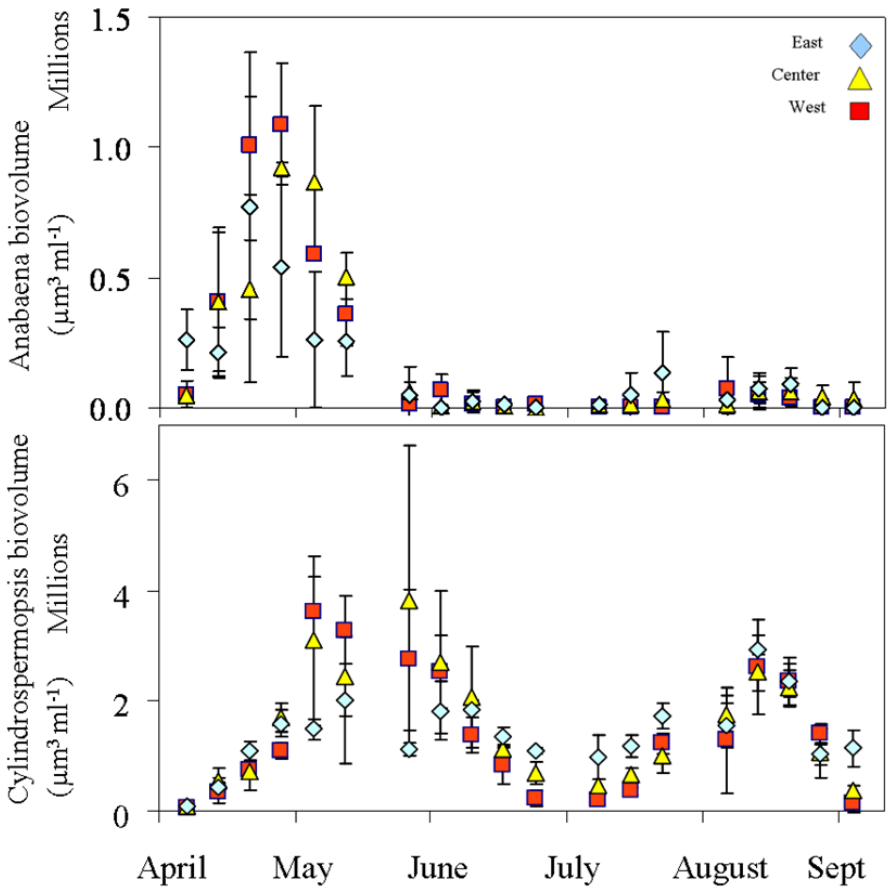


Figure 2-9. Spatial and temporal patterns of heterocystous cyanobacteria biovolume (μm^{-3} ml^{-1}). Points represent the means of three replicates (± 1 SE) obtained from Lake George water-column. A) *Cylindrospermopsis* .B) *Anabaena*. Microscopic analysis by Cichra and Phlips.

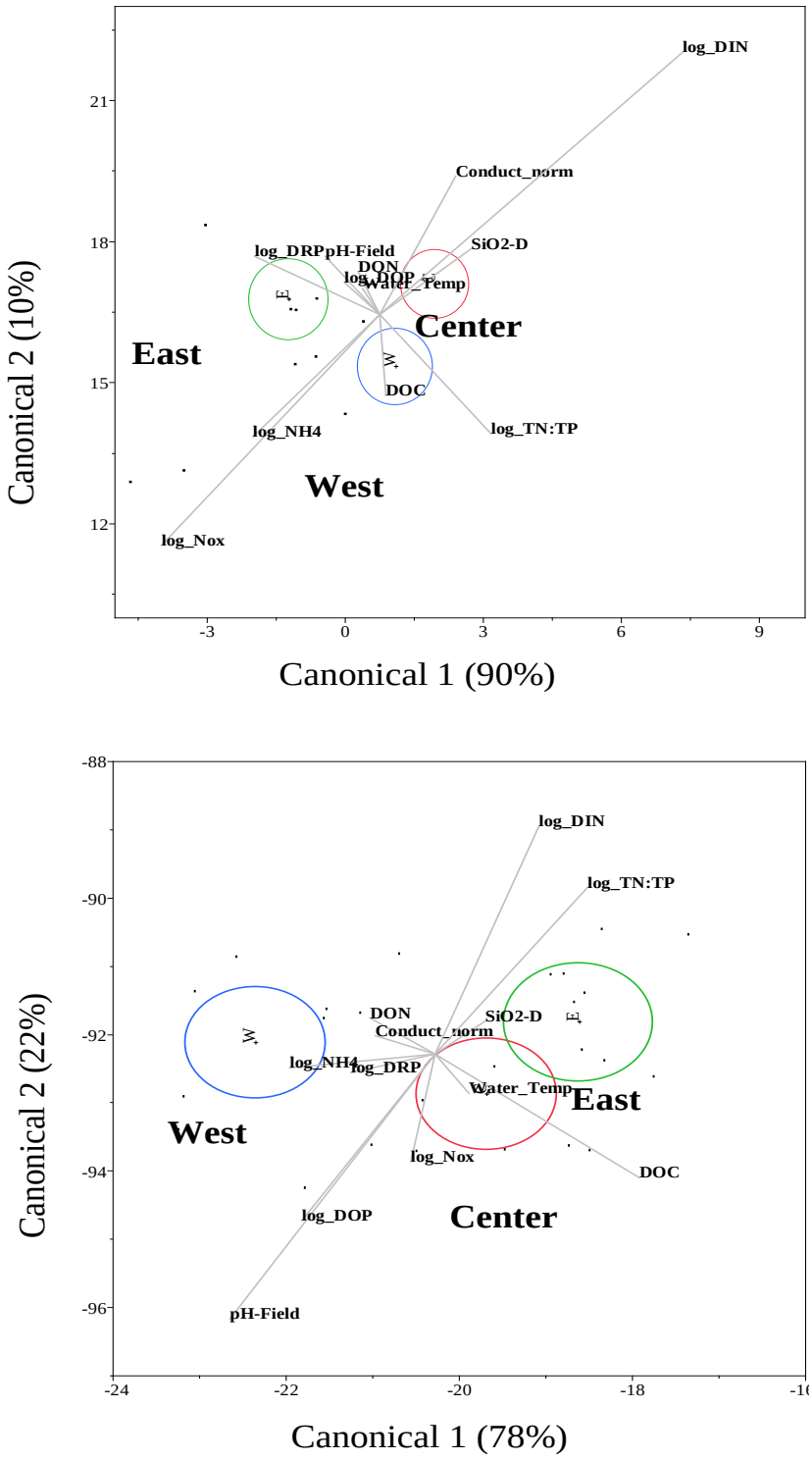


Figure 2-10. Separation of lake transect locations by Canonical analysis based on limnlogical parameters in Lake George water column. A) first sub-period (4/08 – 6/17). B) second sub-period (6/24 - 9/02). The most influential parameters were dissolved organic C (DOC), log NH_4^+, log dissolved reactive P (DRP), dissolved organic N (DON), log TN:TP, and conductivity.

CHAPTER 3
MOLECULAR ANALYSIS

Dinitrogen gas (N_2) is the most abundant gas in the atmosphere comprising about 78% by volume, yet its consumption is limited to an exclusive group of organisms called diazotrophs. The nitrogenase enzyme is a common protein to all diazotrophs due to its role in breaking the triple bond of N_2 to fix (reduces) it into a bioavailable form, NH_4^+. Almost all known nitrogenase enzymes are irreversibly inhibited by oxygen (O_2), yet they are the only known group of enzymes that reduces N_2. Due to nitrogenase importance, N availability is often coupled to biogeochemical processes such as photosynthesis and mineralization, thus shaping ecosystem ecology. Under normal conditions, primary producers are not limited by carbon (C) for photosynthesis, but as an ecosystem becomes more limited by available N, restricted primary production occurs, and N_2 fixation by diazotrophs has the potential to supply the demanded N into the system (Flett et al., 1980; Smith, 1983).

As N limitation increases, higher rates of N_2 fixation are generally observed (Vitousek and Howarth, 1991), and the ability to fix N_2 gives a competitive advantage to diazotrophs over other organisms (Karl et al., 1997). Such conditions promote dominance of diazotrophs, and may result in aquatic systems being sources of N to connected systems (Schindler, 1977; Tilman et al., 1982). Nutrient levels (especially N) in the water can be correlated to N_2 fixation rate or to limitation of other essential nutrients (such as phosphorus (P) and iron (Fe)) probably due to microbial activity (Vitousek and Howarth, 1991; Mills et al., 2004).Temporal changes in aquatic systems have both a direct and an indirect impact on nutrient abundance and distributions (Thayer, 1971), thus resulting in seasonal patterns (Baird and Ulanowicz, 1989). These temporal changes are controlled and regulated functions of microbes that are defined by their genetic potential.

Nitrogenase Gene

Nitrogenase is a complex consisting of two enzymes: dinitrogenase; and dinitrogen reductase. Dinitrogenase is a tetramer with molybdenum–iron (MoFe) co-factor composed of two identical subunits encoded by *nifD* (Lammers and Haselkorn, 1984) and the other two identical subunits are encoded by *nifK* (Mazur and Chui, 1982). Dinitrogenase reductase contains an iron cofactor and is composed of two identical subunits encoded by *nifH* (Mevarech et al., 1980). Although the phylogeny of diazotrophs using *nifD*, 16S rRNA genes (rDNA) and *nifH* is conserved, some discrepancies are present suggesting that both 16S rDNA and *nifH* sequences may be ascribed to a lower level of conservation than *nifD*. High levels of conservation make *nifD* and *nifH* good candidate genes for monitoring potential shifts in diazotrophs populations

Although more research was done using *nifH* and its GeneBank library is larger, *nifD* is expected to be more appropriate for systems in which most of the diazotrophic community is composed of cyanobacteria. Moreover, previous studies have indicated that the divergence found in *nifD* is intermediate between *nifH* and *nifK* (Henson, 2005); thus, it is believed that when used as a phylogenetic marker, its resolution among closely-related diazotrophic microorganisms is higher and might suffice to distinguish *nifD* gene family members as well as alternative nitrogenases such as the vanadium-containing enzyme (Zehr et al., 2003; Henson et al., 2004a).

The nitrogenase gene operon may be located on plasmids (for example, *Rhizobium meliloti* carries two megaplasmids (Honeycutt et al., 1993)) or on the chromosome (for example, in *Azorhizobium caulinodans*). From an evolutionary point of view, it is not clear if the symbiotic genes were originally located on the chromosome and evolved through excision, or if they were originally on a plasmid that became incorporated into the chromosome. For example, gene analysis of *Klebsiella pneumoniae* identified 20 adjacent *nif* genes that were organized in eight operons within ca. 24 kb (kilo base pairs) of DNA (Arnold et al., 1988). The most common

nitrogenase complex employs a MoFe cofactor (the non-protein part of nitrogenase) that exhibits a low efficiency level when fixing N_2.

As expected, some diazotrophs are able to adapt to different conditions by expressing alternative nitrogenase systems that can result in similar structures of nitrogenase. Although similar in structure, the alternative proteins are less efficient than the Mo protein (Robson et al., 1986). Both *vnf* and *anf* are two similar sets of genes which modified their N_2 fixation mechanism (*vnf* assembled a (vanadium) VFe cofactor to produce a haloenzyme with similar structure to nitrogenase). For example, when Mo is not available *Azobacter* used V to create a less efficient form of the nitrogenase protein; when Mo became available, it inhibited the *vnf* mechanism and resumed its Mo utilization (Robson et al., 1986). In addition, other environmental conditions and nutrient (among them N, P and O_2) concentrations are critical signals for the regulation of *nif* gene expression in most of the diazotrophs.

The extremely high energy demand of N_2 fixation (about 40 moles of ATP for the reduction of one mole of N_2 to ammonia) requires efficient regulation of *nif* (Witz et al., 1966). The expression of N_2 fixation genes is controlled by cascades of hierarchically organized regulatory genes which enable the diazotrophs to sense environmental conditions required for N_2 fixation and transmit this information to their gene expression. For example, *Azospirillum lipoferum* is able to regulate its nitrogenase activity at the posttranslational level in response to the presence of combined N (Ludden et al., 1989). While such a control is based on the reversible ADP-ribosylation of nitrogenase reductase, a similar mechanism that can switch on and off the nitrogenase gene was identified in *A. caulinodans* (Kush et al., 1985). Diazotrophs that cannot adjust to new conditions would disappear causing a community shift toward more fit

organisms; thus, their community composition may infer something about their environmental conditions.

In the past, the Polymerase Chain Reaction (PCR) was used extensively as a molecular approach to amplify the 16S rDNA of many bacteria and helped characterization of their composition and evolutionary relationships. Although this approach established their relative diversity in general, in most cases its use as a marker did not identify a specific diazotrophic cyanobacteria. In his works, Zehr used *nifH* genes to identify several probes and primers that were able to target the subunits of the nitrogenase gene (Zehr and McReynolds, 1989). Although *nifH* of cyanobacteria clustered closely together and were expected to facilitate the design of specific PCR primers, it was not possible to design primers for specific cyanobacterial *nifH* genes (Zehr and McReynold, 1989). Later, Henson et al., (2002) used complete *nifD* sequences to evaluate differentiation between two genera of heterocystous cyanobacteria (*Nostoc* and *Anabaena*) and concluded that they were indeed separated. Later, during the development of molecular techniques that would detect N_2 fixing cyanobacteria in environmental samples (complex microbial community), it was found that gene sequences of *nifD* contained conserved regions that permited the design of PCR primers specific for cyanobacterial *nifD* (Roselers et al., 2007). For the above reason, we decided to use *nifD* primers to specifically identify spatial and temporal distribution of diazotrophs in complex microbial communities that may be dominated by cyanobacteria.

Cyanobacteria

Cyanobacteria are gram negative bacteria that have a thicker peptidoglycan layer, although their polysaccharides are similar to those of gram positive cells (Weckesser and Jurgens, 1998). Although unicellular organisms, the majority of cyanobacteria are aerobic photoautotrophs and their morphology covers unicellular, colonial and multicellular filamentous forms. Even though,

photosynthesis is their principal mode of energy metabolism and C source, some species were able to survive long periods in complete darkness, and others exhibit diverse heterotrophic nutrition patterns (Fay, 1965). Their oxygenic photosynthesis activity is responsible for the majority of dissolved oxygen (DO) in their water column (especially in the absence of macrophytes) due to low diffusion rate of atmospheric O_2 into water.

Cyanobacteria are superior competitors under eutrophic conditions for several reasons and frequently thrive in nutrient-enriched waters (Vincent, 1987). Among the major reasons that could explain their success are: a) capable of optimal growth under high light conditions; b) prefer vertically stratified (via either temperature or salinity) conditions (Paerl et al., 1985); c) many genera (including pests) are buoyant (Reynolds 1987; Reynolds and Walsby, 1975); d) their growth rate is relatively slow, making them favorable under long-residence time conditions (Paerl, 1998). Although extensively studied, classification of many members of the cyanobacteria has not been completed (Litvaitis, 2002).

The phylum Cyanobacteria branches into five taxonomic subsections based mostly on morphological characteristics. The simplest forms are unicellular while the more complex are highly structured and may exhibit morphology of branching filaments and even differentiation into specialized cells. Out of the five major subsections, two are those having vegetative cells versus those that form heterocysts. Subsection I is composed of unicellular cyanobacteria that reproduce by budding or binary fission. Subsection II is composed of unicellular cyanobacteria as well but reproduce by internal multiple fissions. Subsection III is composed of non-heterocystous diazotrophs that form filaments and reproduce by binary fission with unbranched trichomes. Both subsection IV and V are composed of filamentous strains of heterocystous diazotrophs that reproduce by binary fission. Subsection IV reproduce in one plane without true

branching of trichomes, while subsection V reproduce in more than one plane, forming “true branching” (Rippka et al., 1979; Castenholz & Waterbury, 1989). Both subsections IV and V have the ability to differentiate their cells into specialized N_2 compartments called heterocysts. These specialized cells enabled N_2 fixation during the day and under aerobic conditions.

Heterocystous Cyanobacteria

The heterocystous structure protects the O_2-sensitive nitrogenase from O_2 that may enter from the environment, or from nearby vegetative cells that produce O_2 under photosystem II. In general, heterocysts are larger than vegetative cells and although their differentiation results from change in gene expression, their frequency may not be solely related to nutrient limitation (Vintila and El-Shehawy, 2007). Outside their cell wall, the heterocyst structure consists of three layers which form a hydrophobic barrier that prevent O_2 diffusion. Like other vegetative cells, heterocystous cells can store N in specialized polymers (cyanophycin), yet in addition they contain a honeycomb membrane that exhibits high respiratory activity (Murry et al., 1981); as a result of higher respiration, O_2 is depleted rapidly, and more ATP is synthesized for N reduction (Fay, 1992).

Their polar region, where they join vegetative cells, is narrow relative to other cells, and the outer cell membrane surrounds the entire filament to permit access of other cells to the periplasm space (Wolk, 1968; Drews and Weckesser, 1982); by using this structure, other members of the filament (vegetative cells) supply needed substrate generated by photosynthesis. It is possible such transfer between cells of the same filament was the evolutionary path to a multicellular organism. Heterocystous cells cannot photoreduce CO_2 and use RPP (reductive pentose phosphate) pathway to supply C skeletons for assimilation of fixed N, because they lack photosystem II activity and ribulose bisphosphate carboxylase (Wolk, 1982). Different levels of labile N (most common are, ammonium, nitrate, nitrite, and urea (Florres and Herrero, 1994))

inhibit heterocyst differentiation, although concentration thresholds vary by species. For example, differentiation of *Anabaena* sp. PCC 7120 under ammonium concentration of 3 to 7 μM is inhibited.

Nostocaceae

Anabaena sp. PCC 7120 is probably the most well-studied heterocyst-forming cyanobacteria. Other important heterocystous cyanobacteria are: *Anabaenopsis, Nodularia, Cylindrospermum, Cylindrospermopsis, Scytonema, Calothrix*, and *Fischrella* (Rippka et al., 1979). *Cylindrospermopsis* appeared in many aquatic systems in the past 30 years, and their increased production was associated with long periods of growth in high light intensity conditions (Dyble et al., 2006). *Microcystis* and the genus *Anabaena* are among the most important cyanobacteria in many other eutrophic Florida lakes (e.g., Lake Apopka, Griffin and Okeechobee (Chapman and Schelske, 1997; Cichra, 1995)); in these systems, heterocysts were the predominant sites for N_2 fixation and their establishment was probably regulated by several variables including external fixed N_2. As mentioned above, cyanobacteria are not the only diazotrophs in aquatic systems.

Proteobacteria

Several members of the phylum *Proteobacteria* that carry the nitrogenase enzyme were found in aquatic systems. The species *Rhodospirillum centenum* (also known as *Rhodocista centenaria*) is a member of the class *alphaproteobacteria* that is capable to fix N_2 under aerobic growth conditions. We chose *R. centenum* as the root in the construction of phylogetic analysis, because it clustered outside of the cyanobacterial sequences. As heterotrophic, they could metabolize a unique set of C sources, yet could not use C-4 dicarboxylic acids as a C source. On the other hand, Fogg (1969) found that most cyanobacteria that formed blooms prefer organic matter (OM) enriched conditions, including OM that comes from agricultural runoff, urban

wastewater and soil erosion products (Paerl, 1988a). Many of the above mentioned conditions are related to increased rates of urbanization, and affected many aquatic systems in Florida including the St. Johns River (SJR) watershed (Hendrickson and Konwinski, 1998). In fact, US Southeast riverine and estuarine waters (including the SJR) are considered to be both nutrient-enriched and N-limited (due to low N:P ratio), which favor cyanobacterial dominance in the phytoplankton community (Smith, 1983).

Lake George

Historically, around 1900, macrophytes (water hyacinth) dominated and covered great areas of the St. Johns River (SJR) in Florida. Later, the US Army Corps of Engineers used concentrated spraying of herbicides around 1940 to control them (Simberloff and Schmitz, 1997). The adverse effects of decayed macrophytes and introduced herbicides into the system resulted in the release of nutrients back into the water column that promoted algal proliferation (also, due to less competition with macrophytes for nutrient acquisition) (Moody, 1970). In addition, without macrophytes, more heat and light could penetrate greater areas of the SJR providing better conditions and energy to cyanobacteria that became dominant. This was seen in many waterbodies including Lake George, which is the biggest water body on the SJR.

The composition of diazotrophs in Lake George is of importance due to the coupling of their activity to nutrient levels (mostly P) and their impact on the N budget. In addition, their distribution may imply different inflow sources and niches in the lake and possible relationships with other members of the lake community. Dominance of heterocystous cyanobacteria reinforced expected conditions of relatively high dissolved oxygen (DO) levels and light penetration through most of the water column. Distribution of non-heterocystous diazotrophs is more effected by phototrophic activity that introduced DO into water and possible association with primary producers (especially true for heterotrophs).

Studies conducted to understand the composition and function of phytoplankton community at Lake George have focused on measuring limnological parameters, enzyme assays (e,g. photosynthesis), and characterizing community composition using microscopic analysis. Because cyanobacteria are located at the base of the food web, changes in nutrient content and taxonomic composition may be used as an early indicator of trophic state and nutrient limitation. In addition, documenting the compositional changes in diazotrophic assemblages in response to temporal and varying nutrient concentrations is important in understanding similar systems and enables their comparison.

Thus far, only microscopic methods have been used to identify the diazotrophic community in Lake George and correlate it to seasonal and environmental conditions. Use of molecular approaches is expected to permit greater resolution inside families or genera and identify organisms not identified by microscopy. This work identifies the genetic potential and variation of diazotrophs in an attempt to investigate how environmental factors affect their community composition and how their composition determines N_2 fixation at Lake George. We hypothesized that *nifD* diversity will vary with nutrient levels that are mostly effected by temporal changes such as precipitation, temperature, wind, salinity, lake water residence time and light intensity.

Materials and Methods

Site Description and Sample Collection

Lake George is approximately 21,000 hectares in size, making it the second-largest freshwater lake in Florida, and the largest on the SJR (Figure 3-1). Lake George is shallow relative to its size, and a thermocline seldom forms in the lake, and its water dynamics appear to be dominated by subtidal variability of the Atlantic Ocean water level (Morris, 1995). Between the years 1996 to 2005, the SJR Water Management District (SJRWMD) estimated the mean

turnover rate of the lake to be 84 days. They concluded that peaks in algal biomass were partially controlled by flushing, and that water quality was significantly influenced by local groundwater sources entering by springs.

Integrated water samples were collected weekly from nine locations in Lake George during the summer of 2008 (22 weeks starting April 8 to September 2). The sites represented the range of water chemistry parameters at Lake George. Water samples were transferred in three 1-L carboys to the lab where they were homogenized and split into three samples, based on sites, and vacuum filtered through 0.7 μm glass fibre prefilters (Millipore CAT No. APFF04700) to concentrate water colmn biomass . Filters were frozen at -20^{o}C until DNA extraction.

DNA Extraction

Each filter was first thawed on ice, then its contents were washed with double distilled water or Tris-acetate-EDTA (TAE) buffer into an Eppendorf tube, and centrifuged to keep precipitate. DNA was extracted from approximately 0.05 g of precipitate using an UltraClean Plant DNA Isolation Kit (MoBio, Solana Beach, CA; catalog # 13000- 50) and following the kit instructions. Extracted DNA was divided into two; one half was stored at -20^{o}C and the second half was combined with 50% ethanol and stored in -80^{o}C until further analysis.

Amplification of nifD by Polymerase Chain Reaction (PCR)

PCR amplification was conducted using a degenerated primer set that flanks a conserved region of the *nifD* gene (from position 552 to 861 in the *nifD* sequence of *Anabaena cylindrica* PCC 7122, AF442506) and designed by Henson et al., (2002). The nucleotide sequences for the primers we used were: forward primer nifD552-F, 5′ TCCGKGGKGTDTCTCAGTC 3′; and reverse primer nifD861-R 5′ CGRCWGATRTAGTTCAT 3′ (MWG Biotech, Huntsville, AL).

The reaction mixture used for PCR amplification was composed of 25 µl GoTaq Green Master Mix (Promega, Medison, WI), 1 µl of each primer (100 pmol μl^{-1}), 13 µl of distilled water, and 10 µl of diluted DNA solution. PCR amplification was done using an iCycler thermal cycler (BIORAD, Hercules, CA) with the following conditions: initial enzyme activation and DNA denaturation of 10 min at 95°C, followed by 35 cycles of 1 min at 95°C for denaturation, 1 min at 52°C for annealing, and 1 min of extension at 72°C, with a final extension of 72°C for 7 min. After PCR, products were evaluated on 1.5% (wt/vol) agarose gel made in TAE buffer (Sambrock et al., 1989) to confirm the expected fragment size (~ 310 bp). For cloning, pGEM®-T and pGEM®-T Easy Vector Systems (Promega WI) were used with a small modification: ligation reaction mix volume was 12 µl (instead of 10) and contained 6 µl of 2X Rapid Ligation Buffer (instead of 5) and 4 µl of fresh PCR-amplicons (instead of 3). Reaction mix was ligated into pCRII-TOPO cloning vector and transformed into chemically competent XL10-Gold® Ultracompetent Cells (Stratagene, CA) according to the manufacture's protocol. Inserts within white colonies were evaluated by PCR amplification using the same primer set and PCR protocol described earlier, and their size was confirmed by agarose gel electrophoresis.

Sequencing and Phylogenetic Analysis

Four sampling dates (4/22, 7/22, 8/19 and 9/02/08) were chosen for phylogenetic analysis based on measured nitrogenase activity (NA, see Chapter 2 for details) A total of 12 clones (three per day) were sequenced at the DNA Sequencing Core Laboratory at the University of Florida using internal vector primers (M13). Each DNA sequence of *nifD* was compared to recorded sequences (GeneBank) from previous studies using BLAST queries (http://www.ncbi.nlm.nih.gov) and revealed that only 11 clone libraries were acceptable. Next,

the sequences were aligned with related sequences and one outgroup using ClustalX2 (Larkin et al., 2007).

For community analyses, operational taxonomic units (OTUs) were generated using DOTUR under furthest neighbor algorithm and a threshold of 10% difference in nucleic acid sequences. The frequency of each OTU was used to construct a rarefraction analysis by comparing obtained versus cumulative expected phylotypes and was used to evaluate its diversity and richness. Thus, non-parametric estimates of richness and diversity were evaluated using DOTUR (Schloss and Handelsman, 2005) and included OTU, Chao1, Shannon index, and Simpson index (calculated using default parameters of the program DOTUR).

Phylogenetic trees were conducted using *MEGA* version 4 (Tamura et al., 2007) with a neighbor joining analysis using a Tamura 3-parameter method for distance estimation of bootstrap analysis. Our selected outgroup was *Rhodospirillum centenum*, which is a non-sulfur purple photosynthetic bacterium that prefers to grow in anoxic zones. ∫-Libshuff (Schloss et al., 2004) was employed to evaluate whether large differences observed between diazotrophic communities in chosen dates represent statistically different populations. The program ∫-Libshuff evaluates community relationship (the existence of individuals in each community), and is considered to be less sensitive to library size than similar tests (Schloss et al., 2004).

Well-aligned *nifD* sequences were used to construct a distance matrix, using Jukes-Cantor corrected pairwise distance, by PHYLIP suite (Felsenstein, 2004). Next, the analysis was done using ∫-Libshuff (Schloss et al., 2004) with the Monte Carlo method and 10,000 permutations to calculate the integral form of the Cramérvon Mise statistic by constructing random sub-set populations from the entire data set and comparing the coverage of the generated populations to coverage in the experimentally obtained data set. Populations were considered significantly

different with P value below 0.01 after a Bonferroni correction for multiple pairwise comparisons (α=0.05, n = 132).

Evaluation of environmental parameters that may affect community composition was performed using the Mantel test (Mantel, 1967; Mantel and Valand, 1970). This test estimates correlations between observed differences in *nifD* diversity between transects and measured environmental parameters of chosen dates to acquire an understanding of factors that may control community composition in Lake George. Mantel test is executed in R (R Development Core Team, 2008) using the package Vegan (Oksanen et al., 2010) and is based on a nonparametric general regression model which employs squared Euclidean distance matrices between variables to test significance of and degree of predictability one variable has on another (Dutilleul et al., 2000).

Eleven *nifD* clone libraries with 198 clones were chosen to represent the diazotrophic community composition during the summer of 2008 (Figures 3-2 to 3-12; Table 3-1). On the third week of study (4/22), 22 clones were selected from the eastern and central transects and 21 from the western transect and were sent to sequence (Figure 3-14). Unifrac PCA analysis of clone libraries (Lozupone and Knight, 2005) was used to evaluate diazotrophic community composition at different dates and sites in order to establish their relationships (Figure 3-15). The clone library that was constructed from the central sites on 9/02 contained only several inserts that were doubtable sequences (possibly chimeras) and were discarded.

Results

The number of groups of operational taxonomic units (OTU) varied between sites but was not significantly different and showed no trends. Based on rarefaction analysis that used the numbers of clones per OTU, a nearly complete coverage of diazotroph diversity in all clone libraries was reached when defining 90% similarity of sequences as comprising an OTU (Figure

3-13). All indexes including the number of OTUs varied between sites and dates (Table 3-1). Three indexes were used to rank community diversity. Shannon diversity index was chosen to estimate the diversity and evenness of the species (increased by unique species, or greater species evenness). Simpson evenness estimation predict the probability that two randomly selected clones belong to the same species (accentuates abundant diazotrophs thus less affected by sample size). The Chao1 richness estimator is particularly useful for data sets that are skewed toward low-abundance classes (Chao, 1984) (Table 1).

Phylogenetic analysis of individual sequences cloned from PCR products identified distinct lineages of bacteria that possessed a *nifD*. The majority of clones were related to cyanobacterial species (mostly filamentous and few unicellular) while the most diverse were heterocystous clustered within the family *Nostocaceae* (subsection IV). Only several free living proteobacteria that might be associated with phytoplankton or with invertebrates and sediments were identified. Our phylogenetic analysis of *nifD* was consistent with *nifD* literature that was relatively similar to cyanobacterial *nifH* and 16S rDNA gene sequences (Henson et al., 2003; Givoannoni et al., 1988; Roeselers et al., 2007).

During the entire study period, *nifD* diversity was the highest in the east transect on the beginning (4/22) and end (9/02) of study. Although both dates were relatively highly diverse (8 and 7 OTUs, respectively), than in the rest of the lake, they were quite different: on April the entire clone library clustered within heterocystous cyanobacteria, while in September it clustered also within *Rhodospirillum* (bootstrap value of 99) and the gram positive order *Actinomycetales*. As mentioned, most clones clustered within cyanobacteria in two major clades: the bigger cluster was highly similar to *Anabaena cylindrical* PCC 7122 (93% similarity to known sequences in the

database), while the other to *Calothrix* sp. 7101 (84% similarity to known sequences in the database).

In some samples like the one taken from east transect on 8/19, one clone was believed to be unicellular cyanobacteria from the family *Pleurocapsales* (subsection II) and more specifically the order *Chroococcidiopsis* (bootstrap value 99); on the other hand, on 7/29, five highly related (bootstrap 100) clones were sequenced from the central transect, and branched inside *Chroococcidiopsis* (bootstrap 98). *Chroococcidiopsis* PCC 7293, closely related here, is a nonheterocystous strain capable of forming specialized "survival" cells (Fewer et al., 2002) that are similar to the akinetes found only in the heterocystous cyanobacteria (Rippka et al., 1979). These clones might be *Microcystis*, (belonging to *Chroococcidiopsis*), even though their sequences showed the highest similarity (84%) to the genus *Leptolyngbya* sp. PCC 7004 which is in the order *Oscillatoriales* (subsection III). This order does not form heterocysts but rather uses different mechanism to fix N_2 under aerobic condition. In fact, in Lake Griffin Florida, the filamentous *Oscillatoriales* outcompeted other cyanobacteria and dominated the diazotrophic community between 2001-2002 (Frost, 2005).

On 9/02, three clones from the west transect clustered close to the genus *Trichodesmium* (also from the order *Oscillatoriales*) but with low bootstrap value (29). These sequences showed high similarity to *Calothrix* sp. 7101 and *Anabaena cylindrical* PCC 7122 (84% and 83% similarity to known sequences in the database). In addition, one clone clustered outside the cyanobacteria (bootstrap 87), probably within the *Proteobacteria* or within the order of the gram positive *Actinomycetales* (due to its clustering in proximity to *Frankia).* Another interesting clone was found on the west transect on 7/29; which despite having only 5 OTUs, they belonged to different groups: in addition to the three clusters of cyanobacteria (one appeared as a

unicellular) and the *Actinomycetales*, one clone was highly related to our outgroup root *Rhodospirillum*. Surprisingly at this day, the center transect had four OTUs (heterocystous and unicellular cyanobacteria), but the east transect had only three OTUs, which were composed solely of heterocystous cyanobacteria; it was the lowest number calculated (assuming the diversity in the east transect on the 7/29, which was also equal to three, was higher than captured).

According to the Mantel Test, the following environmental parameters were positively correlated to community composition particulate organic N (PON), total N (TN), total Kjeldahl N (TKN) and chlorophyll-*a* (*chl-a*) (Table 3-3). Results from Unifrac PCA analysis of *nifD* clone libraries suggests that community composition is more affected by seasonality than location (Figure 3-15). Comparisons of *nifD* DNA gene libraries done by ∫-Libshuff suggest that diazotrophic communities in Lake George were not significantly different from each other, except in two cases: the first in the west transect on 4/22 and 8/19 and the second between 8/19 west and 9/02 west (Table 3-2).

Discussion

DNA-based molecular characterization of *nifD* showed that the majority of sequences were distributed amongst cyanobacterial clades. More specifically, most clones showed similarity to filamentous heterocystous cyanobacteria and clustered in two main groups that appeared in all samples. A distinct group of cyanobacteria clustered within unicellular but were found only in several samples. According to nucleotide sequence, the potential diazotrophic cyanobacterial genera that were the most related were *Anabaena* PCC 7120, *Calothrix* sp., *Chroococcidiopsis* sp. and *Leptolyngbya* sp. This domination of cyanobacteria was expected in eutrophic systems like Lake George, due to nutrient levels and subtropical climate (Vincent, 1987). In addition, the

lake's shallow water, which permitted light penetration through most of its water column, and the small amount of macrophytes, favored cyanobacterial growth.

Several clusters remained unidentified but the fact they clustered within the branch of cyanobacteria suggest they may be novel groups of diazotrophic cyanobacteria. A possible source for this variation can be due to gene duplication and a secondary nitrogenase system that some cyanobacterial generas carry (Young, 1992). These additional systems evolved possibly due to a lateral transfer or gene duplication. It was not clear how these systems are distributed, but such a system was found in vegetative cells of *Anabaena variabilis* strain ATCC 29413 and showed similarity to *nif* of *Anabaena* PCC 7120 or to non-heterocyctous cyanobacteria (Thiel, 1993; Thiel et al., 1995). For this reason we can not conclusively identity those cyanobacterial clones, especially the ones showing low similarity to known sequences.

The other diazotrophs were not observed using microscopy, but most of their clones clustered close to *Actinobacteria* and *Alphaproteobacteria* and showed high similarity to their specie level (*Rhodosperillium* sp. and *Frankia* sp.). *Rhodospirillum* forms colonies that migrate toward or away from light, depending on the wavelength, by using surface-induced lateral flagella, chemotaxis, and a photosynthetic apparatus (Jiang et al., 1997). Both *Rhodosperillium* and *Frankia* may be inhabitants of the lake that associate with other phytoplankton. Among them are heterotrophs that might be important to nutrient cycling and readily available organic C sources. On the other hand, if some entered the system recently, they may be used to trace water sources or give information about their origin type (ex, leaching) or micro niches (ex, anoxic zone for anaerobic diazotrophs).

Because of their lower energetic levels compared to primary producers, and their sensitivity to UV radiation and DO in the water, heterotrophic organisms probably do not

account for a major portion of the fixed N_2 in the lake. Thus, an ideal time for their fixation is during night (dark conditions) after photosynthesis, the main source of DO in the water, has ceased as respiration and bacterial decomposition have lowered remaining DO levels. Due to Lake George shallow water and its possible mixing by wind, some heterotrophs may be connected to sediment or benthic layer that can provide them with UV protection and possibly a DO gradient. Regardless of their quantitatively small contribution to Lake George N budget, they should be further studied, because they may be more sensitive to environmental changes than cyanobacteria; thus, understanding of these organisms might reveal their role as indicators of the lake condition or its phytoplankton dynamics. The fact that they were harder to cultivate and identify using microscopy, favored a molecular approach for their study.

After the examination of the diazotrphoic community composition at all clone libraries, the results confirmed the presence of an active phototrophic and heterotrophic diazotrophic community. This suggested a heterogeneous community that evolved to fit Lake George and some of its different niches. It is likely that heterotrophic metabolic processes done by diazotrophs were strongly tied to phototropic activities that supplied them with photosynthate. Nevertheless, classification of some *nifD* clones into a strong phylogeny were not supported by high bootstrap values, mostly because they showed low similarity to sequences associated with previously characterized organisms. This limited the confidence of our characterization, but since in most cases the clones formed strong clusters, their existence could not be ignored.

Similar to other ecological studies, the amplified *nifD* sequences supplied high taxonomic resolution in our study of Lake's George diazotrophic community. Although *nifD* was more suited to characterization of cyanobacteria, GeneBank was lacking *Cylindrospermopsis raciborskii* sequences. This fact hindered our ability to differentiate between the two members of

the family *Nostocaceae* (*Cylindrospermopsis* and *Anabaena*) that were the most abundant according to microscopic analysis (Chapter 2). Clones that clustered under cyanobacteria showed varying degree of similarity to known organisms.The most diverse clade was related to *Anabaena* PCC 7120 and, in few samples, was divided into several related sub-clades, which was believed to include both *Anabaena* and *Cylindrospermopsis*. The second largest clade branched outside *Nostocaceae* and showed similarity with *Calothrix*. The third cyanobacterial clade was observed only twice and clustered within the unicellular group that was related to *Chroococcidiopsis*, but might actually be *Microcystous* (according to microscopy). Several studies of unicellular non-heterocystous cyanobacteria demonstrated that some can fix N_2 under fully oxic conditions and while oxygenic photosynthesis was taking place by employing different strategies to protect their nitrogenase enzyme during daytime. For example, some diazotrophs use temporal separation, as their main strategy, to fix N_2 during the day (Stal, 1995; Fay, 1992).

The results demonstrated the genetic dynamics and potential of diazotrophic diversity was not very high as supported by ∫-Libshuff analysis that demonstrated that most communities were not significantly different. Currently, we could not relate actual N_2 fixation rates to each organism or clone. Such measurements can be beneficial and might explain the shift in community and role of its members. This analysis can be performed by Real Time Polymerase Chain Reaction (RT-PCR) using *nifD* primers, preferably combined with *nifH*; by comparing both results under different conditions, our analysis would show the organisms that have the potential to fix N_2 (i.e., posses nitrogenase genes), while RT-PCR would identify the ones that were active (i.e., expressing nitrogenase).

Table 3-1. Values of *nifD* diversity and richness in Lake George water, as estimated by Shannon diversity index, Simpson index, and Chao1 richness calculated using DOTUR (Schloss and Handelsman, 2005).

Date	Site	No. of clones sequenced	No. of OUT's	Shannon index	Diversity	Richness
4/22/2008	East	22	8	1.3 (0.9, 1.7)	0.4	7.5 (6, 21)
4/22/2008	West	21	5	1.3 (1, 1.6)	0.3	6 (5, 19)
4/22/2008	Center	22	4	1.1 (0.7, 1.5)	0.5	5 (4, 17)
7/29/2008	East	8	3	0.7 (0.1, 1.4)	0.5	4 (3, 16)
7/29/2008	West	17	5	1.2 (0.6,2)*	0.4	6.5 (5, 20)
7/29/2008	Center	15	4	1.1 (0.7, 1.5)	0.4	5 (4,17)
8/19/2008	East	21	3	0.9 (0.6, 1.2)	0.5	3 (3, 3)
8/19/2008	West	17	6	1.6 (1.3, 1.9)	0.2	7 (6, 20)
8/19/2008	Center	19	6	1.3 (0.8, 1.8)	0.3	8 (6, 21)
9/2/2008	West	19	5	1.4 (1.1, 1.7)	0.8	5 (5,NA)
9/2/2008	East	17	7	1.7 (1.3, 2.1)	0.8	8 (7, 18)

Table 3-2. Population similarity *P* values for comparison of *nifD* clone libraries determined using Cramer-von Mises test statistic, implemented in ∫-Libshuff (Schloss et al., 2004).

Homologous Library (X)	*P*- Values comparison of heterologous library										
	4/22 East	4/22 Center	4/22 West	7/29 East	7/29 Center	7/29 West	8/19 East	8/19 Center	8/19 West	9/02 East	9/02 West
4/22 East		0.5363	0.3228	0.0894	0.0155	0.4943	0.0443	0.8441	**0.0000**	0.0051	0.0150
4/22 Center	0.2536		0.8145	0.1207	0.0224	0.1458	0.0328	0.8615	**0.0000**	0.0031	0.0084
4/22 West	0.1278	0.0045		0.1229	0.0065	0.0380	0.3659	0.9647	0.0016	0.0002	0.0055
7/29 East	0.1876	0.4110	0.1103		0.2699	0.2878	0.7621	0.8534	0.8851	0.8682	0.7757
7/29 Center	0.0092	0.0216	0.0723	0.3475		0.0600	0.9313	0.5032	0.0057	0.0018	0.0165
7/29 West	0.3743	0.0251	0.1589	0.1749	0.1931		0.1404	0.2473	0.0059	0.0023	0.0280
8/19 East	0.0040	**0.0000**	0.0628	0.2010	0.0257	0.0059		0.8429	0.0048	0.0001	**0.0000**
8/19 Center	0.0006	**0.0000**	0.0021	0.0428	0.0006	**0.0000**	0.1901		0.0057	**0.0000**	**0.0000**
8/19 West	**0.0000**	**0.0000**	**0.0000**	0.0106	**0.0000**	**0.0000**	0.0084	0.2076		0.0957	0.0001
9/02 East	**0.0000**	**0.0000**	0.0002	0.0040	**0.0000**	**0.0000**	0.0039	0.1526	0.0077		0.0022
9/02 West	**0.0000**	**0.0000**	0.0001	0.0055	0.0005	**0.0000**	0.0005	**0.0000**	**0.0000**	0.0002	

Values in bold indicate significant *P* values ($P < 0.004$) after Bonferroni correction for multiple pairwise comparisons. Libraries are distinct from one another if both comparisons (*X* versus *Y* and *Y* versus *X*) are significant. Comparisons were made using ∫-Libshuff (Schloss et al., 2004) with 10,000 randomizations. The margin of error for the *P* value's 95% confidence interval for the *P* values near 0.05 was 0.004.

Table 3-3. Correlation between diazotrophic composition and environmental parameters (using Mantel Test).

Parameter	Mantel statistics (R)	Significance
TKN-T	0.3523	0.018*
Chl-a	0.3993	0.022*
TN	0.3223	0.023*
PON	0.3113	0.029*

Total Kjeldahl nitrogen (TKN-T); chlorophyll-*a* (*chl-a*); total nitrogen (TN); particulate organic nitrogen (PON).
* indicates a statistically significant difference ($P \leq 0.05$).

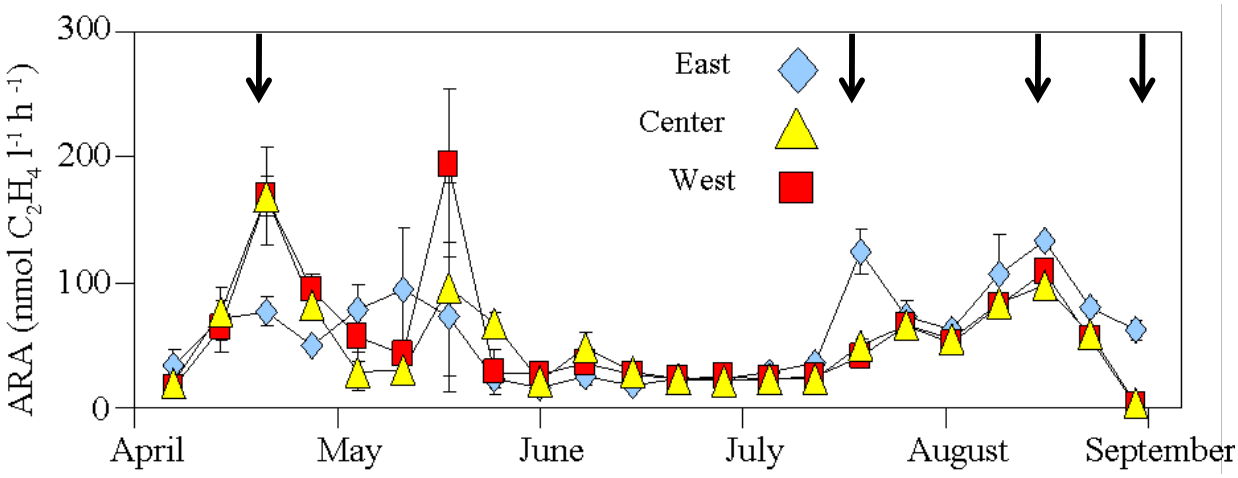


Figure 3-1. Selected dates where samples were obtained for molecular analysis (marked by arrows) based on acetylene reduction assay rate. Three samples were used at each date except to last date (used only the east and west transects).

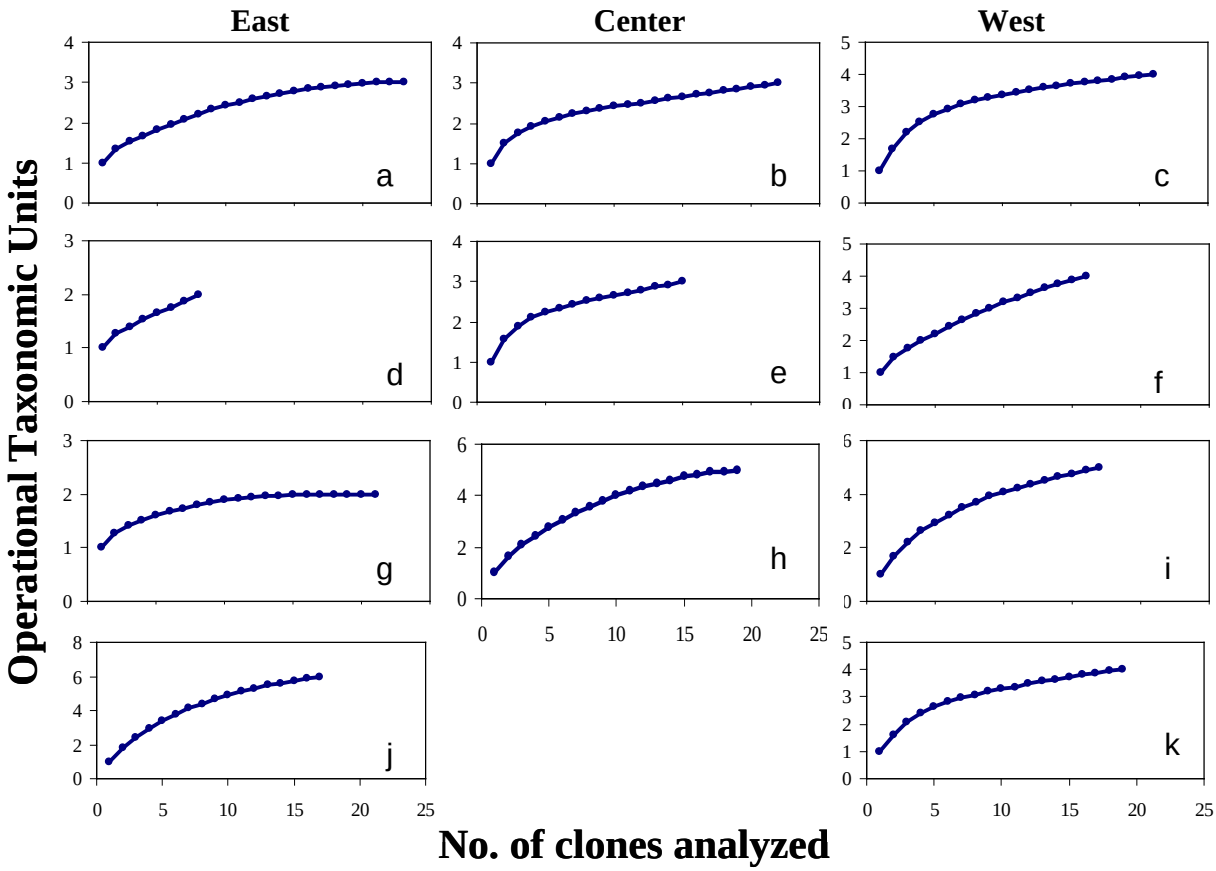


Figure 3-2. Rarefraction analysis for *nifD* collected from Lake George water. A) to C) sampled on 4/22. D to F) sampled on 7/29. G to I) sampled on 8/12. J to k) sampled on 9/22/08. Based on 10% difference.

Figure 3-3. Phylogenetic tree of genomic DNA *nifD* from 4/22/08 east transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.

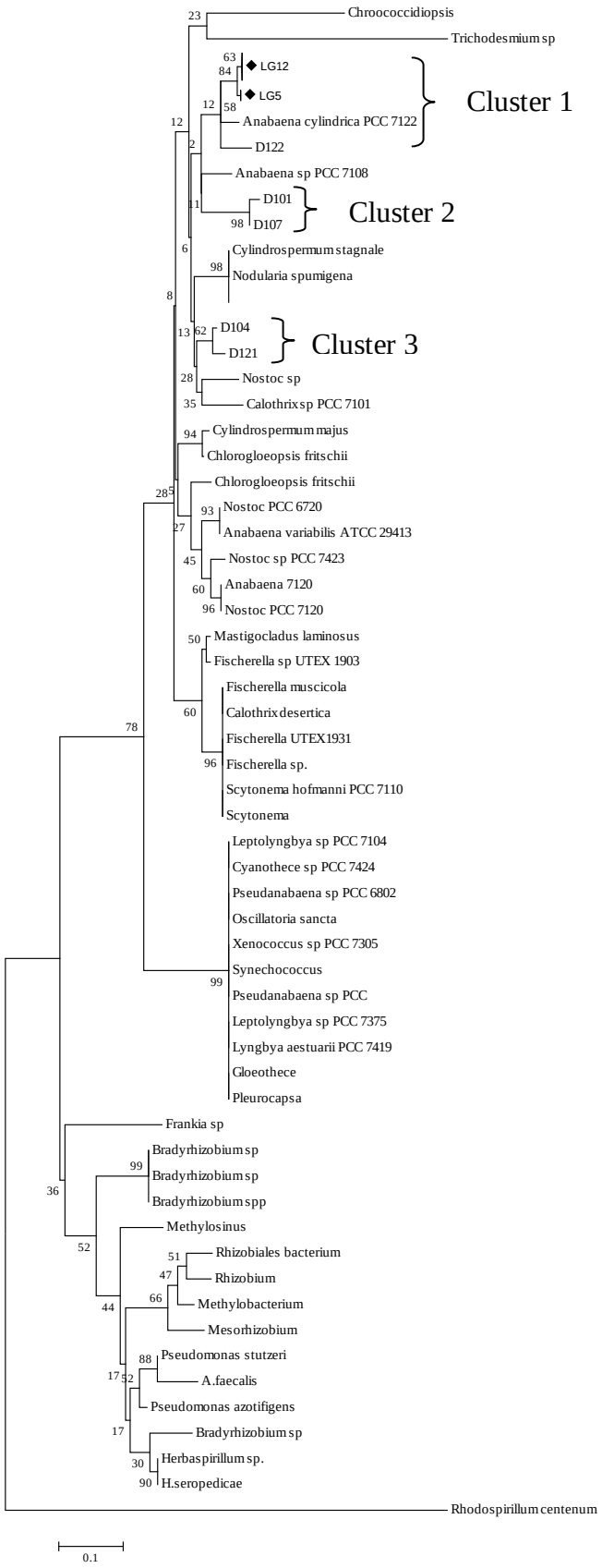

Chroococcidiopsis
Trichodesmium sp
LG12
LG5
Cluster 1
Anabaena cylindrica PCC 7122
D122
Anabaena sp PCC 7108
D101
D107
Cluster 2
Cylindrospermum stagnale
Nodularia spumigena
D104
D121
Cluster 3
Nostoc sp
Calothrix sp PCC 7101
Cylindrospermum majus
Chlorogloeopsis fritschii
Chlorogloeopsis fritschii
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Nostoc sp PCC 7423
Anabaena 7120
Nostoc PCC 7120
Mastigocladus laminosus
Fischerella sp UTEX 1903
Fischerella muscicola
Calothrix desertica
Fischerella UTEX1931
Fischerella sp.
Scytonema hofmanni PCC 7110
Scytonema
Leptolyngbya sp PCC 7104
Cyanothece sp PCC 7424
Pseudanabaena sp PCC 6802
Oscillatoria sancta
Xenococcus sp PCC 7305
Synechococcus
Pseudanabaena sp PCC
Leptolyngbya sp PCC 7375
Lyngbya aestuarii PCC 7419
Gloeothece
Pleurocapsa
Frankia sp
Bradyrhizobium sp
Bradyrhizobium sp
Bradyrhizobium spp
Methylosinus
Rhizobiales bacterium
Rhizobium
Methylobacterium
Mesorhizobium
Pseudomonas stutzeri
A.faecalis
Pseudomonas azotifigens
Bradyrhizobium sp
Herbaspirillum sp.
H.seropedicae
Rhodospirillum centenum
0.1

Figure 3-4. Phylogenetic tree of genomic DNA *nifD* from 4/22/08 center transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.

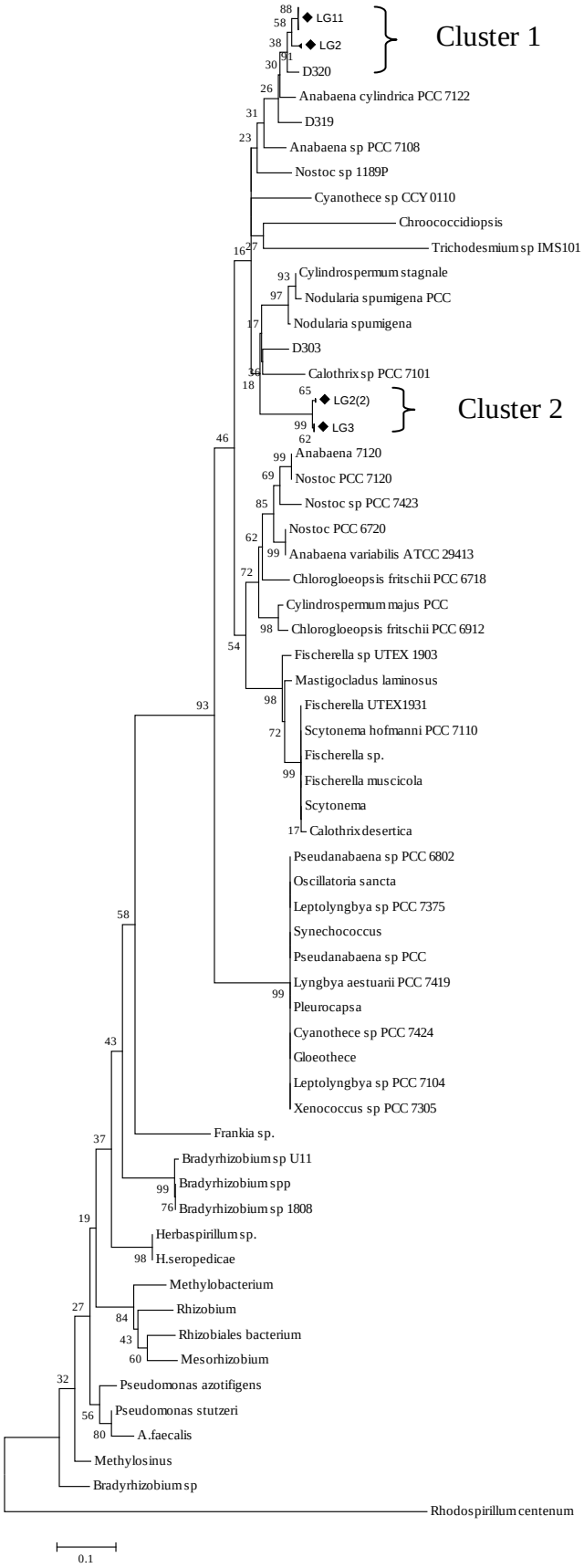

LG11
LG2
D320
Cluster 1
Anabaena cylindrica PCC 7122
D319
Anabaena sp PCC 7108
Nostoc sp 1189P
Cyanothece sp CCY 0110
Chroococcidiopsis
Trichodesmium sp IMS101
Cylindrospermum stagnale
Nodularia spumigena PCC
Nodularia spumigena
D303
Calothrix sp PCC 7101
LG2(2)
LG3
Cluster 2
Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Chlorogloeopsis fritschii PCC 6718
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Fischerella sp UTEX 1903
Mastigocladus laminosus
Fischerella UTEX1931
Scytonema hofmanni PCC 7110
Fischerella sp.
Fischerella muscicola
Scytonema
Calothrix desertica
Pseudanabaena sp PCC 6802
Oscillatoria sancta
Leptolyngbya sp PCC 7375
Synechococcus
Pseudanabaena sp PCC
Lyngbya aestuarii PCC 7419
Pleurocapsa
Cyanothece sp PCC 7424
Gloeothece
Leptolyngbya sp PCC 7104
Xenococcus sp PCC 7305
Frankia sp.
Bradyrhizobium sp U11
Bradyrhizobium spp
Bradyrhizobium sp 1808
Herbaspirillum sp.
H.seropedicae
Methylobacterium
Rhizobium
Rhizobiales bacterium
Mesorhizobium
Pseudomonas azotifigens
Pseudomonas stutzeri
A.faecalis
Methylosinus
Bradyrhizobium sp
Rhodospirillum centenum
0.1

Figure 3-5. Phylogenetic tree of genomic DNA *nifD* from 4/22/08 west transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling

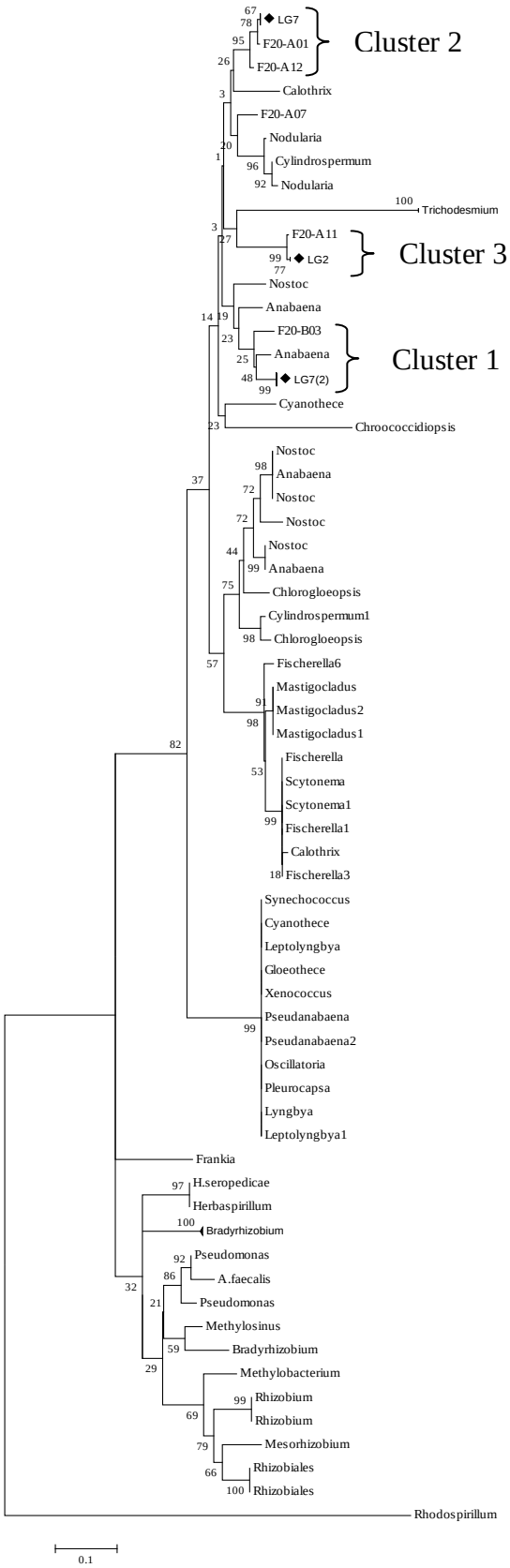

LG7
F20-A01
F20-A12
Cluster 2
Calothrix
F20-A07
Nodularia
Cylindrospermum
Nodularia
Trichodesmium
F20-A11
LG2
Cluster 3
Nostoc
Anabaena
F20-B03
Anabaena
LG7(2)
Cluster 1
Cyanothece
Chroococcidiopsis
Nostoc
Anabaena
Nostoc
Nostoc
Nostoc
Anabaena
Chlorogloeopsis
Cylindrospermum1
Chlorogloeopsis
Fischerella6
Mastigocladus
Mastigocladus2
Mastigocladus1
Fischerella
Scytonema
Scytonema1
Fischerella1
Calothrix
Fischerella3
Synechococcus
Cyanothece
Leptolyngbya
Gloeothece
Xenococcus
Pseudanabaena
Pseudanabaena2
Oscillatoria
Pleurocapsa
Lyngbya
Leptolyngbya1
Frankia
H.seropedicae
Herbaspirillum
Bradyrhizobium
Pseudomonas
A.faecalis
Pseudomonas
Methylosinus
Bradyrhizobium
Methylobacterium
Rhizobium
Rhizobium
Mesorhizobium
Rhizobiales
Rhizobiales
Rhodospirillum
0.1

Figure 3-6. Phylogenetic tree of genomic DNA *nifD* from 7/29/08 east transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.

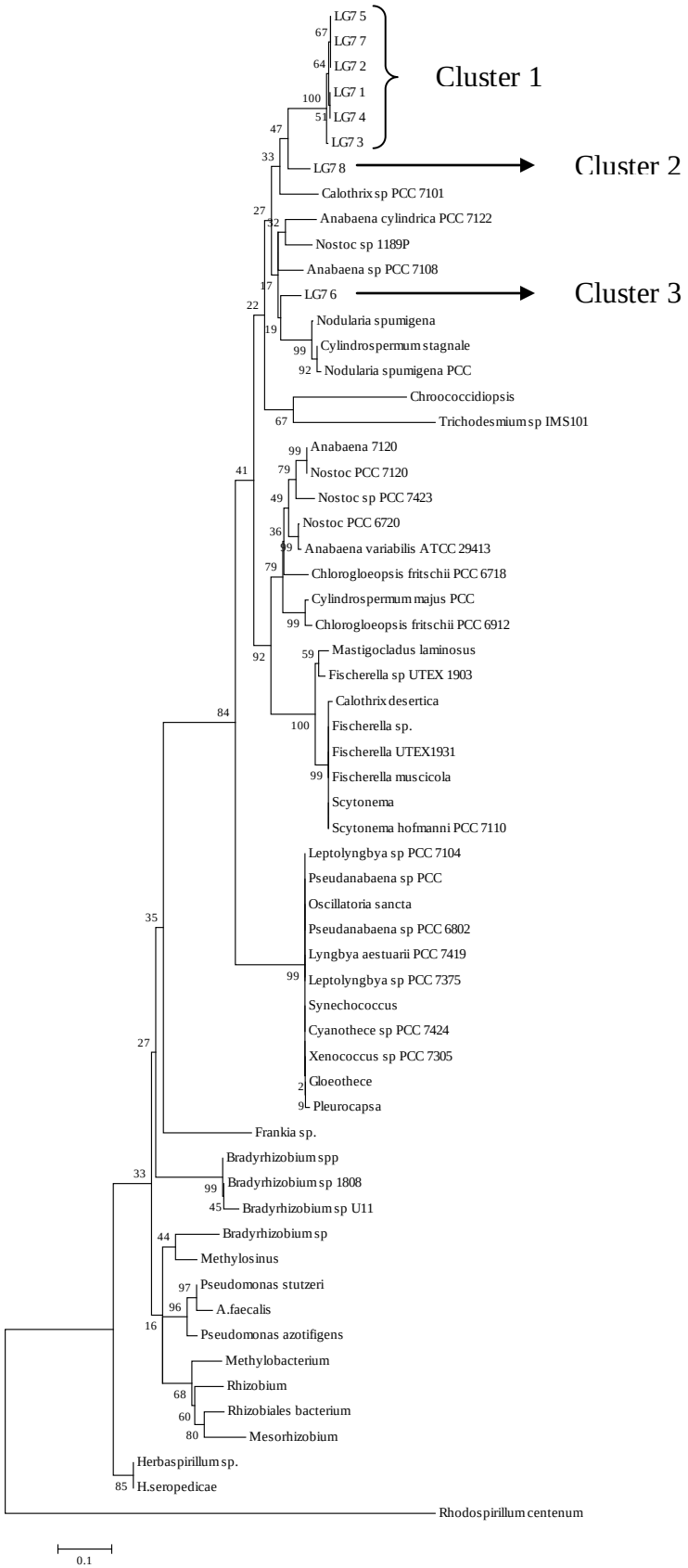

LG7 5
LG7 7
LG7 2
LG7 1
LG7 4
LG7 3
Cluster 1
LG7 8
Cluster 2
Calothrix sp PCC 7101
Anabaena cylindrica PCC 7122
Nostoc sp 1189P
Anabaena sp PCC 7108
LG7 6
Cluster 3
Nodularia spumigena
Cylindrospermum stagnale
Nodularia spumigena PCC
Chroococcidiopsis
Trichodesmium sp IMS101
Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Chlorogloeopsis fritschii PCC 6718
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Mastigocladus laminosus
Fischerella sp UTEX 1903
Calothrix desertica
Fischerella sp.
Fischerella UTEX1931
Fischerella muscicola
Scytonema
Scytonema hofmanni PCC 7110
Leptolyngbya sp PCC 7104
Pseudanabaena sp PCC
Oscillatoria sancta
Pseudanabaena sp PCC 6802
Lyngbya aestuarii PCC 7419
Leptolyngbya sp PCC 7375
Synechococcus
Cyanothece sp PCC 7424
Xenococcus sp PCC 7305
Gloeothece
Pleurocapsa
Frankia sp.
Bradyrhizobium spp
Bradyrhizobium sp 1808
Bradyrhizobium sp U11
Bradyrhizobium sp
Methylosinus
Pseudomonas stutzeri
A.faecalis
Pseudomonas azotifigens
Methylobacterium
Rhizobium
Rhizobiales bacterium
Mesorhizobium
Herbaspirillum sp.
H.seropedicae
Rhodospirillum centenum
0.1

Figure 3-7. Phylogenetic tree of genomic DNA *nifD* from 7/29/08 center transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.


LG5
LG3 9
LG3 2
LG3 8
Cluster 1
Calothrix sp PCC 7101
LG3 15
Cluster 2
Nostoc sp 1189P
Anabaena sp PCC 7108
Anabaena cylindrica PCC 7122
Nodularia spumigena
Cylindrospermum stagnale
Nodularia spumigena PCC
Trichodesmium sp IMS101
Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Chlorogloeopsis fritschii PCC 6718
Mastigocladus laminosus
Fischerella sp UTEX 1903
Scytonema
Fischerella muscicola
Scytonema hofmanni PCC 7110
Fischerella UTEX1931
Fischerella sp.
Calothrix desertica
Chroococcidiopsis
LG5
Cluster 3
Leptolyngbya sp PCC 7375
Oscillatoria sancta
Leptolyngbya sp PCC 7104
Xenococcus sp PCC 7305
Synechococcus
Cyanothece sp PCC 7424
Gloeothece
Pseudanabaena sp PCC
Lyngbya aestuarii PCC 7419
Pseudanabaena sp PCC 6802
Pleurocapsa
Frankia sp.
Rhizobiales bacterium
Mesorhizobium
Rhizobium
Methylobacterium
Bradyrhizobium sp 1808
Bradyrhizobium sp U11
Bradyrhizobium spp
Methylosinus
Bradyrhizobium sp
Herbaspirillum sp.
H.seropedicae
Pseudomonas stutzeri
A.faecalis
Pseudomonas azotifigens
Rhodospirillum centenum
0.1

Figure 3-8. Phylogenetic tree of genomic DNA *nifD* from 7/29/08 west transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling

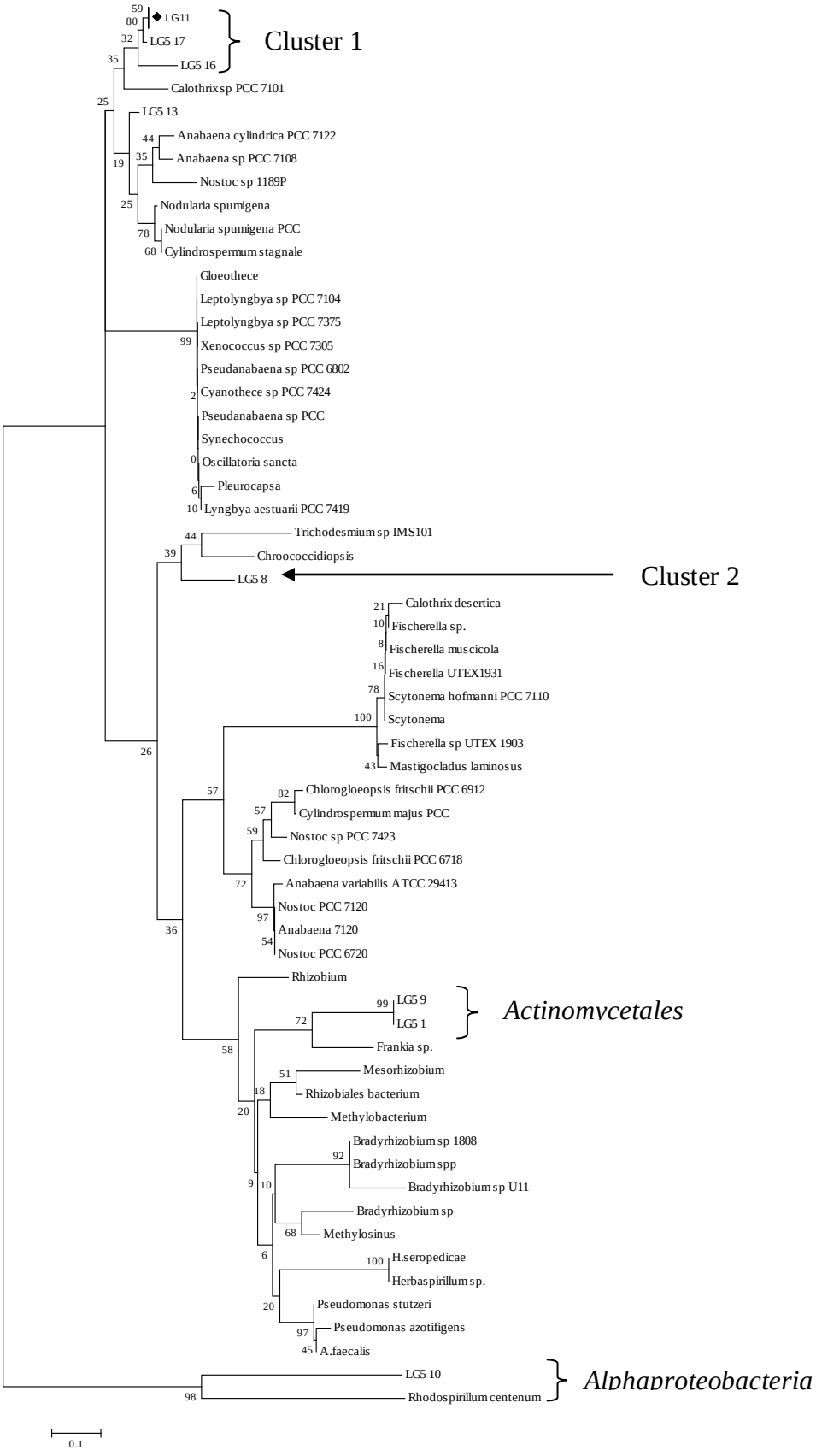

LG11
LG5 17
LG5 16
Cluster 1
Calothrix sp PCC 7101
LG5 13
Anabaena cylindrica PCC 7122
Anabaena sp PCC 7108
Nostoc sp 1189P
Nodularia spumigena
Nodularia spumigena PCC
Cylindrospermum stagnale
Gloeothece
Leptolyngbya sp PCC 7104
Leptolyngbya sp PCC 7375
Xenococcus sp PCC 7305
Pseudanabaena sp PCC 6802
Cyanothece sp PCC 7424
Pseudanabaena sp PCC
Synechococcus
Oscillatoria sancta
Pleurocapsa
Lyngbya aestuarii PCC 7419
Trichodesmium sp IMS101
Chroococcidiopsis
LG5 8
Cluster 2
Calothrix desertica
Fischerella sp.
Fischerella muscicola
Fischerella UTEX1931
Scytonema hofmanni PCC 7110
Scytonema
Fischerella sp UTEX 1903
Mastigocladus laminosus
Chlorogloeopsis fritschii PCC 6912
Cylindrospermum majus PCC
Nostoc sp PCC 7423
Chlorogloeopsis fritschii PCC 6718
Anabaena variabilis ATCC 29413
Nostoc PCC 7120
Anabaena 7120
Nostoc PCC 6720
Rhizobium
LG5 9
LG5 1
Frankia sp.
Actinomycetales
Mesorhizobium
Rhizobiales bacterium
Methylobacterium
Bradyrhizobium sp 1808
Bradyrhizobium spp
Bradyrhizobium sp U11
Bradyrhizobium sp
Methylosinus
H.seropedicae
Herbaspirillum sp.
Pseudomonas stutzeri
Pseudomonas azotifigens
A.faecalis
LG5 10
Rhodospirillum centenum
Alphaproteobacteria
0.1

Figure 3-9. Phylogenetic tree of genomic DNA *nifD* from 8/19/08 east transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.

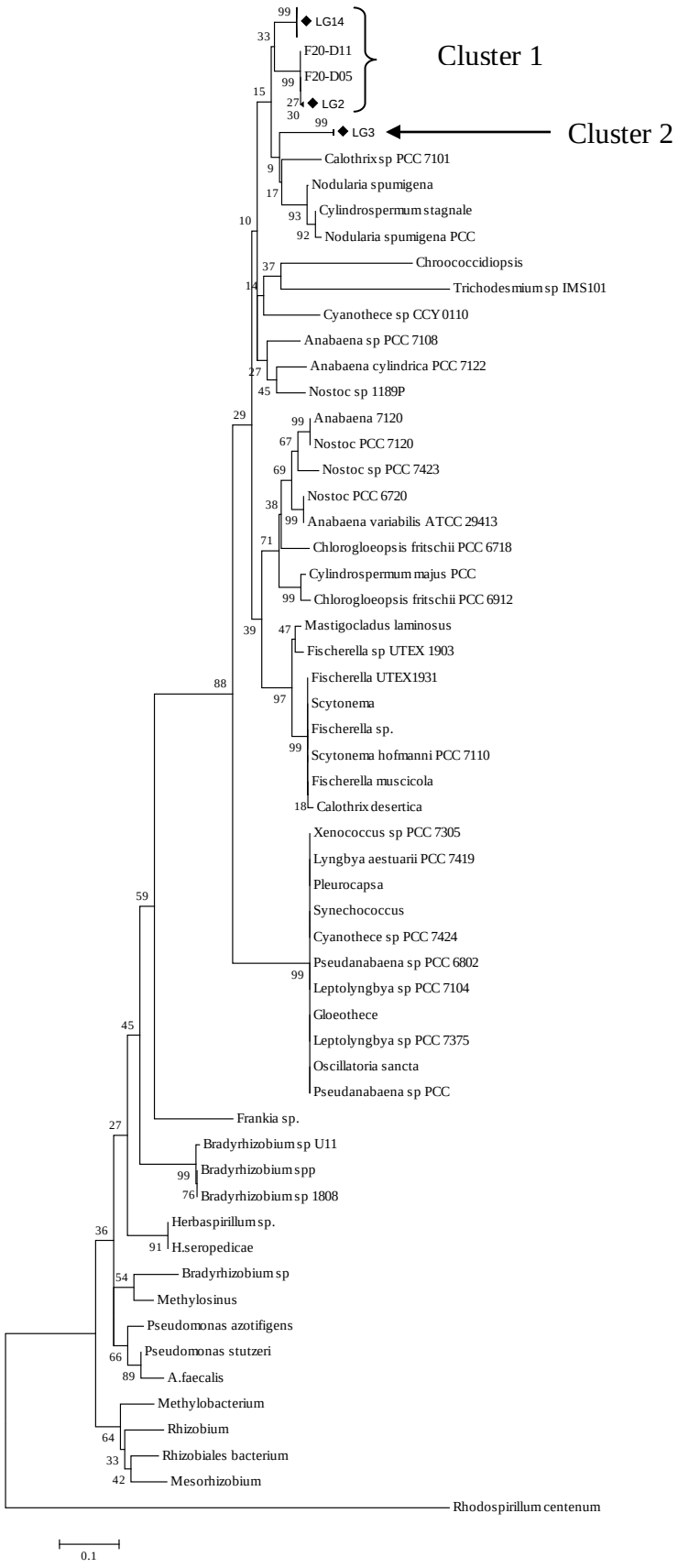

LG14
F20-D11
F20-D05
LG2
LG3
Cluster 1
Cluster 2
Calothrix sp PCC 7101
Nodularia spumigena
Cylindrospermum stagnale
Nodularia spumigena PCC
Chroococcidiopsis
Trichodesmium sp IMS101
Cyanothece sp CCY 0110
Anabaena sp PCC 7108
Anabaena cylindrica PCC 7122
Nostoc sp 1189P
Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Chlorogloeopsis fritschii PCC 6718
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Mastigocladus laminosus
Fischerella sp UTEX 1903
Fischerella UTEX1931
Scytonema
Fischerella sp.
Scytonema hofmanni PCC 7110
Fischerella muscicola
Calothrix desertica
Xenococcus sp PCC 7305
Lyngbya aestuarii PCC 7419
Pleurocapsa
Synechococcus
Cyanothece sp PCC 7424
Pseudanabaena sp PCC 6802
Leptolyngbya sp PCC 7104
Gloeothece
Leptolyngbya sp PCC 7375
Oscillatoria sancta
Pseudanabaena sp PCC
Frankia sp.
Bradyrhizobium sp U11
Bradyrhizobium spp
Bradyrhizobium sp 1808
Herbaspirillum sp.
H.seropedicae
Bradyrhizobium sp
Methylosinus
Pseudomonas azotifigens
Pseudomonas stutzeri
A.faecalis
Methylobacterium
Rhizobium
Rhizobiales bacterium
Mesorhizobium
Rhodospirillum centenum
0.1

Figure 3-10. Phylogenetic tree of genomic DNA *nifD* from 8/19/08 center transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.

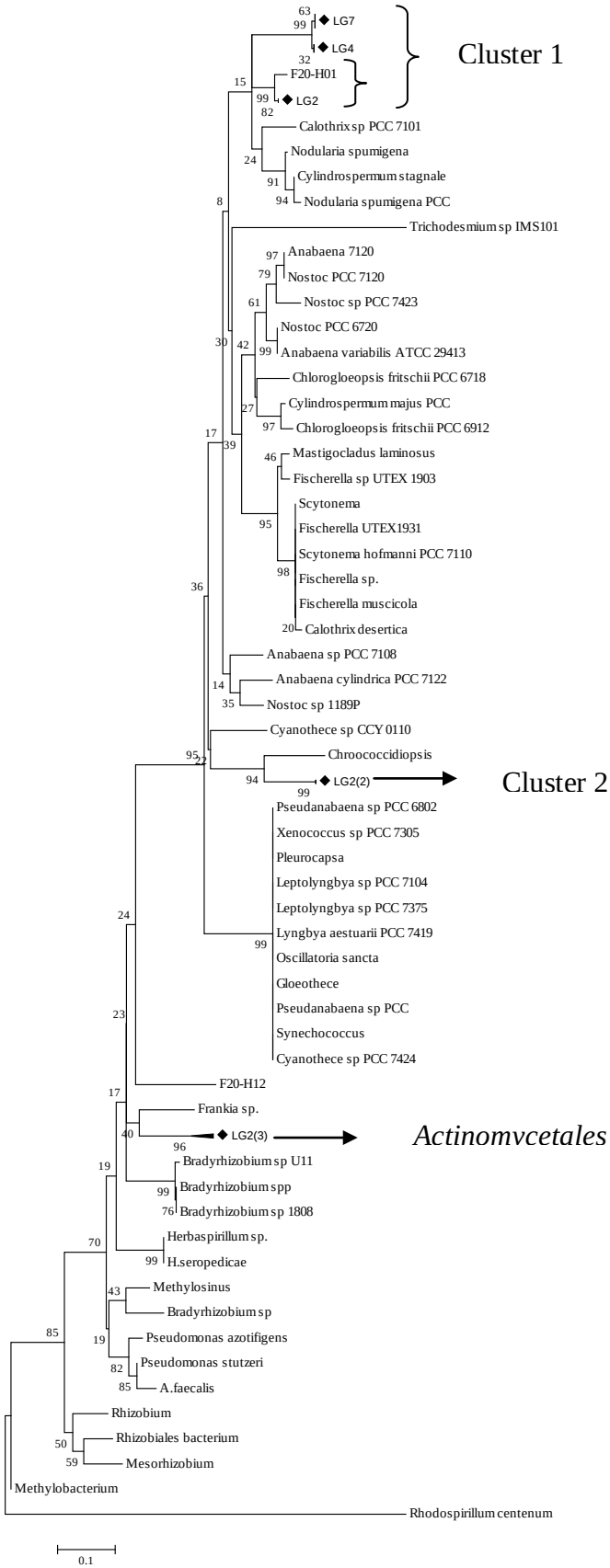

LG7
LG4
F20-H01
LG2
Cluster 1
Calothrix sp PCC 7101
Nodularia spumigena
Cylindrospermum stagnale
Nodularia spumigena PCC
Trichodesmium sp IMS101
Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Chlorogloeopsis fritschii PCC 6718
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Mastigocladus laminosus
Fischerella sp UTEX 1903
Scytonema
Fischerella UTEX1931
Scytonema hofmanni PCC 7110
Fischerella sp.
Fischerella muscicola
Calothrix desertica
Anabaena sp PCC 7108
Anabaena cylindrica PCC 7122
Nostoc sp 1189P
Cyanothece sp CCY 0110
Chroococcidiopsis
LG2(2)
Cluster 2
Pseudanabaena sp PCC 6802
Xenococcus sp PCC 7305
Pleurocapsa
Leptolyngbya sp PCC 7104
Leptolyngbya sp PCC 7375
Lyngbya aestuarii PCC 7419
Oscillatoria sancta
Gloeothece
Pseudanabaena sp PCC
Synechococcus
Cyanothece sp PCC 7424
F20-H12
Frankia sp.
LG2(3)
Actinomycetales
Bradyrhizobium sp U11
Bradyrhizobium spp
Bradyrhizobium sp 1808
Herbaspirillum sp.
H.seropedicae
Methylosinus
Bradyrhizobium sp
Pseudomonas azotifigens
Pseudomonas stutzeri
A.faecalis
Rhizobium
Rhizobiales bacterium
Mesorhizobium
Methylobacterium
Rhodospirillum centenum
0.1

Figure 3-11. Phylogenetic tree of genomic DNA *nifD* from 8/19/08 west transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.

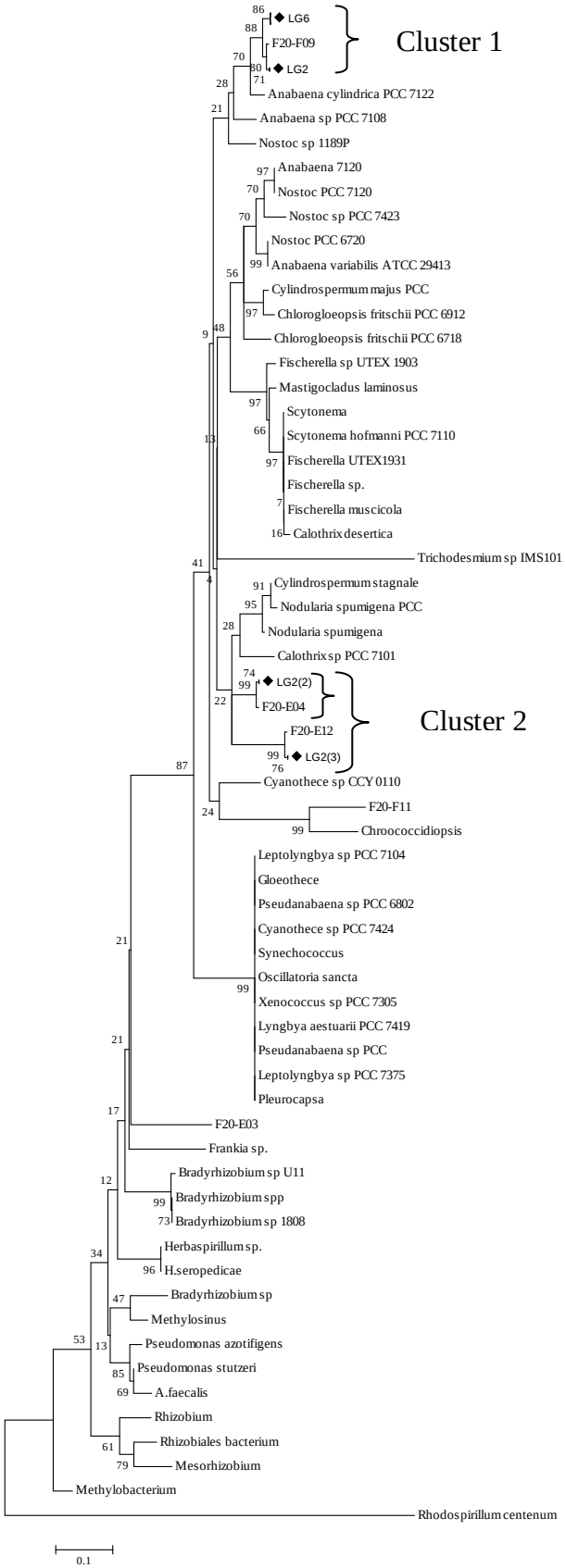

LG6
F20-F09
LG2
Cluster 1
Anabaena cylindrica PCC 7122
Anabaena sp PCC 7108
Nostoc sp 1189P
Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Chlorogloeopsis fritschii PCC 6718
Fischerella sp UTEX 1903
Mastigocladus laminosus
Scytonema
Scytonema hofmanni PCC 7110
Fischerella UTEX1931
Fischerella sp.
Fischerella muscicola
Calothrix desertica
Trichodesmium sp IMS101
Cylindrospermum stagnale
Nodularia spumigena PCC
Nodularia spumigena
Calothrix sp PCC 7101
LG2(2)
F20-E04
F20-E12
LG2(3)
Cluster 2
Cyanothece sp CCY 0110
F20-F11
Chroococcidiopsis
Leptolyngbya sp PCC 7104
Gloeothece
Pseudanabaena sp PCC 6802
Cyanothece sp PCC 7424
Synechococcus
Oscillatoria sancta
Xenococcus sp PCC 7305
Lyngbya aestuarii PCC 7419
Pseudanabaena sp PCC
Leptolyngbya sp PCC 7375
Pleurocapsa
F20-E03
Frankia sp.
Bradyrhizobium sp U11
Bradyrhizobium spp
Bradyrhizobium sp 1808
Herbaspirillum sp.
H.seropedicae
Bradyrhizobium sp
Methylosinus
Pseudomonas azotifigens
Pseudomonas stutzeri
A.faecalis
Rhizobium
Rhizobiales bacterium
Mesorhizobium
Methylobacterium
Rhodospirillum centenum
0.1

Figure 3-12. Phylogenetic tree of genomic DNA *nifD* from 9/02/08 east transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.

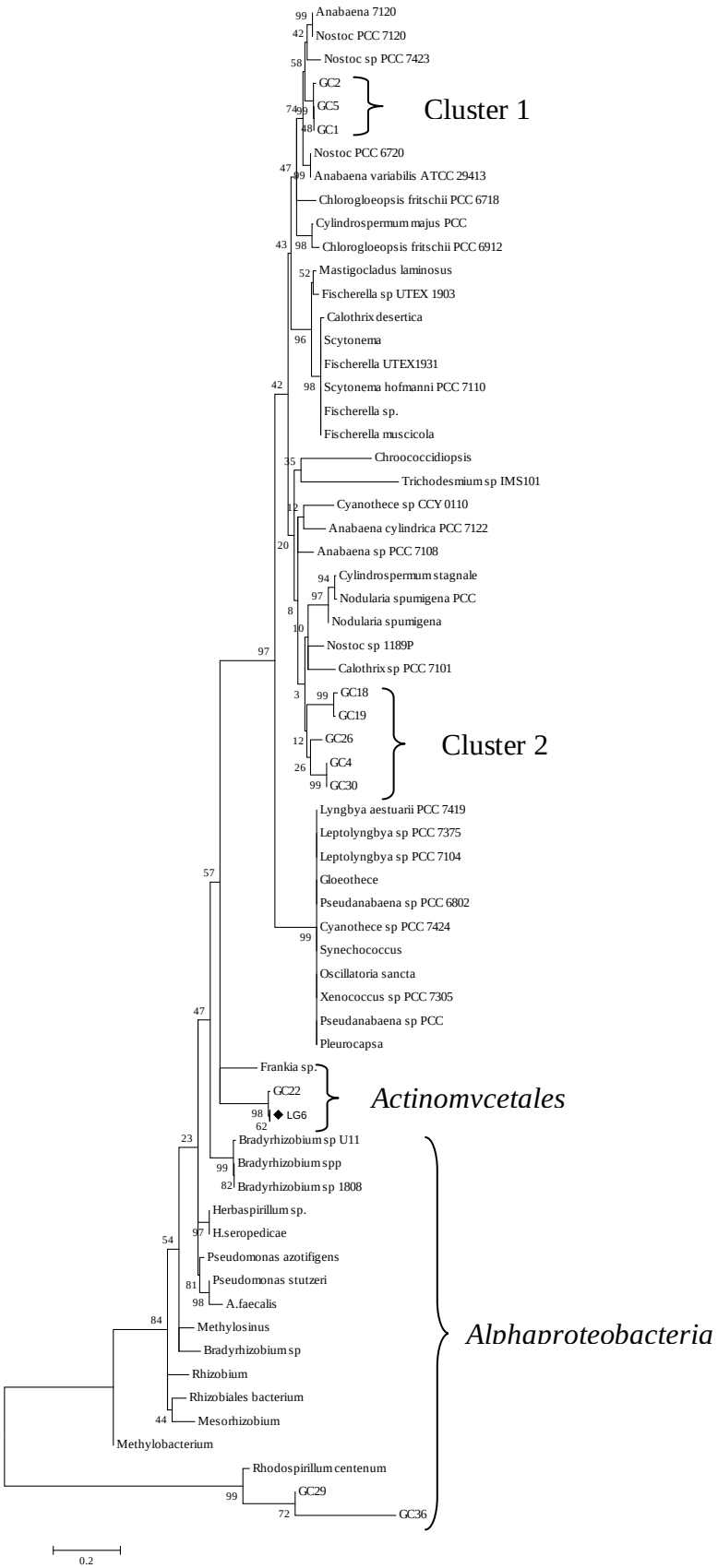
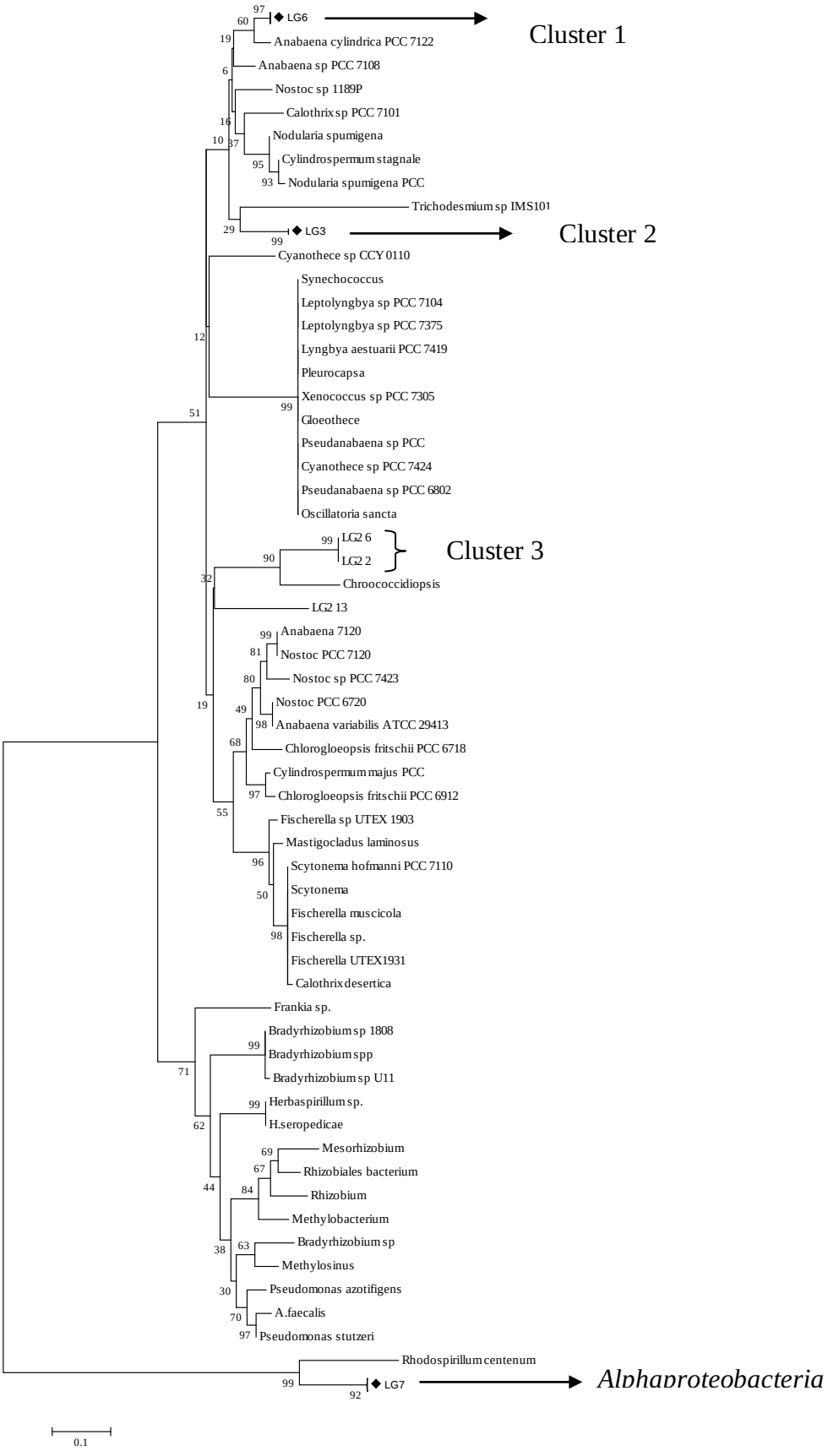

Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
GC2
GC5
GC1
Cluster 1
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Chlorogloeopsis fritschii PCC 6718
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Mastigocladus laminosus
Fischerella sp UTEX 1903
Calothrix desertica
Scytonema
Fischerella UTEX1931
Scytonema hofmanni PCC 7110
Fischerella sp.
Fischerella muscicola
Chroococcidiopsis
Trichodesmium sp IMS101
Cyanothece sp CCY 0110
Anabaena cylindrica PCC 7122
Anabaena sp PCC 7108
Cylindrospermum stagnale
Nodularia spumigena PCC
Nodularia spumigena
Nostoc sp 1189P
Calothrix sp PCC 7101
GC18
GC19
GC26
GC4
GC30
Cluster 2
Lyngbya aestuarii PCC 7419
Leptolyngbya sp PCC 7375
Leptolyngbya sp PCC 7104
Gloeothece
Pseudanabaena sp PCC 6802
Cyanothece sp PCC 7424
Synechococcus
Oscillatoria sancta
Xenococcus sp PCC 7305
Pseudanabaena sp PCC
Pleurocapsa
Frankia sp.
GC22
LG6
Actinomycetales
Bradyrhizobium sp U11
Bradyrhizobium spp
Bradyrhizobium sp 1808
Herbaspirillum sp.
H.seropedicae
Pseudomonas azotifigens
Pseudomonas stutzeri
A.faecalis
Methylosinus
Bradyrhizobium sp
Rhizobium
Rhizobiales bacterium
Mesorhizobium
Methylobacterium
Rhodospirillum centenum
GC29
GC36
Alphaproteobacteria
0.2

Figure 3-13. Phylogenetic tree of genomic DNA *nifD* from 9/02/08 west transect. Numbers at branch points refer to bootstrap analysis based on 1000 resampling.


LG6
Cluster 1
Anabaena cylindrica PCC 7122
Anabaena sp PCC 7108
Nostoc sp 1189P
Calothrix sp PCC 7101
Nodularia spumigena
Cylindrospermum stagnale
Nodularia spumigena PCC
Trichodesmium sp IMS101
LG3
Cluster 2
Cyanothece sp CCY 0110
Synechococcus
Leptolyngbya sp PCC 7104
Leptolyngbya sp PCC 7375
Lyngbya aestuarii PCC 7419
Pleurocapsa
Xenococcus sp PCC 7305
Gloeothece
Pseudanabaena sp PCC
Cyanothece sp PCC 7424
Pseudanabaena sp PCC 6802
Oscillatoria sancta
LG2 6
LG2 2
Cluster 3
Chroococcidiopsis
LG2 13
Anabaena 7120
Nostoc PCC 7120
Nostoc sp PCC 7423
Nostoc PCC 6720
Anabaena variabilis ATCC 29413
Chlorogloeopsis fritschii PCC 6718
Cylindrospermum majus PCC
Chlorogloeopsis fritschii PCC 6912
Fischerella sp UTEX 1903
Mastigocladus laminosus
Scytonema hofmanni PCC 7110
Scytonema
Fischerella muscicola
Fischerella sp.
Fischerella UTEX1931
Calothrix desertica
Frankia sp.
Bradyrhizobium sp 1808
Bradyrhizobium spp
Bradyrhizobium sp U11
Herbaspirillum sp.
H.seropedicae
Mesorhizobium
Rhizobiales bacterium
Rhizobium
Methylobacterium
Bradyrhizobium sp
Methylosinus
Pseudomonas azotifigens
A.faecalis
Pseudomonas stutzeri
Rhodospirillum centenum
LG7
Alphaproteobacteria
0.1

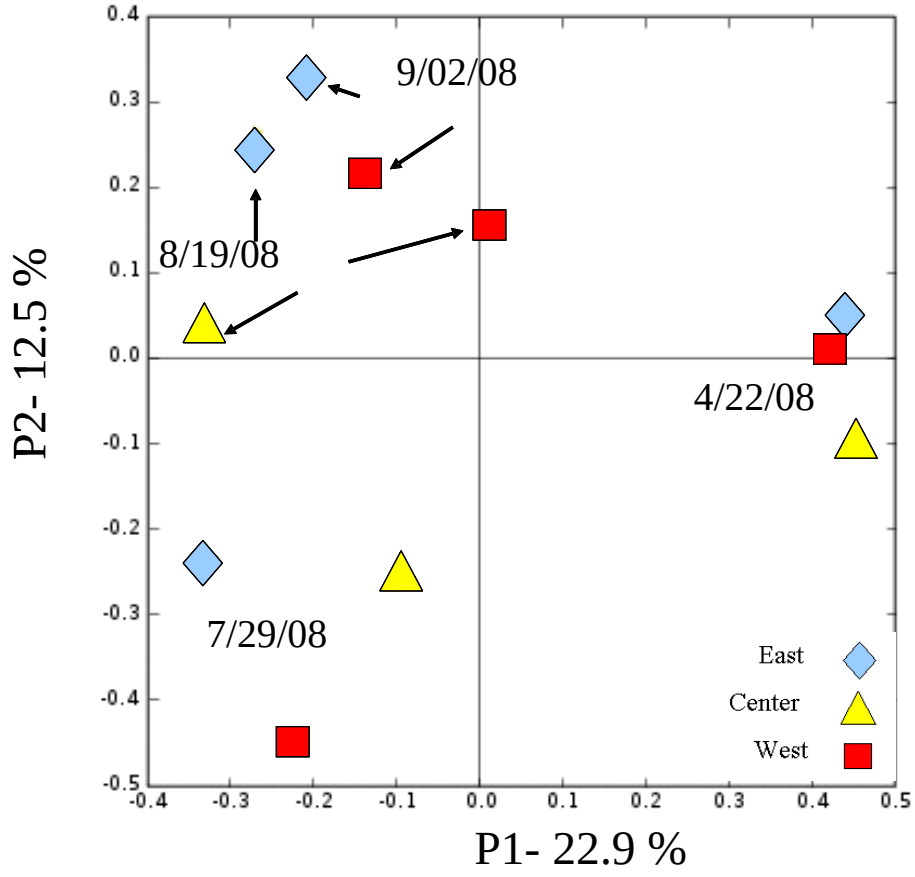


Figure 3-14. Separation of samples by principle component analysis based on the diazotrophic community composition (analyzed genetic clades using Unifrac).

CHAPTER 4
SUMMARY AND CONCLUSION

Eutrophication of aquatic systems is drawing increasing amounts of interest, and one major reason of concern is their potential as pollution sources to other systems (especially downstream systems) that may be irreversibly impacted. Although eutrophic systems are rich in nutrients and experiencing high productivity levels, primary production is usually limited by nutrients. Many previous studies have concluded that primary production in fresh water systems is mostly limited by phosphorus (P) following nitrogen (N) compared to marine systems and estuaries that are primarily limited by N.

This work focused on measuring biological N_2 fixation in the watercolumn of a shallow, subtropical lake with the potential to export significant quantities of N to hydrologically connected systems downstream. Lake George is a shallow, eutrophic lake system located on a geologically P-rich region that has the potential to shift limitation of primary production to N. This nutrient shift causes increased demand for N that can be satisfied by biological N_2 fixation and grants a competitive advantage to diazotrophic organisms. Downstream of Lake George, low levels of dissolved oxygen (DO) are measured annually with periods of hypoxia that may collapse biological systems and alter biogeochemical cycles.

Despite regular monitoring of the surface import/export N balance of Lake George, it was not clear how significant biological N_2 fixation is for the system N budget and what mechanisms may convert the lake into a pollution source. Therefore, the objectives of this research were to: 1) characterize and quantify N_2 fixation spatially, seasonally and under different light levels, 2) identify environmental parameters related to N_2 fixation and their patterns, 3) characterize diazotrophic community composition by examining the effects of location, date, nutrients and N_2 fixation rate.

The acetylene reduction assay (ARA) was selected to measure nitrogenase activity in Lake George, identify its regulators, and estimate the quantity of N_2 that was fixed including its contribution to the lake's N export. The importance of regulators of N_2 fixation and N export in this system can be used for management purposes and also may be adapted to similar systems. By this method, the measured rate of N_2 fixation was expressed in volume (550 µg N m^{-3} h^{-1}, or a mean of 20 nmol N_2 l^{-1} h^{-1} and a range of 0 -55), area (mean 1.4 mg N m^2 h^{-1}), and heterocystous biovolume (ranged from 10 to 18 picomol N_2 μm^{-3} heterocystous- h^{-1}). These potential rates agreed with previous measurement in St. Johns River Estuary and similar systems that showed that N_2 fixation may be a significant component of the N budget (Pearl et al., 2003).

The increasing rates and abundance of eutrophication seen globally and their importance to exported N, which leads to eutrophication of other systems, have called for actions, including demands for their regulation and control. Eutrophication is a broad and complex process, but shallow lakes represent a relative simple system (because most experience little strafication and are easier to sample and extrapolate) that may be ideal for the study of eutrophication.

With assumptions regarding light penetration and day length, it was also possible to use the rates recorded during this study in 2008 to roughly quantify the total amount of N that was fixed in Lake George water during our study period and examine its contribution to yearly N output. Based on this calculation, it was estimated that during the study (late Spring through early Fall), N_2 fixation rate in the dark amounted to an estimated 135 metric tones (MT, or 10^6 g) of N, while daytime N_2 fixation accounted for a potential of 462 MT N to the lake. For the rest of the year (outside the study period) an additional 131 MT N was estimated (based on an assumed constant rate of 28 ng $l^{-1}h^{-1}$). Thus, the total N that was fixed during our study period is estimated to range

from 600-730 MT during the entire year 2008. This quantity fit the lake's N budget and was a little larger than the estimated yearly amount of exported N (600 MT).

It was evident that ARA had seasonal pattern that was consistent between sites and various light treatments. This pattern indicated the importance of seasonality as a regulator of N_2 fixation and diazotrophic growth and also supported the mixing of water sources expected in shallow lakes. The pattern showed four peak events that were separated by low activity and coincided with cyanobacterial and heterocystous abundance and with measured patterns of watercolumn nutrient concentrations (N and P). Nitrogen fixation rate was positively influenced by light which reinforced the assumption that N_2 fixation is photosynthetically derived through heterocystous cyanobacteria in Lake George; ARA showed insignificant difference between low (66 µM photons m^{-2} s^{-1} PAR (photosynthetically active radiation) and high light (115 µM photons m^{-2} s^{-1} PAR) incubation that supported the ability of these organisms to photosynthesis under low light levels.

Seasonality and water source affected nutrient abundance yet N was the limiting nutrient during most of the study period, as indicated by high rate of AR and by weight ratio of dissolved inorganic N: total P (DIN:TP). Several of the biggest difficulties were connecting between nutrient forms to cyanobacterial shifts and species domination and the ability to assess environmental impacts caused by algal blooms. Seasonal patterns of N and P, including ammonium (NH_4^+), nitrate/nitrite (NO_x), particulate organic N (PON) and total N (TN):TP ratio, showed similarity to ARA pattern and reinforced the assumption that nutrients lead to cyanobacterial dominance and thus, regulate N_2 fixation rates.

After dividing the study period into two sub-periods (based on ARA peaks and measured abundance of heterocystous cyanobacteria), statistical analyses revealed that several selected

nutrients (NH_4^+, DO, dissolved organic carbon (DOC)) were positively correlated to ARA, and stepwise regression analysis selected particulate organic C (POC):PON ratio (negatively correlated with ARA) as the strongest correlated parameter in a model that explained 98% of variance in ARA rate. The negative correlations implied that N level may be either inhibitory or may be the outcome of N_2 fixation.

Canonical correlation analysis was also used to identify environmental parameters that could explain spatial N_2 fixation pattern within the lake. The analysis showed the importance of the St. Johns River as well as land uses around the lake on the variability of lake water composition. Results indicated that DOC, NH_4^+, dissolved reactive P (DRP), dissolved organic N (DON), TN:TP, and conductivity were the most different between the east and west lake regions during the study period, while during the first sub-period the central transect was similar to the east, and shifted to be more like the west during the second sub-period. Seasonally, these shifts appeared to control nutrient patterns and supported a N-limited system in which P was an important regulator of N_2 fixation.

Patterns of algal blooms and nutrients composition were affected by seasonality and showed a spatial heterogeneity of nutrients and N_2 fixation. Although, the effect of environmental conditions on the diazotrophic community clearly indicated a shift toward heterocystous cyanobacteria, it was not clear how N and P, or which mechanisms control diazotrophic composition and domination of single species. These blooms are common in shallow lakes and other freshwater systems and indicate N limitation, but their ability to act as sources of N to downstream ecosystems may be controlled only after achieving greater understanding about their mechanisms.

The goal of the second part of the study was to characterize the diazotrophic community composition in Lake George using a non-culture based method. We chose to use the conserved subunit of nitrogenase (*nifD* sequences) to characterize the diazotrophic community composition in order to better understand their relationship between structure and function in Lake George. This was the first ever attempt to characterize the phylogeny of diazotrophs in Lake George using *nifD*, a preferred biomarker for characterizing cyanobacteria.

After identifying all organisms that had the genetic potential to fix N_2, phylogenetic analysis was used to infer their evolutionary relation and find distributional patterns. Several methods were chosen in our attempts to correlate our characterized diazotrophic community to ARA, location, date and environmental parameters. We found that seasonality was the only apparent factor controlling distribution of diazotrophs. And both N and P were controlling community composition. It is important to note that ARA measured the N_2 fixation potential rate of the entire community without the ability to relate fixation rate to each group present and actively fixing N_2. Never the less, it demonstrated that the diazotrophic community at Lake George is active and diverse.

Using this approach, the diazotrophic community in Lake George was found to be similar to communities of other subtropical, eutrophic systems, although novel strains were also recorded in this study. While most clones were unidentified or showed low percentage of similarity, the heterocystous family *Nostocaceae* was found in all samples and was clearly the most diverse group. This family includes two of Lake George's dominant diazotrophs from the genus *Anabaena* and *Cylindrospermopsis* that expected to dominate such systems as supported by microscopic analysis. Heterocystous cyanobacteria (especially *Cylindrospermopsis*) invaded into many systems and became sources of concern (e.g. fix N_2 and may release toxic). Other

diazotrophic members that were also identified included other cyanobacteria (both heterocystous and non-heterocystous), *Alphaproteobacteria,* and the gram positive *Frankia* (order *Actinomycetales*). Knowledge about the diazotrophic composition gives essential information about the system that otherwise may be hard to achieve, including the possible origin, controls, and activities of these organisms).

Except in two cases that could not be explained, ∫-Libshuff analysis suggested that population membership in most clone libraries was related and did not differ significantly. Principle component analysis of *nifD* sequences showed highest separation between clone-libraries based on date with a minor effect of site location within the lake. Although more information is need about the mechanisms by which nutrients affected community shifts, Mantel Test results suggested that N (TN and PON) were the primary factors affecting diazotrophic community composition. As expected, this analysis demonstrated that as the concentration difference of each of these nutrients increased, the diversity of the diazotrophic community also increased, however, it was not clear how these nutrients affected community composition.

Although previous work concluded that Lake George is a N-limited system controlled by P, AR rate and cyanobacterial biovolume were not highly correlated to P levels. Other limitations were connected to the chosen methods and assumptions that were taken and to the complexity of system. In future research, more sites should be used to potentially resolve the difficulties in identifying cause and effect of peaks. Also, in order to encompass all diazotrophic activity at Lake George, other ecosystem components (e.g. sediment, macrophytes, etc) must be measured, and other N cycle processes (e.g. denitrification) should be measured in order to better constrain the N budget and increase its accuracy. Increased sampling during other seasons and better

estimates of diel patterns may give more information about the activities and diazotrophic community and increase accuracy of estimated yearly fixed N.

For most samples, the diazotrophic composition was not rich, comprised mainly of cyanobacteria. In order to increase our confidence in the phylogenetic analysis, *nifD* taken from pure cultures of *Anabaena* and *Cylindrospermopsis* from Lake George should be sequenced and recorded in Genebank; in fact, using the combination of both *nifD* and *nifH* would offer higher resolution within cyanobacteria, may identify additional diazotrophs, and thus increase the confidence of phylogenetic analysis. Several molecular techniques that measure expression of *nif* can be employed to measure activity by each clone and so estimate the importance of each member in the community. Phylogenetic analysis of the diazotrophic community at the lake's sediment can be used to estimate its environmental conditions (For example, presence of cyanobacteria indicates light penetration and relatively high DO levels during light periods or association with other organisms); in addition, it can shed light on phytoplankton migration and interaction with the sediments and their communities.

Lake George, like many shallow lakes may act as a source of N to downstream systems and its destructive impact on the adjacent marine systems is a good example of the importance of controlling algal blooms in inland waters. Thus far, most research on Lake George focused on nutrient manipulation bioassays (identified nutrient limitation and their controls on activities), presence and controls of cyanobacteria toxins, and phytoplankton abundance including their predators. In the past, ARA was used in Lake George to measure N_2 fixation under different nutrient additions and as a function of growth and salinity. This study is the first attempt to quantify N_2 fixation in Lake George water and estimate its significance to the N budget. In addition, *nifD* was used in this system for the first time to characterize the diazotrophic

community composition in the lake. Our results clearly showed that N and P affected N_2 fixation in two levels: 1) directly affecting N_2 fixation rate and 2) indirectly by affecting diazotrophic community composition.

The molecular analysis work was important for the construction of genetic database of the diazotrophic community at Lake George and could be used as a preliminary data to support additional investigations. Our genetic database represented organisms that had the "potential" to fix N_2 and may be used as a foundation in the study of the expression of *nifD* in Lake George. By combining information on potential gene expression with environmental parameters leading to expressed N_2 fixation rates, it may be possible to identify the precise controls on N_2 fixation during particular blooms. Furthermore, it may also assist in the search for early warning indicators that can detect ecosystem changes in advance of bloom formation. Additional work will increase our ability to predict, explain, and control nutrients/environmental conditions leading to algal blooms in Lake George and similar systems. Thus, this work can be viewed as a significant step toward the development of effective strategies to reduce N_2 fixation and minimizing the environmental impacts of N export from lakes.

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BIOGRAPHICAL SKETCH

Moshe Doron was born in Tel- Aviv, Israel. He received his bachelor degree in Microbiology and Cell Science on 2007 from the University of Florida, USA. He joined the Department of Soil and Water Science, which had an emphasis on ecology, because they gave him an opportunity to research genetics of microorganisms and their influences on the environment. During his volunteering time at Dr. Andrew V. Ogram lab, he was fascinated with the studies and research conducted in the lab. At first, he assisted the research on restoration of the "Hole-in-the-Donut" (due to the invasion of the exotic Brazilian Pepper (*Schinus terebinthifolius*)). The following semester, he received his own project about carbon as a final electron acceptor. Unfortunately, he had to stop it after a couple of months because there were not enough funds for the project. Nevertheless, his hard work was noticed and he got accepted to UF University Scholars Program (USP). In this program, USP co-sponsored his research on biodegradation of naphthalene (naphthalene is a toxin that forms naturally from byproducts of organic matter decomposition and from petroleum contamination). Since he enjoyed working with professor Ogram and loved conducting research with microorganisms, he was looking to expand his scope of research to the entire system in which they operate. Biogeochemistry attracted him because it enabled him to see the entire picture from the smallest details (e.g., genes) up to the biggest ones (the entire system). Furthermore, he believed our planet has environmental problems that must be controlled or repaired through a better understanding of the biogeochemical processes. On 2007, he joined the MS program at UF in the Soil and Water Science Department with Dr. W. Patrick Inglett as his advisor. He wanted to learn more about biogeochemistry and how to apply this knowledge to the management and recovery of our planet.