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Chapter 2

Nitrogen Fixing Cyanobacteria: Future Prospect

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Additional information is available at the end of the chapter

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1. Introduction

Cyanobacteria are often called "blue-green algae", this name is convenient for talking about organisms in water that make their own food, but does not reflect any relationship between the cyanobacteria and other organisms called algae. Cyanobacteria are relatives to bacteria, not eukaryotes, and it is only the chloroplast in eukaryotic algae to which cyanobacteria are related. Some cyanobacteria are aquatic and photosynthetic, that is, they live in water, and can manufacture their own food. They are quite small and usually unicellular, though they often grow in colonies large enough to see. In fact, it may surprise you then to know that the cyanobacteria are still around; they are one of the largest and most important groups of bacteria on earth (Berry *et al*, 2008). The great contribution of cyanobacteria is the origin of plants chloroplast with which plants make food for themselves is actually a cyanobacterium living within the plant's cells. Sometime in the late Proterozoic or in the early Cambrian, cyanobacteria began to take up residence within certain eukaryote cells, making food for the eukaryote host in return for a home. This event is known as endosymbiosis, and is also the origin of eukaryotic mitochondrion (Issa *et al*., 2002). Majority of cyanobacteria are aerobic photoautotrophs, their life processes require only water, carbon dioxide, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism. In the natural environment, however, it is known that some species are able to survive long periods in complete darkness. Furthermore, certain cyanobacteria show a distinct ability for heterotrophic nutrition (Fay, 1965). Cyanobacteria might be the first plants to colonies bare areas of rock and soil. Adaptations, such as ultraviolet absorbing sheath pigments, increase their fitness in the relatively exposed land environment. Many species are capable of living in soil and other terrestrial habitats, where they are important in the functional processes of ecosystems and cycling of nutrient elements (Whitton, 1992). The prominent habitats of cyanobacteria are limnic and

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marine environments. They flourish in water that is salty, brackish or fresh, in cold and hot springs, and in environments where no other microalgae can exist. Most marine forms (Humm and Wicks, 1980) grow along the shore as benthic vegetation in the zone between high and low tide marks. Cyanobacteria comprise a large component of marine plankton with global distribution (Gallon *et al.,* 1996). A number of freshwater species are also able to withstand relatively high concentrations of sodium chloride. It appears that many cyanobacteria isolated from coastal environments tolerate saline environments (i.e. are halotolerant) rather than require salinity (i.e. are halophilic). As frequent colonisers of euryhaline (very saline) environments, cyanobacteria are found in salt works and salt marshes, and are capable of growth at combined salt concentrations as high as 2-3 (%) (Reed *et al.,* 1984). Freshwater localities with diverse trophic states are prominent habitats for cyanobacteria. Numerous species characteristically inhabit, and can occasionally dominate, both near-surface epilimnic and deep, euphotic, hypolimnic waters of lakes (Whitton, 1973). Others colonise surfaces by attaching to rocks or sediments, sometimes forming mats that may tear loose and float to the surface. Cyanobacteria have an impressive ability to colonise infertile substrates such as volcanic ash, desert sand and rocks (Dor and Danin, 1996). They are extraordinary excavators, boring hollows into limestone and special types of sandstone (Weber *et al.,* 1996). Another remarkable feature is their ability to survive extremely high and low temperatures. Cyanobacteria are inhabitants of hot springs (Castenholz, 1973), mountain streams (Kann, 1988), Arctic and Antarctic lakes (Skulberg, 1996) and snow and ice (Kol, 1968; Laamanen, 1996). The cyanobacteria also include species that run through the entire range of water types, from polysaprobic zones to katharobic waters (Van Landingham, 1982).

Once known as blue-green algae, cyanobacteria are the most diverse photosynthetic bacteria. The gram negative bacteria have chlorophyll *a* and photosystems I and II that allow them to perform oxygenic photosynthesis. Unlike most bacteria, cyanobacteria lack α-ketoglutarate dehydrogenase and therefore do not use the citric acid cycle for carbohydrate metabolism, but the pentose phosphate pathway. With such great diversity there has been some controversy on how to classify cyanobacteria. *Bergey's Manual* has divided the organism into five subsections. The classical taxonomy of cyanobacteria divides these organisms into five 'subsections' or orders, three for non-heterocystous types and two for heterocystous types (Castenholz, 2001; Castenholz and Waterbury, 1989). The non-heterocystous cyanobacteria comprise Subsection I (Chroococcales), which are unicellular cyanobacteria that reproduce by binary fission; Subsection II (Pleurocapsales) are unicellular cyanobacteria that produce daughter cells smaller than the parent; and Subsection III (Oscillatoriales) consists of cyanobacteria that produce filaments of cells known as trichomes. All three subsections have N_2-fixing representatives (Bergman*etal.*, 1997). The classification of N_2-fixing cyanobacteria based on behaviour are shown in table (1) and photographed in figure (1). Heterocyst formation is an important aspect to nitrogen fixation. The filamentous cells differentiate into heterocysts when the cells are deprived of dissolved inorganic nitrogen. A heterocyst consists of a thick cell wall and only contains photosystem I for ATP production. Photosystem II is degraded to prevent O_2 production. O_2 inhibits nitrogenase, the enzyme responsible for N_2-fixation. The proposal chapter we will discuss the contribution of cyanobacterial nitrogen fixer organisms' in ecosystem and future prospects.

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A. Cyanobacteria that can fix N_2 aerobically
A1.Cyanobacteria that separate N_2 fixation from oxygenic photosynthesis in space. Includes heterocystous genera, for example, *Anabaena*.
A2.Cyanobacteria that separate N_2fixation from oxygenic photosynthesis in time. Includes non-heterocystous genera, such as *Gloeothece*, *Cyanothece* and *Lyngbya*
A3.Cyanobacteria that separate N_2 fixation from oxygenic photosynthesis both in space and in time. Includes non-heterocystous genera, such as *Trichodesmium* and *Katagnymene*
B. Cyanobacteria that can fix only N_2 either anaerobically or microaerobically
Many non-heterocystous cyanobacteria,forexample, *Plectonemaboryanum*.

Table 1. A classification of N_2-fixing Cyanobacteria based on behavior

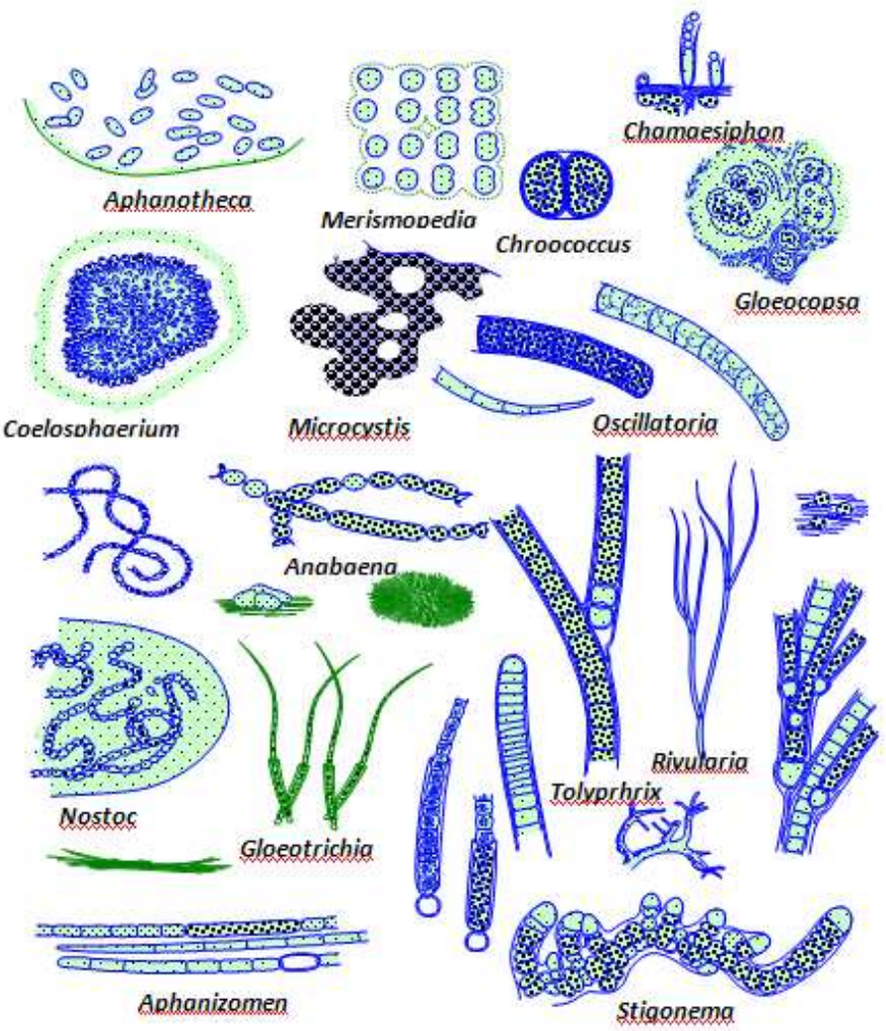


Figure 1. N2-fixing cyanobacteria

2. Effect of abiotic and biotic factor's on population and survival of Cyanobacteria

Cyanobacteria, a group of prokaryotic, oxygen-evolving, photosynthetic Gram-negative bacteria, survive in a wide variety of extreme environmental conditions; they are exposed to various types of natural stresses, such as nutrient limitation, pesticides, pollution, drought, salinity, temperature, pH, light intensity and quality, etc. (Herrero *et al.,* 2001). A Protein in the cyanobacterial thylakoid membranes was identified as a sensitive protein to environmental stress conditions: under various unfavorable conditions like drought, nutrition deficiency, heat, chemical stress, ozone fumigation as well as UV-B and visible light stresses can influence the turnover of protein (Giardi *et al*., 1997). Many species are capable of not only surviving, but thriving in conditions previously thought to be inhabitable, tolerating desiccation, high temperatures, extreme pH, high salinity and pesticides illustrating their capacity to acclimate to extreme environments (Stal 2007). The major abiotic factor affecting the distribution of algae in soils is solar radiation, moisture, temperature, nutrients, and pH, organic matter content and soil texture are less important. Generally, the higher the soil moisture, soil temperature, and sunlight penetration to the soil surface, the greater the population and activities of algae. Increased levels of both organic and inorganic nutrients also enhance the growth and activity of terrestrial algae. Soil pH also affects the activities of certain types of algae. For example, cyanobacteria thrive best in alkaline soils (pH 7.0 and above) whereas green algae do best in more acidic soils (pH 5.5 and below). In fact, in many cases, moisture levels can be excessive, creating anaerobic conditions that favor the growth of some cyanobacterial species. On these types of sites, temperature appears to be the overriding factor influencing algal growth and activity. It was found that species of *Oscillatoria* were the predominant algae in Bermuda grass greens whereas species of *Anacystis* were the dominant algae in bent grass greens. This distribution can vary too according to the time of year and geographical location. Pesticides are another factor affecting the distribution and activity of cyanobacteria. In general, most herbicides, fungicides, and soil fumigants are toxic, whereas insecticides generally are not. Nearly all of our knowledge about pesticide toxicity to cyanobacteria comes from either laboratory culture studies or from a limited number of field studies on agricultural crops (Mahmoud et al, 1992; Issa, 1999; Issa *et al.,* 2013).

The physico-chemical changes in the environment may affect particular species and induce the growth and abundance of other species, which leads to the succession of several species in a course of time (Muthukumar *et al*., 2007). High temperatures favour both the phytoplankton productivity and blue green algae (Roger and Reynaud, 1979). Cyanobacteria grew rapidly in the rice fields that contained ample organic matters in the soil and water as well as conditions such as pH, temperature, and organic sources in various rice fields (Choudhury and Kennedy, 2004). Among soil properties, pH is a very important factor in growth, establishment and diversity of cyanobacteria, which have generally been reported to prefer neutral to slightly alkaline pH for optimum growth (Koushik, 1994). The cyanobacteria *Alosira ferrilissitiia* and *Calothrix brevessima* have been reported to be ubiquitous in Kerala rice fields with pH from 3.5 to 6.5. Subhashini and Kaushik (1981) reported that the pH of the alkaline soil decreased when treated with cyanobacteria. Also, Nayak and Prasanna (2007) investigated the cyanobacteria were more in number at high pH in rice fields. Cyanobacteria

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have been found not only to grow in highly saline-alkali soils, but also improve the physico-chemical properties of the soil by enriching them with carbon, nitrogen and available phosphorus (Kaushik, 1994).

Many species of cyanobacteria not only fix carbon in CO_2 through photosynthesis, but they can also fix atmospheric nitrogen. Both of these processes also play an important role in humus formation. In natural soils, cyanobacteria produce considerable amounts of polysaccharide that helps to aggregate soil colloids and improve soil structure while at the same time improving water infiltration and percolation. Subsurface soil cyanobacteria are also known to associate with plant roots, producing hormones that stimulate root growth and enhance the activities of other beneficial root-associated microorganisms (Issa *et al.*, 1994). In fact in many of the rice growing regions of the world some cyanobacterial species are inoculated into soils to enhance rice yields by as much as 36%. Soil cyanobacteria also commonly interact with other microorganisms in soil. Many soil cyanobacteria excrete avariety of antimicrobial compounds that affect the activities of other microorganisms, including plant pathogens. In this case, a species of *Nostoc* was used for the biological control of a seedling disease of millet. Upon the death of nearly all algae, they serve as an important food source for many important bacteria and fungi in soils (Issa, 1999). While living, soil algae serve as food sources for protozoa, earthworms, nematodes, and micro arthropods. A number of associations of algae with othermicroorganisms in soil can result in enhanced algal growth resulting in detrimental effects on turfgrass growth and quality. On the other hand, a number of cyanobacteria species have been shown to inhibit root growth of a number of crop plants by producing antibiotic substances that also inhibit bacterial growth. It is well known in the floriculture industry that algal proliferation (usually cyanobacteria) on subirrigation mats and on roots of potted plants in greenhouses can lead to reductions in plant growth and quality.

In the Arctic, cyanobacteria are the primary source of newly fixed nitrogen (Hobara *et al*. 2006; Solheim *et al*. 2006) and form many associations with vegetation including epiphytic and endophytic facultative associations with bryophytes (Turetsky, 2003) and the lichen symbioses and soil surface colonies that are components of Biological Soil Crusts (Belnap *et al*. 2001). Bryophyte-associated cyanobacteria are an important source of N_2 within many terrestrial ecosystems, for example, a high abundance of feather moss-cyanobacterial associations occur innorthern boreal forests, where they contribute 1.5 to 2.0 kg N $ha^{-1}yr^{-1}$ (DeLuca *et al*.2002; Houle *et al*.2006; Lagerström *et al*.2007; Zackrisson *et al*.2009).While variation is often high within and between bryophyte species, the highest rates of N_2-fixation in arctic landscapes are often associated with cyanobacteria bryophyte associations (Alexander and Schell1973; Henry and Svoboda1986; Solheim et al.1996). Cyanobacterial symbioses with lichens are also a major source of fixed N_2 as they often have N_2-fixation rates exceeding that of other cyanobacterial symbioses (Schell and Alexander1973; Kallio and Kallio 1975; Crittenden and Kershaw 1978; Gunther 1989; Hobara *et al.* 2006). Finally, the prevalence of Biological Soil Crusts in many arctic ecosystems ensures that the cyanobacteria associated with those crusts are major contributors to arctic N_2 inputs (Alexander *et al*.1978). Although many environmental factors could potentially determine the microbial community present in these multidimensional ecosystems, changes in the diversity of cyanobacteria in rice fields was correlated to salinity Low salinity favored the presence of heterocystous cyanobacteria,

while very high salinity mainly supported the growth of non-heterocystous genera. High nitrogen content in the low salt soils is proposed to be a result of reduced ammonia volatilization in comparison to the high salt soils. Cyanobacterial mats are dense, stratified microbial agglomerations that develop well in hypersaline habitats because of the limited grazing activities (Javor and Catenholz 1984; Cohen 1989; Farmer 1992). These mats are composed of different physiological groups of microbes such as photoautotrophic, photoheterotrophic, chemoautotrophic, and heterotrophic organisms (van Gemerden 1993; Stal 1995). Oxygenic photosynthesis is mainly performed by cyanobacteria in the top few millimeters of the mats, resulting in the development of strong oxygen gradients and the production of organics that are utilized by heterotrophic bacteria (Jonkers et al. 2003).

3. Effect of adverse soil condition on heterocyst formation and nitrogenase activity in hetreocystous cyanobacteria

Many free-living blue-green algae (cyanobacteria) fix atmospheric nitrogen and since they are photosynthetic, they do not compete neither with crop plants nor with heterotrophic soil microflora for carbon and energy. Nitrogen-fixing ability has not only been shown by heterocystous Cyanobacteria (*Nostoc*, *Anabaena, Aulosira,* etc.) but also by several non-heterocystous unicellular (*Gloeocapsa, Aphanothece, Gloeothece*, etc.) and filamentous (*Oscillatoria, Plectonema*, etc.) cyanobacteria (Table 2). In non heterocystous forms, the oxygenic photosynthesis was found to be separated from nitrogen fixation either temporally or spatially. In temporal separation, nitrogen fixation predominantly occurs during the dark period and photosynthesis during the light; in these forms in terms of energy the anaerobic dark conditions are not very favourable for the process of nitrogen fixation. In spatial separation, the central non-photosynthetic cells get engaged in nitrogen fixation, whereas, the outer green cells are photosynthetically active. The species with bio fertilizer potential are the heterocystous, filamentous forms belonging to the order Nostocales and Stigonematales in which the nitrogenase activity and oxygenic photosynthesis are separated spatially and nitrogenase activity is usually light-dependent. Species of *Nostoc, Anabaena, Tolypothrix, Aulosira, Cylindrospermum, Scytonema,* and several other genera are widespread in rice fields and contribute significantly to their fertility. Cyanobacteria can contribute about 20-30 kg N ha^{-1} $season^{-1}$ as well as organic matter to the soil which is quite significant for the economically weak farmers who are unable to invest on costly chemical nitrogen fertilizer. Often blooms of free-living cyanobacteria are favoured in tropical regions and inoculation of paddy fields with cyanobacteria is traditionally applied in most of the Asian countries. Besides rice, other crop plants like vegetables, wheat, sorghum, maize, cotton, sugarcane, etc. also respond to cyanobacterial biofertilizer (Abd-Alla and Issa, 1994; Abd-Alla *et al*., 1994). In sub-tropical regions *Azolla*, a fern within the leaf cavity of which is found the heterocystous cyanobacterium-*Anabaena azollae*, is the traditional biofertilizer (Kimura, 2000; Kirk, 2004)

Biological nitrogen fixation, and specifically the nitrogenase enzyme, is notorious for its sensitivity to molecular oxygen. Moreover, high oxygen stress causes proteolysis of nitrogenase subunits (Durner *et al*., 1996), suppresses nitrogenase synthesis, and leads to a shortage of

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respiratory substrates and reductants necessary for nitrogen fixation and assimilation (Gallon, 1992). Inhibitory effects of moderate levels of oxygen, or short exposure times, *in vivo* may be reversed, leading to an increase in nitrogen fixation rates (Yakunin *et al*., 2001) and, in some diazotrophs, post-translational modification of the Fe protein from an inactive to active form (Zehr *et al*., 1993). Furthermore, diazotrophic cyanobacteria, which provide the bulk of fixed nitrogen to the surface oceans, are the only diazotrophs that actively produce oxygen *via* photosynthesis and must contend with further restrictions on the nitrogen (Berman-Frank *etal*., 2003). Thus, nitrogenase in the real-world operates at only a fraction of its potential activity, yet is a major elemental taxation on diazotrophic cyanobacteria both for scarce trace elements, such as iron, and in the costs of protein synthesis. These taxes have, in turn, led to a global limitation of fixed nitrogen in the oceans (Falkowski, 1997). In addition to contributing nitrogen, cyanobacteria benefit crop plants also by producing various growth promoting substances, like gibberellins, auxins like indole-3-acetic acid, indole-3-propionic acid, etc., vitamin B12, free amino acids like serine, arginine, glycine, aspartic acid, threonine, glutamic acid, etc., extra- and intra-cellular polysaccharides like xylose, galactose, fructose, etc. Such substances have several beneficial effects like improved soil structure, stimulation of growth of crop plants as well as useful bacteria, chelation of heavy metals (El-Enany and Issa, 2000). Cyanobacteria are primary colonizers and many have been shown to possess the property of tricalcium phosphate solubilization. Abundantly available rock phosphate, being insoluble, is unavailable to crop plants. Some of the cyanobacteria like, *Tolypothrix, Scytonema, Hapalosiphon*, etc. have been reported to solubilize rock phosphate.

Unicellular group	Unicellular strains growing on BG II medium without nitrogen *(Aphanothece,Gloeothece,*)
Anabaena group:	Heterocystous strains with a thin sheath, without branching, do not form mucilaginous colonies of definite shape *(Anabaena,Nodularia, Cylindrosperinurn, Anabaenopsis* etc.)
Nostoc group:	Heterocystous strains with a thick sheath, without branching, forming mucilaginous colonies of definite shape *(Nostoc)*
Aulosira group:	Heterocystous strains with a thick sheath, usually without branching, do not form diffuse colonies on agar medium *(Aulosira)*
Scytoneina group:	Heterocystous strains with false branching, without polarity, forming velvet-like patches on agar medium *(Scytonema)*
Calothrix group:	Heterocystous strains with false branching, with polarity, forming velvet-like patches on agar medium *(Calothrix, Tolypotkrix, Hassalia*)
Gloeofrichia group:	Heterocystous strains, with polarity, forming mucilaginous colonies of definite shape *(Gloeotrichia, Rivularia*)
Fischerella group:	Heterocystous strains with true branching *(Fischerella, Westiellopsis, Stigonerna*)

a All features refer to strains grown from soil or water sample dilutionsplated on agarized BGII medium without nitrogen.

Table 2. Table2. Definition of the taxa of N_2-fixing cyanobacteria

4. Nitrogenase

The enzyme complex nitrogenase (E.C.1.18.6.1) consists of a dimeric Fe-protein (the dinitrogenase reductase) functioning as an electron carrier to the tetrameric MoFe-protein (the dinitrogenase) which reduces molecular nitrogen to ammonia. Both enzymes are highly oxygen-sensitive. The intrinsically anaerobic character of the nitrogenase complex requires special adaptation in cyanobacteria which produce oxygen in a plant-type photosynthesis. Filamentous heterocystous cyanobacteria provide such an anaerobic environment by creating a diffusion barrier for gases, enhanced respiratory activity and the lack of the oxygenic photosystem I1 (Scherer *et al*., 1988).Reductant supply of nitrogenase *via* ferredoxin is provided by photosynthates transported from vegetative cells to the heterocysts and ATP is generated by photosystem-l activity or oxidative phosphorylation in the heterocysts (Stewart and Rowell, 1986). Under a light-dark regime most heterocystous strain described so far preferentially fix nitrogen in the light (Khamees *et al*., 1987). Natural blooms also, dominated by heterocystous cyanobacteria exhibit higher nitrogenase activity in the light than in the dark (Horne, 1979). The low activity of nitrogenase in darkness was assigned to the inability of metabolism to sufficiently generate reductants under these conditions (Ernst and Bohme, 1984). In many respects this modification resembles the ADP-ribosylation of Fe-protein of nitrogenase observed in *Rhodospirillaceae* after transfer from light to darkness or after addition of ammonia (Kanemoto and Ludden, 1984).Heterocysts have thick multilayered wall preventing the entry of oxygen, high rate of respiration which utilizes the defused oxygen, and they lack photosystem II so that there is no photosynthetic evolution of oxygen. The scheme of a heterocyst with adjacent vegetative cells is shown below. The outer and inner layers of the heterocyst envelope consist of polysaccharides and glycolipids, respectively. In this scheme the pore region is not drawn to scale and shown enlarged to accommodate metabolite exchange between the cells. Cell wall and cell membranes are not drawn separately. Heterocysts import carbohydrates from vegetative cells, with glutamine moving in the opposite direction. In a cell-free system derived from heterocysts, the following substrates supported nitrogenase activity: glycogen, maltose, sucrose (less active), glucose and fructose; glucose 6-phosphate (G6P) and other intermediates of the oxidative pentose-phosphate cycle (PPC), including dihydroxyacetone phosphate (DAP), glyceraldehyde 3-phosphate (GAP) and fructose-1,6-bisphosphate (FBP), were particularly active. Glycolytic substrates, such as phosphoenolpyruvate (PEP) and pyruvate (Pyr) were inactive or inhibitory in acetylene reduction by the heterocyst extract. In the dark, reductant for nitrogen and oxygen is generated by the activity of the oxidative PPC and possibly by isocitrate dehydrogenase. NADPH thus formed donates electrons via ferredoxin: NADP reductase (FNR) to a heterocyst-specific ferredoxin (FdxH) and then to the two components of nitrogenase (Fe-protein and FeMo-protein) as indicated. NAD(P)H and hydrogen are also electron donors to the respiratory electron transport (RET) generating the necessary ATP for the nitrogenase reaction. In the light, ATP is formed by cyclic photophosphorylation mediated by photosystem I (a PSI-dimer, as indicated). Ferredoxin could be also photoreduced by PSI at the expense of hydrogen and NAD(P)H as electron donors (Figure 2). Wyatt and Silvey (1969)

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for the first time reported that non-heterocystous cyanobacteria have also the ability to fix nitrogen in which all the vegetative cells contain nitrogenase but due to thepresence of oxygen the enzyme gets inactivated. Nitrogen fixation in these organisms is light stimulated process. Cyanobacteria fix nitrogen only under combined nitrogen deficient conditions and in the presence of combined nitrogen source the enzyme nitrogenase remains repressed which, similar to oxygen effect, is a reversible inhibition. Inoculation of rice fields with cyanobacteria reduces the nitrogen losses through metabolization of the applied combined nitrogen forms. The metabolized combined nitrogen as well as the biologically fixed nitrogen becomes available gradually through exudation and decomposition of these algae. The nitrogen fixation by cyanobacteria depends upon the various biotic and abiotic factors. Nitrogenases are highly sensitive to oxygen and hydrogen production catalyzed by the nitrogenase / hydrogenases can only function under anaerobic conditions because of its extreme sensitivity to oxygen. Some cyanobacteria have solved this problem by developing specialized thick walled cells known as heterocysts which maintain low oxygen tension inside, thereby facilitating nitrogenase activity, which produces hydrogen during N_2 fixation (Issa 1995). A wide range of nitrogenase activity has been reported in cyanobacteria (Table 3).

Name of the species	**Nitrogenase activity (nmol C_2H_4 mg dry wt.$^{-1}$ h^{-1})**	**Reference**
Anabaena sp. Strain CA	0.17	Antal et al. 2005
Anabaena sp. Strain N9AR	0.13	Antal et al. 2005
Phormidium valderianum	2.2	Kiran, 2007
Nostoc calcicola	9.021	Bolton, 1996
Anabaena cylindrica	5.579	Bolton,1996
Anabaena oryzae	5.076	Bolton,1996
Nostoc commune	6.346	Anjana et al. 2012
Nostoc muscorum	8.5	Issa, 1995

Table 3. Nitrogenase activity of various cyanobacterial species

5. Effect of severe conditions on nitrogenase activity in non heterocystous cyanobacteria

Cyanobacteria are oxygenic phototrophic microorganisms, usually living in aerobic and oxygen-supersaturated environments (Stanier and Cohen-Bazire 1977). Many cyanobacteria, filamentous as well as unicellular species, synthesize the enzyme nitrogenase and are able to

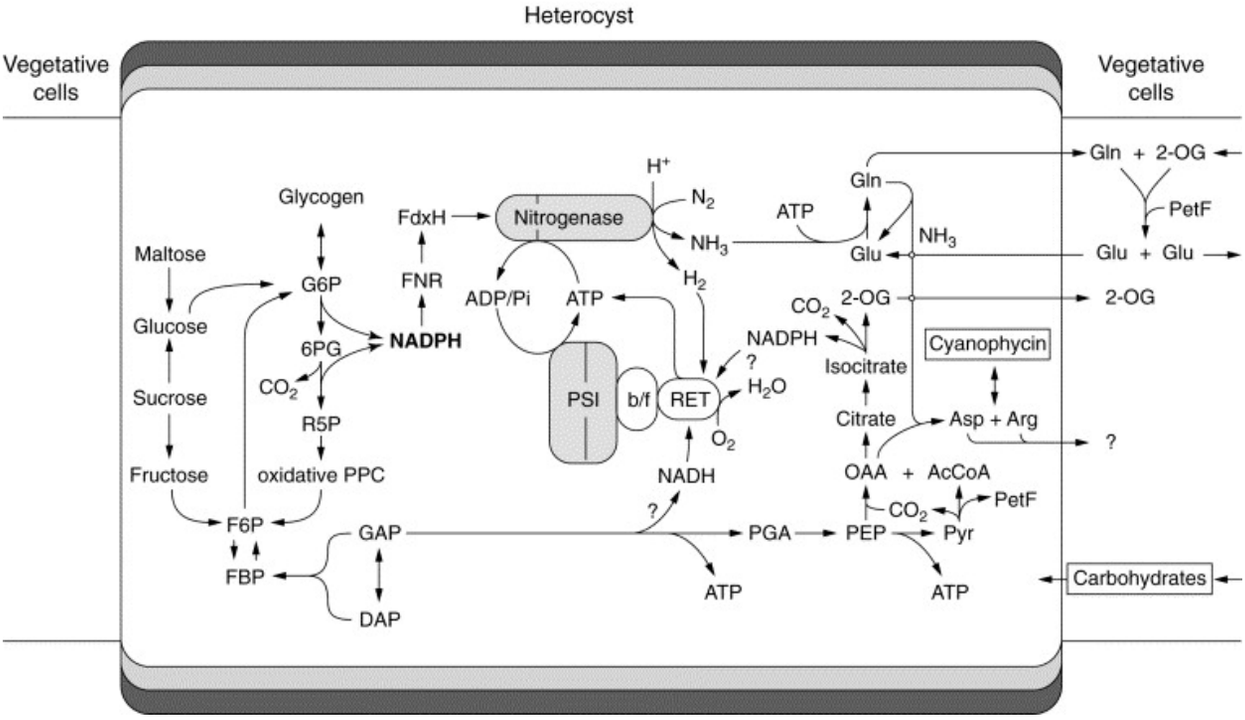


Figure 2. Heterocyst metabolism and nitrogen fixation. Abbreviations: AcCoA, acetyl coenzyme A; Arg, arginine; Asp, aspartate; b/f, cytochrome b6f complex; F6P, fructose 6-phosphate; PetF, vegetative cell type ferredoxin; Glu, glutamate; Gln, glutamine; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; 6PG, 6-phosphogluconate; PGA, 3-phosphoglycerate; Pi, inorganic phosphate; R5P, ribose 5-phosphate. (Böhme,1998)

fix molecular nitrogen (Stewart 1980). This phenomenon seems to be in contradiction with the extreme sensitivity of nitrogenase towards molecular oxygen (Robson and Postgate 1980).Therefore, nitrogen-fixing cyanobacteria have developed mechanisms to protect nitrogenase from inactivation by oxygen. Thus far, these mechanisms are largely unknown. Photosynthetic oxygenevolution and nitrogen fixation cannot occur simultaneouslyin one single cell (Mitsui *et al*. 1986).Several filamentous cyanobacteria develop heterocysts.Heterocysts are non-dividing cells which have lost thecapacity of oxygenic photosynthesis and which contain theenzyme nitrogenase (Fay et al. 1968); hasorganisms thus have solved the problem by spatial separation of the incompatible processes of oxygen evolution and nitrogen fixation. In non-heterocystous filamentous and unicellular nitrogen-fixing cyanobacteria nitrogenase and photosynthesis apparently occur in the same cell. It has been suggested that in such organisms nitrogen fixation is separated from oxygenic photosynthesis temporary (Stal and Krumbein 1985a). When grown under light-dark cycles, non-heterocystous cyanobacteria show nitrogenase activity only during the dark period (Huang and Chow 1986). However, when cultures were synchronized or previously adapted to light-dark cycles, also in continuous light a cyclic pattern of nitrogenase activity can be observed (Grobbelaar et al. 1986). The strategy by which non-heterocystous cyanobacteria protect nitrogenase from deterioration by atmospheric and photosynthetic evolved oxygen, it has been shown fora variety of non-heteroeystous cyanobacteria, that when grown underlight-dark cycles, nitrogenase activity predominantly occurs during the dark period. However, all these or-

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ganisms are able to grow in continuous light at the expense of molecular nitrogen, showing nitrogenase activity under such conditions. Using synchronized cultures of *Synechococeus* sp., Mitsui *et al.* (1986) showed that N_2-ase and oxygen evolution followed a reciprocal pattern, even in continuous light. They also showed that the capacity of oxygen evolution decreased to virtually zero at the maximum of nitrogenase activity and in one case even became negative (respiration exceeded possible oxygen production). These authors, however, measured photosynthesis at far higher (more than 6 times) light intensity than applied for growth and acetylene reduction. This phenomenon is most pronounced in continuous light. However, this observation cannot be taken as evidence for the coexistence of N_2-ase and photosynthesis in one single cell. Weare and Benemann (1974) provided evidence that a cyclic degradation and resynthesis of phycobiliprotein regulated photosynthetic activity. When phycobiliprotein of the cell was low, oxygen evolution ceased and nitrogenase was induced. On the other hand, Giani and Krumbein (1986) found that this phenomenon strongly depended on light intensity. At low light intensity (500 lux or less) phycobiliprotein was constant. From epifluorescence microscopy and microfluorimetry it was concluded that under nitrogen-fixing conditions, the oxygenic photosystem was still intact. However, oxygen evolution was not measured and the possibility that photosynthesis was switched off could not be excluded. In *Oscillatoria,* the phycobiliprotein content is lower in nitrogen-fixing cells than in nitrate grown cultures (Stal and Krumbein 1985b). This may result in a lower activity of the photosynthetic apparatus. However, phycobiliprotein and total protein remained constant during growth on N_2. The question arises whether this is also the case in the light (Maryan et al. 1986). Both in the dark and in the light a very high rate of respiration was observed here. The nitrogenase activity in the light, however, was about six times the dark maximum rate. Thus, light clearly stimulated nitrogenase activity. Therefore it was concluded that, in the light, respiration more likely fulfilled a protection function than that it provided energy for nitrogenase. In the dark, respiration provides both energy and a protective role. Respiration of cyanobacteria in the light has been shown for several species and the highest rates have been found in nitrogen-fixing species (Scherer and Böger 1982). However, in none of these organisms, respiration exceeded photosynthesis.

Trichodesmium spp. is non-heterocystous cyanobacteria found in tropical and subtropical seas which are important in mediating a flux of reduced nitrogen from the atmosphere to the ocean. The organism fixes nitrogen when grown with N_2 as the sole inorganic nitrogen source (Ohki and Fujita 1988). The nitrogen-fixing system of this algae is regulated at two levels: (1) the synthesis of enzyme is regulated at a transcriptional or post-transcriptional level by the presence of urea, and (2) the activity of the Fe protein is correlated with a shift in electrophoretic mobility, which is believed to be a post-translational modification (Ernst et al. 1990a, b). *Trichodesmium* lacks both akinetes and heterocystsand shows no ability to produce hormogonia. *Trichodesmium* has therefore been classified as anundifferentiated filamentous cyanobacterium exclusivelycomposed of photosynthetic vegetative cells, defined as cyanobacteria group III (Rippka etal. 1979). However, it is now becoming increasingly evidentthat *Trichodesmium* is differentiated, secludingthe nitrogen-fixing enzyme nitrogenase into a low number of cells (Fredrikssonand Bergman 1995). *Trichodesmium* istherefore the first detected non-heterocystous cyanobacterium with cells specialised for nitrogen fixa-

tion. Cyanobacteria develop specialised nitrogen-fixing cells in order to solve their problem of managing theco-existence of oxygen labile nitrogen fixation andoxygen producing photosynthesis (Fay 1992, Gallon1992). Heterocysts provide the ability to perform aerobic nitrogen fixation through the exclusion of atmosphericoxygen and oxygenic photosynthesis. *Trichodesmium* practises a different, hitherto unknown typeof specialisation to accommodate the oxygen sensitive nitrogenase (Fredriksson and Bergman 1995). The present study further characterises the nitrogenase containing cells in *Trichodesmium* by comparing theultrastructure of these cells with those lacking nitrogenase. Indeed, nitrogenase-containing cells exhibited structural modifications indicative of additional changes in gene expression. The functional implication of these changes is interesting considering *Trichodesmium's* unusual ability to perform oxygenlabile nitrogen fixation under fully aerobic and oxygen producing photosynthetic conditions withoutheterocyst formation (Saino and Hattori 1978). The differentiation of cells specialised for nitrogen fixation also questions the taxonomic affiliation of *Trichodesmium* within cyanobacteria.

The crystal structure of the enzyme one can see that nitrogenase is multisubunit enzyme. The FeMo protein is the site for N_2 reduction. The other subunit is the Fe protein, encoded by the highly conserved *nif*H gene used for sequencing and identification. The Fe protein has a Fe_4S_4 complex where ATP is hydrolyzed, providing the necessary electrons to the FeMo active site. Oxygen inhibition of the enzyme occurs in this subunit where the molecule can interact with the Fe_4S_4 complex (see Figure 3). Conversion of N_2 to ammonia is no easy process; Schindelin *et al*. (1997) concluded that once ATP binds, the Fe protein goes through a substantial conformational change in order to efficiently feed electrons to the FeMo redox site. This site also goes through some reorientation, maximizing the energy put into the system. For every electron that is fed to the FeMo protein active site, two ATP are hydrolyzed. The overall reaction formula is,

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi.$$

Nitrogen assimilation in natural populations of *Trichodesmium* spp. proceeds via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. GS is necessary for NH_4 assimilation regardless of the primary form of N being used. High rates of GS transferase activity relative to rates of total Nuptake have been observed in natural and cultured populations of *Trichodesmium* spp. (Mulholland et al., 1999; Mulholland and Capone, 1999). Rates of both GS transferaseand GS biosynthetic activity (which approximates *in vivo* forward reaction activity) in *Trichodesmium* spp. increase in the afternoon during the period when rates of N_2 fixation are highest. The ratio of GS transferase:biosynthetic activity decreases during the period of maximum N_2 fixation, indicating that the proportion of the GS poolthat is biosynthetically active increases during the day. The biosynthetic capacity of GS is sufficient to allow *Trichodesmium*spp. Colonies to turnover their cell N at least three times per day, suggesting that N assimilation does not limit the rate of N utilization by cells, even during midday when N_2 fixation rates are highest. Cells appear to have sufficient capacity to assimilate all of theintracellular N substrates derived from N_2 fixation and N uptake incultures growing on media with or without added N (Mulholland et al., 1999). Excess GS activity is characteristic of cells limited by N orusing N_2 as their N source. A positive correlation be-

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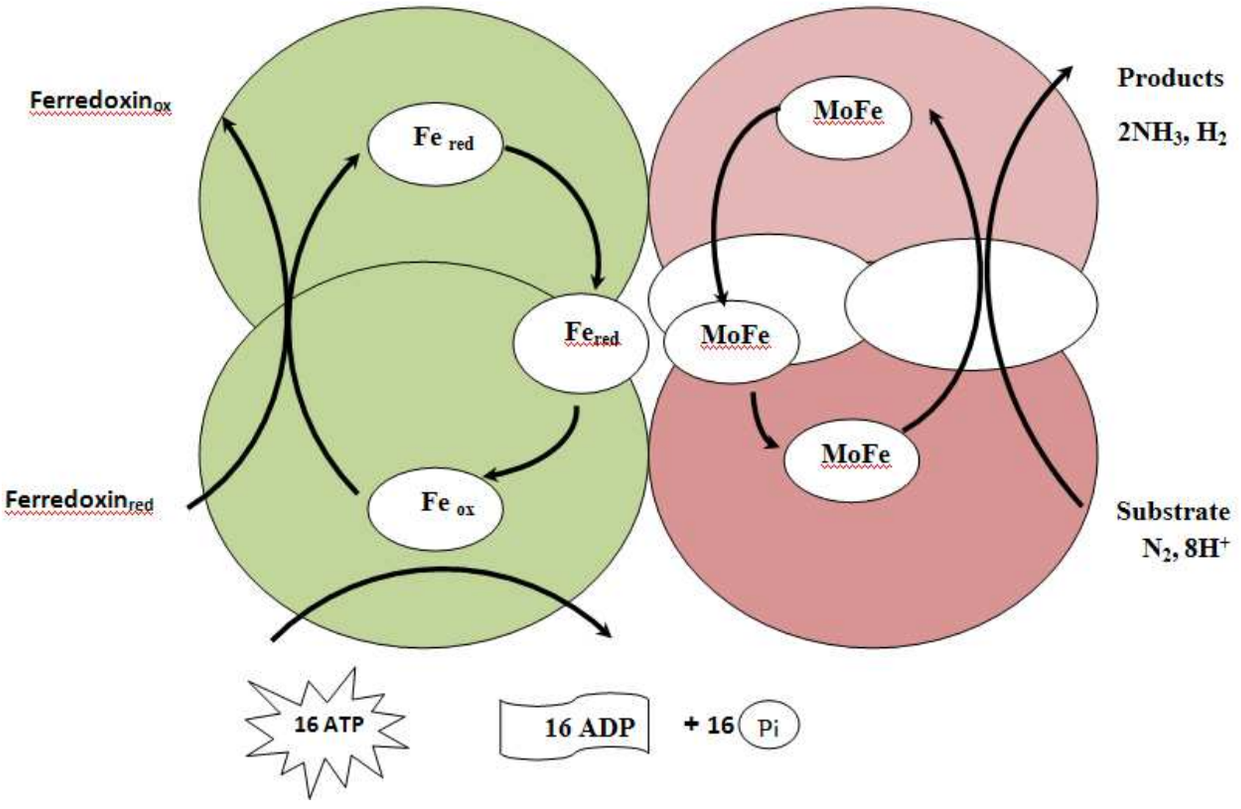


Figure 3. The structure of nitrogenase

tween GS andnitrogenase enzyme abundance and distribution has been observedin a variety of heterocystous and non-heterocystous cyanobacteria including *Trichodesmium* spp. Thus, there might be both a constitutive pool of GS, regulated for the general assimilation of N derived from various N sources, and a nitrogenase-linked pool co-regulated specifically with nitrogenase under low N conditions. Both regulatory mechanisms appear to be important. A global N-regulating gene, *ntcA*, has been identified in a *Trichodesmium* spp. isolated from the Red Sea.

6. Symbiotic cyanobacteria

Symbiotically competent cyanobacteria have some excellent features that make them particularly significant in any attempt to extend the list of N_2-fixing symbioses to include plants of commercial interest, such as cereals. Unlikerhizobia, most symbiotic cyanobacteria carry their own mechanism for protecting nitrogenase from inactivation by oxygen (heterocysts). Cyanobacteria have anunmatched host range (fungi sponges, protists and angiosperms), are not restricted to roots but may form symbiosis withvarious plant parts, and do not need to be located intracellularly within the host plant (Adams et al., 2006; Bergman et al., 2007). Cyanobionts generally supply their hosts with fixed nitrogen, although they can also provide fixed carbon to non-photosynthetic hosts. The major plant hosts are bryophytes, cycads, the

angiosperm *Gunnera*, the water-fern *Azolla,* and fungi (to form lichens). Although all cyanobacteria are photoautotrophs, many are also facultative heterotrophs and so are not restricted to the areas of the plant that receive light, and can be found in roots, stems, leaves, and thalli. This review will concentrate on the cyanobacteria–bryophyte symbioses, focusing in particular on the importance of pili and gliding motility in plant infection (Meeks 2003). Plant cyanobionts all have two major characteristics in common: (i) the ability to differentiate both specialized nitrogen-fixing cells known as heterocysts (Zhang et al., 2006) and (ii) short, motile filaments known as hormogonia, which lack heterocysts and provide a means of dispersal for otherwise immotile cyanobacteria (Meeks, 1990,1998). Heterocysts usually occur singly in a semi-regular spacing within filaments of vegetative cells (Golden and Yoon, 2003; Zhang et al., 2006). The infective agents in most plant symbioses are hormogonia and some, perhaps all, plants produce chemical signals that trigger their formation and chemoattractants that guide them into the plant tissue (Figure 4). The plant cyanobionts are members of the genus *Nostoc*, which is commonly found free-living in nature (Dodds *et al.*, 1995; Rai *et al.*, 2002). However, in the laboratory, other hormogonium-developing cyanobacterial genera, such as *Calothrix* and *Chlorogloeopsis*, may infect liverworts (West and Adams, 1997). Members of the genus *Nostoc* are primarily non-motile, but a characteristic of the genus is the ability to produce specialized motile filaments known as hormogonia which serve as a means of dispersal as well as plant infection (Meeks and Elhai, 2002). Hormogonia development is triggered by a variety of environmental factors, including plant-derived chemical signals. The development of hormogonia in heterocystous cyanobacteria results from a round of rapid, synchronous cell divisions which result in a decrease in cell size (Meeks and Elhai, 2002). This is followed by fragmentation of the filament at the heterocyst–vegetative cell junctions, releasing short, motile hormogonia. Hormogonia lack heterocysts and are a temporary stage in the *Nostoc* life-cycle, soon returning to vegetative growth and developing heterocysts once more. For hormogonia to locate the symbiotic tissue of a plant host they must attach to the surface and both extracellular polysaccharides and pili (fimbriae) are thought to play a role in this process (Adams, 2000). Type IV pili are required for gliding in some unicellular cyanobacteria (Bhaya, 2004), and the cell surface of hormogonia of the symbiotically competent *Nostoc punctiforme* is covered with pili (Duggan *et al.*, 2007). Plant hosts increase the likelihood of infection by cyanobacteria by both stimulating the formation of hormogonia in potential cyanobionts and by guiding the hormogonia to the symbiotic tissues by chemotaxis. Hormogonia formation is stimulated by hormogonia-inducing factors (HIFs). HIF production has been found in the hornwort *Anthoceros punctatus* (Meeks, 2003), as well as cycads and the angiosperm *Gunnera* (Rasmussen *et al.*, 1994; Ow *et al.*, 1999). *Anthoceros punctatus* HIF is a small, heat-labile product released by the hornwort when starved of combined nitrogen (Meeks and Elhai, 2002; Meeks, 2003). The liverwort *Blasia* also releases HIF when nitrogen-starved (Adams, 2002). *Nostoc punctiforme* mutants with increased sensitivity to *Anthoceros* HIF, also show a greater initial frequency of infection of the hornwort than the wild type (Cohen and Meeks, 1997).

The infection of hornworts *via* the stomata-like opening to the slime cavity has interesting parallels with the likely method of entry of cyanobacteria into the primitive, extinct land plant *Aglaophyton major*. This symbiosis is only known from fossil evidence, but an

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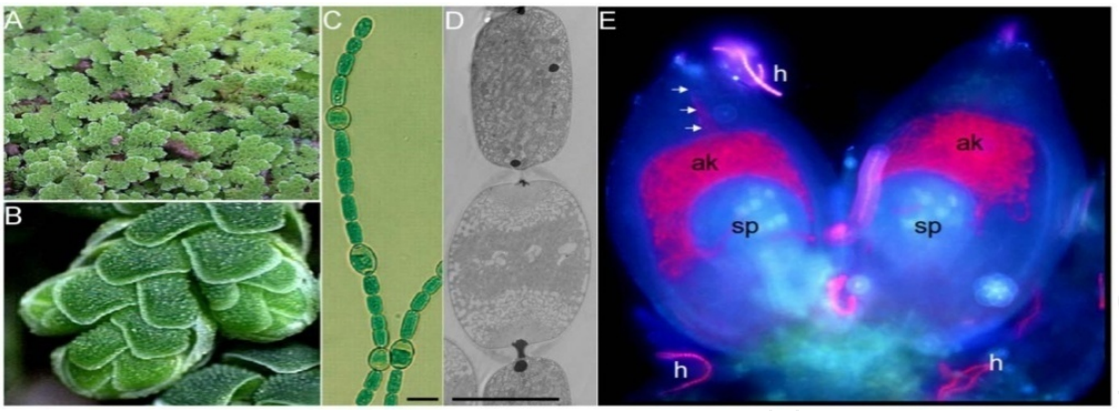


Figure 4. The partners in the Azolla symbiosis. A) Fronds of the Azolla filiculoides Lam. plant. B) Close up of an Azolla branch showing the apex and the alternating 'stacked' dorsal leaves, each containing a cavity in which the cyanobiont (NoAz) filaments reside. C), D), E) Light micrograph of the cyanobiont. Pairs of megasporocarps (blue) develop at the underside of the cyanobacterial colonized Azolla leaves. Filaments of the motile cyanobacterial cell stage (red), the hormogonia (h), are attracted to the sporocarps, gather at the base and subsequently move towards the tip, before entering the sporocarps via channels (white arrows). Once inside the sporocarp the hormogonia differentiate into individual thick walled resting spores (or akinetes; ak), seen as the intensively red fluorescing small inoculum on top of the megaspores (sp). (Ran et al., 2010)

Archaeothrix-type filamentous cyanobacterium is thought to have entered the plant via stomatal pores (Taylor and Krings, 2005). The cyanobacteria are thought to have initially colonized the substomatal chambers and then spread throughout the outer cortical tissue, where they can be seen in fossil specimens of the plant. This is somewhat similar to the infection process in the extant hornwort *Leiosporoceros dussii* in which the cyanobacteria are found in mucilage-filled 'canals' (Villarreal *et al.*, 2005; Villarreal and Renzaglia, 2006). Once the cyanobacterium has entered the host plant a number of morphological, developmental, and physiological changes occur. The development of hormogonia is repressed, whereas the development of heterocysts is greatly stimulated. The rate of cell division is reduced, ensuring that the cyanobiont does not outgrow the host. The rate of CO_2 fixation is greatly reduced, whereas nitrogen fixation is stimulated and ammonium assimilation down-regulated (Figure 5).

The nitrogen fixation rates for cyanobacteria symbiotically associated with bryophytes are several-fold higher than for the same free-living cyanobacteria. This increase is due to a greatly elevated heterocyst frequency, which may be 6–10-fold higher than in the free-living state (As little as 20% of the nitrogen fixed is retained by the cyanobiont, the remainder being transferred as ammonia to the host (Meeks and Elhai, 2002). The primary route of ammonia assimilation in cyanobacteria is the GS-GOGAT (glutamine synthetase–glutamate synthase) pathway. The level of GS protein in *Anthoceros*-associated *Noctoc* is similar to that in free-living cyanobacteria, but GS activity is reduced implying that activity is regulated by an unknown, and presumably plant-regulated, post-translational modification of the enzyme.

Close examination of an Azolla leaf reveals that it consists of a thick, greenish (or reddish) dorsal (upper) lobe and a thinner, translucent ventral (lower) lobe emersed in the water. It is

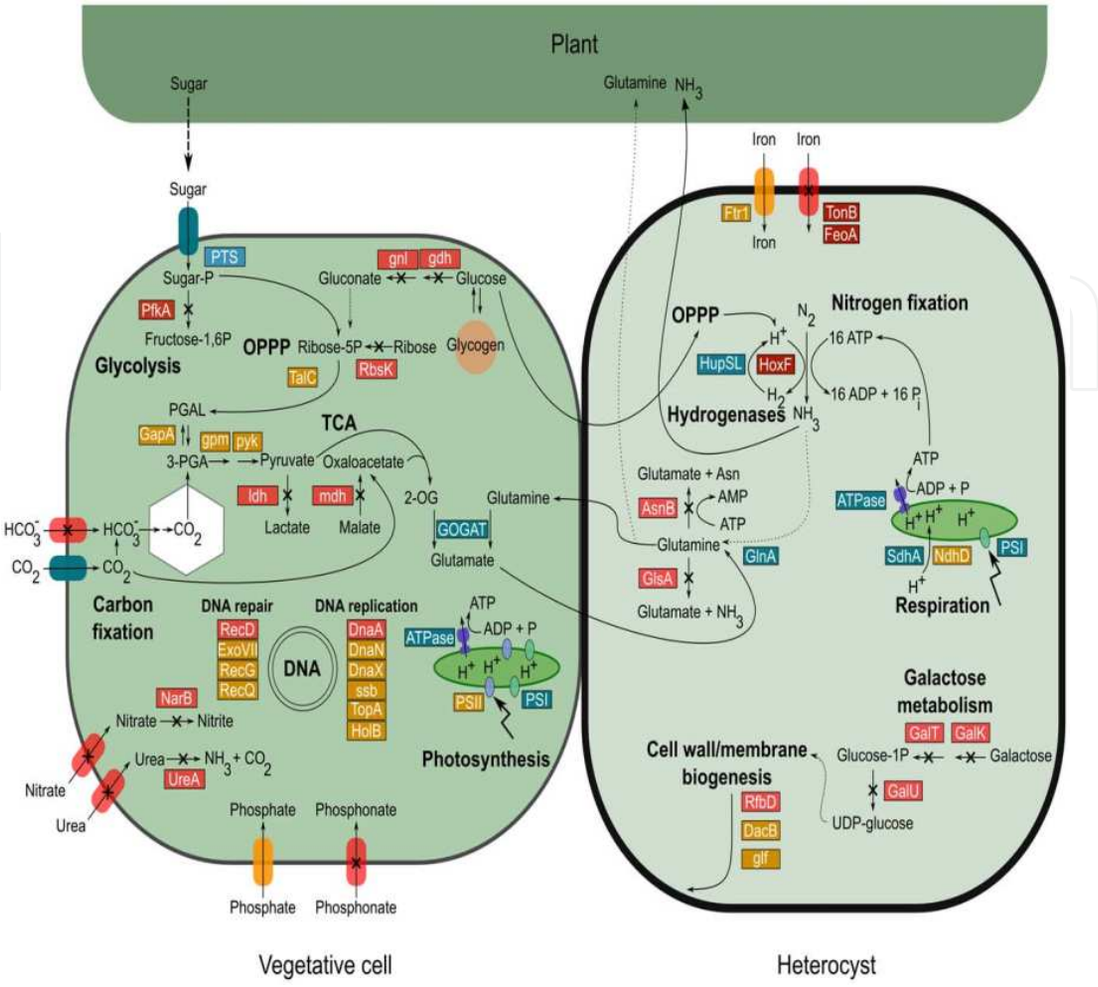
the upper lobe that has an ovoid central cavity, the "living quarters" for filaments of Anabaena. Probably the easiest way to observe *Anabaena* is to remove a dorsal leaf lobe and place it on a clean slide glass with a drop of water. Then apply a cover slip with sufficient pressure to mash the leaf fragment. Under 400X magnification the filaments of *Anabaena* with larger, oval heterocysts should be visible around the crushed fern leaf. The thick-walled heterocysts often appear more transparent and have distinctive "polar nodules" at each end of the cell. The "polar nodules" may be the same composition as cyanophycin granules (co-polymer of arginine and aspartic acid). Cyanophycin granules occur in many cyanobacteria and may serve as a nitrogen storage product.

Although Azolla can absorb nitrates from the water, it can also absorb ammonia secreted by Anabaena within the leaf cavities. Rice is the single most important source of food for people and Azolla plays a very important role in rice production. For centuries Azolla and its nitrogen-fixing partner, *Anabaena,* have been used as "green manure" in China and other Asian countries to fertilize rice paddies and increase production. Republic of China has 3.2 million acres of rice paddies planted with Azolla. This provides at least 100,000 tons of nitrogen fertilizer per year worth more than $50 million annually. Extensive propagation research is being conducted in China to produce new varieties of Azolla that will flourish under different climatic and seasonal conditions. According to some reports, Azolla can increase rice yields as much as 158 percent per year. Rice can be grown year after year, several crops a year, with little or no decline in productivity; hence no rotation of crops is necessary. In addition to nitrogen fixation, Azolla has a number of other uses. Several California aquafarms grow Azolla in large vats of circulating fresh water. Apparently fish and shrimp relish the Azolla. In fact, Azolla was grown for fish food and water purification at the Biospere II project in Arizona (a 2.5 acre glass enclosure simulating an outer space greenhouse). Fresh Azolla and duckweed (Wolffia) can also be used in salads and sandwiches, just as alfalfa and bean sprouts are used. Dried, powdered Wolffia and Azolla make a nutritious, high protein powder similar to the popular alga (cyanobacterium) *Spirulina* that is sold in natural food stores. Azolla has also proved useful in the biological control of mosquitos.The mosquito larvae are unable to come up for air because of the dense layer of Azolla on the water surface. Azolla grows very quickly in ponds and buckets, and in makes an excellent fertilizer (green manure) and garden mulch.

7. Future challenges—Prospects

The nitrogen cycle of Earth is one of the most critical yet poorly understood biogeochemical cycles. Current estimates of global N_2 fixation are approximately 240 Tg N y^{-1} with a marine contribution of 100–190 Tg N y^{-1}. Of this, a single non-heterocystous genus, *Trichodesmium* sp. contributes approximately100 Tg N y^{-1} (Capone pers. comm.). Geochemical evidence suggests that, on a global scale, nitrogen fixation does not always keep pace with denitrification on time scales of centuries to millenia (Falkowski and Raven, 1997), yet it remains unclear what process (es) limits nitrogen fixation in the oceans. More importantly, given the potential for heterocystous cyanobacteria to outcompete organisms such as *Trichodesmium*, it

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The left cell represents a vegetative cell while the right a nitrogen-fixing heterocyst. Red color indicates pseudogenes lacking functional counterpart in the No Azgenome. Orange indicates pseudogenes where a functional counterpart is present elsewhere in the genome. Fully functional gene(s) are illustrated (blue) only if their function is linked to other processes in the figure.The localization of pathways in vegetative cells or heterocysts is representative only for nitrogen fixation (heterocysts) and PSII activity(vegetative cells).Note that only amin or part of the nitrogen fixed in heterocysts is incorporate dusing the GS-GOGAT pathway and used for synthesis of amino acids,while most is exported to the plant as NH3.Sugar is provided by the plant *via* the sugar phosphotransferase system (PTS). Function has been lost int he glycolyticpathway as the pfkA gene, encoding 6-phosphofructokinase, is a pseudogeneand sugar metabolism in the Azolla cyanobiont probably proceeds via the Oxidative Pentose Phosphate Pathway (OPPP). Extensive loss of function is evident among genes involved in uptake and transport of nutrients and NoAzhas lost the capacity to both import and metabolise alternative nitrogen sources, (Ran et al., 2010).

Figure 5. Schematic illustration of important metabolic and geneticin formation pathways in NoAz.

is unclear why the apparent tempo of evolution of marine diazotrophic cyanobacteria is so slow. Diazotrophic cyanobacteria have effectively become the "gate keepers" of oceanic productivity, yet despite the rapid radiation of eukaryotic oxygenic photoautotrophs throughout the Phanaerozoic eon marine cyanobacteria seem like living fossils (Berman-Frank et al., 2003). Finally, some Questions needs answering, Are there N_2-fixing picoplankton? What limits the growth of N_2-fixing microorganisms in the open ocean? Is N_2 fixation associated with zooplankton?

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