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# $N_2$-Fixing Cyanobacteria: Ecology and Biotechnological Applications

*Kirsten Heimann*[1,2,3], *Samuel Cirés*[1,2,3]

[1]James Cook University, College of Marine and Environmental Sciences, Townsville, QLD, Australia;
[2]James Cook University, Centre for Sustainable Fisheries and Aquaculture, Townsville, QLD, Australia;
[3]James Cook University, Comparative Genomics Centre, Townsville, QLD, Australia

## 1. PHYSIOLOGY AND GENETICS OF NITROGEN FIXATION

### 1.1 Introduction

As an essential component of biomolecules, including amino acids and nucleic acids, nitrogen (N) is one of the key elements for life. Together with phosphorus, N is considered to be the main limiting factor in aquatic ecosystems. Photoautotrophic prokaryotic cyanobacteria assimilate N from combined nitrogen sources (ammonium, urea, nitrite, and nitrate) and, in diazotrophic species, from atmospheric nitrogen gas ($N_2$). Ammonium and urea are readily incorporated into organic compounds as $NH_4^+$, which is the preferred intracellular form of N, whereas intracellular nitrate ($NO_3^-$) requires reduction to nitrite ($NO_2^-$) and subsequently to $NH_4^+$ by the enzymes nitrate reductase and nitrite reductase, respectively.

Given the scarcity of $NH_3/NH_4^+$ and $NO_3^-$ in many aquatic and terrestrial ecosystems, the ability to fix atmospheric $N_2$ (comprising 78% of the atmospheric gas) became one of the most advantageous physiological strategies developed by microorganisms. In cyanobacteria, $N_2$-fixation occurs via nitrogenase complexes, the central enzyme of which is denominated nitrogenase 1. Nitrogenase 1 is a molybdenum-dependent adenosine triphosphate (ATP)-hydrolyzing complex of two metalloproteins: a MoFe-protein (dinitrogenase $\alpha 2\beta 2$ heterotetramer) that contains the active site for the reduction of $N_2$ and a Fe-protein (dinitrogenase reductase $\gamma 2$ homodimer) that transfers high-energy electrons to MoFe-containing dinitrogenase. Nitrogenase reduces one molecule of $N_2$ into two molecules of $NH_3$ (Eqn (1)) at a high-energy cost (16 ATP molecules), due to the need to break the stable triple bond between the N atoms in $N_2$- and generating $H_2$ as a byproduct (Eqn (1)):

$$N_2 + 8H^+ + 8e^- + 16\,ATP \rightarrow 2NH_3 + H_2 + 16\,ADP + 16\,Pi \quad (1)$$

Nitrogenase 1 is inactivated upon oxygen binding. This suggests that nitrogenase originated before the Great Oxygenation Event,

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more than 2200 million years ago. According to Latysheva et al. (2012), cyanobacteria were the first organisms releasing oxygen to the atmosphere as a byproduct of photosynthesis. To overcome the incompatibility of $N_2$-fixation in an oxygenic atmosphere, cyanobacteria have developed a number of strategies to counteract the inactivation of nitrogenase by photosynthetic $O_2$, including the separation of photosynthesis and $N_2$-fixation in space (e.g., in the heterocystous cyanobacterium *Anabaena*) and in time (e.g., in the nonheterocystous *Lyngbya*), as well as the restriction of $N_2$-fixation to microaerobic or anaerobic conditions (e.g., in nonheterocystous *Leptolyngbya boryanum*; Table 1).

## 1.2 Separation of $N_2$-Fixation and Photosynthesis in Space: Heterocystous Cyanobacteria

Following the traditional classification of cyanobacteria into five orders, heterocystous cyanobacteria are located within orders Nostocales and Stigonematales (Table 1), equivalent to subsections IV and V in the bacteriological classification. To date, more than 100 heterocystous genera have been described; some of the most common are the nostocalean *Anabaena*, *Aphanizomenon*, *Nostoc*, *Calothrix*, and *Tolypothrix* and the true-branching stigonematalean *Fischerella* and *Mastigocladus*.

The heterocyst and vegetative cells of cyanobacteria are examples of cellular specialization normally associated with higher organisms (plants and animals). Heterocystous cyanobacteria are filamentous organisms capable of aerobic $N_2$-fixation due to the production of specialized, differentiated, nonphotosynthetic cells called heterocysts. Heterocysts possess only photosystem I providing ATP for $N_2$ fixation, but not the water-splitting and oxygen-producing photosystem II; in addition, they are surrounded by a thick wall to limit the entry of oxygen. The heterocyst wall is a thick double layer, with the

TABLE 1 Classification on $N_2$-Fixing Cyanobacteria

| Group | $N_2$-fixation behavior | Separation of photosynthesis and $N_2$-fixation | Morphology | Orders | Some genera |
|---|---|---|---|---|---|
| Heterocystous | Aerobic | Spatial | Filamentous | Nostocales | *Anabaena*<br>*Nostoc* |
| | | | | Stigonematales | *Fischerella*<br>*Mastigocladus* |
| Non-heterocystous | Aerobic | Temporal | Unicellular | Chroococcales | *Gloeothece*<br>*Cyanothece* |
| | | | | Pleurocapsales | *Chroococcidiopsis* |
| | | | Filamentous | Oscillatoriales | *Lyngbya*<br>*Microcoleus* |
| | Aerobic | Spatial and temporal | Filamentous | Oscillatoriales | *Trichodesmium*<br>*Katagyneme* |
| | Anaerobic | - | Filamentous | Oscillatoriales | *Leptolyngbya* |

Bergman, B., Gallon, J., Rai, A., Stal, L., 1997. $N_2$ Fixation by non-heterocystous cyanobacteria. FEMS Microbiol. Rev. 19, 139–185.
Gallon, J., 2005. $N_2$ Fixation by Non-Heterocystous Cyanobacteria, Genetics and Regulation of Nitrogen Fixation in Free-Living Bacteria. Springer, pp. 111–139.

first polysaccharide layer being for mechanical support. The second "laminated" layer limits oxygen diffusion into the cell and is composed of unusual glycolipids (heterocyst glycolipids) (Wörmer et al., 2012). Heterocysts also lack ribulose-1,5-biphosphate carboxylase (RuBisCO), thus being incapable of fixing $CO_2$ and instead relying on adjacent photosynthetic vegetative cells for their carbon supply (Figure 1). Organic carbon is delivered to the heterocyst in the form of disaccharides (e.g., maltose), whose metabolism supplies the reducing power (NADPH) required for $N_2$ reduction via the oxidative pentose phosphate pathway enzymes (glucose-6-phosphate and 6-phosphogluconate dehydrogenases). Glutamine synthetase (GS) catalyzes the incorporation of ammonia, produced by nitrogen fixation, to form glutamine. The heterocystous glutamate pool is generated either by endogenous synthesis or by transport from the vegetative cell. In return, glutamine is exported to the vegetative cell, where it forms glutamate via glutamate synthase (GOGAT) for incorporation into vegetative cell metabolites.

The heterocysts store fixed N in cyanophycin polar bodies (Figure 1), which are clearly visible under the light microscope (Figure 2(a)).

## 1.3 Nonheterocystous Cyanobacteria

Until the 1960s, only heterocystous cyanobacteria were believed to fix $N_2$. Since then, $N_2$-fixation has been described in about 17 genera, including 70 strains of nonheterocystous cyanobacteria within orders Chroococcales, Pleurocapsales, and Oscillatoriales (Table 1), in most cases when incubated in micro-oxic or anoxic conditions (Bergman et al., 1997). Although less conspicuous than their heterocystous counterparts, nonheterocystous cyanobacteria are important contributors to global $N_2$-fixation. The highly important nonheterocystous *Trichodesmium* alone is estimated to contribute 42% (240 Tg $N_2$ $year^{-1}$) of newly fixed $N_2$ to the earth's nitrogen budget, with the total input through nonheterocystous cyanobacteria believed to exceed 50% of the annual global $N_2$-fixation rate (Berman-Frank et al., 2003; Gallon, 2001).

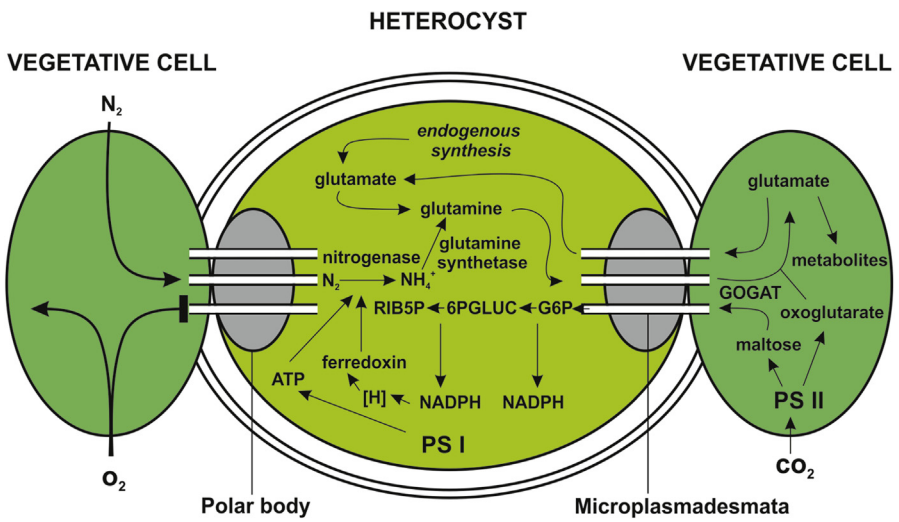


FIGURE 1 Schematic view of $N_2$-fixation in heterocysts and carbon–nitrogen exchanges with vegetative cells in diazotrophic filamentous cyanobacteria. PS I, photosystem I; PS II, photosystem II; RIB5P, ribulose-5-phosphate; 6PGLUC, 6-phosphogluconic acid; G6P, glucose-6-phosphate; GOGAT, glutamate synthase. *Modified from Lea (1997).*

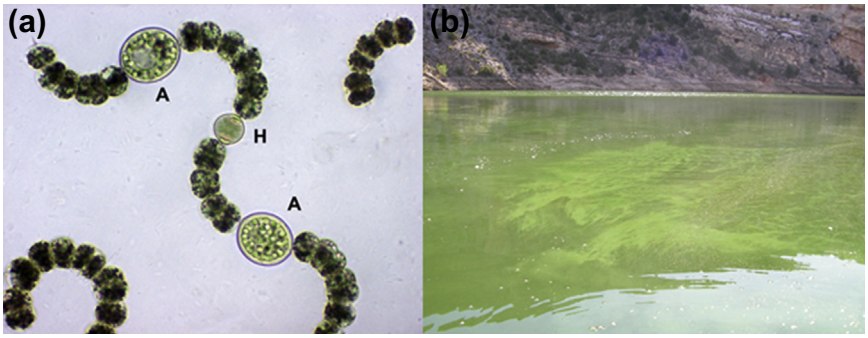


FIGURE 2 Planktonic $N_2$-fixing cyanobacteria in temperate freshwaters. (a) *Anabaena crassa* (syn. *Dolichospermum crassum*), one of the most common species in temperate freshwaters. H, heterocyst ($N_2$-fixing cell); Ak, akinete (resting stage). (b) Mass proliferation (bloom) of *Anabaena flos-aquae* in a Mediterranean freshwater body (Cueva Foradada reservoir, northeast Spain).

The filamentous nonheterocystous *Leptolyngbya boryanum* (previously known as *Plectonema boryanum*) can fix $N_2$ under continuous illumination at low-light intensity if an $O_2$-free atmosphere is generated by sparging gas (Ar, Ar/$CO_2$, or $N_2/CO_2$) or by adding inhibitors of photosynthetic $O_2$ production (DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)) or sodium sulfide) (Bergman et al., 1997). When transferred to $N_2$-free medium, $N_2$ fixation is derepressed for 2–24 h in *L. boryanum*, after which temporal separation of $O_2$ production and $N_2$ fixation is effected under continuous illumination, but not when grown in dark/light cycles (Bergman et al., 1997; Gallon, 2005). Although the physiology of anaerobic $O_2$ fixation is not fully understood, experiments suggest that during the photosynthetic phase light energy is transferred to the $O_2$-evolving photosystem II, whereas during the $N_2$-fixing phase light energy is transferred preferentially to photosystem I, which does not generate $O_2$ (Gallon, 2005).

Although more restricted, aerobic nonheterocystous $N_2$-fixation has been described in several unicellular and filamentous species capable of circadian separation of photosynthesis and $N_2$ fixation (e.g., unicellular *Gloeothece* and filamentous *Lyngbya*) and/or containing certain cellular adaptations to create microaerobic environments (e.g., filamentous *Trichodesmium*) (Table 1). With the exception of *Trichodesmium*, *Katagyneme*, and *Symploca*, which confine nitrogenase to specialized but not differentiated cells (see Section 2.3 for details on *Trichodesmium* $N_2$ fixation), nonheterocystous cyanobacteria fix $N_2$ during the dark phase of an alternating light/dark cycle. Circadian control of $N_2$ fixation is achieved through differential expression of the nitrogenase-encoding genes (*nif* genes, see Section 1.3), with transcription being restricted to the night phase and destruction of the enzyme during the day phase in the unicellular *Gloeothece* and *Cyanothece*. In contrast, genes encoding reaction center proteins of the $O_2$-releasing photosystem II (*psb* genes) and photosystem I (*psa* genes) are only transcribed during the light phase (Gallon, 2005). In *Lyngbya*, nitrogenase levels persist during the light period and activity is controlled through modification of the Fe-protein of nitrogenase to a larger form, which is presumably inactive under aerobic growth conditions (Gallon, 2005). Thus, a reciprocal rhythm of photosynthetic $O_2$ production and $O_2$ consumption to support $N_2$-fixation is created, with increased respiratory requirements for $N_2$ fixation supported by enhanced carbohydrate breakdown (Gallon, 2005).

## 1.4 *Nif* Genes: Phylogeny and Molecular Methods

As detailed in Section 1.1, $N_2$ fixation in cyanobacteria is carried out by the central enzyme nitrogenase 1, a molybdenum-dependent complex of two metalloproteins: a FeMo-dinitrogenase heterotetramer whose α and β subunits are encoded by the *nifD* and *nifK* genes, respectively, and a Fe-dinitrogenase reductase homodimer encoded by the gene *nifH*. Other than the *nifHDK* genes, at least 13 other genes are involved in $N_2$-fixation, 10 of which are also denoted as *nif* genes (*nifWX-NEUSBVZT*) arranged in a tight 15-kb cluster (Stucken et al., 2010). In addition to nitrogenase 1, some heterocystous cyanobacteria, such as *Anabaena variabilis* ATCC29413, contain a second alternative Mo-nitrogenase (encoded by the *nif*2 operon) that is transcribed in both vegetative cells and heterocysts under anaerobic conditions and/or an alternative vanadium-dependent nitrogenase (encoded by the *vnf* genes) that is transcribed in heterocysts under molybdenum deficiency (Thiel and Pratte, 2013).

Although the origin of *nif* genes has not been fully determined yet, phylogenetic studies using 49 cyanobacterial genomes suggest that they may have a very ancient precyanobacterial origin, arising approximately 3 billion years ago under the Early Earth atmosphere (Latysheva et al., 2012). Despite their long evolutionary history, the apparently low rates of horizontal gene transfer resulted in highly conserved sequences, thus making *nif* genes suitable as molecular markers. Among the *nif* operon, *nifH* is the most sequenced. For example, 23,847 *nifH* sequences are available in the database by Gaby and Buckley (2012), hence becoming the marker gene of choice for researchers studying the phylogeny, diversity, and abundance of nitrogen-fixing microorganisms, including cyanobacteria. Furthermore, a wide range of oligonucleotides for polymerase chain reaction (PCR) amplification of cyanobacterial *nifH* in cultures and environmental samples exist (Table 2). Generally, *nifH*-based phylogenetic trees show an overall separation of nonheterocystous and heterocystous

TABLE 2 Selection of PCR Primers Suitable for *nifH* Amplification in Cyanobacteria

| Primer | Sequence (5′−3′) | Product size (bp) | Specificity | References |
|---|---|---|---|---|
| *nifH3* | ATRTTRTTNGCNGCRTA | 473 | Universal, external | Zehr and Turner (2001)[c] |
| *nifH4* | TTYTAYGGNAARGGNGG | | | |
| *nifH1* | TGYGAYCCNAARGCNGA | 359 | Universal, internal | |
| *nifH2* | ADNGCCATCATYTCNCC | | | |
| CNF | CGTAGGTTGCGACCCTAAGGCTGA | 375 | Cyanobacteria-specific | Olson et al. (1998)[b] |
| CNR | GCATACATCGCCATCATTTCACC | | | |
| cylnif-F | TAARGCTCAAACTACCGTAT | 220 | *Cylindrospermopsis*-specific | Dyble et al. (2002)[a] |
| cylnif-R | ATTTAGACTTCGTTTCCTAC | | | |

*Note:* NifH3 and nifH4 are often used as external primers in combination with internal nifH1 and nifH2 in a nested PCR approach (Zehr and Turner, 2001).

[a]*Dyble, J., Paerl, H.W., Neilan, B.A., 2002. Genetic characterization of Cylindrospermopsis raciborskii (Cyanobacteria) isolates from diverse geographic origins based on nifH and cpcBA-IGS nucleotide sequence analysis. Appl. Environ. Microbiol. 68, 2567−2571.*

[b]*Olson, J., Steppe, T., Litaker, R., Paerl, H., 1998. $N_2$-fixing microbial consortia associated with the ice cover of Lake Bonney, Antarctica. Microbial Ecol. 36, 231−238.*

[c]*Zehr, J., Turner, P., 2001. Nitrogen fixation: Nitrogenase genes and gene expression. Methods Microbiol. 30, 271−286.*

cyanobacteria, with subgroups within heterocystous (branching Stigonematales vs. Nostocales) and nonheterocystous (filamentous Oscillatoriales vs. unicellular Chrococcales). However, *nifH* trees are often more convoluted than this, because some nonheterocystous members cluster close to heterocystous taxa. There are also many cyanobacterial species containing two to three nonidentical *nifH* copies, leading to clustering in different parts of the trees (Yeager et al., 2007).

Besides its generalized use in phylogeny, *nifH* has been widely used in studies of $N_2$-fixing cyanobacterial diversity in environmental samples, such as by density gradient gel electrophoresis and clone libraries of DNA (Ininbergs et al., 2011; Omoregie et al., 2004) as well as in reverse-transcriptase PCR approaches to evaluate gene expression (*nifH* transcription levels) in a range of aquatic and terrestrial ecosystems (Omoregie et al., 2004; Zani et al., 2000) or in response to environmental factors in cultures (Vintila and El-Shehawy, 2007).

## 2. $N_2$-FIXING CYANOBACTERIA IN THE ENVIRONMENT

### 2.1 Soils and Agroecosystems

$N_2$-fixing cyanobacteria can be considered one of the greatest natural biological fertilizers in soils with varied agricultural uses. In those ecosystems, $N_2$-fixing cyanobacteria thrive in benthic, epiphytic, and subaerophytic habitats, as free forms or in symbiotic association with plants such as *Azolla*.

Within the variety of crop-derived ecosystems, rice fields are particularly remarkable given their economic relevance and the ecological importance of $N_2$-fixing cyanobacteria for rice field sustainability. Approximately 90% of the rice production occurs in wetland fields, constituting the most dominant anthropogenic wetland ecosystem worldwide (Scott and Marcarelli, 2012) and a major habitat for $N_2$-fixing cyanobacterial communities. The diversity of those communities is illustrated by a study of 38 soil samples from rice fields in Bangladesh, where 84 species of cyanobacteria, 42 of which were heterocystous diazotrophic species, of 14 different genera were identified (Khan et al., 1994). Additional studies of 102 soils from four countries found that half of the cyanobacterial genera reported were heterocystous (*Anabaena*, *Aulosira*, *Calothrix*, *Cylindrospermum*, *Fischerella*, *Gloeotrichia*, *Nostoc*, *Scytonema*, *Tolypothrix*, *Wollea*); their presence was strongly correlated with pH and P concentrations (Whitton, 2002 and references therein). Within those genera, *Fischerella*, *Nostoc*, and *Calothrix* are considered widespread, often persisting as epiphytic communities, such as *Nostoc* sp. growing on the macrophyte *Chara vulgaris*. Additionally, the symbiosis between the freshwater fern *Azolla* and the $N_2$-fixing cyanobacterium *Anabaena azollae*—used for centuries as a natural biofertilizer for rice fields in China and Vietnam—is widely distributed in Asian paddy fields.

A number of in situ investigations have revealed the enormous magnitude of cyanobacterial $N_2$-fixation in rice fields. Roger and Ladha (1992) estimated the N contribution from cyanobacteria to $\sim$80 kg N $ha^{-1}$ crop, clearly outperforming N input from heterotrophic N-fixing bacteria in the rice rhizosphere (31 kg N $ha^{-1}$ crop). In Spanish rice fields, indigenous cyanobacterial $N_2$-fixation seems to follow a crop-cycle seasonal pattern, with low values at the beginning of the crop (May), maximum values at the end of the tillering stage (June), and declining again with the end of the cultivation cycle (September) (Quesada et al., 1998). Over the annual cycle, $N_2$-fixation by indigenous cyanobacteria has been estimated to provide 0.23–75.5 kg N $ha^{-1}$ $year^{-1}$, representing a remarkable natural N input that could potentially reduce the need for urea fertilizer by 25–35% (Whitton, 2002 and references therein). In a pilot study in Chile, Pereira et al. (2009)

demonstrated that the use of biofertilizer from indigenous *Anabaena iyenganii* and *Nostoc* spp. reduced the amount of synthetic nitrogen fertilizer (50 kg N $ha^{-1}$ $year^{-1}$) by 50% while providing the same yield of 7.4 t rice $ha^{-1}$. The agricultural tradition in Asia (e.g., in Bangladesh) includes growing cyanobacteria in open cultures settled on ground parcels with nutrients and afterward draining the parcels and drying the biomass under the sun or directly applying it as fertilizer to the rice field. This potential of cyanobacterial biofertilizers has been overlooked in rice fields elsewhere in favor of using synthetic fertilizers, which cause serious eutrophication of water systems and gravely compromise the environmental sustainability of rice production.

## 2.2 Freshwaters

$N_2$-fixing cyanobacteria are widely distributed in lentic freshwater ecosystems (lakes, dammed rivers, lagoons, wetlands) and running waters of all latitudes. Investigations since the early 1980s have traditionally linked the dominance of $N_2$-fixing cyanobacteria to low nitrogen:phosphorus ratios in water. Nevertheless, extensive monitoring data seem to contradict this commonly accepted dogma, confirming a lack of relationship between N:P ratios and the abundance of $N_2$-fixing cyanobacteria in 102 German lakes (Dolman et al., 2012). Diazotrophic cyanobacteria are of crucial importance for the N cycle in freshwaters, where $N_2$-fixation can provide up to 1.5 g N $m^{-2}$ $year^{-1}$ (Scott and Grantz, 2013), compensating for up to 35% of N-deficiency in freshwater ecosystems (Wiedner et al., 2014). Other than nutrient availability, temperature seems to be directly correlated with the efficiency of freshwater cyanobacterial $N_2$-fixation (Scott and Marcarelli, 2012).

Deep thermally stratified lakes and reservoirs in temperate regions host important populations of planktonic $N_2$-fixing cyanobacteria, such as the bloom-forming nostocalean genera *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, and *Cylindrospermopsis*, with benthic members developing mostly in shallow waters. $N_2$-fixing planktonic nostocalean contain intracellular gas vesicles (aerotopes), enabling them to regulate their position in the water column in response to environmental gradients. During certain periods of high temperatures and calm winds, these taxa generate mass proliferations (blooms), which visibly accumulate in the water surface due to overbuoyancy (Figure 2(b)) and on occasion produce cyanotoxins (microcystins, anatoxin-a, saxitoxins, cylindrospermopsin). Most $N_2$-fixing planktonic nostocalean differentiate akinetes (Figure 2(a)), resting stages that are able to survive desiccation and low temperatures, which germinate when water conditions improve (Cirés et al., 2013). Akinetes remain viable after transport by winds or migratory birds, allowing for the invasion of new habitats (Sukenik et al., 2012).

In running freshwaters, $N_2$-fixing cyanobacteria develop as benthic forms in epilithic habitats, forming mats together with other filamentous and unicellular cyanobacteria. Some of the most common $N_2$-fixing taxa are the heterocystous *Calothrix*, *Nostoc*, *Rivularia*, and *Tolypothrix*, and nonheterocystous *Schizothrix* (Mateo et al., 2010). $N_2$ fixation rates by benthic diazotrophic cyanobacteria can reach 8.4 mg $m^{-2}$ $h^{-1}$, with spatial and temporal differences driven by nutrient availability (Scott and Marcarelli, 2012). Additionally, some of these $N_2$-fixers (e.g., *Rivularia*, *Tolypothrix*, *Nostoc*, *Schizothrix*) are important contributors to the recycling of organic P in oligotrophic streams due to the production of extracellular phosphatase enzymes (Mateo et al., 2010).

## 2.3 Oceans: *Trichodesmium*

*Trichodesmium* is an important tropical, filamentous, nonheterocystous cyanobacterium, providing new nitrogen to the oligotrophic seas. Filaments can cluster into puff or tuff colonies; the reasons for these filament bundlings are

poorly understood and not species specific. *Trichodesmiun* can occur at a depth of 200 m and can represent up to 60% of the chlorophyll *a* in the top 50 m of the water column. It also contributes more than 20% of the primary productivity of the area. *Trichodesmium* forms extensive annual blooms (Figure 3(a)), stretching for 1600 km along the Queensland coast and covering an area of up to 52,000 $km^2$, as observed by Joseph Banks in 1770 on the *Endeavor* with James Cook "Vast quantities of little substances … floating upon the water in large lines a mile or so long … either immediately upon the surface or not many inches under it. The seamen … began to call it Sea Sawdust" (Walsby, 1978).

Blooms can be terminated through cyanophage infection, grazing by the harpacticoid copepod *Macrosetella gracilis*, oxidative stress, ultraviolet (UV) damage, or nutrient limitation (N and P). Nutrient limitation activates programmed cell death via the proteolytic caspase pathway in *Trichodesmium*, a pathway typically thought to be confined to higher order plants and animals (Berman-Frank et al., 2004). As the blooms decay, they also provide an important source of organic carbon (Figure 3(b)) and phosphate (P). The end of a *Trichodesmium* bloom closes the nutrient recycling cycle and ensures proliferation of the food web; thus, *Trichodesmium* blooms make a direct contribution to high tropical ecosystem biodiversity.

*nifH* gene sequences are unique for the genus *Trichodesmium*, containing roughly seven recognized species identified by filament width and cell length:width ratios (Janson et al., 1995). The 16S sequences place them close to species in the order Oscillatoriales (Capone et al., 1997). *Trichodesmium* contains the strongest gas vesicles discovered in cyanobacteria, withstanding pressures of ~3.5 MPa in *T. contortum* and *T. thiebautii*, 14 times stronger than those of freshwater cyanobacteria, which presumably represents and adapts to occurrence at great depths (200 m = 2 MPa) (Walsby, 1978). Gas vesicle collapse studies showed that large colonies (larger filament diameter species of *Trichodesmium*) had greater floating velocities and sank faster after gas vesicle collapse induction at 6 MPa, with initial floatation speed to sinking speeds after pressurization ratios of 1.17, 0.61, and 0.54 for *T. erythraeum*, *T. thiebautii*, and *T. contortum* (Walsby, 1978).

Trichodesmium shows Diel vertical migration, retreating to nutrient-rich lower water

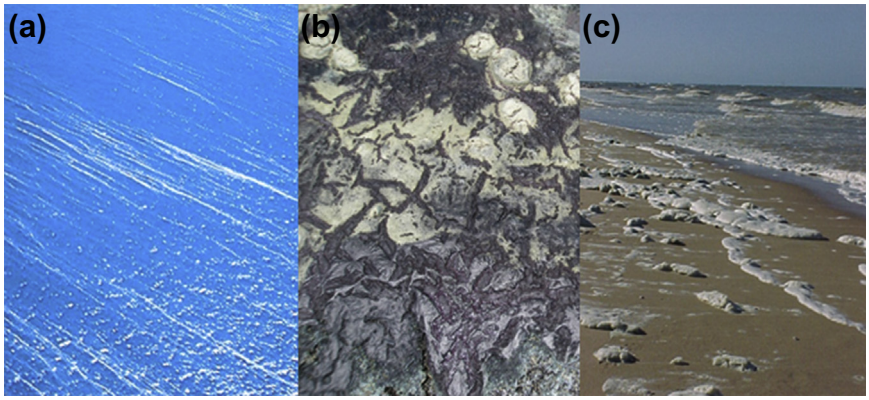


FIGURE 3 *Trichodesmium* blooms. (a) Bloom streaks in open Queensland water. (b) *Trichodesmium* crust of dried washed-up material (*Courtesy of John Webster, EPA*). (c) *Trichodesmium* bloom decay (foam on the beach) at Saunders Beach, Townsville, Queensland Australia. *Courtesy of John Webster, EPA.*

during the night and migrating to the surface at the onset of day. This is achieved through carbohydrate and polyphosphate ballasting. Cyanophycin content does not appear to have a functional role (Romas et al., 1994).

*Trichodesmium* is a nonheterocystous cyanobacterium where $N_2$-fixation is also under circadian control. However, in contrast to $N_2$-fixation by other nonheterocystous diazotrophic cyanobacteria, nitrogenase activity is downregulated during the night and is established 1–3 h after the transition to the light phase, reaching peak activity at midday (Ohki and Fujita, 1988). The oxygen sensitivity of the nitrogenase complex is also solved through cell differentiation, with the production of diazocytes instead of heterocysts. Unlike heterocysts, diazocytes are indistinguishable from vegetative cells at the light microscopical level but are ultrastructurally different; they are characterized by dense thylakoid networks partitioning the vacuole-like space, less extensive gas vacuoles, and fewer and smaller cyanophycin granules (Fredericksson and Bergman, 1997). In addition, *Trichodesmium* is capable of both temporal and spatial segregation of oxygenic photosynthesis and $N_2$-fixation; photosynthetic activity is highest in the early morning and lowest at midday, when nitrogenase activity is highest and/or diazocytes may be aggregated in clusters, allowing for simultaneous $N_2$ fixation and oxygenic photosynthesis (Berman-Frank et al., 2001). Large blooms of *Trichodesmium* are supported by an organism's capability to simultaneously utilize additional nitrogen sources, such as ammonium generated by grazers and other mat-associated organisms with cell surface amino acid oxidases (Mulholland and Capone, 2000).

Dissolved inorganic P is typically low in tropical waters, but alternative pathways exist for phosphate acquisition, such as the use of dissolved organic P pools (DOP), which are a significant portion of the total dissolved P pool (TDP) or alkaline-phosphate liberated P, as realized in many microalgal species. DOP exists as monophosphate esters, making up 75% of the TDP, with the rest being phosphonates (25%; many herbicides are phosphonates). *Trichodesmium* is supposed to have acquired the genes necessary for the phosphonate-lyase pathway, typically present in bacteria, through lateral gene transfer before speciation occurred (Dyhrman et al., 2006). Harboring the phosphonate-lyase pathway is a unique feature of *Trichodesmium*, as it has not been found thus far in other cyanobacteria, and it is nutritionally activated.

## 3. BIOTECHNOLOGICAL APPLICATIONS OF $N_2$-FIXING CYANOBACTERIA

### 3.1 Bioremediation of Wastewaters

$N_2$-fixing cyanobacteria have been the subject of broad research for wastewater bioremediation, given their natural presence in wastewaters (e.g., *Nostoc* sp. found in municipal wastewater from Brazil; Furtado et al., 2009), their well-known metabolic flexibility, and their tolerance to harsh conditions. Studies have used cultures and consortia, including diazotrophic Nostocales (*Anabaena*, *Calothrix*, *Cylindrospermum*, *Nostoc*, *Rivularia*, and *Tolypothrix*), Stigonematales (*Hapalosiphon*, *Mastigocladus,* and *Stigonema*), and less frequently, Chroococcales (*Gloeocapsa* and *Cyanothece*), to remove contaminants from a variety of domestic, industrial, and synthetic wastewaters (Table 3). Contaminants remediated included N ($NO_2^-$, $NO_3^-$, $NH_4^+$) and P ($PO_4^{3-}$) sources responsible for eutrophication of aquatic systems, organic matter (chemical oxygen demand), and persistent and highly toxic pesticides (e.g., lindane) and heavy metals (e.g., Cd, Cr, Hg, Pb). Removal efficiencies obtained were generally high (>80%), although with wide variations (13–100%) depending on the target contaminant, water characteristics, and the species used (Table 3).

TABLE 3 Wastewater Bioremediation by $N_2$-Fixing Cyanobacteria

| Type of wastewater | Species | Target contaminants | Removal efficiency (%) |
|---|---|---|---|
| Open fish pond effluent | *Aulosira fertilissima* | $NO_2^-$, $NH_4^+$ | 100 |
| Primary treated effluent | *Anabaena* sp., *Westelliopsis* sp, *Fischerella* sp.[a] | $NO_3$–N, $NH_4$–N<br>$PO_4$–P | 90–100<br>98 |
| | | COD | 87 |
| Mixed domestic–industrial | *Anabaena oryzae* | COD | 74 |
| | *Tolypothrix ceytonica* | Cu, Zn | 86–94 |
| Industrial (plating industry) | *Nostoc* sp. PCC7936 | Cr(III), Cr(VI) | 60–99 |
| Industrial (paper production) | *Anabaena subcylindrica* | Cu, Co, Pb, Mn | 33–86 |
| | *Nostoc muscorum* | Cu, Co, Pb, Mn | 22–85 |
| Synthetic (pesticide-containing) | *Nostoc* sp. | Lindane[b] | 87–94 |
| | *Nodularia* sp. | Lindane | 76–98 |
| Synthetic metal mixtures | *Anabaena* spp. | Ag, Au, Cd, Cu, Hg, Pb, Zn | 29–85 |
| | *Calothrix* spp. | Ag, Au, Cd, Cu, Hg, Pb, Zn | 13–88 |
| | *Cylindrospermum* sp. | Cd, Pb | 52–65 |
| | *Gloeocapsa* sp. | Cd, Pb | 96 |
| | *Hapalosiphon* spp. | Cd, Hg, Pb | 13–90 |
| | *Mastigocladus* spp. | Cd, Hg, Pb, Zn | 29–89 |
| | *Nostoc* spp. | Cd, Cr, Cu, Hg, Ni, Pb, Zn | 22–94 |
| | *Rivularia* sp. | Cd, Hg, Pb | 76–88 |
| | *Scytonema schmidlei* | Cd | 98 |
| | *Stigonema* sp. | Cd, Hg, Pb | 80–89 |
| | *Tolypothrix tenuis* | Cd, Cu, Hg, Pb, Zn | 53–94 |

Removal efficiency is expressed as a percentage of contaminant removed compared to the initial concentration (growth periods varying between 2 and 15 days). COD, chemical oxygen demand.
[a]*Consortium includes $N_2$-fixing and non-fixing strains (Renuka et al., 2013).*
[b]*Lindane [γ-hexachlorocyclohexane (γ-HCH)].*
*Information from De Philippis and Micheletti (2009), El-Bestawy et al. (2007), Brayner et al. (2007), Colica et al. (2010), El-Sheekh et al. (2005).*

The capacity of $N_2$-fixing species to remove metals and metalloids from synthetic metal mixtures is particularly remarkable (Table 3). Although mechanisms behind this phenomenon are not fully understood, a big part of the metal retention is attributed to the exopolysaccharides (EPS) usually produced in high amounts by many $N_2$-fixing strains. Cyanobacterial EPS are exocellular polysaccharidic layers classified according to their features, such as sheaths (thick layers with high mechanical and physicochemical stability), capsules

(a gelatinous layer associated with the cell surface), and slime (an amorphous mucilaginous material loosely dispersed around the microorganism) (De Philippis and Micheletti, 2009). The complex chemical composition of EPS seems to be strain dependent. Metal sorption occurs by binding of cationic metals to the negatively charged surface of EPS, mainly via carboxylic groups (in particular at low pH), with a minor role for other functional groups (e.g., sulfonate and amino groups, particularly at high pH). Besides EPS, the intracellular polyphosphate granules and the metal-chelating proteins metalothionines are also involved in the sequestration of metals by cyanobacteria (Turner and Robinson, 1995).

## 3.2 Bioproducts and Bioenergy

Cyanobacteria are a prolific source of bioproducts, derived from their very active and versatile metabolisms. Within cyanobacteria, diazotrophic species share most applications with their non-nitrogen-fixing counterparts but with the added advantage of potentially lower costs (and/or carbon and water footprints) for mass production. $N_2$-fixing cyanobacteria offer potentially reduced culturing costs (no nitrogen fertilizer necessary) and harvesting−dewatering costs. Many species are self-settling, thus reducing centrifugation/filtration costs, and/or can be grown in low-water containing biofilms. However, these applications have not been fully explored on a commercial scale.

Cyanobacterial bioproducts include high-value compounds with an ongoing worldwide market, such as phycocyanin and fatty acids (Table 4); these are currently produced commercially from non-nitrogen-fixing microorganisms, such as the cyanobacterium *Spirulina* (phycocyanin), or eukaryotic microalgae, such as *Nannochloropsis* (fatty acids) (Borowitzka, 2013). In addition, $N_2$-fixing cyanobacteria produce uncountable products that may reach high economic value but whose markets are still to be developed, including cyanotoxin standards and cyanotoxin-derived pharmaceuticals, UV sunscreens, anticancer compounds, EPS-derived cosmetics, and metal nanoparticles. Furthermore, $N_2$-fixing cyanobacteria represent an environmentally friendly source for bioplastics and biofertilizers, which may reach an enormous market as substitutes for traditional highly energy-consuming and contaminant petrol-derived plastics and chemical fertilizers (Table 4).

Furthermore, $N_2$-fixing cyanobacteria may also play a role in the increasing field of biofuel and bioenergy. The production of biohydrogen, with applications for transport and electricity, has been described in at least 14 cyanobacterial genera, including the $N_2$-fixing *Anabaena*, *Calothrix*, *Chroococcidiopsis*, *Cyanothece*, *Gloeobacter*, and *Nostoc*. Cyanobacteria generate hydrogen ($H_2$) either as a byproduct of $N_2$ fixation using nitrogenase (see Eqn (1) in Section 1.1) or by a reversible NADPH-dependent [NiFe]-hydrogenase (Peters et al., 2013); however, more research is still needed to understand the metabolic pathways that influence $H_2$ rates and yields in these organisms. In addition, $N_2$-fixing cyanobacteria are often rich in carbohydrates (starch), therefore representing a good feedstock for bioethanol generation by yeast fermentation (John et al., 2011). Studies also demonstrate the natural production of hydrocarbons (alkanes and alkenes) by at least 13 $N_2$-fixing cyanobacterial genera (Coates et al., 2014). Some of these hydrocarbons (e.g., pentadecane) could be directly used as biofuel without the need for transesterification, as required for fatty-acid-based microalgal biodiesel. Additionally, the feedstock-agnostic process of hydrothermal liquefaction is opening promising avenues for the use of wet cyanobacterial biomass to generate cheaper jet biofuel, which remains to be explored in $N_2$-fixing cyanobacteria.

TABLE 4 Bioproducts and Bioenergy from $N_2$-Fixing Cyanobacteria

| Product | Applications | Main $N_2$-fixing producers | Price ($/kg) | Global market (million US$/annum)[c] |
|---|---|---|---|---|
| Phycobiliproteins (phycocyanins, phycoerythrin) | Biomedicine (fluorescent markers)<br>Food coloring<br>Pharmaceuticals<br>Cosmetics | *Nostoc*, *Anabaena*[a] | 50,000 | 60 |
| Fatty acids (omega-3 and omega-6) | Neutraceuticals<br>Animal feed (aquaculture) | *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Calothrix*, *Nodularia*, *Nostoc* | 0.88–3.8 | 700 |
| Bioplastics (Polyhydroxyalkanoates) | Substitute for nonbiodegradable petrochemical-based plastics | *Anabaena*, *Aulosira*, *Chlorogloea Gloeocapsa*. *Gloethece*, *Nostoc*, *Scytonema*, *Trichodesmium* | 1.5 | – |
| Metal nanoparticles (Ag, Au) | Chemical industry (catalysis)<br>Environmental remediation<br>Biomedicine (gene therapy, biomarkers) | *Anabaena*, *Calothrix* | – | – |
| Ultraviolet sunscreens (Scytonemin, mycosporine-like aminoacids) | New-generation sunscreens<br>Pharmaceuticals (anti-inflammatory, antiproliferative,) and cosmetics | *Anabaena*, *Aphanizomenon*, *Aphanothece*, *Calothrix*, *Chlorogloeopsis*, *Gloecapsa*, *Gloethece*, *Scytonema* | – | – |
| Cytotoxic (anticancer) compounds | Alternative anticancer therapies (leukemia cell-apoptogens, protection against chemoresistance) | *Anabaena*, *Calothrix*, *Nostoc*, *Nodularia* | – | – |
| Biofertilizers and phytohormones (gibereline-like compounds, indole-3 acetic acid) | Agriculture (N, P, and trace element sources; soil conditioners; enhancement of plant growth) | *Anabaena* spp, *Aulosira*, *Chroococcidiopsis*, *Nostoc*, *Tolypothrix*., *Scytonema* sp. | – | $5 \times 10^9$ |

| | | | | |
|---|---|---|---|---|
| Exopolysaccharides | Cosmetics<br>Biomedicine (antioxidants, blood-clotting agents)<br>Chemical industry (thickening agents) | *Anabaena*, *Calothrix*, *Cylindrospermum*, *Gloeocapsa*, *Nostoc*, *Tolypothrix* | – | – |
| Cyanotoxins (microcystins, anatoxins, saxitoxins, cylindrospermopsin) | Analytical standards<br>Biomedicine (potential pharmaceutical applications) | *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Raphidiopsis*, *Umezakia* | – | 1–3 |
| Hydrogen | Bioenergy | *Anabaena*, *Calothrix*, *Chroococcidiopsis*, *Cyanothece*, *Gloeobacter*, *Nostoc* | – | – |
| Bioethanol (from yeast-fermented cyanobacteria) biomass) | Bioenergy (liquid biofuel) | *Nostoc*[b] | – | – |

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[a]*Phycobiliproteins ubiquitous in cyanobacteria, only high yield (8–17% dry weight) phycocyanin-containing $N_2$-fixers specified (Moreno, J., Rodríguez, H., Vargas, M.A., Rivas, J., Guerrero, M.G., 1995. Nitrogen-fixing cyanobacteria as source of phycobiliprotein pigments. Composition and growth performance of 10 filamentous heterocystous strains. J. Appl. Phycol. 7, 17–23.).*
[b]*Potentially from all high carbohydrate $N_2$-fixers, but only genera with high-starch content after oil extraction are included (John, R.P., Anisha, G., Nampoothiri, K.M., Pandey, A., 2011. Micro and macroalgal biomass: a renewable source for bioethanol. Biores. Technol. 102, 186–193).*
[c]*Actual market values for phycobiliproteins and fatty acids in 2013 (Markou, G., Nerantzis, E., 2013. Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. Biotechnol. Adv. 31, 1532–1542.) and projected market estimates for biofertilizers (soil conditioner from $N_2$-fixers) and toxins (Sharma, N.K., Rai, A.K., Stal, L.J., 2014. Cyanobacteria: An Economic Perspective. John Wiley & Sons, UK.).*

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