The Effect of Nitrates, pH, and Dissolved Inorganic Carbon Concentrations on the Extracellular Polysaccharide of Three Strains of Cyanobacteria Belonging to the Family *Nostocaceae*

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ABSTRACT

Three strains of cyanobacteria (*Anabaena PCC7120, A. variabilis and Nostoc commune*), all belonging to the family *Nostocaceae,* were found to be capable of modulating the production and chemical composition of extracellular polysaccharides (EPS) in response to carbon and nitrogen availability as well as pH. While the carbohydrate compositions of the glycans produced by the different organisms were indicative of their recent evolutionary divergence, there were measurable differences that were dependent upon growth conditions. The EPS resulting from biofilm growth conditions was reduced in glucuronic acid levels in both *Anabaena variabilis* ATCC 29413 and *Anabaena* PCC 7120. Under planktonic conditions, the glycan from *A. variabilis* contained glucuronic acid when grown in nitrate-free BG-11_0 medium whereas *A.* PCC 7120 produced similar levels in standard BG-11 medium. This suggests that phylogenetically-related cyanobacteria respond very differently to changes in their local environment. The pH of BG-11 cultures increased to 9-10 for all three strains of cyanobacteria. The increase resulted in an increase in the amount of dissolved inorganic carbon available in the medium, creating an imbalance in the carbon-nitrogen ratio, with the complete consumption of 17.65 mmol L^{-1} nitrates raising the pH to near 10 in BG-11 medium. While increased carbon availability has been shown to induce capsulated morphologies in strains of cyanobacteria, only *Nostoc commune* DRH-1 exhibited this behavior, and only when grown in BG-11 medium.

Carbon and nitrogen availability as well as pH modulate the monosaccharide composition of the glycan generated by cyanobacteria investigated. The different characteristics of the glycans produced can affect the survivability of the organisms and the community structure of cyanobacterial biofilms and microbial mats found in nature. As cyanobacteria are ubiquitous organism both now and in the past, they play a pivotal role in the biological and geological processes of the Earth, controlling the availability and cycling of carbon and nitrogen both actively and passively.

DEDICATION

I would like to dedicate this work to my wonderful wife, Christy, for her unrelenting encouragement, unconditional love, and our beautiful son, Alexander.

Christy, I love you always and forever.

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LIST OF ABBREVIATIONS

kDa: Kilodalton

EPS: Extracellular Polysaccharide

Pco_2: partial pressure of carbon dioxide

RuBisCO: ribulose 1,5-bisphosphate carboxylase/oxygenase

TFA: trifluoroacetic acid

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CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

Cyanobacteria are a group of Gram negative photoautotrophic microorganisms capable of oxygenic photosynthesis (Whitton and Potts, 2002). These ancient organisms (Schopf, 1993, 2000, 2006) have minimal nutrient requirements. They are capable of fixing carbon dioxide and utilizing light as an energy source and water as an electron donor (Stal, 2003). In addition, many strains possess the ability to fix atmospheric nitrogen (Stal, 2003; Whitton and Potts, 2002). Such minimal requirements enable cyanobacteria to inhabit nearly every illuminated environment on Earth, aquatic or terrestrial, from the arctic deserts, to hydrothermal hot springs, to oceans, to freshwater environments (de los Rios *et al.*, 2007; Gorbushina, 2007; Whitton and Potts, 2002). Cyanobacteria are able to compensate for changes in the Earth's atmosphere and local environments by investing in a carbon concentrating mechanism (CCM) to adjust to changes in carbon dioxide concentrations, and some are able to form heterocyst for changes in nitrogen availability. Most cyanobacteria produce and release an extracellular polymeric substance composed primarily of polysaccharides (De Philippis and Vincenzini, 1998). The extracellular polysaccharides (EPS) play an important role in the survivability of cyanobacteria and can strongly influence their habitats and community structures. The characteristics of the EPS produced can also be altered by nutrient availability and other environmental perturbations. Of particular emphasis in this study is how the dissolved inorganic carbon concentrations and nitrogen source affect the EPS.

1.2 Evidence of Ancient Cyanobacteria

Fossil evidence suggests that cyanobacteria originated over 3500 million years ago (Schopf, 1993, 1999, 2000). This is significant because 3500 million years ago the Earth was a little over 1000 million years old itself with conditions that were vastly different from those of modern day. Fossils of such an extreme age are met with some skepticism, and the actuality of these fossils being cyanobacteria has been disputed (Brasier *et al.*, 2002). Additional controversy comes from the incongruency of the molecular clock and the dates given to the fossils (Doolittle *et al.*, 1996; Schopf, 2000). More evidence, however, continues to be found that supports the 3500 million-year-old date for cyanobacteria (Schopf, 2006). The microfossils of ancient cyanobacteria are

predominantly found in macrofossils called stromatolites, structures that were initially formed by microbial mat communities. Even though stromatolites date back to 3500 million years ago (Altermann *et al.*, 2006; Hofmann *et al.*, 1999), they only became abundant around 2300 million years ago (Stal, 2000, 2003).

1.3 Microbial Mats and Stromatolites: Formation and Calcification

The laminar layering and general structure of stromatolites is generated from filamentous cyanobacteria (Douglas and Beveridge, 1998; Stal, 2000). Through hydrophobic interactions of the EPS, cyanobacteria colonize a benthic surface (De Philippis *et al.*, 2005). Sediment becomes trapped in the EPS generated by the cyanobacteria and other organisms which collect and differentiate to layers within the mat (Stal, 2003). The accumulated sediment on the surface of the mat diminishes the light that reaches the cyanobacteria. To obtain more light the cyanobacteria exhibit phototaxis by moving up through the layers of polysaccharide and sediment to the surface (Stal, 2000). This process repeats itself several times to generate the laminated aggregate.

Calcification within the microbial mats plays an essential role in the development of stromatolites. Microbial mat calcification occurs through active and passive means within the community. For cyanobacteria to induce calcification there must be a supersaturation of calcium carbonate minerals, a shift in carbonate equilibrium that surpasses the critical supersaturation for calcium carbonate, and there must be a seed crystal formation (Arp *et al.*, 2001). As cyanobacteria fix carbon from the water, usually in the form of bicarbonate in a microbial mat, they change the pH and solution content of their environment (Altermann *et al.*, 2006; Stal, 2000). The net result of the process is an increase in sugars, oxygen, and calcium carbonate (eq. 1.1) (Garcia-Pichel *et al.*, 2004);

$$Ca^{2+} + 2HCO_3^- \rightarrow Ca^{2+} + CO_3^{2-} + CO_2 + H_2O \rightarrow CaCO_3(s) + CH_2O + O_2 \quad \text{(eq. 1.1)}$$

As nutrients are removed from the solution, primarily bicarbonate in this case (Kupriianova *et al.*, 2004), the pH increases promoting the precipitation of calcium carbonate (Douglas and Beveridge, 1998; Schultze-Lam and Beveridge, 1994; Schultze-Lam *et al.*, 1992). When cyanobacteria are grown at high densities, the pH can increase to 10.5 in 48 hours (Douglas and

Beveridge, 1998). The pH rise in a microbial mat will fluctuate on a daily cycle (Stal, 2000). Garcia-Pichel *et al.* observed that calcification of freshwater stromatolites reached maximum rates under low light intensities (Garcia-Pichel *et al.*, 2004). Zavarzin *et al.* have demonstrated that cyanobacteria can induced calcification in the laboratory (Zavarzin *et al.*, 2003).

The EPS plays an additional role in not only aggregating sedimentary particles (Douglas and Beveridge, 1998) but also in the collection of ions (particularly magnesium and calcium) necessary for mineral formation (Altermann *et al.*, 2006). Even though the EPS is a chelator for ions with the potential to be mineralized, the EPS acts as a kinetic inhibitor to mineralization (Stal, 2000). The deeper layers of heterotrophic organisms in the microbial mat consume kinetic inhibitors such as the EPS, sulfates, and phosphates and promote precipitation of carbonate minerals in the mat (Altermann *et al.*, 2006).

Stromatolites are identified as a macro structure that may contain microfossils of the organisms that formed them. However, not all stromatolites contain identifiable cyanobacterial microfossils. Carbonate stromatolites of the Precambrian rarely exhibit carbonate microfossils of cyanobacteria (Arp *et al.*, 2001). Modern stromatolites demonstrate that low dissolved inorganic carbon and high calcium concentration induce calcium carbonate precipitation. Arp *et al.* concluded that the low abundance of Precambrian microfossils of cyanobacteria would have been a result of high concentration of dissolved inorganic carbon in the oceans (Arp *et al.*, 2001). The high concentration of dissolved inorganic carbon would not only have affected the kinetics of calcium carbonate precipitation but also the metabolism of the cyanobacteria, which in return would have had additionally affected the kinetics of calcium carbonate precipitation. This makes it necessary for one to consider not only the kinetics for carbonate mineral precipitation, but also the metabolic state of the cyanobacteria.

1.4 Evolutionary pressures

Cyanobacteria have participated in the evolution of the Earth for at least the past 3500 million years (Schopf, 1993, 2000, 2006). Throughout this time cyanobacteria have remained relatively unchanged when compared to the morphology of other organisms in the fossil record. This relatively slow rate of mutation provides strong evidence for the ability of cyanobacteria to cope with diverse conditions (Schopf, 1994). Cyanobacteria have not played a passive role in simply being subject to their environments but have actively been altering the Earth. This includes the

production of oxygen (Allen and Martin, 2007) and actively and passively modulating the global carbon cycle (Schneider and Campion-Alsumard, 1999). Cyanobacteria have endured changes in atmospheric oxygen, a general decrease in atmospheric carbon dioxide, appearance of predatory grazers, and fluctuations in light wavelength and intensity all with little change in morphology and potentially gene sequence.

The only appreciable source of oxygen on the earth comes from oxygenic photosynthesis (Allen and Martin, 2007). It is generally agreed that cyanobacteria were the first in this process and that the creation of the oxygen atmosphere is primarily attributed to them. The need of electrons for Photosystem II (PSII) comes from the calcium-manganese complex. This complex restores its electrons by taking four from two water molecules thus generating the highly reactive oxygen gas. By generating oxygen, however, cyanobacteria were essentially poisoning their own environment.

Geologic evidence shows that a significant rise in oxygen had occurred by 2320 million years ago (Bekker *et al.*, 2004). Production would have begun before then and is suggested to have begun even as early as 4000 million years ago (Allen and Martin, 2007). No evidence of such an early age of oxygen evolution has been found since any oxygen produced at the time would have been rapidly consumed in Earth's early reducing atmosphere. With the rise in oxygen cyanobacteria would need to have mechanisms that protect important oxygen sensitive processes. Cyanobacteria have also managed to deal with a large fluctuation in their primary carbon source. These pressures would have first occurred not by exposure to a general oxygen atmosphere, but within microbial mats, where pockets of high concentrations of oxygen and low concentrations carbon dioxide would have developed (Stal, 2003).

1.5 Carbon Concentrating Mechanism

The rise in atmospheric oxygen created a problem for the main carbon assimilatory enzyme of cyanobacteria and other photoautotrophs, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Both oxygen and carbon dioxide compete for the active site of RuBisCO, providing the oxygenase and carboxylase activities, respectively. The carboxylase reaction of RuBisCO utilizes ribulose 1,5-bisphosphate, carbon dioxide and water to form two molecules of glycerate-3-phosphate (eq. 1.2) (Giordano *et al.*, 2005).

ribulose 1,5-bisphosphate + CO_2 + H_2O → 2glycerate-3-P (eq. 1.2)

The oxygenase reaction leads to cleavage of the same carbon-carbon bond in ribulose 1,5-bisphosphate, but provides one molecule of glycerate-3-phosphate and the two-carbon molecule glycolate-2-phosphate (eq. 1.3) (Giordano *et al.*, 2005).

ribulose 1,5-bisphosphate + O_2 → glycerate-3-P + glycolate-2-phosphate (eq. 1.3)

The efficiency of RuBisCO is also impaired by a low affinity for its substrate carbon dioxide. On a geologic scale the current atmosphere is one of low carbon dioxide (Badger and Price, 2003). As far back as the early Phanerozoic carbon dioxide levels were 15-20 times what they currently are. Even with an oxygen concentration near current conditions, it has been suggested that modern RuBisCO enzymes would have been able to carry out efficient photosynthetic fixation of carbon (Badger *et al.*, 1998; Badger and Price, 2003). Under current conditions, however, RuBisCO is less than half-saturated with carbon dioxide (Badger and Price, 2003).

In order to increase the efficiency of the carboxylase reaction, cyanobacteria and algae have developed a way of increasing carbon dioxide in the vicinity of RuBisCO through what is known as carbon concentrating mechanism (CCM) (for reviews see (Badger and Price, 2003; Beardall and Giordano, 2002; Giordano *et al.*, 2005; Kaplan and Reinhold, 1999)). This mechanism is inducible under low concentrations of carbon dioxide and increases the efficiency of the carboxylation reaction. In cyanobacteria, the carbon concentrating mechanism consists of carboxysomes, bicarbonate transporters, carbon dioxide transporters, and carbonic anhydrase enzymes. The carboxysome is a protein body similar to a virus protein coat. Crystal structures of proteins of the carboxysome have recently been characterized enabling the atomic modeling of the carboxysomal shell (Tanaka *et al.*, 2008). The carboxysome contains most if not all of the cellular RuBisCO in a paracrystalline array. Here bicarbonate ions are converted back to carbon dioxide in the presence of RuBisCO (Badger and Price, 2003).

There are two known types of bicarbonate transporters in cyanobacteria. They are located on the plasma membrane and shuttle extracellular bicarbonate ions into the cytosol (Badger and Price, 2003). The first is a high affinity, ATP driven transporter called BCT1 (bicarbonate transporter 1), which is inducible under low carbon dioxide concentrations (current atmospheric

partial pressure) (Omata *et al.*, 1999). The second is a sodium-dependent transporter of lower affinity than BCT1 (Badger and Price, 2003). The bicarbonate transporters are responsible for increasing internal bicarbonate concentration. Two carbon dioxide transporters, NDH-1_4 and NDH-1_3, are located on the cytosolic side of the thylakoid membrane. They are coupled with the electron/proton transport associated with the thylakoid membrane and convert carbon dioxide to bicarbonate. NDH-1_3 possesses a higher substrate affinity of the two and is inducible under low concentration of carbon dioxide (current atmospheric conditions). NDH-1_4 and NDH-1_3 both function as carbon dioxide scavengers to increase internal bicarbonate concentration. (Badger and Price, 2003; Giordano *et al.*, 2005; Kaplan and Reinhold, 1999)

Carbonic anhydrase is known to play two roles in the carbon concentrating mechanism. The carboxysome-specific carbonic anhydrase is responsible for the rapid conversion of bicarbonate to carbon dioxide in the vicinity of RuBisCO thus saturating the carboxylase enzyme with substrate (Badger *et al.*, 1998; Giordano *et al.*, 2005; Kaplan and Reinhold, 1999). The elevated concentration of carbon dioxide also serves to drive out competing oxygen. An extracellular α-type carbonic anhydrase has been identified in the glycocalyx of *Microcoleus chthonoplastes* (Kupriianova *et al.*, 2004) and in the extracellular fraction of *Anabaena* sp. strain PCC 7120 and *Synechococcus* sp. strain PCC 7942 (Soltes-Rak *et al.*, 1997). Soltes-Rak *et al.* also demonstrated that this enzyme was up-regulated under high carbon dioxide conditions (Soltes-Rak *et al.*, 1997). In the extracellular fraction the carbonic anhydrase rapidly established equilibrium between dissolved carbon dioxide and carbonate.

The carbon concentrating mechanism begins in the extracellular space, where carbonic anhydrase converts dissolved carbon dioxide to bicarbonate. Bicarbonate is actively taken up by either bicarbonate transporter and carbon dioxide freely diffuses through the plasma membrane, whereupon it is converted by the carbon dioxide transporters to bicarbonate. By this process, the internal bicarbonate concentration can be increased by three orders of magnitude relative to extracellular bicarbonate concentrations. Bicarbonate diffuses to the carboxysome where it is converted back to carbon dioxide in the presence of RuBisCO and subsequently fully assimilated through the reductive pentose phosphate pathway (Badger and Price, 2003; Giordano *et al.*, 2005; Kaplan and Reinhold, 1999).

As implied previously, certain aspects of the carbon concentrating mechanisms are subject to regulation depending on carbon dioxide availability and other nutrients. Cyanobacteria

supplemented with carbon dioxide show increased growth rates (Kang *et al.*, 2005; Otero and Vincenzini, 2004). Kaplan *et al.* demonstrated how carbon dioxide supplementation affected *Anabaena variabilis* when grown in the presence of 5% carbon dioxide. No change in K_m the inorganic carbon transport occurred versus that of low (atmospheric) carbon dioxide levels. However, the inorganic carbon transport V_{max} was 10 times greater in cells grown in low carbon dioxide. *Anabaena variabilis* was accumulating more inorganic carbon internally under low partial pressure of carbon dioxide (Kaplan *et al.*, 1980). Nieva and Valiente compared *Anabaena variabilis* grown mixotrophically and autotrophically. Those grown mixotrophically showed reduced ability to fix inorganic carbon and had lower carbonic anhydrase and RuBisCO activities (Nieva and Valiente, 1996). Under low light conditions the carbon concentrating mechanism of *A. variabilis* was found not to be repressed (Beardall, 1991). Skleryk *et al.* examined a number of growth conditions (autotrophic, mixotrophic, heterotrophic, high and low carbon dioxide) for *Chlorogloeopsis* sp. ATCC 27193 and noted that each conditions impacted the carbon concentrating mechanisms differently (Skleryk *et al.*, 2002). These studies indicate that careful consideration should be given to nutrient conditions, particularly dissolved inorganic carbon concentrations, when studying cyanobacterial metabolic processes.

1.6 Nitrogen Metabolism

Many cyanobacteria are diazotrophic, having the ability to fix atmospheric nitrogen. The process of fixing nitrogen is costly requiring 8 electrons and at least 16 ATP (eq. 1.4) (Stal, 2003).

$$N_2 + 8[H] + 16ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16Pi \qquad \text{(eq. 1.4)}$$

Nitrogenase, the enzyme that reduces nitrogen to ammonia, is very sensitive to oxygen (Fay, 1992). This is a problem for cyanobacteria, which produce oxygen as a byproduct of photosynthesis. Cyanobacteria in the orders *Nostocales* and *Stigonematales* protect nitrogenase by specialized cells called heterocysts (Whitton and Potts, 2002). Heterocysts are terminally differentiated cells that are devoid of oxygenic PSII (PSI remains active to provide energy) and lose the ability to fix inorganic carbon (Wolk, 1996). Additionally, heterocysts have a polysaccharide envelope that aids in preventing oxygen from entering the cell (Cardemil and Wolk, 1981; Wolk *et al.*, 1988). In a filament, heterocysts intersperse vegetative cells by every

tenth to fifteenth cell. Heterocysts receive nutrients from adjacent vegetative cells and in return provide a source of fixed nitrogen (for reviews see (Fay, 1992; Wolk, 1996)).

As a means of conserving cellular energy, all forms of diazotrophs prefer to use other forms of available fixed nitrogen before utilizing N_2. Nearly all cyanobacteria are capable of utilizing nitrate by reducing it to ammonia. Other nitrogen sources that can be assimilated by cyanobacteria are ammonia, nitrite, urea, and amino acids. Heterocysts do not form until the cells have been starved for fixed nitrogen for a period of approximately 20 hours. Ammonia is an inhibitor of nitrogen fixation in fully formed heterocysts (Fay, 1992; Stal, 2003).

Brewer and Goldman recognized that the assimilation of some fixed forms of nitrogen by marine phytoplankton can result in changes of pH and alkalinity exterior to the cells (Brewer and Goldman, 1976). They documented that the uptake of ammonia resulted in a decrease in pH and alkalinity. Conversely the uptake of nitrate led to an increase in pH and alkalinity. In a later study it was shown that assimilation of urea did not affected pH or alkalinity, indicating that the phenomenon was associated with the charged species of fixed nitrogen (Goldman and Brewer, 1980). The increase in pH in relation to nitrate uptake has also been observed with filamentous cyanobacteria (Otero and Vincenzini, 2004).

1.7 EPS Synthesis

A conspicuous characteristic of most cyanobacteria is the ability to produce large amounts of extracellular polymeric substance composed primarily of polysaccharides (EPS). The EPS can be observed in the form of sheaths, capsules, slimes, and released polysaccharides (RPS). A sheath is a thin, electron-dense layer, which surrounds a cell or cell groups loosely. Capsules are thick, structurally coherent layers that are tightly associated with the surface of the cell and have an identifiable sharp outline. Slimes do not reflect the shape of the cells and are dispersed unevenly around the organisms. Released polysaccharides (RPS) are water soluble materials that are shed from capsules and slimes. Most extracellular polysaccharides contain uronic acids making them anionic/acidic (De Philippis and Vincenzini, 1998).

The presence of the EPS is thought to perform several functions. The material can provide a repository for water and protect against desiccation (Hill *et al.*, 1994; Potts, 1994; Shaw *et al.*, 2003), prevent membrane fusion upon desiccation (Hill *et al.*, 1997; Potts, 1994), facilitate biofilm formation (De Philippis *et al.*, 2005; De Philippis and Vincenzini, 1998; Fattom

and Shilo, 1984), distribute filaments and cells in the medium to provide maximum light collection (Martin and Wyatt, 1974), function as a chelator for ions whether as potential nutrients or sequestering toxins (De Philippis *et al.*, 2006; De Philippis and Vincenzini, 1998; Shah *et al.*, 2000), may aid in symbiotic attachments (De Philippis and Vincenzini, 1998), and protect against grazing protozoa (De Philippis and Vincenzini, 1998; Garcia-Pichel *et al.*, 2004).

Not all strains of cyanobacteria are capable of generating RPS in significant amounts. De Philippis *et al.* examined 40 strains of the genus *Nostoc* from the Pasteur Culture Collection (De Philippis *et al.*, 2000b). Of these only 25 showed capsules, sheaths, or slime surrounding the cell filaments along with RPS. The other 15 strains did not generate appreciable amounts of RPS and were of mixed origins from soils, freshwater, plant symbiont, or unknown. The monosaccharide composition of the RPS from the 25 strains characterized by extracellular glycan consisted of glucuronic and galacturonic acids, galactose, glucose, mannose, arabinose, xylose, ribose, fucose, and rhamnose (De Philippis *et al.*, 2000a). Similar monosaccharide units have been found in *Nostoc commune* DRH-1 with the addition of the novel nosturonic acid (Helm *et al.*, 2000).

In 1998 De Philippis reviewed the known monosaccharide compositions of RPS for several strains of cyanobacteria (De Philippis and Vincenzini, 1998). Among the order *Nostocales* the monosaccharide ratios varied between strains and consisted of those listed above with some methyl and amino sugars. Glucose was the most common monosaccharide appearing in 90% of all cyanobacteria strains examined. These compositions are not static and can change with cell cycle or nutrient conditions. For instance, *Anabanea cylindrica* monitored in BG-11, BG-11_0, BG-11_0 without phosphate, and with various organic carbon sources varied in the amount of EPS generated (BG-11 medium produce 7.5 time greater EPS than BG-11_0 medium) and in the internal monosaccharide ratios between growth conditions (Lama *et al.*, 1996). In another study, *Cyanospira capsulata* was found to produce a RPS with a monosaccharide composition that was independent of growth phase and tested growth conditions (Vincenzini *et al.*, 1990). Thus variation in monosaccharide compositions of extracellular glycan is strain dependent.

Whether cyanobacteria grown planktonically or attached to surfaces on biofilms produce polysaccharides of different composition is not known. The most recent study comparing biofilm and planktonic EPS has been done with a non-cyanobacterial organism. Kives *et al.* (Kives *et al.*,

2006) characterized the monosaccharide composition of the extracellular glycans of *Pseudomonas fluorescens* grown on glass, stainless steel, and in liquid medium. Analysis showed that uronic acids (mainly glucuronic and galacturonic) were a major component of both planktonic and biofilm EPS. Differences in EPS were noted between biofilm surfaces, in that stainless steel promoted more glucuronic acid in the EPS. Biofilm EPS also contained proportionately more non-uronic acid monosaccharides than planktonic EPS. Planktonic and biofilm generated EPS were classically thought to be the same, but these findings press the need to consider differences in EPS monosaccharide content in other organisms, such as cyanobacteria, in relation to growth conditions.

In addition to changes in composition, EPS morphology (capsule, slime, sheath, or RPS) depends on nutrient conditions. Otero and Vincenzini (Otero and Vincenzini, 2003) studied the effect of light intensity and nitrogen source on three strains of the filamentous cyanobacteria *Nostoc* from the Pasteur Culture Collection. *Nostoc* sp. strains PCC 7413, 7936, and 8113 showed strain specific variations in cell growth, EPS production and morphology. It was observed that the capsulated strains PCC 7936 and PCC 8113 became uncapsulated when grown in the presence of fixed nitrogen (BG-11 medium). They also demonstrated that the capsulated morphology could be reestablished by returning the strain to nitrate-free medium showing that the nude morphology was not due to the selection of an uncapsulated mutant. The strain PCC 7413 was never capsulated regardless of nitrates or light intensity. PCC 7413 was shown to release copious amount of EPS into the medium under all conditions, greatly contributing to the viscosity of the medium. It was the only strain of the three to generate RPS of appreciable amounts in BG-11 medium. Increased light intensity resulted in an increase in total carbohydrates for PCC 7936 and PCC 8113. Otero and Vincenzini's findings support the concept that EPS morphology is not a static characteristic of cyanobacteria but subject to nutrient conditions. They also concluded that the capsulated morphology of *Nostoc* sp. is strongly related to diazotrophic conditions (Otero and Vincenzini, 2003).

In a subsequent study, Otero and Vincenzini (Otero and Vincenzini, 2004) addressed the issue of capsulated morphologies in relation to nitrogen source, carbon availability, and pH using PCC 7936 as the model organism. The pH changed little in diazotrophic cultures but was observed to increase to nearly 10 in BG-11 cultures grown in low carbon dioxide. Growth was impaired in cultures that approached pH 10, and such cultures were shown to contain short

damaged filaments. The increase in pH was a result of nitrate uptake, and was corrected by increasing the partial pressure of carbon dioxide as well as the addition of 20 mmol L^{-1} of HEPES buffer. Supplementation of carbon dioxide enhanced the growth of the cultures as did the presence of nitrates. In total eight different conditions were tested for the capsulated morphology of which four showed this phenotype. Capsulated morphologies were observed in diazotrophic cultures supplemented with carbon dioxide with and without HEPES buffer, cultures grown non-diazotrophically under low carbon dioxide without HEPES buffer where the pH approached 10, and cultures grown non-diazotrophically with HEPES and supplemented with carbon dioxide. The microscopic morphologies were confirmed with protein to carbohydrate ratios less than or equal to 1.5. Otero and Vincenzini concluded that the capsule serves as a carbon sink when the availability of carbon and nitrogen is unbalanced. Of concern was that the capsulated morphology was generated in the presence of nitrates where the pH had increased. They reasoned that the increase in pH was related to an inhibition of nitrate uptake while carbon fixation continued (Otero and Vincenzini, 2004).

A proper understanding of the nature of extracellular polymeric substances, their composition, physical properties, and means of production has great applications to both an ecological understanding and to industrial applications. The structures of biofilms, microbial mats, and planktonic community behavior are highly dependent on the content and nature of the EPS. Survivability of cyanobacteria is also tightly associated with the EPS. Potts commented on the importance of Otero and Vincenzini's findings stating that they have shown how to reproduce capsulated morphologies which enable further study of the capsule (Potts, 2004). Other strains should also be examined for capsular morphology under similar conditions, such as non-heterocystous strains which are dominant in benthic mats.

1.8 Research Objectives

The nutrients available to cyanobacteria influence EPS production rates and composition modulating community structures. Hence it is important to understand the role of cyanobacterial EPS in ecological processes. As demonstrated in previous work, variations in growth conditions can greatly alter the abundance, composition, and nature of EPS (Kang *et al.*, 2005; Kives *et al.*, 2006; Lama *et al.*, 1996; Otero and Vincenzini, 2003; Otero and Vincenzini, 2004). Otero and Vincenzini concluded that the carbon-nitrogen ratio range to produce capsulated morphologies may be narrow (Otero and Vincenzini, 2004). Therefore, it is important to know and consider the exact availability cells have to carbon and nitrogen and how the availability may change with length of culturing and growth. I apply Stumm and Morgan's (Stumm and Morgan, 1970) calculation for aquatic systems for calculating dissolved inorganic carbon available in the traditionally used BG-11 and BG-11_0 medium (Rippka *et al.*, 1979), and demonstrate morphological and chemical differences resultant of the presence or absence of nitrates in three different strains of cyanobacteria belonging to the order *Nostocales*.

CHAPTER 2: METHODS

2.1 Methods for Cell Culture: General.

Three filamentous strains of cyanobacteria belonging to order Nostacales, *Nostoc commune* DRH-1, *Anabaena* PCC7120, and *Anabaena variabilis* ATCC 29413 (Potts, 2000) were cultured in liquid medium at room temperature. Cells were grown in 50 ml of media (250 ml erlenmeyer flasks) on a shaker table rotating at a constant 75 revolutions min^{-1}. Illumination was provided by cool white fluorescent lights at a flux of $1.5x10^{15}$ Quanta sec^{-1} cm^{-2}, and a 12 h/12 h on/off light cycle was used. Inoculants for biological triplicates were taken from the same nurse cultures (one nurse culture to three identical experimental cultures) that were acclimated to appropriate growth medium. The filamentous strains of cyanobacteria were grown in both BG-11_0 and BG-11 liquid growth medium at an initial pH of 7.4 (Rippka *et al.*, 1979). BG-11 medium differs from BG-11_0 by containing a substantial fixed source of nitrogen, 17.65 mM of sodium nitrate. It was observed that the pH does not remain at 7.4 after a few days growth in BG-11/BG-11_0 medium. To keep pH from drifting, 20 mM HEPES was added to both BG-11 and BG-11_0 media as described by Otero *et al.* (Otero and Vincenzini, 2004). Inoculants originate from BG-11 or BG-11_0 cultures without HEPES buffer. Cultures were grown without HEPES buffer for various periods of time: 26, 38, 41, 57, and 61 days. Cultures with HEPES buffer were grown for 13 days before imaging.

The composition of BG-11 medium is $MgSO_4 \cdot 7H_2O$, 0.3 mM; $CaCl_2 \cdot 2H_2O$, 0.25 mM; $NaNO_3$, 17.65 mM (omitted for BG-11_0); K_2HPO_4, 0.18 mM; Citric Acid, 0.03 mM; Ferric Ammonium Citrate, 0.03 mM; Na_2Mg EDTA, 0.003 mM; Na_2CO_3, 0.19 mM; H_3BO_3, 0.0463 mM; $MnCl_2 \cdot 4H_2O$, 0.00914 mM; $ZnSO_4 \cdot 7H_2O$, 0.00137 mM; $CuSO_4 \cdot 5H_2O$, 0.000316 mM; $Co(NO_3)_2 \cdot 6H_2O$, 0.000168 mM (Rippka *et al.*, 1979).

2.2 Biofilm Growth

Microscope glass cover slips (24 x 60 mm) (VWR) were suspended vertically in liquid cultures of *Anabaena variabilis* ATCC 29413 and *Anabaena* PCC 7120 grown in both BG-11 and BG-11_0 medium as explained above. The glass cover slips traversed the air-water interface of the liquid culture similar to Kives *et al.* (Kives *et al.*, 2006) where biofilm attachment and growth

has previously been observed on the sides of culture flasks (**Fig. 2.1**). After 32 days the microscope cover slips were removed with special attention not to disturb biofilm. Biofilm was imaged macroscopically, and with confocal laser scanning microscopy.

2.3 Imaging

Macroscopic images were photographed with an Olympus SP-310 digital camera (Olympus Corporation). Microscopic images of cells from culture were obtained with a Carl Zeiss Axiovert 200 inverted microscope using bright field microscopy, phase contrast microscopy, and epifluorescence microscopy with a Rhodamine filter. Images were acquired and processed with Carl Zeiss Axiovision software release 4.6. Confocal microscopy was performed with a Carl Zeiss 510 Confocal Laser Scanning Microscope (CLSM). CLSM images were obtained in multi-track mode with excitation wavelengths of 488 nm, 543 nm, and 633 nm with corresponding emission band passes of 505-550 nm, 560-615 nm, and 655-719 nm. Images were collected with Carl Zeiss LSM imaging software.

2.4 Extracellular Polymeric Substance (EPS) Isolation and Analysis

Cells were removed from culture medium by centrifugation at 3000 x g. Centrifugal filtration (Amicon; 10kDa molecular weight cut-off, MWCO---3000 x g) provided a high molecular weight fraction that was then diluted with 15 mL of distilled/deionized water and centrifuged again. The high molecular weight EPS (greater than 10kDa fraction) was freeze-dried and weighed (freeze-dried weight).

The high molecular weight EPS fractions were hydrolyzed using one-step and two-step procedures to determine the monosaccharide compositions (Talaga *et al.*, 2002). For the two step hydrolysis one to four milligrams of freeze-dried high molecular weight EPS was methylated in methanolic 2N HCl (1 mL) at 80 °C in sealed glass ampoules. After 24 hours, samples were dried under reduced pressure with either a rotary evaporator or a centrifugal vacuum apparatus and subsequently hydrolyzed with 2N TFA (trifluoroacetic acid) for 2 hours at 121 °C in sealed glass ampoules. Samples were dried and rinsed twice with isopropanol with drying after each rinse. Samples were then placed on a lyophilizer for 24 hours. The 2N methanolic HCl was excluded from the one step hydrolysis (*i.e.* TFA hydrolysis only).

2.5 Monosaccharide Analysis by Anion-Exchange Chromatography and Pulsed Amperometric Detection

EPS hydrolysates were analyzed on a Dionex LC system using a CarboPac PA 10 (4mm x 250 mm) anion-exchange analytical column (Dionex Corp., Sunnyvale, CA) with a guard column (4mm x 50mm). Mobile phases were A: 100mM NaOH, B: 1M NaAc and 100mM NaOH, C: distilled and deionized water, and D: 100mM NaOH. The mixing of the mobile phases generated the following NaOH gradients: 0-5 min, 12 mM; 5-15 min, 20-50 mM; 15-20 min, 50 mM; 20-22 min, 50-90 mM; 22-24 min, 90-100 mM; 24-40 min, 100 mM; 40-46 min, 100-50 mM; 46-48 min, 50-20 mM; 48-53 min, 20 mM. For NaAc the gradients were: 0-22 min, 0 mM; 22-24 min, 0-100 mM; 24-25 min, 100 mM; 25-31 min, 100-200 mM; 31-38 min, 200-500 mM; 38-40 min, 500-0 mM; 40-53 min, 0 mM. Flow rate was 1 ml/min. Dried hydrolysates were resuspended in 1 ml distilled/deionozed water and diluted by a factor of 10 before loading. Sample injection volume was 25 μL. Monosaccharides were detected with pulsed-amperometric detector (PAD) with an AgCl electrode. Data was collected and analyzed with Dionex PeakNet software. EPS monosaccharide concentrations were quantified against external monosaccharide standards that were subjected to identical hydrolysis conditions.

2.6. Dissolved Inorganic Carbon Calculations

Carbon dioxide (CO_2) and nitrogen (N_2) are not available in liquid medium in the same ratios as they are in the atmosphere. Carbon dioxide has a greater ability to dissolve in water than nitrogen. The ratio between available carbon and available nitrogen affect rates of metabolism and growth in cyanobacteria. Thus it is of importance to estimate the amount of carbon and nitrogen available under the culturing conditions used. The relationship between the partial pressure of a gas and concentration of the dissolved gas in water is given by Henry's Law. This natural law states that the concentration of gas dissolved into an aqueous medium is directly proportional to the partial pressure of the gas by a constant (Stumm and Morgan, 1970) (see equation 1).

$$P_x \; K_H = [x_{(aq)}] \qquad \text{(eq.2.1)}$$

Where 'Px' is the partial pressure of the gas 'x', 'K_H' is Henry's constant, and '$[x_{(aq)}]$' is the concentration of the dissolved gas. Henry's Constant is specific to the gaseous species and temperature (Stumm and Morgan, 1970). With this relationship we can calculate the concentration of N_2 and inorganic carbon available to cells in aqueous solutions.

At 1 atmospheric pressure the partial pressure of nitrogen is about 0.77 atm. The concentration of nitrogen dissolved in water at equilibrium with the atmosphere is $5x10^{-4}$ M. Carbon dioxide dissolves in water in two forms: aqueous carbon dioxide ($CO_{2(aq)}$) and the hydrated carbonic acid (H_2CO_3) species. For simplicity we treat aqueous carbon dioxide and carbonic acid as the same entity ($[H_2CO_3^*]$) and use a corresponding K_H.

$$Pco_2\ K_H.co_2 = [CO_{2(aq)}] + [H_2CO_3] = [H_2CO_3^*] \qquad \text{(eq. 2.2)}$$

The $K_H = 10^{-1.5}$ for equation above (Stumm and Morgan, 1970). At atmospheric Pco_2 ($10^{-3.5}$ atm) the $[H_2CO_3^*]$ is approximately 10^{-5} M. As carbonic acid is a weak acid, it dissociates to bicarbonate and carbonate ions in relation to pH.

$$[H_2CO_3^*] = [HCO_3^-] + [H^+] \quad \text{(eq. 2.3)}$$

$$[HCO_3^-] = [CO_3^{2-}] + [H^+] \qquad \text{(eq. 2.4)}$$

The equilibrium for these equations are given by

$$[HCO_3^-][H^+][H_2CO_3^*]^{-1} = Ka_1 = 10^{-6.3} \qquad \text{(eq. 2.5)}$$

$$[CO_3^{2-}][H^+][HCO_3^-]^{-1} = Ka_2 = 10^{-10.25} \qquad \text{(eq. 2.6)}$$

This means the total inorganic carbon (C_i) is the summation of dissolved carbon dioxide, carbonic acid, bicarbonate ion, and carbonate ion.

$$[C_i]_T = [CO_{2(aq)}] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}] \qquad \text{(eq. 2.7)}$$

In an open system with the current atmospheric partial pressures the total dissolved inorganic carbon will never drop below 10^{-5}M (Stumm and Morgan, 1970). This means the lowest $C_{i(aq)}/N_{2(aq)}$ molar ratio is around $2x10^{-2}$. Compare this to an atmospheric $C_{i(gas)}/N_{2(gas)}$ molar ratio of $4.1x10^{-4}$ in air. The traditional method for increasing the available dissolved inorganic carbon for autotrophic growth is by increasing the percentage of carbon dioxide in the open system. As the partial pressure of carbon dioxide of a system is increased, the dissolved $H_2CO_3^*$ increases, producing a further equilibrium shift in equations 2.3 and 2.4 to generate more HCO_3^- and CO_3^{2-}. By combining equations 2.2, 2.5, 2.6, and 2.7 we generate the relationship between Pco_2 and total dissolved inorganic carbon C_i.

$$[C_i]_T = Pco_2 K_{H\text{-}CO2} (1 + Ka_1 [H^+]^{-1} (1 + Ka_2 [H^+]^{-1})) \qquad \text{(eq. 2.8)}$$

From the working equation we see that the total dissolved inorganic carbon is dependent upon the pH of the solution. If we enabled the system to maintain a pH of 7.5 the dissolved inorganic carbon would be related to the partial pressure of carbon dioxide by a factor of 0.534mol l^{-1} atm^{-1}.

$$[C_i]_T = 0.534\ Pco_2 \qquad \text{(eq. 2.9)}$$

This yields a dissolved inorganic carbon concentration of 0.169 mmol L^{-1} at the current partial pressure of carbon dioxide (3.16×10^{-4}). The molar ratio between the dissolved inorganic carbon and dissolved nitrogen ($C_i/N_{2(aq)}$) at atmospheric conditions is 0.4. It is common practice to supplement cultures with 1%, 5%, and 10% CO_2 mixed with air. The corresponding dissolved inorganic carbon concentrations at pH 7.5 and 1 atm are 5.34 mmol L^{-1}, 26.7 mmol L^{-1}, and 53.4 mmol L^{-1} respectively. At a constant pressure as the Pco_2 increases the partial pressure of nitrogen will decrease. This give us the respective $C_i/N_{2(aq)}$ ratios are 11.3, 59.0, and 124.6. So only a slight increase in the Pco_2 of a system dramatically increases the availability of dissolved inorganic carbon over that of aqueous N_2.

All of the dissolved inorganic carbon to dissolved nitrogen ratios listed and equation 2.9 are valid only if the system is maintained at a constant pH of 7.5. However, the increase of Pco_2 in an aqueous system also generates more protons in solution resulting in drop in pH. The

relationship between proton concentration and Pco_2 is obtained by using the charge balance equation for all ions in solution. The charge balance for BG-11 medium (Rippka *et al.*, 1979) modified with the addition of HEPES buffer (Otero and Vincenzini, 2004) is given in equation 10.

$$[H^+] + 2[Ca^{2+}] + 2[Mg^{2+}] + [K^+] + 3[Fe^{3+}] + [Na^+] + [NH_4^+] = [OH^-] + [HCO_3^-] + 2[CO_3^{2-}] + [Cl^-] + 2[SO_4^{2-}] + [NO_3^-] + [H_2PO_4^-] + 2[HPO_4^{2-}] + 3[PO_4^{3-}] + [H_2A^{-1}_{citrate}] + 2[HA^{-2}_{citrate}] + 3[A^{-3}_{citrate}] + [A^-_{HEPES}] + [EDTA^-] + 2[EDTA^{2-}] \quad \text{(eq. 2.10)}$$

Some species (NH_4^+, PO_4^{3-}, $EDTA^-$, $EDTA^{2-}$) are of little abundance within the pH range of interest and can be ignored to simplify the calculation. Other species (Ca^{2+}, Mg^{2+}, K^+, Fe^{3+}, Na^+, Cl^-, SO_4^{2-}) are ions that do not change concentration significantly within the pH range of interest and can be combined into a single constant (K_{si}) for the purpose of this calculation. The NO_3^- concentration does not change in response to pH within the range of interest and would be considered part of the K_{si} constant however, it is left in the equation for an application that will be explained later. The simplified charge balance equation is:

$$[H^+] = [OH^-] + [HCO_3^-] + 2[CO_3^{2-}] + [NO_3^-] + [H_2PO_4^-] + 2[HPO_4^{2-}] + [H_2A^{-1}_{citrate}] + 2[HA^{-2}_{citrate}] + 3[A^{-3}_{citrate}] + [A^-_{HEPES}] + K_{si} \quad \text{(eq. 2.11)}$$

K_{si} can be calculated by knowing the initial concentrations of the medium, the pH, and the Pco_2. For BG-11 at pH 7.5 and an atmospheric Pco_2 of 3.16×10^{-4} atm the K_{si} is (-0.0183 mol l^{-1}).

The relationship between $[H^+]$ and $[H_2CO_3^*]$ is calculated by substituting in the carbonic acid dissociation equations (eq. 2.5 and 2.6) then solving for $[H_2CO_3^*]$.

$$[H_2CO_3^*] = ([H^+]^3 - (\textit{sum of charge concentrations of all other charged species})[H^+]^2 - K_W[H^+])(K_{a1}([H^+] + 2K_{a2}))^{-1} \quad \text{(eq.12)}$$

We then substitute in Henry's law (eq. 2.1) for $[H_2CO_3^*]$ to obtain the final relationship between $[H^+]$ and Pco_2.

$$Pco_2 =$$
$$([H^+]^3 - (\textit{sum of charge concentrations of all other charged species})[H^+]^2 - K_W[H^+])(K_{a1}([H^+] + 2\,K_{a2})\,K_{H\text{-}CO2})^{-1} \qquad \text{(eq. 2.13)}$$

The coefficient of the '$[H^+]^2$' term is the sum of charge concentrations of all negatively charged species (other than carbonate and bicarbonate) minus the charge concentration of positive ions in solution. For species of little abundance or which do not change within the pH range of interest we summed those charges into a single term 'K_{si}'. For other species that change within the pH of interest, such as phosphates and biological buffers, the concentration of the charged species will be written as their dissociation equilibria solved for the charged species. The equations used for our calculations are listed below:

$$[A^-_{HEPES}] = K_{a1\text{-}HEPES}\,[A_{HEPES}]_{total}\,([H^+] + K_{a1\text{-}HEPES})^{-1} \qquad \text{(eq. 2.14)}$$

$$[H_2PO_4^-] = [H^+]\,[A_{PO4}]_{total}\,(K_{a2\text{-}PO4} + [H^+])^{-1} \qquad \text{(eq. 2.15)}$$

$$[HPO_4^{-2}] = K_{a2\text{-}PO4}\,[A_{PO4}]_{total}\,(K_{a2\text{-}PO4} + [H^+])^{-1} \qquad \text{(eq. 2.16)}$$

$$[H_2A^{-1}_{citrate}] = K_{a1\text{-}citrate}\,[A_{citrate}]_{total}\,[H^+]^2\,([H^+]^3 + K_{a1\text{-}citrate}\,([H^+]^2 + K_{a2\text{-}citrate}\,([H^+] + K_{a3\text{-}citrate}))^{-1} \qquad \text{(eq. 2.17)}$$

$$[HA^{-2}_{citrate}] = K_{a1\text{-}citrate}\,K_{a2\text{-}citrate}\,[A_{citrate}]_{total}\,[H^+]\,([H^+]^3 + K_{a1\text{-}citrate}\,([H^+]^2 + K_{a2\text{-}citrate}\,([H^+] + K_{a3\text{-}citrate}))^{-1} \qquad \text{(eq. 2.18)}$$

$$[A^{-3}_{citrate}] = K_{a1\text{-}citrate}\,K_{a2\text{-}citrate}\,K_{a3\text{-}citrate}\,[A_{citrate}]_{total}\,([H^+]^3 + K_{a1\text{-}citrate}\,([H^+]^2 + K_{a2\text{-}citrate}\,([H^+] + K_{a3\text{-}citrate}))^{-1} \qquad \text{(eq. 2.19)}$$

Without writing out the entire equation (leaving the buffers in concentrations of charged species) the Pco_2 - $[H^+]$ relationship appears as:

$$Pco_2 = ([H^+]^3 - ([NO_3^-] + [A^-_{HEPES}] + [H_2PO_4^-] + 2[HPO_4^{-2}] + [H_2A^{-1}_{citrate}] + 2[HA^{-2}_{citrate}] + 3[A^{-3}_{citrate}] + K_{si})[H^+]^2 - K_W[H^+])(K_{a1\text{-}CO2}([H^+] + 2K_{a2\text{-}CO2})K_{H\text{-}CO2})^{-1} \quad \text{(eq. 2.20)}$$

Otero and Vincenzini noticed that when the filamentous nitrogen fixing cyanobacteria *Nostoc* PCC 7936 was grown in the presence of nitrate as a nitrogen source (BG-11 medium), the pH increased (Otero and Vincenzini, 2004). This phenomenon has also been observed with the three filamentous strains examined in this study. Brewer and Goldman (Brewer and Goldman, 1976) describe the basic net metabolic reactions of the phytoplankton studied in the following equation.

$$106\,CO_2 + 138\,H_2O + 16NO_3^- \rightarrow (CH_2O)_{106} + (NH_3)_{16} + 16OH^- + 138O_2 \quad \text{(eq. 2.21)}$$

In a later study Goldman and Brewer confirmed that when phytoplankton is grown with nitrate as a nitrogen source the alkalinity of the medium increases as nitrate is consumed in a one to one ratio. In other words, the uptake of nitrate from the medium is countered by the release a strong base (hydroxide ions) into the medium (Brewer and Goldman, 1976; Goldman and Brewer, 1980). From this relationship the nitrate concentration of the medium can be inferred by the change in alkalinity. If no other mechanism perturbs the system, the concentration of nitrate can be calculated simply by using the charge balance equation (eq. 2.11) solved for $[NO_3^-]$ (eq. 2.22).

$$[NO_3^-] = [H^+] - ([OH^-] + [HCO_3^-] + 2[CO_3^{2-}] + [H_2PO_4^-] + 2[HPO_4^{2-}] + [H_2A^{-1}_{citrate}] + 2[HA^{-2}_{citrate}] + 3[A^{-3}_{citrate}] + [A^-_{HEPES}] + K_{si}) \quad \text{(eq. 2.22)}$$

With the above equations we can now calculate the total dissolved inorganic carbon available to cells and remaining nitrate concentration in BG-11 medium by knowing the pH and Pco_2 of the system.

Table 2.1 Constants used in calculations

$K_{a1\text{-}CO2}$ =	$10^{-6.3}$
$K_{a2\text{-}CO2}$ =	$10^{-10.25}$
$K_{H\text{-}CO2}$ =	$10^{-1.5}$ mol L^{-1} atm^{-1}
$K_{H\text{-}N2}$ =	$10^{-3.21}$ mol L^{-1} atm^{-1}
$K_{a1\text{-}HEPES}$ =	$10^{-7.55}$
$K_{a2\text{-}PO4}$ =	$10^{-7.21}$
$K_{a1\text{-}citrate}$ =	$10^{-3.13}$
$K_{a2\text{-}citrate}$ =	$10^{-4.76}$
$K_{a3\text{-}citrate}$ =	$10^{-6.4}$
K_{si} (BG-11 with 20mM HEPES @ pH 7.5) =	-0.0277 mol L^{-1}
K_{si} (BG-11 without HEPES @ pH 7.5) =	-0.0183 mol L^{-1}
K_W =	10^{-14}

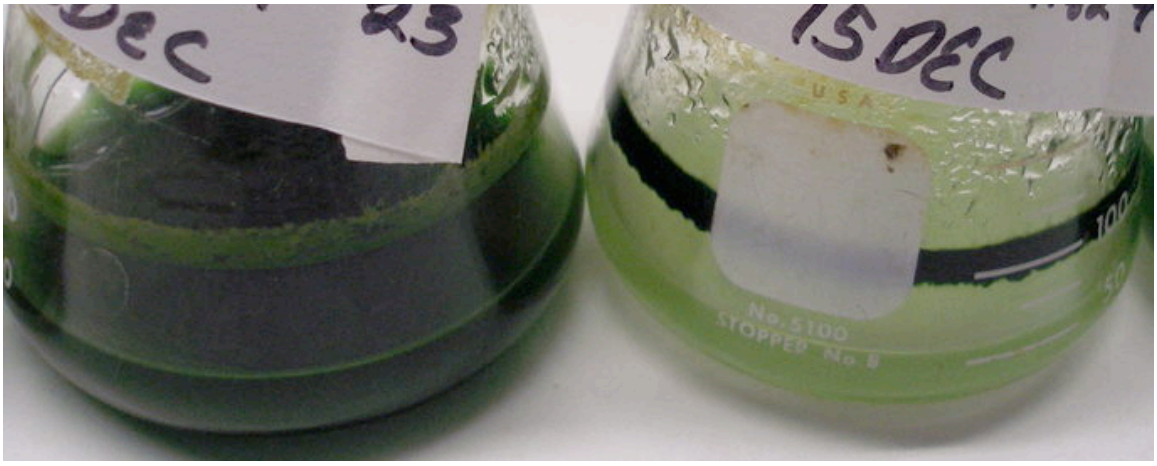

Figure 2.1 Cultures of *Anabaena* PCC 7120. Cultures of *Anabaena* PCC 7120 grown in BG-11 (left) and BG-11_0 (right). The biofilm ring of cells around the flask is thicker in the BG-11_0 culture with a visually lesser density of cells in the medium.

CHAPTER 3: RESULTS

3.1 Introduction

Three strains of cyanobacteria (*Anabaena variabilis* ATCC29413, *Anabaena* PCC 7120, and *Nostoc commune* DRH-1) demonstrated different characteristics when grown with (BG-11) and without (BG-11_0) nitrates in the medium. These differences were observed in cell and EPS yields, biofilm development and planktonic behavior, and monosaccharide composition and morphology of the extracellular glycans. The addition of HEPES buffer to the medium created an unexpected morphology which resulted in the author's decision not to pursue further analysis using HEPES buffer in this context. Dissolved inorganic carbon concentrations were calculated for conditions which cyanobacteria are cultured.

3.2 Final Cell and EPS Yields

The cell mass obtained in 50 mL cultures are shown in **Fig 3.1**. Some variation was noticed in cell pellet masses obtained by freeze-dried weights. Changes appeared dependent on both length of incubation and nitrogen source. *Anabaena* PCC 7120 showed the greatest variations with regards to length of time for growth. Masses presented are total masses obtained from 50 milliliter cultures of which those cultures of the same strain, medium, and days of incubation were inoculated from the same nurse culture (**Fig. 3.1**).

Greatest growth was achieved for *Anabaena* PCC 7120 in BG-11 medium. Freeze-dried cell masses ranged from 79.9±15.9 milligrams at 26 days, 117.2±7.5 milligrams at 38 days, and 169.4±8.4 milligrams at 57 days. From 26 to 57 days of culture incubation the growth appears to be linear. *Anabaena* PCC 7120 grown in BG-11_0 showed little change in mass between 26 and 57 days of growth.

Like *Anabaena* PCC 7120, *Nostoc commune* showed significantly greater growth in the presence of fixed nitrogen. *Nostoc commune* grown in BG-11 medium for 61 days had a freeze dried cell mass 148.7±28.5 milligrams which was 3.7 times greater than that grown in BG-11_0 medium which had a freeze-dried mass of 40.0±0.7 milligrams. *Nostoc commune* grown for 41 days yielded a greater mass of cells grown in BG-11 medium than that grown for 61 days. The decrease in mass may have been the result of shedding more EPS from the filament or cell death and lysis due to the age of the culture. It may also be related to differences in the quantity of cells

used for inoculation. Important to note is that *Nostoc commune* cultured in BG-11 medium produced significantly more viscous cultures over those grown in BG-11_0 medium.

Anabaena variabilis showed comparatively little variation between medium used and time of growth. Average culture freeze-dried cell mass was approximately 66.7 milligrams with the minimum mass of 56.6±2.7 milligrams at 38 days in BG-11_0 medium. The maximum freeze dried cell mass was 78.6±6.1 milligrams at 57 days also in BG-11_0 medium.

Freeze-dried masses were also obtained for released EPS after filtration through a 10 kDa centrifugal filter for *Anabaena variabilis* and *Anabaena* PCC 7120 cultures grown for 57 days and for *Nostoc commune* grown for 61 days (**Fig. 3.2**). Higher amounts of released polymeric material were obtained from both *Anabaena* strains when grown in the presence of nitrates. *Anabaena variabilis* and *Anabaena* PCC 7120 produced over 3 and 6 times more polymeric material (respectively) when grown in BG-11 vs. BG-11_0. On the other hand *Nostoc commune* produced approximately the same amounts with and without the presence of nitrates. This may be a result of bulk glycan structure, which appears to be different in the two medium preparations (**Fig. 3.3**). If cells adhere to the bulk glycan more tightly, it would result in an increased total freeze-dried cell mass, since no measures were taken to remove the tightly associated EPS fraction. This would make the comparison of BG-11 and BG-11_0 grown *Nostoc commune* freeze-dried cells incorrect as the BG-11 cells would include a considerable amount of glycan.

Comparison of released EPS freeze-dried masses and cell freeze-dried masses indicates that cell growth and EPS production differentiated when grown in the presence of nitrates. While *Anabaena variabilis* did not show a significant increase in freeze-dried cell mass when grown in BG-11 or BG-11_0, it did show greater production of released EPS per freeze-dried cell mass. *Anabaena variabilis* produced 0.5 mg released EPS/mg of freeze dried cells when grown in BG-11 and 0.14 mg released EPS/mg of freeze dried cells when grown in BG-11_0. *Anabaena* PCC 7120 ratios were 0.19 mg released EPS/mg of freeze dried cells and 0.12 mg released EPS/mg freeze-dried cells for BG-11 and BG-11_0, respectively.

3.3 Biofilms of *Anabaena variabilis* and *Anabaena* PCC 7120

Biofilms of *Anabaena variabilis* ATCC 29413 and *Anabaena* PCC 7120 were established on partially immersed glass cover slips at the air/water interface grown in both BG-11 and BG-11_0

medium (**Fig. 3.4**). The thickness and density of biofilm on the glass slide was dependent on both the strain and the presence or absence of nitrates. *A. variabilis* biofilms were perceptively denser when grown in the presence of nitrates. The biofilms of *A.* PCC 7120 grown in the presence of nitrates were visibly less dense than all other biofilms. However, glass cover slips immersed in $BG11_0$ cultures of *A.* PCC 7120 developed the thickest biofilms with also the greatest density. Medium of *A.* PCC 7120 cultures would retain a low optical density unless the biofilm on the wall of the flask was agitated back into solution by vigorous swirling (**Fig. 2.1 right side**).

Confocal Laser Scanning Microscopy showed differences in biomass, density, and thickness of the biofilms (**Fig. 3.5**), although uneven distribution of the biofilms made it difficult to obtain a truly representative image for the entire biofilm. The thickness and density of BG-11_0 biofilms of *A.* PCC 7120 made quantitative comparative analysis impossible. The density of the biofilm was too great to allow penetration of the excitation laser and the thickness of the biofilm, from thinnest point to thickest point, exceeded the scope of the field of view. Nonetheless, CLSM did show that biofilms of *Anabaena variabilis* grown in BG-11 and BG-11_0 differed in structure. BG-11 biofilms were comprised of filaments that were relatively parallel to one another, whereas in BG-11_0 biofilm filaments frequently crossed over one another. Biofilm biomass appeared greater for *Anabaena variabilis* grown in BG-11 and for *Anabaena* PCC 7120 grown in BG-11_0 the latter showing the greatest biofilm biomass.

3.4 Planktonic Cell Microscopy

Nostoc commune was the only strain to exhibit capsulated and 'nude' filaments. In the presence of nitrates *Nostoc commune* produced a thick capsule around the filaments visible with phase contrast microscopy (**Fig. 3.3a**). *Nostoc commune* produced long nude filaments with heterocysts when grown diazotrophically in BG-11_0 medium (**Fig. 3.3b**). Unexpectedly the filaments grown in the presence of nitrates also contained numerous heterocysts. The capsule appeared to thin in the vicinity of the heterocysts, only to encapsulate vegetative cells.

Anabaena variabilis and *Anabaena* PCC 7120 did not produce capsulated filaments as seen with phase contrast microscopy. It can be assumed that all EPS generated by these two strains was released into the medium.

Under diazotrophic conditions *Anabaena variabilis* grown for fifty-seven days produced long moderately dispersed filaments with heterocysts seen at the filament terminii (**Fig. 3.6a**). Several cells (assumed to be detached heterocysts) were present as non-filamentous aggregates that were slightly larger than vegetative filamentous cells. These cells did not appear to contain phycobiliproteins since they did not fluorescence in the range indicative of these proteins (Sidler, 1994). Weak fluorescent emission was seen from the clumped cells between 505 and 530 nanometers when excited with light at 488 nanometers. When grown in the presence of nitrates *A. variabilis* was dominated by aggregates of phycobili-protien-containing cells that were either unicellular or binary (**Fig. 3.6b**). Very few filaments of even short length were present.

Anabaena PCC 7120 produced morphologies similar to *Anabaena variabilis* but under the opposite growth conditions. In BG-11 medium *Anabaena* PCC 7120 produced long dispersed filaments of vegetative cells absent of heterocysts (**Fig. 3.6c**), whereas in BG-11_0 medium cells aggregated in clumps of short filaments approximately seven or less cells in length (**Fig. 3.6d**). As observed with *A. variabilis*, the aggregates contain cells that are devoid of phycobiliproteins and are assumed to be detached heterocysts. Weak fluorescence in the green bandwidth was also observed for many of these cells but not all.

3.5 Modified BG-11 and BG-11_0 medium with HEPES Buffer

The pH in unmodified BG-11 cultures would increase with growth while the pH in BG-11_0 cultures would remain between 6 and 7.4. HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (20 mmol L^{-1}) was added to BG-11 and BG-11_0 medium in an attempt to buffer the cultures (Otero and Vincenzini, 2004). Cultures of *Anabaena variablis* and *Anabaena* PCC 7120 grown for thirteen days in medium buffered with HEPES produced unique phenotypes that were strain and medium-dependent. Both *A. variabilis* grown in BG-11_0 medium and *Anabaena* PCC 7120 grown in BG-11 medium developed enlarged cells. The thylakoid membranes appeared to be sequestered to half the cell or less by a large vacuole (**Fig. 3.7a and d**). Filaments of *Anabaena* PCC 7120 that were not enlarged were still present in the medium while cultures of *A. variabilis* contained most entirely short filaments with enlarged cells. *Anabaena* PCC 7120 grown in BG-11_0 medium still aggregated together as described previously but filaments were longer and heterocysts were more abundant. The longer length of filaments over that described previously may be a result of the age of the culture rather than the presence

of the HEPES buffer (13 vs. 57 days). *Anabaena variabilis* grown in BG-11 medium produce long dispersed filament of vegetative cells with no aggregating. *Nostoc commune* morphology was not affected by the presence of HEPES buffer and was the same as described previously. The only exception was for BG-11 medium, where the cell capsule was not as apparent (data not shown).

3.6 Monosaccharide Composition Analysis of Released EPS

Composition analysis of the released extracellular glycans was performed by acid hydrolysis and subsequent liquid chromatography (Table 3.1). One-step and two-step hydrolyses were used to obtain maximum yields for both uronic acid-containing and non-uronic acid-containing polysaccharides. The-two step method has been shown to release more uronic acids from the polysaccharide however; the two-step method also results in increased degradation of monosaccharides (Talaga *et al.*, 2002). As some polysaccharides only exhibited traces of glucuronic acid, both methods were used. One step trifluoroacetic acid (TFA) hydrolysis of the released EPS from *Nostoc commune* DRH-1 generated six identifiable monosaccharides. These were in order of relative abundance glucose, galactose, ribose, xylose, arabinose, and glucuronic acid. This is consistent with previous studies of the released polysaccharides from *N. commune* DRH-1 (Helm *et al.*, 2000). For both *Anabaena variabilis* and *Anabaena* PCC 7120 there were six carbohydrates present as shown by both methods of hydrolysis: galactose, glucose, arabinose, glucuronic acid, mannose, and an unidentified monosaccharide. All monosaccharide concentrations were calculated relative to galactose which was frequently the most abundant monosaccharide present and resulted in less error than compared to total carbohydrate because of fluctuations in trace monosaccharides. The amount of each monosaccharide varied depending on the growth medium used (either BG-11 or BG-11_0) and varied somewhat with the age of the culture.

Both methods showed the Ara:Gal and GlcA:Gal ratios for the released EPS of *A. variabilis* were lower when grown in the presence of nitrates, whereas the Glc:Gal ratio increased. Mannose was detected in trace amounts regardless of the growth medium and was occasionally below detection limits. The released EPS of *A.* PCC 7120 exhibited increases in the Ara:Gal and GlcA:Gal ratios when grown with nitrates. The Glc:Gal ratios decreased in nitrate grown cultures from approximately 1.25 in BG-11_0 to 0.45 in BG-11 for cultures grown for 26

days. The difference the Glc:Gal ratio appeared to decrease with the age of the culture. Cultures grown for 38 days showed Glc:Gal ratios of 0.78 and 0.45 for BG-11_0 and BG-11 respectively and then again at 57 days the Glc:Gal ratios were 0.49 and 0.42 for BG-11_0 and BG-11 respectively. Again mannose was not always detected and inconsistently varied with growth medium. All monosaccharide to galactose ratios decreased in cultures of *N. commune* grown in BG-11 medium over those grown in BG-11_0 with exception of GlcA:Gal which remained relatively the same.

3.7 Dissolved Inorganic Carbon Concentrations in BG-11 and BG-11_0 Media.

Dissolved inorganic carbon present in BG-11 and BG11_0 media differs greatly depending on the partial pressure of carbon dioxide and the pH of the medium. The consumption of nitrates in BG-11 medium results in an increase in pH (Brewer and Goldman, 1976; Goldman and Brewer, 1980). Cultures that experience an increase in pH also increase the available dissolved inorganic carbon. An increase in pH from 7.5 to 9.5, under the current partial pressure of carbon dioxide of 3.16×10^{-4} atm, will provide dissolved inorganic carbon available at levels comparable to a system maintained at pH 7.5 with the partial pressure of carbon dioxide increased to 3.5×10^{-2} atmospheres.

The relationship of Pco_2 and pH of equation 2.13 is shown in **Figure 3.8a/b** dashed lines and solid symbols. The calculations were done with an initial pH of 7.5, Pco_2 of 3.16×10^{-4} atm and total air pressure at 1 atm. As the partial pressure of carbon dioxide approaches zero the concentration of dissolved oxygen and nitrogen rapidly decreases. With 20 mmol L^{-1} HEPES present in the medium (**Fig. 3.8b** dashed lines and solid symbols), the pH of the medium initially set at 7.5 will at 1%, 5%, and 10% CO_2 become ~7.2, ~6.9, and ~6.7 respectively. If no HEPES or other additional buffer is used in the medium (**Fig. 3.8a** dashed lines and solid symbols), the effect reduces the pH even further to ~6.3, ~5.6, and ~5.3 respectively. Such pH changes can be severely detrimental to growth.

If the pH of a culture is allowed to rise by consumption of nitrates in the medium, the relationship is determined using equation 2.22 with a constant Pco_2 of 3.16×10^{-4} atmospheres (**Fig. 3.9a/b** dotted lines and solid symbols). The primary dissolved inorganic species for the entire range is bicarbonate. As the initial 17.65 mmol L^{-1} concentration of nitrate is consumed the bicarbonate concentration rises rapidly above both the dissolved nitrogen and oxygen. When

the nitrate is fully consumed the bicarbonate concentration is approximately $1.5x10^{-2}$ mol L^{-1}. This concentration is a hundred times greater than the initial bicarbonate concentration of $1.5x10^{-4}$. Notice also under this condition of constant partial pressure of carbon dioxide that the concentration of H_2CO_3* does not change.

Otero and Vincenzini compensated for the increase in pH by increasing the partial pressure of carbon dioxide (Otero and Vincenzini, 2004). This will once again change the concentration of available species in solution. This relationship is plotted by using Eq. 2.22 but with a constant pH and a variable carbon dioxide partial pressure (**Figs. 3.8a/b** and **3.9a/b** solid lines and open symbols). In this system the three species of dissolved inorganic carbon, H_2CO_3*, HCO_3^-, and CO_3^{2-} increase in equal molar ratios with bicarbonate still being the dominant species. H_2CO_3* becomes a species of significant concentration under these conditions of which the vast majority of the H_2CO_3* species is aqueous carbon dioxide and not carbonic acid (Stumm and Morgan, 1970).

The availability of inorganic carbon to cells in liquid medium varies under different partial pressures of carbon dioxide and pH. It must not be assumed that an increase in the gaseous partial pressure of carbon dioxide will also increase the amount of available dissolved inorganic carbon proportionately. Consideration must also be given to the availability of the different species of inorganic carbon available to the cells.

Table 3.1 Monosaccharide to Galactose Ratios of Released EPS. Monosaccharides were identified in > 10 kDa released EPS. Standard deviation based on biological triplicates. Values with no standard are deviation based on single sample. EPS underwent methanolysis followed by TFA hydrolysis (Method 2) or TFA hydrolysis only (Method 1).

				Monosaccharide to Galactose ratios			
strain	Medium	days of growth	Meth	Ara/Gal	Glc/Gal	Man/Gal	GlcA/Gal
A. variabilis	BG-11	26	2	0.29(0.08)	1.03(0.17)	trace	Trace
A. variabilis	$BG\text{-}11_0$	26	2	0.50(0.03)	0.43(0.03)	trace	0.15(0.01)
A. variabilis	BG-11	26	1	0.33(0.02)	0.94(0.18)	trace	Trace
A. variabilis	$BG\text{-}11_0$	26	1	0.51(0.02)	0.43(0.00)	trace	0.19(0.02)
A. variabilis	BG-11	38	2	0.35(-)	1.22(-)	trace	Trace
A. variabilis	$BG\text{-}11_0$	38	2	0.47(0.03)	0.60(0.06)	trace	0.26(0.04)
A. variabilis	BG-11	57	2	0.34(0.03)	0.81(0.17)	trace	Trace
A. variabilis	$BG\text{-}11_0$	57	2	0.44(0.01)	0.53(0.08)	trace	0.22(0.04)
A. variabilis	BG-11	57	1	0.25(0.06)	0.85(0.15)	trace	Trace
A. variabilis	$BG\text{-}11_0$	57	1	0.34(0.05)	0.49(0.03)	trace	0.26(0.04)
Anabaena PCC 7120	BG-11	26	2	0.53(0.01)	0.43(0.05)	trace	0.13(0.02)
Anabaena PCC 7120	$BG\text{-}11_0$	26	2	0.35(0.08)	1.25(0.43)	trace	Trace
Anabaena PCC 7120	BG-11	26	1	0.52(0.03)	0.49(0.02)	trace	0.17(0.01)
Anabaena PCC 7120	$BG\text{-}11_0$	26	1	0.36(0.03)	1.24(0.23)	trace	Trace
Anabaena PCC 7120	BG-11	38	1	0.44(0.01)	0.45(0.04)	trace	0.23(0.03)
Anabaena PCC 7120	$BG\text{-}11_0$	38	1	0.26(0.08)	0.78(0.21)	trace	Trace
Anabaena PCC 7120	BG-11	57	2	0.56(0.05)	0.42(0.02)	trace	0.21(0.01)
Anabaena PCC 7120	$BG\text{-}11_0$	57	2	0.53(0.02)	0.49(0.06)	trace	Trace
Anabaena PCC 7120	BG-11	57	1	0.42(0.03)	0.45(0.02)	trace	0.27(0.02)
Anabaena PCC 7120	$BG\text{-}11_0$	57	1	0.32(0.06)	0.54(0.06)	trace	Trace

				Monosaccharide to Galactose ratios				
strain	Medium	days of growth	Meth	Ara/Gal	Glc/Gal	Xyl/Gal	Rib/Gal	GlcA/Gal
N. commune	BG-11	61	1	0.22(0.05)	1.75(0.07)	0.42(0.04)	0.46(0.11)	0.31(0.10)
N. commune	$BG\text{-}11_0$	61	1	0.52(0.05)	2.20(0.24)	0.81(0.14)	0.82(0.09)	0.26(0.04)

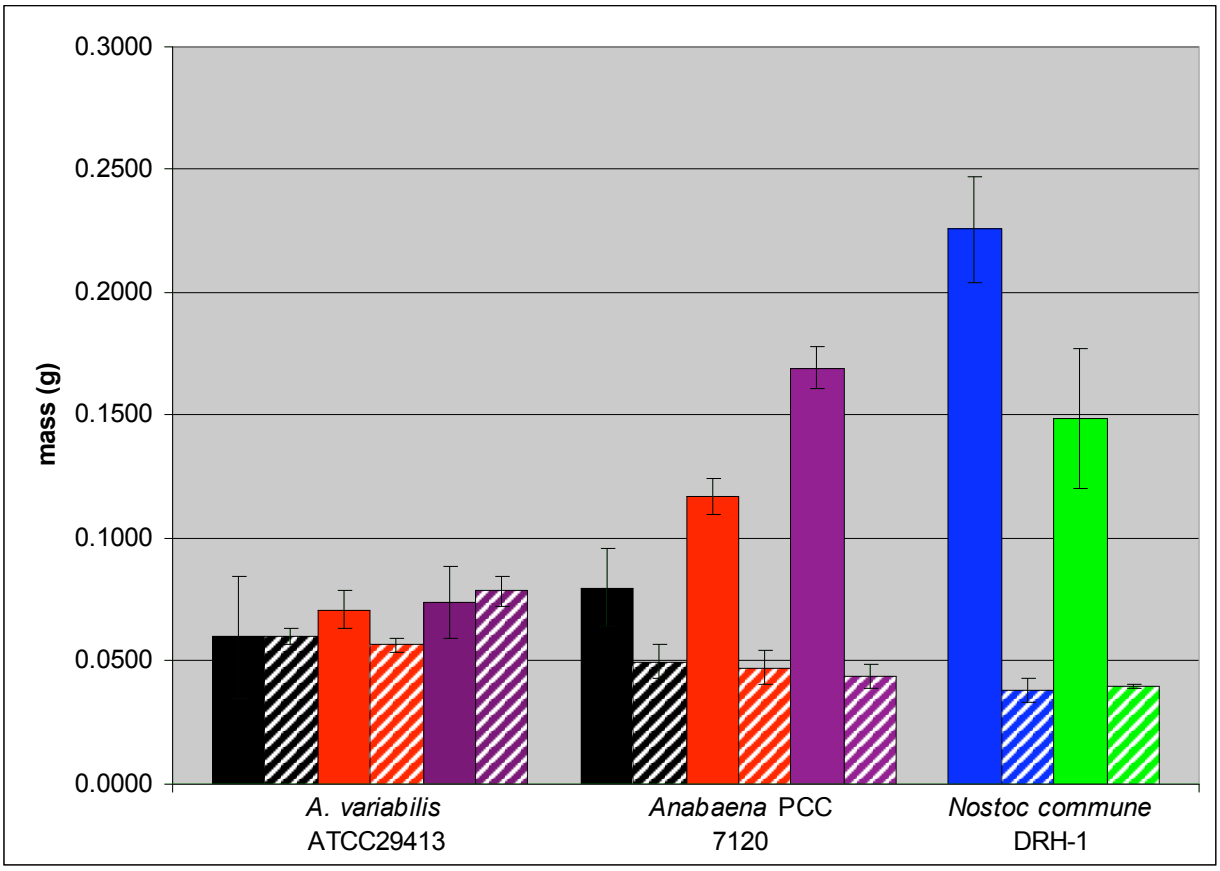

Figure 3.1 Freeze-dried Cell Masses. Comparison of Freeze-dried cell masses from 50 ml cultures of *Anabaena variabilis* ATCC 29413, *Anabaena* PCC 7120, and *Nostoc commune* DRH-1 with regards to time and medium. Growth was generally enhanced in BG-11 medium (solid bars) over growth in BG-11_0 medium (striped bars). Error bars represent standard deviations of biological triplicates. Colors represent days of growth: black = 26 days; red = 38 days; purple = 57 days; blue = 41 days; green = 61 days.

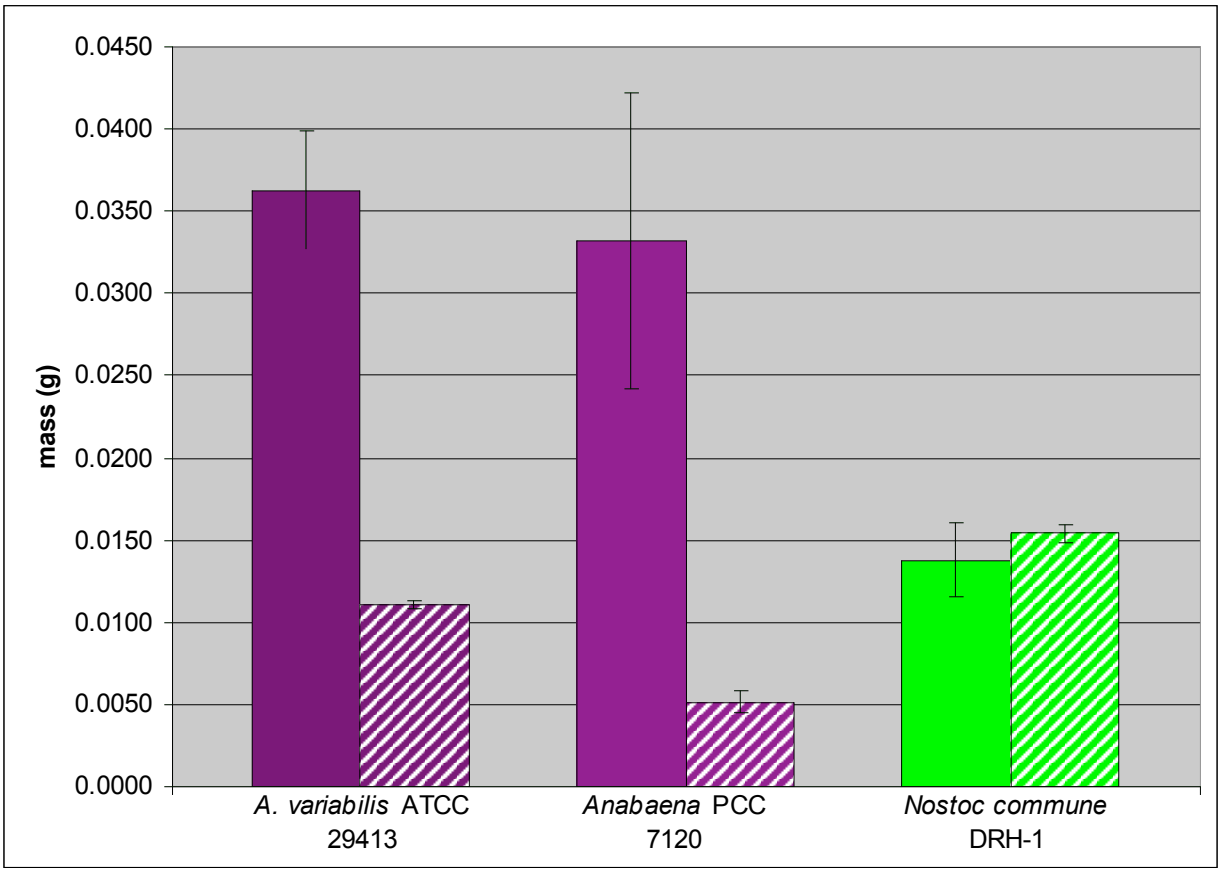

Figure 3.2 Freeze-dried Released EPS Masses >10 kDa. Comparison of Freeze-dried released EPS from >10 kDA obtained from cell free medium of 50 ml cultures of *Anabaena variabilis* ATCC 29413, *Anabaena* PCC 7120, and *Nostoc commune* DRH-1. More released EPS was found in BG-11 medium (solid bars) over growth in BG-11_0 medium (striped bars). Error bars represent standard deviations of biological triplicates. Colors represent days of growth: purple = 57 days; green = 61 days.

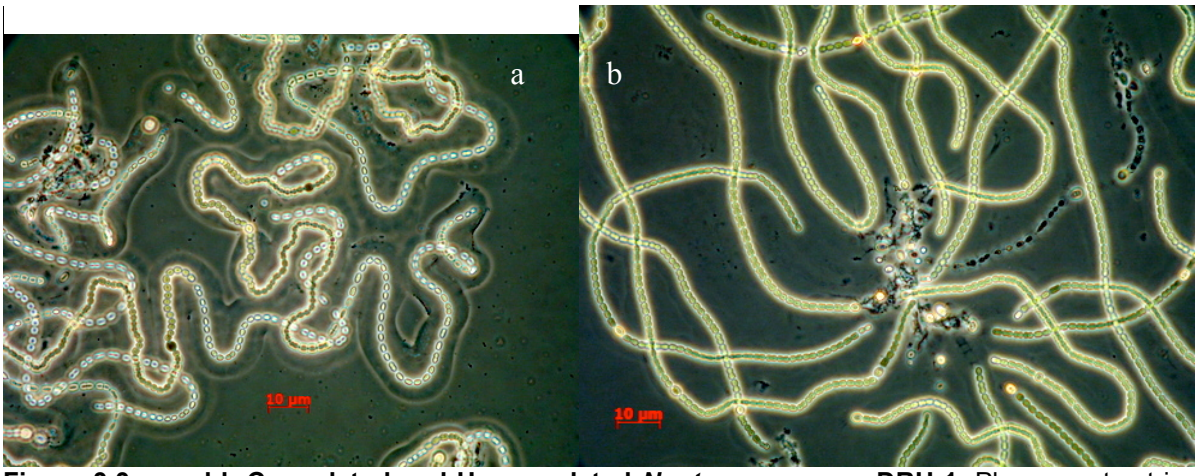

Figure 3.3 a and b Capsulated and Uncapsulated *Nostoc commune* DRH-1. Phase contrast images of 41 day old planktonic cells grown in 50 ml cultures of *Nostoc commune* DRH-1 in BG-11 (a) and $BG11_0$ (b). bar = 10 micrometers

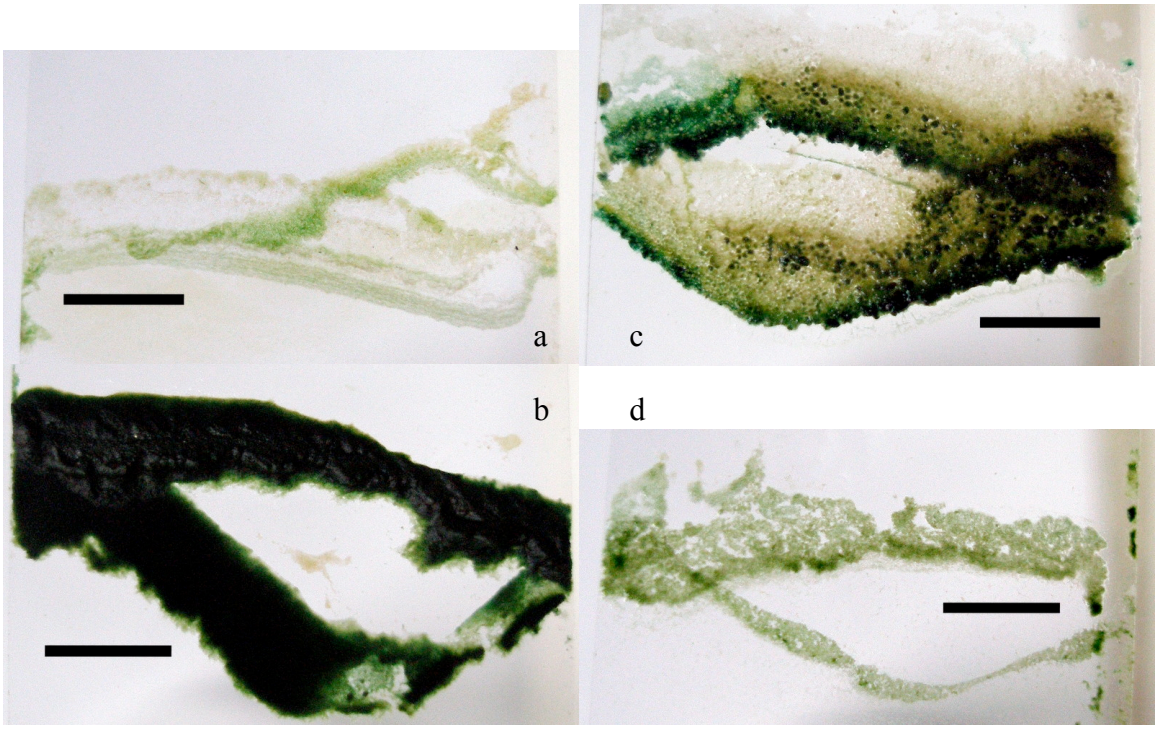

Figure 3.4 a-d Biofilms Established on Glass. Representative images of biofilms established on glass cover-slips that traversed the air-water interface in 50 ml cultures of *Anabaena* PCC 7120 in BG-11 (a) and $BG11_0$ (b), and *Anabaena variabilis* in BG-11 (c) and $BG\text{-}11_0$ (d). Biofilm developed along the air-water interface with the lower half of the slide immersed in growth media. Cultures were grown for 32 days. bar = 5mm.

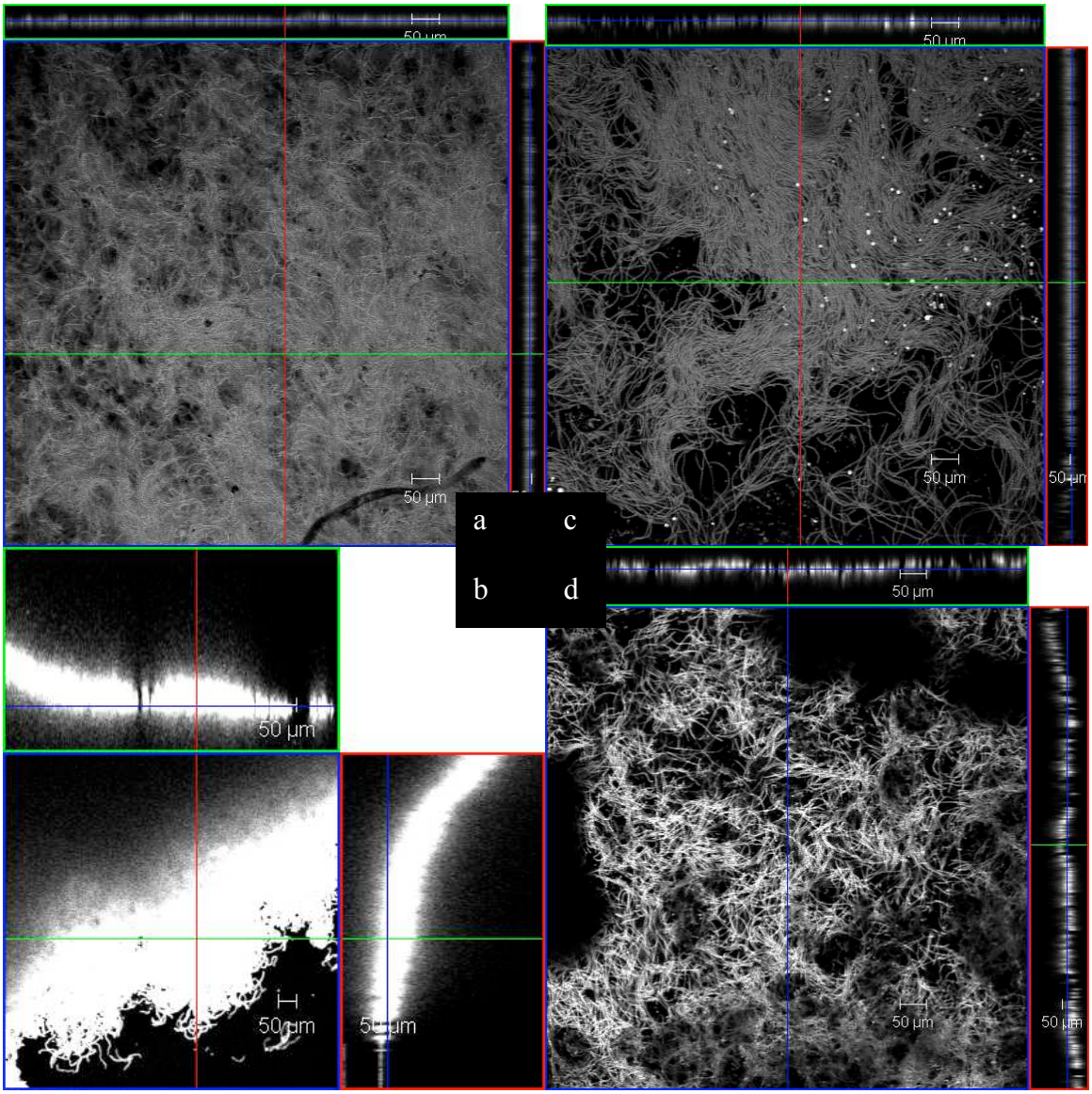

Figure 3.5 a-d CLSM Cross-sections of Biofilms Established on Glass. Cross sections of CLSM image stacks of biofilms established on glass cover-slips that traversed the air-water interface in 50 ml cultures of *Anabaena* PCC 7120 in BG-11 (a) and $BG11_0$ (b), and *Anabaena variabilis* in BG-11 (c) and $BG\text{-}11_0$ (d). Blue, green, and red lines indicated the cross-section plane: blue = x, y plane, green = x, z plane, red = y, z plane. Cultures were grown for 32 days. bar = 50 micrometers.

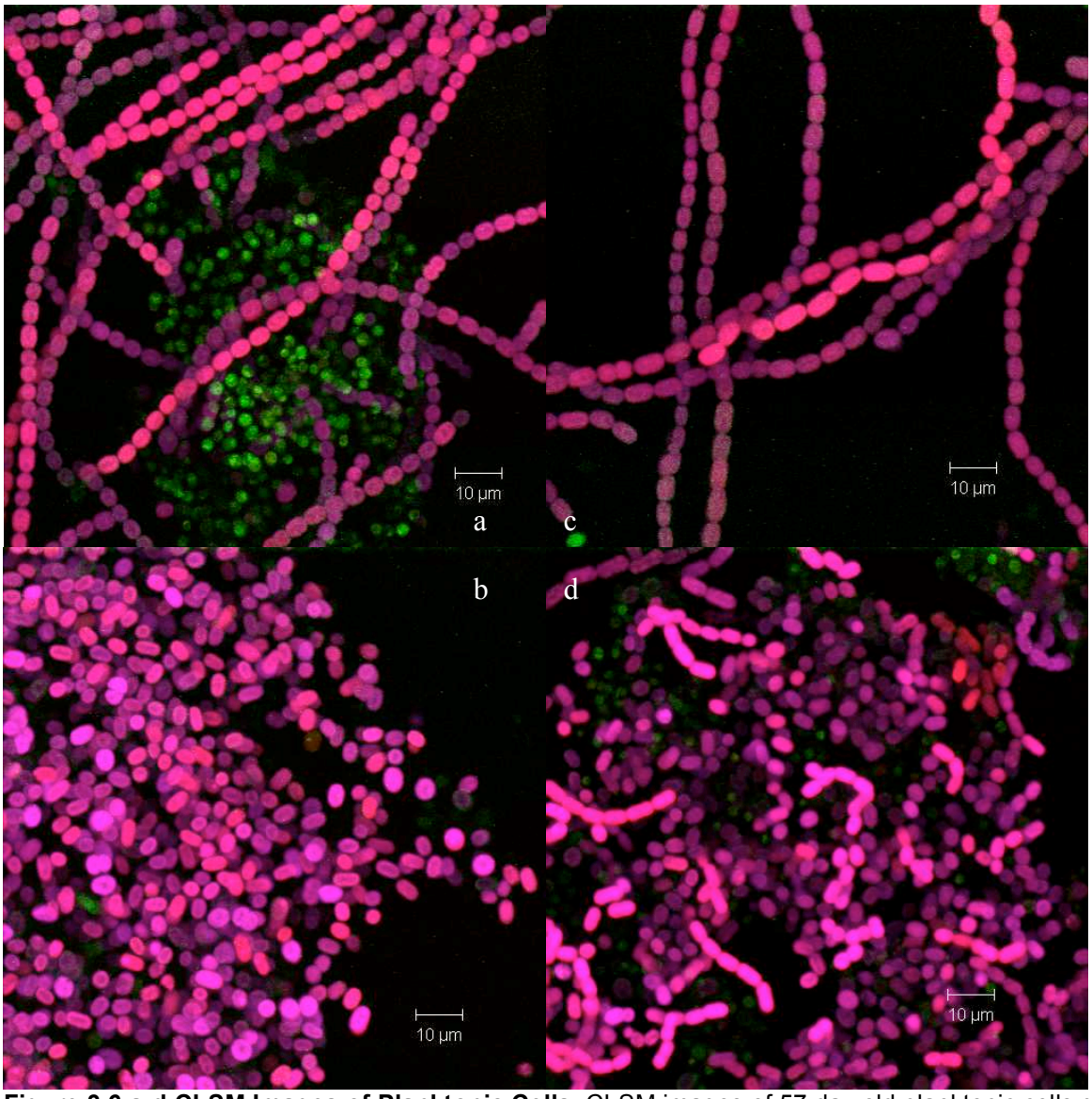

Figure 3.6 a-d CLSM Images of Planktonic Cells. CLSM images of 57 day old planktonic cells grown in 50 ml cultures of *Anabaena variabilis* in BG-11_0 (a) and BG-11 (b) and *Anabaena* PCC 7120 in BG-11 (c) and BG11_0 (d). False coloring corresponds to the excitation wavelengths of 488 nm, 543 nm, and 633 nm with corresponding emission band passes of 505-550 nm (green), 560-615 nm (red), and 655-719 nm (purple). Pink color is a combination of red and purple.

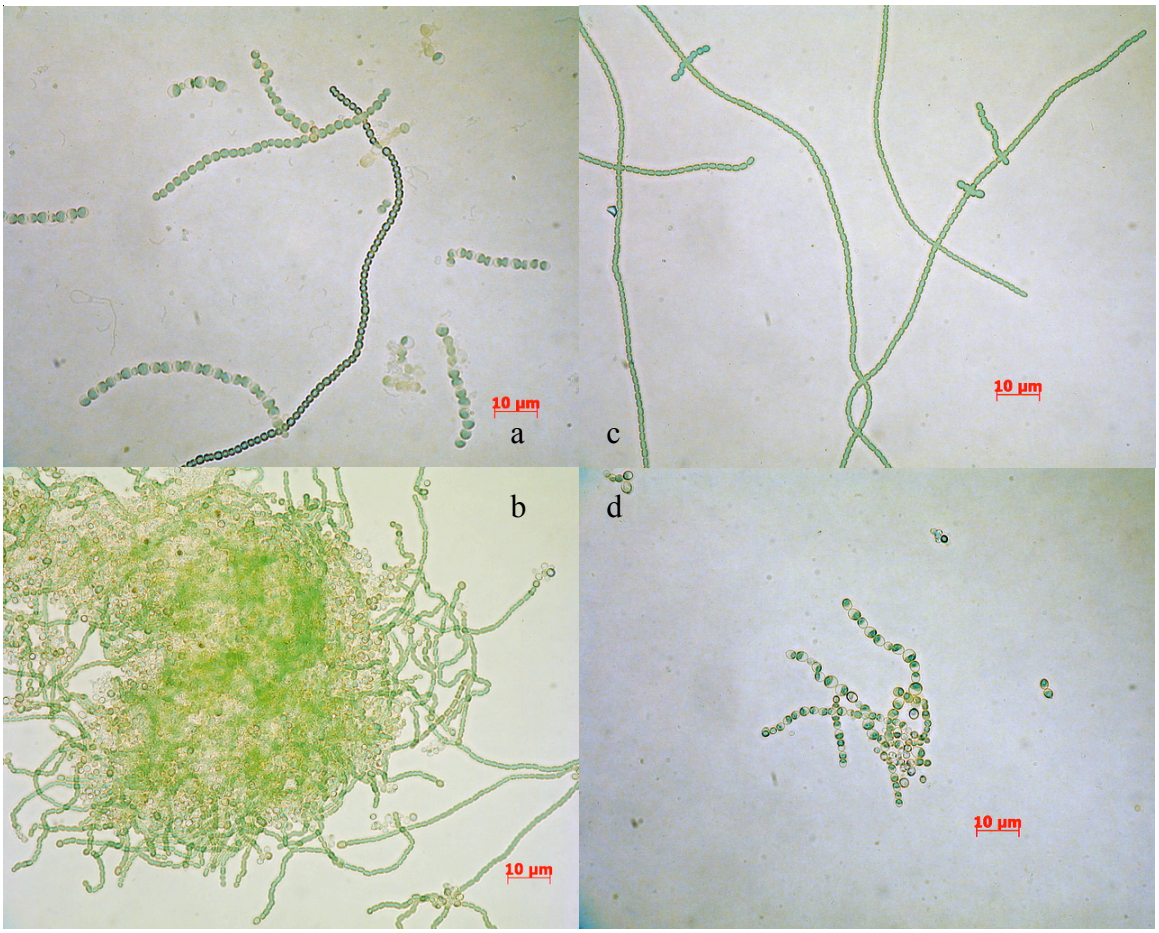

Figure 3.7 a-d Planktonic Cells Grown in Media Modified with HEPES Buffer. Bright-field microscope images of planktonic cells grown for 13 days in media modified with 20 mmol L^{-1} HEPES buffer. *Anabaena* PCC 7120 in BG-11 (a) and $BG11_0$ (b), and *Anabaena variabilis* in BG-11 (c) and $BG\text{-}11_0$ (d). bar = 10 micrometers

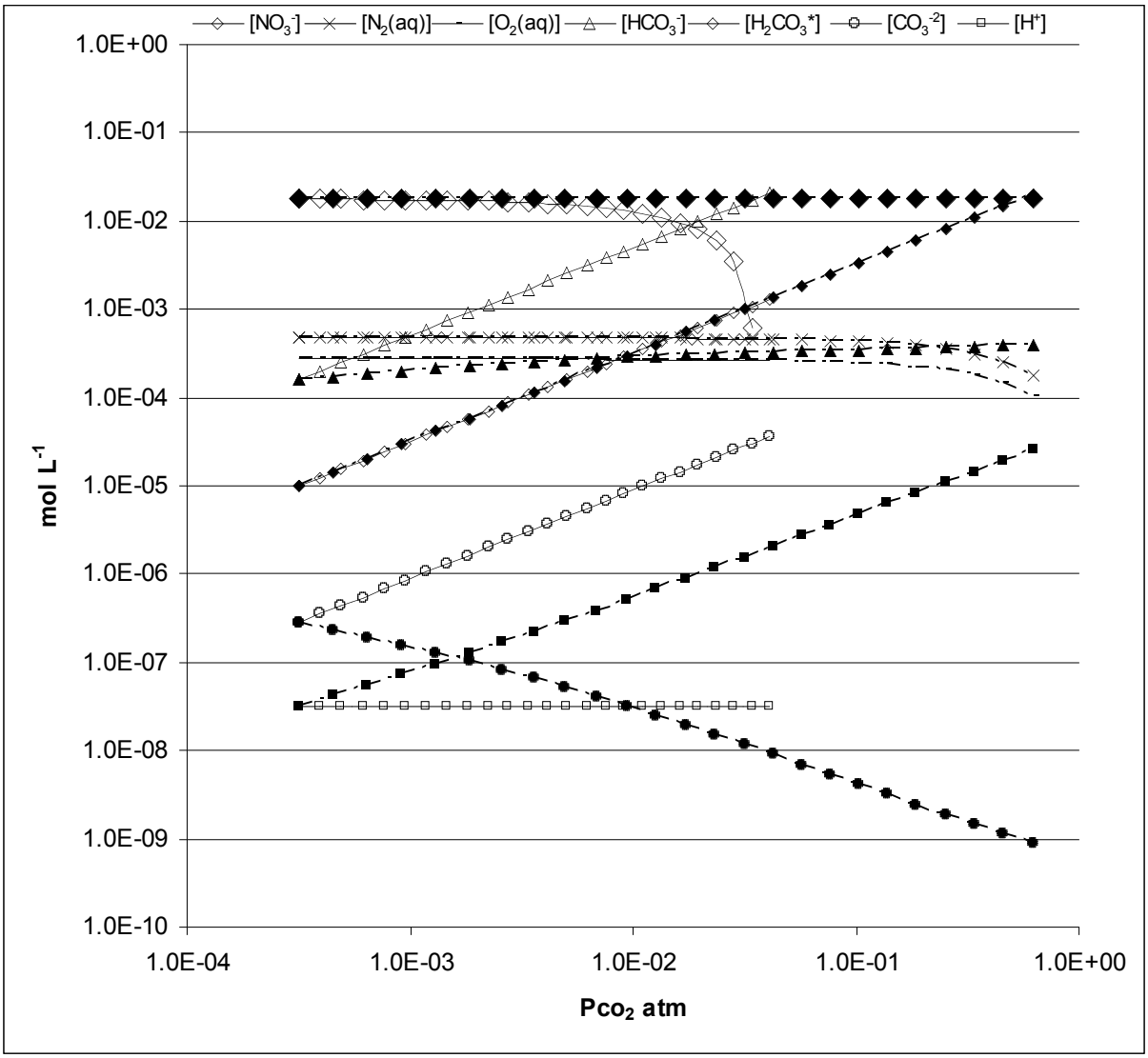

Figure 3.8a Concentrations of Dissolved Inorganic Carbon in BG-11 Medium Against Pco_2. BG-11 concentration of dissolved inorganic carbon species (carbonate (circles), bicarbonate (triangles), H_2CO_3* (small diamonds)), nitrate (large diamonds), dissolved oxygen (dashes) and nitrogen (x's) and H^+ (squares) plotted against the partial pressure of carbon dioxide when pH is allowed to decrease (dashed lines and closed symbols) and pH is maintained at 7.5 (solid lines and open symbols). Near 0.04 atm Pco_2 all nitrates are consumed in the medium making this point the maximum without resulting in a decrease in pH. Temp = 25ºC at 1 atm.

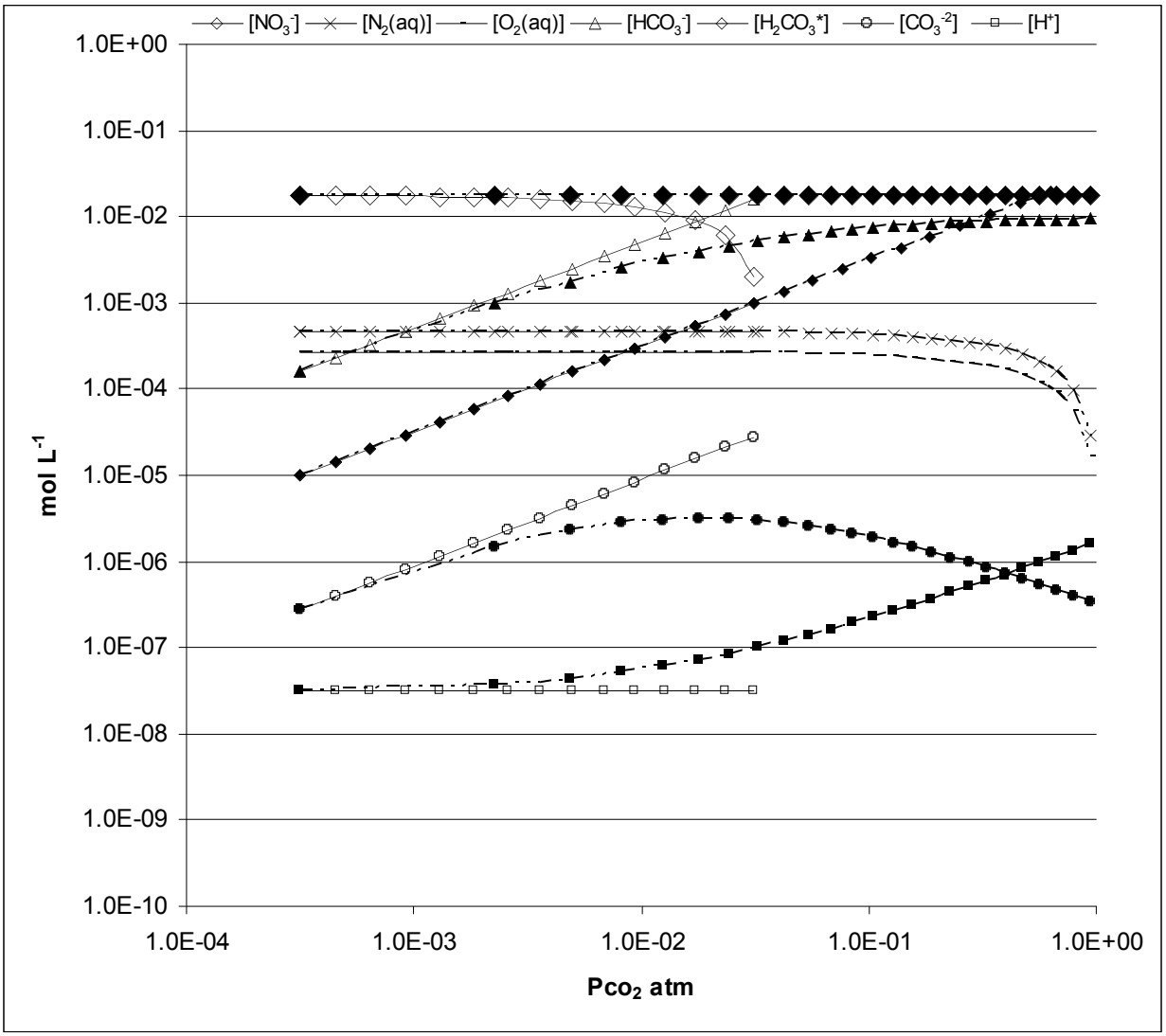

Figure 3.8b Concentrations of Dissolved Inorganic Carbon in BG-11 Medium with 20 mmol L^{-1} HEPES Against Pco_2. BG-11 with 20 mmol L^{-1} HEPES concentration of dissolved inorganic carbon species (carbonate (circles), bicarbonate (triangles), H_2CO_3* (small diamonds)), nitrate (large diamonds), dissolved oxygen (dashes) and nitrogen (x's) and H^+ (squares) plotted against the partial pressure of carbon dioxide when pH is allowed to decrease (dashed lines and closed symbols) and pH is maintained at 7.5 (solid lines and open symbols). Near 0.04 atm Pco_2 all nitrates are consumed in the medium making this point the maximum without resulting in a decrease in pH. Temp = 25ºC at 1 atm.

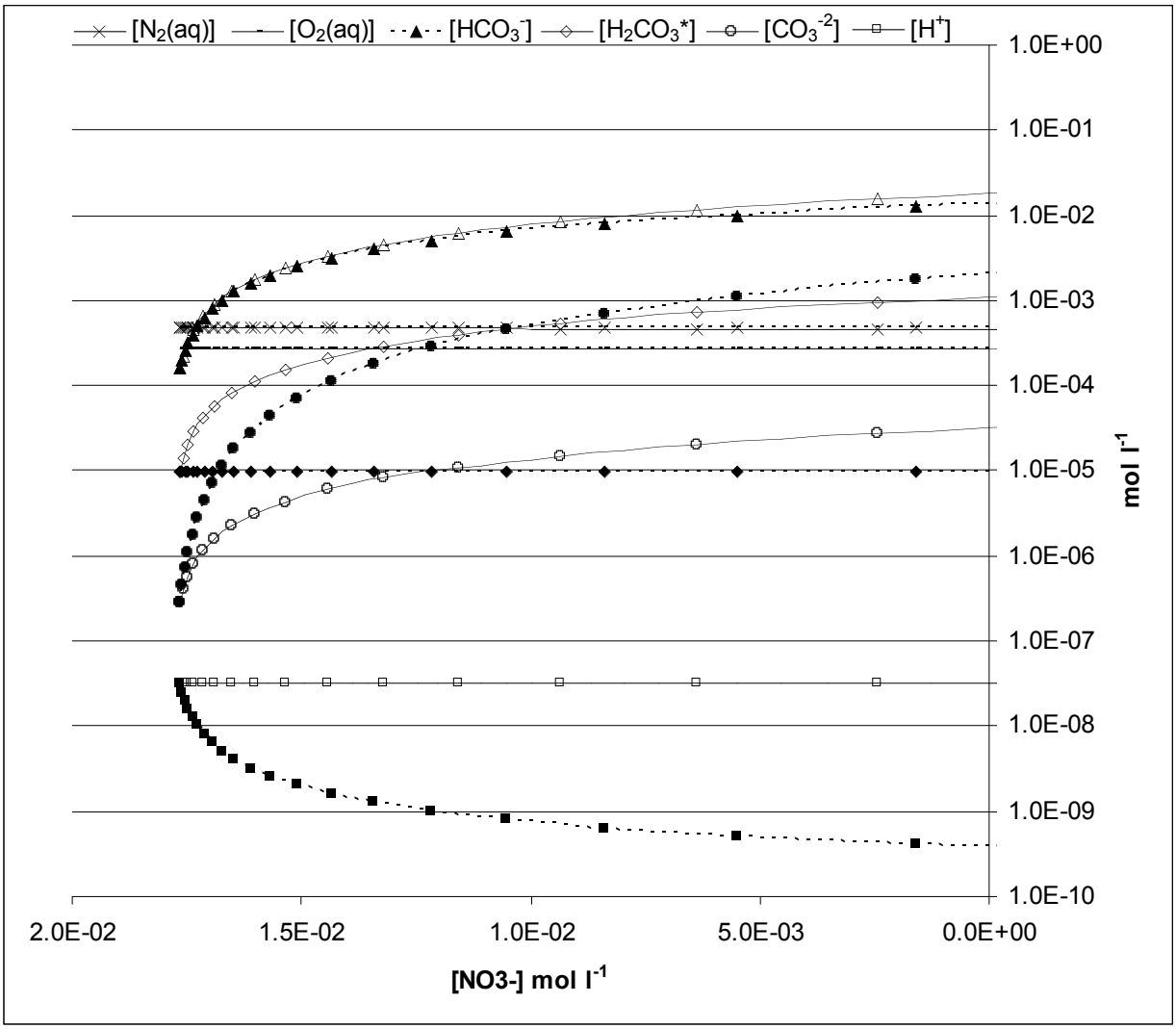

Figure 3.9a Concentration of Dissolved Inorganic Carbon Species in BG-11 Medium Against Nitrate Concentration. BG-11 concentration of dissolved inorganic carbon species (carbonate (circles), bicarbonate (triangles), H_2CO_3* (diamonds)), dissolved oxygen (dashes) and nitrogen (x's) and H^+ (squares) plotted against the concentration of nitrates which had an initial value of 17.65 mmol L^{-1} at pH 7.5. The pH is held at a constant 7.5 by increasing the partial pressure of carbon dioxide (solid lines and open symbols) or the pH is allowed to increase (dashed lines and closed symbols).

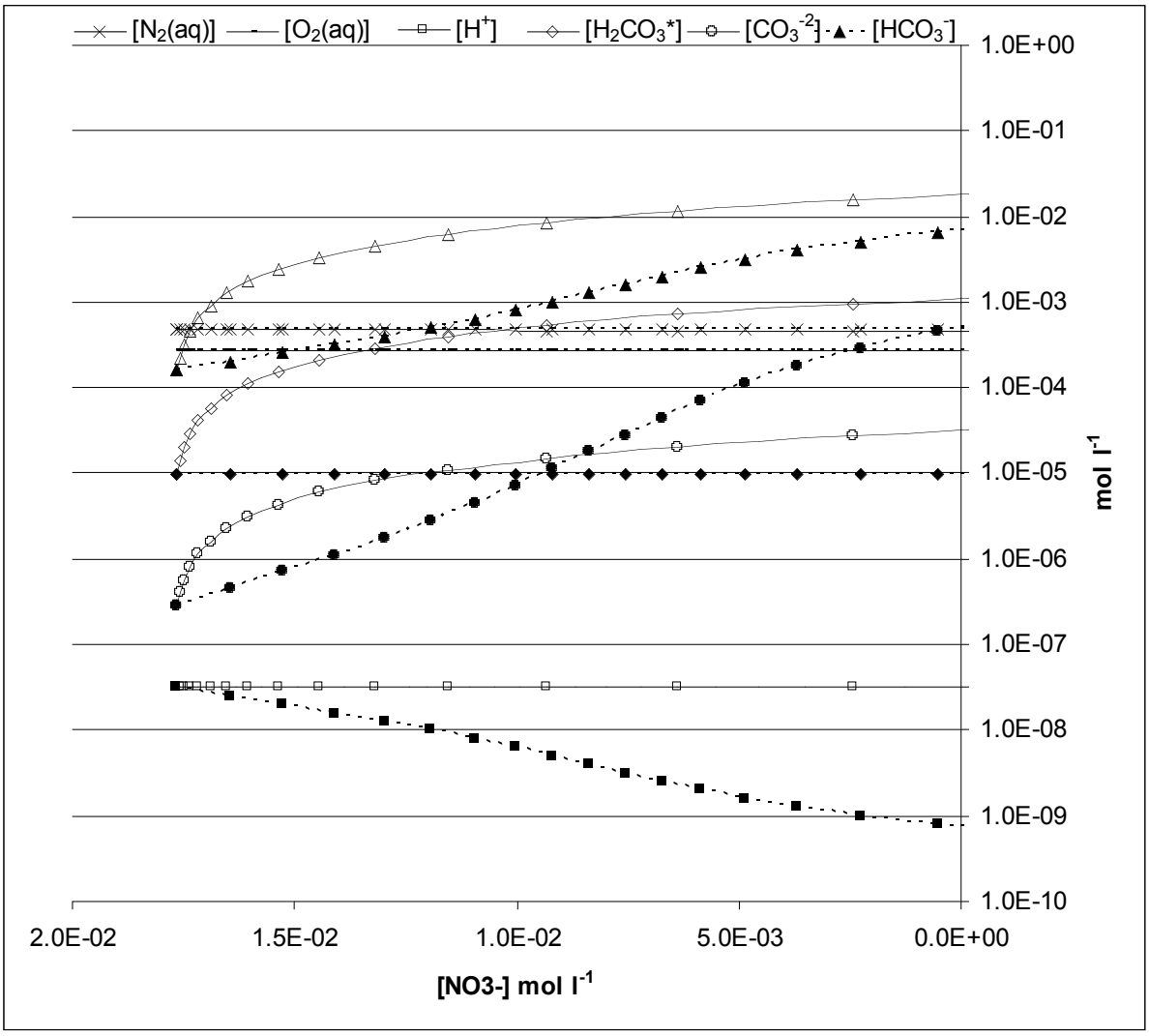

Figure 3.9b Concentration of Dissolved Inorganic Carbon Species in BG-11 Medium with 20 mmol L^{-1} HEPES Against Nitrate Concentration. BG-11 with 20 mmol L^{-1} HEPES concentration of dissolved inorganic carbon species (carbonate (circles), bicarbonate (triangles), H_2CO_3* (diamonds)), dissolved oxygen (dashes) and nitrogen (x's) and H^+ (squares) plotted against the concentration of nitrates which had an initial value of 17.65 mmol l^{-1} at pH 7.5. The pH is held at a constant 7.5 by increasing the partial pressure of carbon dioxide (solid lines and open symbols) or the pH is allowed to increase (dashed lines and closed symbols).

CHAPTER 4: DISCUSSION AND CONCLUSIONS

4.1 Introduction

Cyanobacteria extracellular polysaccharides are subject to changes that are species and nutrient dependent. Changes in EPS monosaccharide composition (particularly glucuronic acid) were associated with biofilm formation along the air-water interface. The change in EPS composition is specific to the cyanobacteria strain and may aid in the survivability of the organism. *Anabaena variabilis* and *Anabaena* PCC 7120 exhibit similar behavior but under opposite conditions. The carbon-nitrogen ratio increases with an increase in pH. In BG-11 medium the consumption of nitrates is assumed to be the primary contributor in increasing the pH since pH increase did not occur in BG-11_0 medium. The different EPS morphologies would affect biofilm formation and microbial mat structure and calcification. Under different partial pressures of carbon dioxide cyanobacteria have different morphologies and carbon fluxes related to EPS production and cyanobacteria induced calcification. Hence, geobiological carbon sequestering and calcification is important geochemical process. It is important to understand how EPS and calcification would be altered by available inorganic carbon when considering the structure and metabolisms of ancient cyanobacteria and how cyanobacteria will influence and respond to the current increases in atmospheric carbon dioxide.

4.2 EPS Composition as it Correlates to Biofilm Formation and Other Characteristics

Glucuronic acid was present in the released EPS of *Anabaena variabilis* and *Anabaena* PCC 7120 which preferred planktonic behavior, whereas the released EPS from cultures that formed planktonic aggregates and biofilms contained only traces of glucuronic acid (**Fig. 3.3b/c** and **Table 3.1**). Suspension of cells would have been facilitated by the hydrophilic nature of an uronic acid-containing EPS, compared to less hydrophilic EPS preferring biofilm formation. A lower percentage of uronic acids were found in the EPS from biofilms of *Pseudomonas fluorescens* relative to the EPS from planktonic cells that were almost entirely composed of uronic acids (Kives *et al.*, 2006). These results indicate that uronic acids influence the solubility of the EPS and cells but are not the only elements present that control solubility. The presence of lipopolysaccharides and surfactants, which were not tested for, would greatly change the surface properties of the cells and therefore influence biofilm vs. planktonic growth.

For the two *Anabaena* strains, the Glc:Gal ratios, when glucuronic acid was present in appreciable amounts, was significantly less than that of the EPS containing only trace amounts of glucuronic acid for both methods of hydrolysis. The reduction in glucose and the increase in glucuronic acid are not equal in molar ratios. Incomplete hydrolysis with both methods should also be taken into account when comparing the two different glycan compositions.

The pH and subsequently the dissolved inorganic carbon concentration has been observed to increase in all cultures of BG-11 as the nitrates were consumed. Therefore, the differences between BG-11 and BG-11_0 glycan composition may have been influenced by pH changes or availability of dissolved inorganic carbon and not just the presence of nitrates. Additional studies where the pH is maintained would need to be done to determine whether the presence or absence of nitrates was the only factor affecting glycan composition. Efforts to address this here were thwarted by the phenotype change in cyanobacterial growth when buffered with HEPES.

Monosaccharide composition of the glycan generated by *Nostoc commune* was different between growth in BG-11 and BG-11_0 and correlated with presence and absence of capsulated filaments respectively. This shows that the monosaccharide content of the glycan reported herein and in other sources (Helm *et al.*, 2000) is specific to the growth condition in which *N. commune* was incubated. The extracellular polysaccharide of *Nostoc commune* has already been shown to play an important role in preventing fusion of membranes under desiccated states (Hill *et al.*, 1997). It would be interesting to see how changes in polysaccharide composition affect desiccation survival and UV stress. Further studies would need to be conducted to examine whether the composition of the glycan changes with temperature, nutrient limitation and cell concentration during these stresses. The physical properties of the differing glycan could then be compared and correlated to the condition that induced the changes to screen for ecological advantages (or disadvantages) that the polysaccharides rheology provides.

4.3 Opposing Responses of *Anabaena variabilis* and *Anabaena* PCC 7120 to Nitrates

Of noticeable interest are the similarities between the aggregating/biofilm behavior and glycan composition of *Anabaena variabilis* and *Anabaena* PCC 7120. The presence of nitrates in the medium resulted in only trace amounts of glucuronic acid and short aggregated filaments for *Anabaena variabilis* whereas similar results were observed for *Anabaena* PCC 7120 but in the absence of nitrates. This difference of behaviors was consistent in the adverse reaction to HEPES

buffer (Fig. 3.7). Both strains showed deformed cell filaments: *A. variabilis* in BG-11_0 and *Anabaena* PCC 7120 in BG-11. *Anabaena variabilis* and *Anabaena* PCC 7120 have been shown to be closely related by phylogenic 16s rRNA analysis (Hess, 2008). Sharing a relatively recent evolutionary divergence provides reason for the overall similarity in EPS composition. The opposite behaviors seem to be the result of a mutation in a regulatory metabolic switch. In one organism nitrates induce the production of a glycan with increased glucuronic acid and in the other nitrates reduce the production of glucuronic acid containing glycan. The phenotypic response influences either planktonic or biofilm community behavior. The advantage of opposite response to nitrates is not clear but may be related to the niches both organisms occupy. This could be a possible future study to examine whether *Anabaena variabilis* and *Anabaena* PCC 7120 separate themselves from one another in culture. Such behavior for several organisms is discussed by Romling and Amikam dealing with the differential and 'fine tuned' behavior in relation to cyclic diguanylate levels (Romling and Amikam, 2006).

4.4 Comments on the Unique Phenotype Associated with HEPES Buffer

The addition of 20 mmol L^{-1} HEPES buffer resulted in enlarged cells present in shortened filaments for *Anabaena variabilis* and *Anabaena* PCC 7120 in BG-11_0 and BG-11 respectively. The thylakoid membranes also appeared to be sequestered to less than half of the cell. Kang *et al.* have grown *Anabeana* PCC 7120 in BG-11 with HEPES and reported consistent heterocyst differentiation when supplemented with carbon dioxide but did not mention anything similar to what was observed here (Kang *et al.*, 2005). Zigler *et al.* described how HEPES exposed to light can have cytotoxic effects (Zigler *et al.*, 1985). If this is the case, the toxin only has an effect on cells made sensitive through the opposing responses of nitrates discussed in 4.2. The enlarged cells resemble heterocysts that may be halted in differentiations. Wolk describes several mutants (*hetR*, *hetP*, and *hetN*) that are capable of forming contiguous filaments of heterocysts, one (*hetR*), even in the presence of combined nitrogen (Wolk, 1996). The enlarged cells may be a response to the HEPES buffer (or toxin from the HEPES buffer) associated with the differential behavior in the presence of nitrates, that has induced heterocyst or partial heterocyst formation. Different polysaccharide composition, which has been shown for non-buffered cultures but was not examined for cultures containing HEPES, may block inducing/signaling molecules from being recognized by cells in the cultures that do not exhibit enlarged cells. Otero and Vincenzini

observed this cell morphology in BG-11 cultures of *Nostoc* PCC 7936 with no HEPES buffer where the pH had risen between 9 and 10 (Otero and Vincenzini, 2004). This added insight strongly suggests that HEPES is not the sole inducer of the phenotype, nor is it directly associated with the phenotype. The appearance of this phenotype is either induced under strain specific conditions or through another variation not presently understood.

4.5 Carbon-Nitrogen Ratio Associated with Capsulated Morphologies

Nostoc commune was observed to generate a capsulated morphology when grown in BG-11 media where the pH has been observed to consistently rise to between 9 and 10. Bicarbonate is the dominant dissolved inorganic carbon species (1.26×10^{-3} mol L^{-1}) at pH 9.4 and is represented **in Figure 3.9a** as dotted lines and closed symbols. The conditions that induced this capsulated morphology is similar to one of the conditions of the capsulated *Nostoc* PCC 7936 (Otero and Vincenzini, 2004). The calculations of dissolved inorganic carbon correlate to the observed capsulated morphologies of *Nostoc* PCC7936 as seen by Otero and Vincenzini (Otero and Vincenzini, 2004). *Nostoc* PCC 7936 was capsulated when grown under the following conditions:

BG-11_0 supplemented with carbon dioxide with and without 20 mmol L^{-1} HEPES

BG-11 supplemented with plain air ($Pco_2 = 3.16 \times 10^{-4}$ atm) and no HEPES

BG-11 supplemented with carbon dioxide with 20 mmol L^{-1} HEPES.

They concluded that PCC 7936 exhibited a capsulated morphology when the carbon-nitrogen ratio was imbalanced and the excess fixed carbon was excreted as extracellular polysaccharide generating the capsule. In the one condition where a capsule was present but no carbon dioxide was supplemented, they speculated that nitrate uptake had been inhibited while carbon assimilation continued. If their conclusion is correct it would be important to know what concentration of dissolved inorganic carbon was available to the cells (Otero and Vincenzini, 2004).

The dissolved inorganic carbon concentration for BG-11_0 supplemented with carbon dioxide without HEPES should theoretically follow the solid symbols and dashed lines of **Figure 3.8a**. The dissolved inorganic carbon concentration can only be implied from the pH, which was maintained above 7, since no partial pressure of carbon dioxide was provided (Otero and Vincenzini, 2004). BG-11_0 supplemented with carbon dioxide without HEPES under these

considerations would have a maximum concentrations of H_2CO_3* and HCO_3^- of 4.09 x 10^{-5} mol L^{-1} and 2.15 x 10^{-4} mol L^{-1} respectively, and the corresponding partial pressure of carbon dioxide would have been 1.3 x 10^{-3} atm. With the addition of HEPES buffer the pH would not have been so sensitive to changes in partial pressure of carbon dioxide and could have reached a partial pressures of 3.3 x 10^{-2} atm with corresponding H_2CO_3* and HCO_3^- values of 1.03 x 10^{-3} mol L^{-1} and 5.21 x 10^{-3} mol L^{-1} respectively. Under these calculated values the medium with HEPES present is capable of dissolving more inorganic carbon than that without HEPES. Since both conditions produced capsulated morphologies; however, the minimum dissolved inorganic concentrations for BG-11_0 can be assumed to be no greater than 4.09 x 10^{-5} mol L^{-1} and 2.15 x 10^{-4} mol L^{-1} for H_2CO_3* and HCO_3^- respectively.

The dissolved inorganic species of BG-11 supplemented with plain air and no HEPES is represented in **Figure 3.9a** dotted lines and solid symbols. At pH 9.4 H_2CO_3* remains unchanged from initial conditions at a concentration of 10^{-5} mol L^{-1}; however, the HCO_3^- concentration increased substantially to 1.26 x 10^{-3} mol L^{-1}, a value greater than the concentration available to BG-11_0 cultures without HEPES and supplemented with carbon dioxide. Thus cultures that increase in pH increase the available dissolved inorganic carbon, not decrease it as stated by Otero and Vincenzini (Otero and Vincenzini, 2003) in citing Brewer and Goldman (Brewer and Goldman, 1976). According to **Figure 3.9a**, at pH 9.4 all nitrates have been consumed. Otero and Vincenzini observed the pH of these cultures to increase above 9 after the first two days (Otero and Vincenzini, 2004). In effect BG-11 cultures created an imbalance in the carbon-nitrogen ratio by consuming nitrates rapidly and increasing dissolved inorganic carbon by increasing the pH.

Anabaena PCC 7120 in BG-11 was observed to have nitrate concentrations above 16 mmol L^{-1} from the initial 17.65 mmol L^{-1} after 6 days and 6 mmol L^{-1} when supplemented with 1 or 3 % carbon dioxide (Kang *et al.*, 2005). The author of this thesis has noticed pH rise above 9 in BG-11 cultures of *Anabaena* PCC 7120 suggesting the complete consumption of nitrates. Kang *et al.* however, reported no pH and used 20mmol L^{-1} HEPES in the media (Kang *et al.*, 2005). This makes it difficult to verify pH change with nitrate consumption for *Anabaena* PCC 7120 without further experimentation. It is not known whether *Nostoc* PCC 7936 may have similar nitrate consumption rates to *Anabaena* PCC 7120 but, the rapid increase in pH above 9 would suggest that the nitrates were consumed within three days of inoculation.

At 41 days of growth BG-11 media is expected to have nutrients such as nitrates and phosphates depleted. This was the case for *Nostoc commune* where heterocyst had formed indicating nitrogen fixation had become the source of nitrogen. Cultures of *Nostoc commune* that went to 61 days in the same medium were at pH 9.5-10 indicating that this range is the maximum pH induced by cell growth. The data also suggest that the primary factor in increasing the pH was the consumption of nitrates.

Kang *et al.* observed that by supplementing carbon dioxide to cells of *Anabaena* PCC 7120, the rate of nitrate reduction decreased and heterocysts were frequently observed (Kang *et al.*, 2005). This suggests that supplementing with carbon dioxide affects the reduction of nitrates and overall nitrogen metabolism. This would also affect the carbon-nitrogen balance of the cell. It would be interesting to see if Kang's *et al.* observation of *Anabaena* PCC 7120 was also applicable to strains such as *Nostoc* PCC 7936 and *Nostoc commune* which are capable of producing a capsulated morphology.

Concentrations of dissolved inorganic carbon species for BG-11 cultures that were supplemented with carbon dioxide (Otero and Vincenzini, 2004) are shown by the solid lines and open symbols of **Figures 3.8a** and **3.9a**. Otero and Vincenzini reported that these cultures did not produce a capsulated morphology (Otero and Vincenzini, 2004). It is a puzzle as to why these cultures did not. If the nitrates were fully consumed they would have had a carbonate concentration similar to those cultures which the pH was between 9 and 10 and which did produce a capsulated morphology. Neither final concentration of nitrates nor the partial pressure of carbon dioxide was reported and this leaves no indication of the actual dissolved inorganic carbon concentration. The same lack of data prevents knowing the dissolved inorganic carbon concentration of BG-11 cultures with HEPES that were supplemented with carbon dioxide and did produce a capsulated morphology (Otero and Vincenzini, 2004). The conclusion remains that the carbon-nitrogen ratio must have been low enough to prevent the formation of a capsulated morphology in the former case and high enough in the latter.

4.6 Conclusions

The structure and composition EPS of cyanobacteria is not static but dependent on nitrogen source, pH, and carbon availability. From these experiments we are unable to narrow down whether it was a change in nitrogen source, pH, or carbon availability that drove the alteration of the EPS since all three were altered by the consumption of nitrates. The absolute minimum dissolved inorganic carbon concentration to generate a capsulated morphology in *Nostoc* PCC 7936 in BG-11_0 is 4.09×10^{-5} mol L^{-1} and 2.15×10^{-4} mol L^{-1} for H_2CO_3* and HCO_3^- respectively. The consumption of nitrates raises both the pH and the availability of dissolved inorganic carbon in the form of bicarbonate and produces capsulated morphologies in both *Nostoc commune* and *Nostoc* PCC 7936. It was not determined whether carbon dioxide stimulates the capsulated morphology equally as bicarbonate since bicarbonate was the dominant species in all experiments.

Biofilm formation is influenced by the composition of the EPS in response to nutrient conditions. The similar but opposite responses of *Anabaena variabilis* and *Anabaena* PCC 7120 to BG-11 and BG-11_0 media may facilitate the ability to occupy different niches. Any ecological advantage to their opposing responses remains yet to be determined.

Slight changes in pH can greatly affect the availability of dissolved inorganic carbon as will changes in partial pressure of carbon dioxide alter the pH. This needs to be considered when selecting BG-11 or BG-11_0 for culturing. Modification to the medium by the addition of a buffer may be necessary to limit variables; however, caution should be used in selecting even traditionally used buffers.

4.7 Suggestions for Future Work

Additional studies where the partial pressure of carbon dioxide, pH, and the nitrate concentrations are closely monitored will help define the threshold for generating a capsulated morphology in different species of *Nostoc* and other cyanobacteria. Consideration should also be given to which species of dissolved inorganic carbon is being absorbed by observing the regulation of the carbon concentrating mechanism. Under high pH the carbon concentrating mechanism should still be active since the primary dissolved inorganic carbon species, bicarbonate, would require active transport by BCT-1 or sodium-dependent transport (Badger and Price, 2003; Giordano *et al.*, 2005; Kaplan and Reinhold, 1999). This would help confirm

the concept that capsulated morphologies are controlled by imbalanced carbon-nitrogen ratios and help determine if these ratios vary between strains of cyanobacteria. The finding could then be applied to modern and ancient environmental conditions dealing with planktonic and biofilm formation and community behavior of microbial mats, calcification within microbial mats.

The alteration of polysaccharide by increasing available dissolved inorganic carbon implies that paleo-atmospheric conditions would have affected polysaccharide production, composition, and morphology of polysaccharides generated depending upon the metabolism and strains of cyanobacteria present. Subsequently, this would have had an impact on microbial mats and calcification of stromatolites. The current low abundance of partial pressure in the atmosphere may, in addition to grazing protozoa, play a role in the growth rates of current stromatolites and influence global carbon dioxide partial pressures.

Special attention should be given to how high amounts of atmospheric carbon dioxide would affect microbial mat growth and calcification. Inglesias-Rodriguez *et al.* provided both laboratory and field evidence that the coccolithophore species *Emiliania huxleyi* increased in calcification and net primary production significantly under high carbon dioxide partial pressure (Iglesias-Rodriguez *et al.*, 2008). Similar results may be found for microbial mat communities and would support reasons for the greater abundance of stromatolites in geologic history as well as provide a methodology for global increases in carbon sequestration.

Carbon-nitrogen ratios also need to be considered when examining polymer production of cyanobacteria for industrial uses. Increased production and varying composition of polymers could be achieved by controlling carbon and nitrogen availability.

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