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N₂ Fixation by non-heterocystous cyanobacteria

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Abstract

Many, though not all, non-heterocystous cyanobacteria can fix N₂. However, very few strains can fix N₂ aerobically. Nevertheless, these organisms may make a substantial contribution to the global nitrogen cycle. In this general review, N₂ fixation by laboratory cultures and natural populations of non-heterocystous cyanobacteria is considered. The properties and subcellular location of nitrogenase in these organisms is described, as is the response of N₂ fixation to environmental factors such as fixed nitrogen, O₂ and the pattern of illumination. The integration of N₂ fixation with other aspects of cell metabolism (in particular photosynthesis) is also discussed. Similarities and differences between different individual strains of non-heterocystous cyanobacteria are highlighted.

Keywords: Non-heterocystous cyanobacteria; N₂ fixation; Nitrogenase; Immunolocalization; ATP and reductant; Diurnal rhythm; Natural environment

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1. Introduction

Cyanobacteria are O₂-evolving photosynthetic prokaryotes, many (though not all) of which can fix N₂. N₂-fixing (diazotrophic) cyanobacteria can be broadly grouped as heterocystous and non-heterocystous forms. All heterocystous forms are capable of aerobic N₂ fixation and, in the absence of combined nitrogen, 5–10% of their vegetative cells differentiate into specialized cells called heterocysts (heterocytes) that provide an environment suitable for the functioning of nitrogenase (the enzyme that catalyses N₂ fixation). Unlike vegetative cells, heterocysts are photosynthetically inactive. They do not fix

CO₂, nor do they produce O₂. They also exhibit a high rate of respiratory O₂ consumption and are surrounded by a thick, laminated cell wall that limits ingress of atmospheric gases, including O₂. The internal environment of heterocysts is therefore virtually anoxic, which is ideal for nitrogenase, a notoriously O₂-sensitive enzyme. In contrast, non-heterocystous cyanobacteria do not show cellular differentiation and until 1960 it was believed that such cyanobacteria were incapable of N₂ fixation. Hints that this belief might be unfounded came from reports of aerobic N₂ fixation by the non-heterocystous marine cyanobacterium *Trichodesmium* [1] and by laboratory cultures of the unicellular organism *Gloeotheca*, then called *Gloeocapsa* [2]. Shortly afterwards, Stewart and Lex (1970) demonstrated N₂ fixation under micro-oxic² conditions by cultures of the filamentous cyanobacterium *Plectonema boryanum* [3], now considered to belong to the genus *Leptolyngbya* [4,5]. Since then, the list of non-heterocystous cyanobacteria that can fix N₂ has been growing at a steady pace, with representatives isolated from marine, freshwater and terrestrial habitats. Currently 17 genera containing over 70 strains of non-heterocystous cyanobacteria are known to be capable of N₂ fixation. Of these, most can fix N₂ only when incubated under micro-oxic or anoxic conditions. However, a few strains can fix N₂ aerobically. Because of their apparent ability to sustain both oxygenic photosynthesis and the O₂-sensitive process of N₂ fixation in the same cell, these partic-

² In this review, the terms 'oxic' and 'anoxic' refer to environments where O₂ is, respectively, present or absent. The term 'aerobic' is used specifically to describe the presence of O₂ at concentrations comparable to those in air. With cyanobacteria, however, strictly anoxic conditions are obtained in the light only when photosynthetic production of O₂ is inhibited. Consistent with previous use of the term 'microaerobic', the expression 'micro-oxic' is therefore used to describe the environment of cultures grown in the absence of exogenous O₂ but which remain capable of generating O₂ photosynthetically. This term implies that O₂ is present at concentrations lower than those in air. However, it must be emphasized that this cannot be stated with certainty since the concentration of O₂ actually experienced by the organism has not usually been measured and may be much greater than realized. As an extreme example, cultures sparged with O₂-free gas at low flow rates, yet exposed to intense illumination, may be exposed to a local concentration of O₂ greater than that encountered by cultures illuminated less strongly and vigorously sparged with air.

Table 1
Genera of non-heterocystous cyanobacteria with N₂-fixing representatives^a

Genera	References
Unicellular forms	
Section I	
<i>Gloeocapsa</i>	[15–17]
<i>Gloeotheca</i> ^b	[2,18,19]; also see [20] (Plate 1a)
Cyanothece ^c	[18,19,21–31]
<i>Synechococcus</i> ^d	[22]
<i>Synechocystis</i> ^e	[32–34]
Section II	
<i>Chroococcidiopsis</i>	[18,22]
<i>Dermocarpa</i>	[18,22]
<i>Myxosarcina</i>	[18,22]
<i>Pleurocapsa</i> group	[18,22]
<i>Xenococcus</i>	[18,22]
Filamentous forms	
LPP Group ^f (Lyngbya , <i>Phormidium</i> , <i>Plectonema</i>)	[3,18,22,35–37] (<i>Plectonema boryanum</i> is illustrated in Plate 1d)
Symploca ^g	[38,39]
<i>Microcoleus</i>	[40,41]
Oscillatoria	[18,22,42–47] (Plate 1c)
<i>Pseudanabaena</i> ^h	[18,22,24]
Trichodesmium	[1,48–52] (Plate 1b)

^aIn grouping these genera the criteria of Rippka (1988) [53], Waterbury and Rippka (1989) [54], Waterbury (1989) [55] and Castenholz (1989) [56] were followed as far as possible. Genera listed in bold type possess strains that are capable of aerobic N₂ fixation. However, with the possible exception of *Gloeotheca*, this does not mean that every member of that genus can fix N₂ aerobically.

^bN₂-fixing strains referred as *Gloeocapsa* prior to 1979 were designated as *Gloeotheca* [22]. The name *Gloeocapsa* is used here for recent isolates identified as belonging to this genus. It should, however, be pointed out that the taxonomic difference between *Gloeotheca* and *Gloeocapsa* is subtle: cells of *Gloeotheca* divide in one plane, those of *Gloeocapsa* divide in two or three planes. This distinction is often difficult to observe in laboratory cultures. For example, the axes of individual cells of *Gloeotheca*, though not the planes of division, can shift during cell division. In field samples identification is even more difficult.

^cIncludes N₂-fixing strains referred to as *Aphanothece* or *Synechococcus* (for example, strains PCC 7418, 7424 and 7425, RF-1, BG 43511 and 43522). The *Cyanothece* group is superficially distinguished from the *Synechococcus* group by cell size (3–6 µm diameter as opposed to usually less than 2 µm diameter) and from the *Gloeotheca* group by the lack of a clearly defined, mucilaginous, multi-layered sheath. However, since sheathless strains have been derived from laboratory cultures of *Gloeotheca* ([57–60]; Shestakov *et al.*, unpublished) the taxonomic difference between *Cyanothece* and *Gloeotheca* is blurred.

^dOnly one strain of *Synechococcus*, strain PCC 7335, can fix N₂.

^eTwo marine *Synechocystis* strains (one previously designated *Erythrosphaera marina* [32]) can fix N₂ aerobically. They have very exacting growth requirements and have not been widely studied.

^fThe LPP group includes the genera *Lyngbya*, *Phormidium* and *Plectonema*. The characteristics that define these individual genera have proved to be variable in culture, so the detailed taxonomy of this group is currently uncertain. *Lyngbya majuscula* [36] and *L. aestuarii* [37] have been reported to fix N₂ aerobically: the latter may be identical to *Oscillatoria limosa* [42]. The marine *Oscillatoria* UCSB25 described by Gallon *et al.* (1991) [46] is probably also a strain of *Lyngbya*. Many strains designated as *Plectonema* or *Phormidium* can fix N₂ under anoxic or micro-oxic conditions. *Plectonema boryanum* and some strains of *Phormidium* have been reassigned to the genus *Leptolyngbya* [4].

^gThe strain of *Microcoleus chthonoplastes* that can fix N₂ aerobically [38] differs from other strains of *Microcoleus* and has recently been assigned to the genus *Symploca*.

^hIncludes LPP PCC 7409 which has been regrouped as *Pseudanabaena* PCC 7409 [24].

ular non-heterocystous cyanobacteria have been subjected to intense laboratory scrutiny. This has been directed especially at the mechanisms by which they protect nitrogenase from inactivation by both atmospheric and photosynthetically generated O₂, but also at the way in which photosynthesis and

respiration might generate the reductant and ATP needed to sustain N₂ fixation.

The present article attempts to review and summarise the current state of knowledge gained from laboratory and field studies of N₂ fixation in non-heterocystous cyanobacteria. This topic has pre-

Plate 1. Some N_2 -fixing non-heterocystous cyanobacteria. (a) *Gloeothece* strain PCC 6909. Bar = 10 μm . (b) *Trichodesmium* strain IMS 101 [61]. Bar = 20 μm . (c) *Oscillatoria limosa*. Bar = 200 μm . (d) *Plectonema boryanum* PCC 73110. Bar = 10 μm .

Table 2
Non-heterocystous cyanobacteria in diazotrophic symbioses

Symbiosis	Cyanobiont	References
<i>Rhopalodia</i> :cyanobacteria	Thin-walled unicells ^a	[75,76]; also see Plate 2
Marine sponges:cyanobacteria	Unicells (<i>Aphanocapsa</i>) ^b	[77,78]
Fungi:cyanobacteria ^c (cyanolichens)	<i>Gloeocapsa</i> , <i>Gloeotheca</i> , <i>Hyella</i>	[79]

^aThese occur intracellularly and resemble unicellular cyanobacteria. None have been cultured or taxonomically characterized.

^b*Aphanocapsa* is not recognized as a distinct genus by Rippka et al. (1979) [22] or Rippka (1988) [53], who designate *Aphanocapsa* strains as *Synechocystis*. However, since these cyanobionts have not been cultured in the laboratory, their taxonomic status is unknown.

^cA large number of cyanolichens, mostly belonging to the Lichinaceae and Heppiaceae, have unicellular cyanobacteria as cyanobionts (see [79]). Most cyanobionts resemble *Gloeocapsa*/*Gloeotheca* [80]. However, it is not certain whether such lichens fix N₂.

viously been reviewed by Gallon and Stal (1992) [6], but a number of other recent reviews are relevant to this topic. These include the reviews of Fay (1992) [7] and Gallon (1992) [8] on the interrelations between N₂ fixation and O₂ in cyanobacteria and other diazotrophs and the more specialized reviews of Huang and Grobbelaar (1995) [9] on *Synechococcus* (*Cyanothece*) RF-1, Bergman et al. (1994) [10], Zehr (1995) [11] and Gallon et al. (1996) [12] on *Trichodesmium*, Stal (1995) [13] on cyanobacteria in microbial mats and Rai (1990) [14] on symbiotic cyanobacteria.

2. Non-heterocystous cyanobacteria capable of N₂ fixation

Diazotrophic non-heterocystous cyanobacteria are conventionally grouped into unicellular and filamentous forms (Table 1). Some representative strains are illustrated in Plate 1. The taxonomy of cyanobacteria has undergone considerable revision over the past few years [22,53–57] and is still by no means definitive. Consequently, many of the organisms referred to in this review have been subjected to at least one change of name since first isolated and studied. Further alterations in nomenclature may reasonably be anticipated. Readers studying the primary literature for the first time are therefore strongly advised to check strain numbers (if quoted) very carefully before assuming that two cyanobacteria with different names are really different organisms. There is also a widespread feeling among workers in the field that some strains, currently believed to be different, may in reality represent independent isolates of the same organism. Use of modern techniques of molec-

ular taxonomy, such as sequencing of 16S rRNA and related hyper-variable internally transcribed spacer regions in the genome, will be invaluable in resolving many of the current uncertainties (for an excellent review, see [62]). Meanwhile, we have opted to use the names by which the organisms are currently most widely known, quoting synonyms where appropriate. For details, please see the footnotes to Table 1.

Unicellular cyanobacteria can be subdivided into two sections. Section I consists of genera reproducing by binary fission or budding while Section II consists of those reproducing either by multiple fission giving rise to small daughter cells (baeocytes) or by both multiple and binary fission. Among members of Section I, aerobic N₂ fixation was first reported in *Gloeotheca* [2] followed by *Aphanothece pallida* (now *Cyanothece* sp) [21,29]. Subsequently, Rippka and Waterbury (1977) [18] reported aerobic N₂ fixation in five strains of *Gloeotheca* (all deposited in the Pasteur Culture Collection as PCC 6501, 6909, 7109, 73107 and 73108); a sixth (PCC 8302) was added later [57]. These strains have all been given the species name *Gloeotheca membranacea*. One such strain is shown in Plate 1a. Since then, aerobic N₂ fixation has been reported in *Gloeocapsa* [15–17], the marine unicellular cyanobacterium *Erythrospira marina* [32] (probably a strain of *Synechocystis*) and several *Synechococcus* strains [23,25,27–30]. The latter are almost certainly *Cyanothece* spp, and include the rice field isolate *Synechococcus* RF-1 [19] and marine strains such as *Synechococcus* strains SF1 [27], BG 43511 and 43522 [23]. Three *Synechococcus* strains, two rice field isolates (PCC 7424 and 7425) and one marine isolate (PCC 7335), were reported to fix N₂ only under anoxic conditions [18].

Plate 2. The freshwater diatom *Rhopalodia gibba*. The photographs show: (a) an epifluorescence micrograph stained with the DNA-binding fluorescent dye 4',6-diamidino-2-phenylindole (DAPI), showing (centre) a diatom with four fluorescing symbionts and a nucleus; (b) an electron micrograph of an intact diatom; and (c) a magnified view of a cyanobacterial-like symbiont. Size bars represent (a) 100 μm , (b) 10 μm and (c) 1 μm . Photographs kindly provided by Dr H.R. DeYoe, National Estuary Program, Texas A and M University, Corpus Christi, TX, USA.

Although one of these, *Synechococcus* PCC 7424 has been reported to be capable of aerobic N₂ fixation (Rippka, unpublished results, quoted in [24]), it appears as a strain capable of N₂ fixation only under anoxic conditions in the classification of Waterbury and Rippka (1989) [54]. Both PCC 7424 and 7425, along with *Synechococcus* PCC 7418 (formerly *Aphanothece halophytica*) are now classified as *Cyanothece* strains [54]. Another two strains of *Cyanothece*, designated BH63 and BH68 (now deposited in the American Type Culture Collection as ATCC 51141 and 51142 respectively), were reported by Reddy et al. (1993) [31]. *Synechococcus* PCC 7335 remains as the only N₂-fixing cyanobacterium currently still assigned to this genus [54].

Two strains of *Synechocystis*, WH 8501 and 8502, both marine, can fix N₂ aerobically. In addition, in at least one freshwater strain, *Synechocystis* BO 8402, synthesis of nitrogenase can be induced by anoxic incubation in nitrogen-free medium [33,34].

Section I of the unicellular cyanobacteria includes an unusual organism, *Gloeobacter violaceus* [63]. This cyanobacterium resembles *Gloeotheca*, but lacks photosynthetic thylakoids: the photosynthetic apparatus is confined to the plasma membrane. *G. violaceus* cannot fix N₂, nor is nitrogenase synthesis induced under anoxic conditions [22,63]. Nevertheless there is a more recent report that this organism can grow aerobically in nitrogen-free medium [64]. This latter observation was not, however, accompanied by measurements of N₂ fixation, so it would be premature to reclassify *G. violaceus* as a diazotroph. It is not, therefore, included in Table 1.

In Section II, 19 strains (10 marine) are known to synthesize nitrogenase but only under anoxic conditions [18,22]. These include all strains of *Chroococcidiopsis* examined (PCC 6712, 7203, 7431, 7432, 7433, 7434, 7436 and 7439), two strains of *Dermocarpa* (PCC 7301 and 7303), one strain of *Myxosarcina* (PCC 7312), one strain of *Xenococcus* (PCC 7305) and seven strains of the *Pleurocapsa* group (PCC 7314, 7320, 7321, 7322, 7324, 7327 and 7516). Of these *Dermocarpa* PCC 7301 and 7303, *Myxosarcina* PCC 7312, *Xenococcus* PCC 7305 and six strains of the *Pleurocapsa* group (PCC 7314, 7320, 7321, 7322, 7324 and 7516) were isolated from marine habitats.

Among filamentous forms, aerobic N₂ fixation has been reported in several species of *Trichodesmium*,

[1,49,51,52,65–68], *Microcoleus chthonoplastes* [38,69], *Oscillatoria limosa* [42], *Oscillatoria* sp. [43], *Oscillatoria* sp. strains UCSB8 and UCSB25 [46], *Lyngbya majuscula* [36] and *L. aestuarii* (probably identical to *Oscillatoria limosa*: see Section 6.2.2 [37]. All are marine forms. A laboratory strain of *Trichodesmium* [61], now designated strain IMS 101, and *Oscillatoria limosa* are shown in Plate 1b,c, respectively. The original N₂-fixing strain of *Microcoleus chthonoplastes* [38] has recently been re-assigned to the genus *Symploca*, whilst *Oscillatoria* sp. UCSB25 has been reidentified as a strain of *Lyngbya* (N. Anand, personal communication). In addition, there are several strains that fix N₂ only under micro-oxic or anoxic conditions [3,18,35,70]. These include *Phormidium foveolarum*, 15 strains of the LPP group (PCC 6306, 6402, 6409, 7004, 7104, 7113, 7124, 7375, 7406, 7408, 7410, 7419, 7424, 7505 and 73110), five strains of *Pseudanabaena* (PCC 6406, 6802, 7403, 7409, and 7429), and five strains of *Oscillatoria* (PCC 6407, 6412, 6506, 6602 and 7515). To these can be added *Oscillatoria chalybea* [47] and the phycoerythrin-rich strains epiphytic on pelagic *Sargassum* [45]. All are freshwater forms except for LPP group strains PCC 7124, 7375 and 6419 and the *Oscillatoria* strains from *Sargassum*, which are marine. One of the LPP group strains, *Plectonema boryanum*, is shown in Plate 1d.

Mutants of heterocystous cyanobacteria have been reported that fail to produce heterocysts (het[−]), yet retain the ability to fix N₂ when incubated under micro-oxic or anoxic conditions [71,72]. Although, taxonomically, these mutant cyanobacteria would not be classified as non-heterocystous, physiologically they may have much in common with their non-heterocystous relatives. However, these het[−] mutants would appear to be unusual. Most het[−] mutants cannot fix N₂ [73,74].

Some non-heterocystous cyanobacteria have been reported to form symbiotic associations (Table 2). The cyanobacterial partner in these symbioses is referred to as a cyanobiont. The freshwater diatom *Rhopalodia* (Plate 2) contains thin-walled unicells resembling cyanobacteria that have been shown to fix N₂ [75,76]. Unicellular cyanobacteria are also known from the marine diatom *Streptotheca* sp., but it is uncertain whether this association fixes N₂ [78]. *Aphanocapsa* (probably *Synechocystis*) and *Phormi-*

Plate 3. Some natural populations of non-heterocystous cyanobacteria. (a) Microbial mats in Baja California, Mexico. The smooth mat (foreground) is composed of cyanobacteria of the *Lyngbya/Oscillatoria* type. (b) Cyanobacteria on the wall of a limestone cave near Swansea, Wales: a population dominated by *Gloeotheca*. (c) An aerial photograph of a bloom of *Trichodesmium* off Hawaii. Photograph courtesy of Dr R. Letelier and Dr D.M. Karl, Hawaii Ocean Time-Series Team, University of Hawaii, Honolulu, Hawaii, USA. (d) Part of a *Trichodesmium* bloom from sea level (acknowledgements as c).

Plate 3 (continued).

dium have been reported as cyanobionts in 38 genera of marine sponges belonging to the Calcarea and

Desmospongia (see [14]). In at least two cases, *Thionella swinhoei* and *Siphonochalina tabernacula*, the

Plate 4.

Plate 4 (*continued*).

Plate 4. Immunolocalization of the Fe-protein of nitrogenase in various N_2 -fixing cyanobacteria. (a) The unicellular cyanobacterium *Gloeotheca* strain PCC 6909. (b) A longitudinal section of the filamentous mat-forming organism *Oscillatoria limosa* [42]. (c) A transverse section of the filamentous marine cyanobacterium *Trichodesmium* sp. (d) For comparative purposes, the cellular location of Fe-protein is also shown in a heterocyst of the heterocystous cyanobacterium, *Nostoc* sp. PCC 9229, isolated from symbiosis with the angiosperm *Gunnera*. Size bar = 1 μm throughout. Note that nitrogenase is evenly distributed within all cells of *Gloeotheca* and *O. limosa* (a,b), but that the intensity of immunogold labelling varies considerably between individual cells of *Trichodesmium*. For example, intense labelling is seen in the cross-sectioned cell in the upper right-hand corner of (c), whilst the cell at the bottom of the photograph shows no labelling at all. In *Nostoc* (d), Fe-protein is detected only in heterocysts.

cyanobiont has been shown to fix N_2 [77]. Unicellular cyanobacteria variously described as *Gloeocapsa*, *Gloeotheca* and *Hyella* (a genus that has been assigned to the *Pleurocapsa* group) have been reported

Fig. 1. Two forms of the Fe-protein of nitrogenase in extracts of (a) *Gloeotheca* and (b) *Rhodospirillum rubrum*. Track a shows an extract of *Gloeotheca* prepared from cells sampled 2 h into the dark phase of a cycle of alternating 12 h light and 12 h darkness after SDS/PAGE and Western blotting. The Fe-protein was detected using antisera raised against the Fe-protein of nitrogenase from the photosynthetic bacterium *Rhodospirillum rubrum*. For further details see [60]. Track b shows a sample of ADP-ribosylated Fe-protein of nitrogenase from *R. rubrum*. The upper band of the Fe-protein from *R. rubrum* represents the ADP-ribosylated subunit; the lower band ($M_r = 32\,500$; [137]) is unmodified. The upper band of the Fe-protein from *Gloeotheca* has an apparent M_r of 40 000 and is presumed to be a modified form of the lower, presumed unmodified, band (apparent $M_r = 38\,500$). The nature of the modification is unknown but is unlikely to involve ADP-ribosylation (see text). Antisera and *R. rubrum* Fe-protein were kindly provided by Prof P. Ludden, University of Wisconsin, Madison, Wisconsin, USA. Photograph courtesy of Dr. J. Cheng, Biochemistry Research Group, University of Wales Swansea, Swansea, UK.

Fig. 2. The pattern of N_2 fixation in (a) laboratory cultures of *Gloeotheca* grown under alternating 12 h light and 12 h darkness and (b) natural populations of *Trichodesmium*, sampled in the Eastern Caribbean Sea in January 1992. In b, zero time corresponds to sunrise. Day length was approximately 10.5 h.

Table 3

Relative amounts and activities of nitrogenase in heterocysts of *Anabaena cylindrica* and in non-heterocystous cyanobacteria

	Amount of nitrogenase ^a	Nitrogenase activity ^b
Heterocysts	50 ± 8 (3–4) ^c	10–12 ^d
<i>Oscillatoria limosa</i>	54 ± 6	12–15
<i>Plectonema boryanum</i>	47 ± 6	10–15
<i>Gloeothoece</i> strain PCC 6909	42 ± 5	6–8

^aValues for the amount of nitrogenase represent labelling densities (number of gold particles per μm^2 area) obtained in immunogold labelling experiments using primary antibodies (1:100 dilution) raised in rabbits against the Fe-protein of the nitrogenase of *Rhodospirillum rubrum*, and secondary antibodies raised in goats against rabbit IgG and conjugated to colloidal gold particles (10 nm diameter).

^bnmol acetylene reduced h^{-1} ($\mu\text{g chl } a$)⁻¹.

^cFigures in brackets represent the value obtained when heterocyst labelling is expressed per unit cell area, taking into account vegetative cells as well as heterocysts.

^dThe specific activity of nitrogenase relates to whole filaments (i.e. to both vegetative cells and heterocysts).

as cyanobionts from several lichens, mainly of the families Lichinaceae and Heppiaceae (see [79,80]). Although the cyanobionts from such lichens have not been tested for nitrogenase activity, several other strains of these genera are known to fix N_2 (see Table 1).

3. N_2 fixation by non-heterocystous cyanobacteria in the natural environment

3.1. Microbial mats

Marine intertidal sediments are often characterized by the development of dense communities of cyanobacteria referred to as microbial or cyanobacterial mats [81]. An example is shown in Plate 3a. Because the marine environment is low in combined nitrogen, the development of microbial mats is possible only due to the activity of N_2 -fixing organisms. The majority of microbial mats are composed of *Microcoleus chthonoplastes*. Because mats of *M. chthonoplastes* have been repeatedly reported to fix N_2 , this cyanobacterium was thought to be the N_2 -fixing agent [38,82–87]. Moreover, it has been suggested that formation of bundles of trichomes enclosed by a polysaccharide sheath, which characterizes *M. chthonoplastes*, protects nitrogenase against inactivation by O_2 [83]. However, cultures of this organism were not capable of diazotrophic growth under oxic conditions [40]. Nevertheless, Pearson et al. (1979) isolated a non-heterocystous diazotrophic cyanobacterium that they identified as *M. chthonoplastes* [38].

They showed that neither bundle formation nor the polysaccharide sheath was necessary for aerobic diazotrophic growth. However, this organism is now considered to be a strain of *Symploca* (Table 1). *M. chthonoplastes* isolated from North Sea microbial mats expressed nitrogenase activity in laboratory culture only under anoxic conditions in the light [40]. Although such behaviour can be found in a large number of cyanobacteria [18], in most cases its ecological relevance is not clear. Nevertheless, preliminary experiments, designed to study microbial mat formation in the laboratory, have provided some evidence that nitrogenase is expressed in *M. chthonoplastes* following immobilization on sea sand (R.T. Carius and L.J. Stal, unpublished observations). It cannot therefore be excluded that *M. chthonoplastes* plays a role in N_2 fixation in the natural environment. Immunogold localization of nitrogenase in natural samples would help to resolve this uncertainty.

Microbial mats with *M. chthonoplastes* as the dominant cyanobacterium often also contain other types of cyanobacteria. For instance in North Sea microbial mats, *Oscillatoria limosa* was usually the pioneer organism [81]. In culture, this cyanobacterium possesses the capacity for diazotrophic growth under fully oxic (aerobic) and photoautotrophic conditions [42]. Furthermore, the rate of acetylene reduction in the mat could be positively correlated with the amount of *O. limosa* present [88]. Although in well-established microbial mats *M. chthonoplastes* becomes the dominant cyanobacterium, *O. limosa* remains in considerable numbers. Indeed, *O. limosa*

Figure 3. 'Autoprotection' of nitrogenase from inactivation by O₂.**a.** An overview **b.** Individual reactionsReaction 1: N₂ fixation [163, 165] Reaction 2: Production of superoxide ($\text{O}_2^{\cdot -}$) [260]

Fig. 3. 'Autoprotection' of nitrogenase from inactivation by O₂. (a) An overview. (b) Individual reactions: Reaction 1: N₂ fixation [163,165]; Reaction 2: production of superoxide ($\text{O}_2^{\cdot -}$) [260]; Reaction 3: 'autoprotective' O₂ consumption [260]. Fd = ferredoxin; $\text{Fe}(\text{ADP}^-)_2$ = Fe-protein (MgADP^-)₂ complex; FeATP^{2-})₂ = Fe-protein MgATP^{2-})₂ complex; MoFe = 1/2 MoFe-protein; P_i^- = H_2PO_4^- ; X (XH) = Oxidized (reduced) form of a peroxidase substrate.

Reaction 3: 'Autoprotective' O₂ consumption [260] **Notes:**

Fd = ferredoxin; Fe(ADP⁻)₂ = Fe-protein (MgADP⁻)₂ complex;

Fe(ATP²⁻)₂ = Fe-protein (MgATP²⁻)₂ complex; MoFe = 1/2 MoFe-protein; P_i⁻ = H₂PO₄⁻;

X (XH) = oxidized (reduced) form of a peroxidase substrate.

Fig. 3 (continued).

may even become dominant in established mats, probably depending on the nitrogen status of the system. Similarly, microbial mats on the North Carolina coast [89] and in intertidal flats near Guerrero Negro, Baja California, Mexico [90] are composed virtually solely of a single morphotype of cyanobacterium (*Oscillatoria-Lyngbya* type). These mats are all characterized by very high nitrogenase activities. Furthermore, a number of other reports on N₂ fixation in marine tidal sediments mention *Oscillatoria* (*Lyngbya*) spp. as important organisms [84,87,91,92]. N₂-fixing *Lyngbya* was also found in submersed mats on coral reefs [93]. From these reports it can be concluded that *Oscillatoria* spp. are probably the most important N₂-fixing agents in marine sediments.

3.2. Terrestrial and freshwater cyanobacteria

The unicellular N₂-fixing cyanobacterium *Gloeotheca* can be found in soils [94], rice fields [19], sphagnum bogs [22] and on limestone cave walls

[92] (see Plate 3b). Very little is known about N₂ fixation by natural populations of this cyanobacterium, though Griffiths et al. (1987) showed that *Gloeotheca* growing on the wall of a limestone cave fixed N₂ only during the night [92].

Several unicellular *Cyanothece* (*Synechococcus* or *Aphanothece*) strains that are capable of aerobic N₂ fixation also occur in soils or freshwater environments [16,19,28,95]. In contrast, those filamentous non-heterocystous cyanobacteria that can fix N₂ aerobically are mainly confined to marine or brackish habitats. However, several representatives of cyanobacterial genera that include strains capable of N₂ fixation only under anoxic or micro-oxic conditions are found in terrestrial and freshwater environments. For example, *Phormidium*, *Plectonema* and *Lyngbya* strains are reported from stagnant pools and waterlogged soils [96]. In such relatively anoxic environments, they may be able to fix N₂, but there have so far been no detailed studies on the environmental significance of N₂ fixation by these strains. *Synechococcus*, *Synechocystis* and *Oscillatoria* strains

are also abundant in fresh waters (see, for example, [97,98]) but, again, there are few reports of N_2 fixation by these strains in situ. In general, the dominant N_2 -fixing cyanobacteria in soils and in fresh waters are heterocystous strains.

The diatom *Rhopalodia*, which lives in symbiosis with a thin-walled coccoid cyanobacterium (Table 2; Plate 2), is found in fresh water. Although this association is diazotrophic, the cyanobiont has not been isolated and it is not known to what extent this symbiotic association contributes to N_2 fixation in the natural environment.

3.3. Marine plankton

Very small unicellular *Synechococcus* spp. are abundant in the marine plankton and make an important contribution to primary production in the oceans. Although a few N_2 -fixing unicellular cyanobacteria have been isolated from the marine environment [32,99] there has been no systematic investigation of their contribution to the nitrogen budget of the marine environment. However, current data point to a minor contribution of picoplankton to N_2 fixation. Virtually all strains that have been isolated are incapable of fixing N_2 . The small size of these organisms is probably an adaptation to the oligotrophic marine environment and their large surface area to volume ratio probably enables them to scavenge combined nitrogen more effectively than larger species.

Trichodesmium spp. are extremely abundant in the tropical and subtropical oceans with a salinity of approximately 35‰ and a water temperature above 25°C. In these waters, this cyanobacterium can form massive blooms covering areas over 2×10^6 km² ([100]; E.J. Carpenter and D.G. Capone, personal communication). Much smaller blooms are illustrated in Plate 3c,d. Blooms of *Trichodesmium* can readily be detected by remote imaging from orbiting satellites, giving *Trichodesmium* the unusual distinction of being a living organism that can be observed from space. There are occasional reports of *Trichodesmium* in the Mediterranean, where the temperature is usually lower than 25°C, but no information is available on its physiological activity, including N_2 fixation. The occurrence of *Trichodesmium* (usually in a moribund state) in the colder North Atlantic

has been explained by transport from the tropical ocean.

Trichodesmium spp. occur as single trichomes [101,102] and also as aggregates of variable size. Large aggregates may measure several millimetres and may contain hundreds of trichomes. The trichomes in the bundles may be orientated parallel to each other ('tufts') or radially ('puffs'). The density of the colony may vary. Although different types of colonies can be found in one population, comparatively little is known about possible differences in their physiology. On the other hand, Carpenter et al. (1993) demonstrated distinct differences in the physiology of two of the more commonly occurring species of *Trichodesmium*, *T. thiebautii* and *T. erythraeum* [50]. In addition, a recent survey clearly demonstrated that several different species of *Trichodesmium* exist and, under natural conditions, they often occur together [103]. Although laboratory cultures of *Trichodesmium* can fix N_2 [61,104], these are not yet axenic. Moreover, in the natural environment, aggregates of *Trichodesmium* may be associated with chemotrophic N_2 -fixing bacteria [105] and with other filamentous non-heterocystous cyanobacteria [106]. However, the amount of nitrogenase detected in these associated organisms is negligible compared with that seen in *Trichodesmium* itself [106,107]. It therefore seems safe to conclude that this cyanobacterium is a true diazotroph.

As with other symbiotic systems, the environmental significance of N_2 fixation in the two marine sponges, *Thionella swinhoei* and *Siphonochalina tabernacula*, remains unknown.

4. Nitrogen budgets and rates of non-heterocystous N_2 fixation

Field studies on N_2 -fixing, non-heterocystous cyanobacteria have focused attention on their potential role in the nitrogen economy of a wide range of habitats in general and in marine ecosystems in particular. However, with the possible exception of *Trichodesmium*, very little is known about the contribution of N_2 fixation by non-heterocystous cyanobacteria to the nitrogen budget of the systems where these organisms occur. The rates that are published are, in general, difficult to compare. For ex-

ample, in most cases N_2 fixation was determined using the acetylene reduction technique. The conversion of acetylene reduction to actual rates of N_2 fixation cannot be done accurately without direct calibration using $^{15}N_2$. The ratio of acetylene reduced to $^{15}N_2$ fixed can vary. Furthermore, published rates of N_2 fixation were usually derived from occasional measurements which did not take into account diel and seasonal variations. In addition, the quantitative measurement of N_2 fixation in the natural environment is beset with methodological problems. We do not propose therefore to give a detailed overview of rates of N_2 fixation and nitrogen budgets in environments dominated by non-heterocystous cyanobacteria. Rather, we confine ourselves to some general remarks.

N_2 fixation by *Trichodesmium* spp. ranges from 0.002–0.04 g N m⁻² yr⁻¹ [108] to up to 0.7 g N m⁻² yr⁻¹ [109]. Carpenter and Capone (1992) [67] calculate a total annual rate of N_2 fixation of 5.4 Tg N for blooms of *Trichodesmium* (assuming 20 blooms per year with an average surface area of 20 000 km²) whilst Capone and Carpenter (1982) [110] have estimated global non-bloom N_2 fixation to be 4.7 Tg N yr⁻¹. Estimates of total N_2 fixation on Earth vary, but that of 170–270 Tg N yr⁻¹ recorded by Paul (1978) [111] appears reasonable. On this basis, it appears that *Trichodesmium* could contribute as much as 6% to global N_2 fixation (see [12]). This compares with an estimated contribution of 20% for N_2 fixation by legumes [112]. However, recent data indicate that estimates of N_2 fixation by *Trichodesmium* could be as much as two orders of magnitude too low (D.G. Capone and E.J. Carpenter, personal communication). If so, the estimate of total N_2 fixation on earth will need revising, and *Trichodesmium* could make a larger contribution to this total than all other sources of N_2 fixation combined. It is also worth mentioning that N_2 fixed by *Trichodesmium* enhances the productivity of other phytoplankton species [113].

Stal et al. (1984) calculated the contribution of N_2 fixation by *O. limosa* on a North Sea intertidal flat to be, on average, 0.8–1.5 g N m⁻² yr⁻¹ [88]. These rates were calculated from acetylene reduction rates using a conversion factor (C_2H_2 reduced: N_2 fixed) of 4:1, which is that predicted by theory. Diel and seasonal variations were also taken into account. For

instance, if only the highest rates of acetylene reduction were used [114] and extrapolated, assuming 12 h day⁻¹ and 200 days yr⁻¹ of activity, a figure of 9.6 g N m⁻² yr⁻¹ can be derived. These rates are among the highest reported [108]. It can therefore be concluded that communities of non-heterocystous cyanobacteria may contribute as efficiently to the nitrogen budget of an ecosystem as do heterocystous cyanobacteria. However, with the possible exception of *Trichodesmium*, the data on the distribution of non-heterocystous N_2 -fixing cyanobacteria and their contribution to the nitrogen budget are unfortunately too scarce to allow global extrapolation of their importance. Nevertheless, in general terms, it is likely that non-heterocystous cyanobacteria make a major contribution to the operation of the nitrogen cycle on earth.

5. Nitrogenase in non-heterocystous cyanobacteria

5.1. Nitrogenase and the *nif* genes

‘Conventional’ molybdenum nitrogenase consists of two proteins, a Fe-protein (dinitrogenase reductase) and a MoFe-protein (dinitrogenase). These are encoded, respectively, by genes designated *nifH* and *nifDK*. However, many diazotrophs also possess genes (*vnf* or *anf*) that encode one or more molybdenum-independent nitrogenase (for details, see [115,116]). Although the presence of molybdenum-independent nitrogenases has been confirmed in heterocystous cyanobacteria [117–120], there are no reports of such enzymes in non-heterocystous cyanobacteria. Indeed, attempts to demonstrate their existence in *Gloeotheca* sp. ATCC 27152 (PCC 6909) and in *Oscillatoria* spp. UCSB8 and UCSB25 have proved unsuccessful (T.S. Page and J.R. Gallon, unpublished observations). Whilst production of ethane from acetylene (one of the preliminary diagnostic tests for the presence of molybdenum-independent nitrogenases) was observed in cultures of *Microcoleus* (*Symploca*) *chthonoplastes* incubated in Mo-free medium, no evidence for the existence of *vnf* or *anf* genes was obtained (T.S. Page, R.R. Eady, R. Pau and J.R. Gallon, unpublished findings).

Several lines of evidence suggest that molybdenum

nitrogenase in non-heterocystous cyanobacteria is similar to that in other diazotrophs.

1. Antibodies raised against the Fe-protein and Mo-Fe proteins of nitrogenases from *Rhodospirillum rubrum*, *Azotobacter*, *Klebsiella pneumoniae*, the non-heterocystous cyanobacterium *Trichodesmium* and the heterocystous cyanobacterium *Anabaena variabilis* have been shown to cross-react specifically with the corresponding nitrogenase proteins from *Plectonema* (PCC 6306 and 73110), various *Trichodesmium* spp., *Oscillatoria limosa*, *Oscillatoria* spp. UCSB8 and UCSB25 *Aphanothece* (*Cyanothece*) *pallida*, *Cyanothece* BH68 and *Gloeotheca* PCC 6909 ([10,51,52,60,107,121–126]; L.A. Sherman, personal communication; our unpublished results).

2. The Fe-protein of *P. boryanum* nitrogenase successfully complements the MoFe-protein of the nitrogenases from a variety of diazotrophs to form catalytically active complexes [127,128]. Similarly, the MoFe-protein of the nitrogenases from *P. boryanum* and *Gloeotheca* have been found to form active complexes with, respectively, the Fe-proteins of nitrogenase from the heterocystous cyanobacterium *Anabaena cylindrica* [128] and the facultative anaerobe *K. pneumoniae* (P.S. Maryan, unpublished results; quoted in [20]).

3. SDS/PAGE-immunoblot analysis of the Fe-protein of nitrogenase from the heterocystous cyanobacteria *Anabaena* and *Nostoc* [129–131], and the non-heterocystous cyanobacteria *Plectonema boryanum* [126] and *Trichodesmium* [107,122,125,132], shows that these proteins all have a similar apparent M_r of approximately 36 000. There are, however, reports of an M_r as high as 40 000 for the Fe-protein in *Synechococcus* (*Cyanothece*) RF-1 [133], *Trichodesmium* [107,134], *Oscillatoria limosa* [124] and *Gloeotheca* sp. ATCC 27152 [60]. However, measurement is complicated by the fact that, in many cyanobacteria, the Fe-protein of nitrogenase can exist in two distinct forms, of different M_r (see Fig. 1a). Moreover, measurement of M_r by SDS/PAGE has an accuracy no better than $\pm 10\%$. It is therefore premature to ascribe any structural or physiological significance to the apparent differences in M_r among Fe-proteins from different cyanobacteria.

In a number of diazotrophs, the Fe-protein of nitrogenase undergoes reversible covalent modification by attachment of an ADP-ribose moiety to a specific

arginine residue. ADP-ribosylation of the protein is catalysed by dinitrogenase reductase ADP-ribosyl transferase (DRAT), and renders the Fe-protein catalytically inactive. Removal of ADP-ribose is catalysed by dinitrogenase reductase activating glycohydrolase (DRAG) and restores activity. Covalent modification of the Fe-protein has been most extensively studied in photosynthetic bacteria such as *Rhodospirillum rubrum* and has recently been reviewed by Ludden and Roberts (1989) [135], Nordlund (1991) [136], Roberts and Ludden (1992) [137] and Zhang et al. (1995) [138]. Covalent modification (and associated inactivation) of nitrogenase is stimulated in the presence of ammonium or certain other nitrogen sources, by transfer of cultures to the dark or, to a lesser extent, by exposure to O_2 (*R. rubrum* fixes N_2 only under anoxic conditions).

A convenient method for studying covalent modification of the Fe-protein of nitrogenase is SDS/PAGE. When subjected to SDS/PAGE, ADP-ribosylated Fe-protein migrates more slowly than unmodified Fe-protein, appearing to have a larger M_r (Fig. 1b). In a number of cyanobacteria, including non-heterocystous strains such as *Gloeotheca* ([8]; Fig. 1a), *Microcoleus* (*Symploca*) *chthonoplastes* (D.P. Singh and J.R. Gallon, unpublished observation), *Oscillatoria limosa* [124], *Synechococcus* (*Cyanothece*) RF-1 [133], *Synechocystis* BO 8402 [34] and *Trichodesmium* [132,139], the Fe-protein can be resolved into two components by SDS/PAGE. Most detailed studies have been performed with the heterocystous cyanobacterium *Anabaena variabilis* ATCC 29413 [140–143] and with *Gloeotheca* ATCC 27152 [8,60,144]. No incorporation of radioactivity from $^{32}PO_4^{3-}$ into the Fe-protein of nitrogenase and no reaction of this protein with antibodies specific to ADP ribose occurred in either *A. variabilis* [143] or *Gloeotheca* (J. Cheng, D. Pederson, H. Hilz and J.R. Gallon, unpublished findings). Similarly, attempts to label the Fe-protein from *Trichodesmium* with ^{32}P proved unsuccessful (unpublished data, cited in [11]). The balance of evidence therefore strongly suggests that the Fe-protein of cyanobacterial nitrogenase is not subjected to ADP ribosylation. On the other hand, although no evidence for the existence of genes encoding DRAT or DRAG was found in *A. variabilis* [143], proteins antigenically similar to both

DRAT and DRAG have been found in *Gloeotheca* [144]. These proteins were much larger than their counterparts in *Rhodospirillum rubrum*, but the fact that they were not produced by cultures of *Gloeotheca* growing in medium containing ammonium suggests that they have a role in N_2 fixation or related metabolism. The recent finding that glutamine synthetase, a key enzyme in the assimilation of nitrogen, is subject to ADP ribosylation in the non-diazotrophic unicellular cyanobacterium *Synechocystis* sp. PCC 6803 [145], raises the possibility that the DRAT- and DRAG-like proteins in *Gloeotheca* may act on this enzyme rather than the Fe-protein of nitrogenase. Unfortunately however, we have so far been unable to detect any incorporation of $^{32}PO_4^{3-}$ into immunologically identifiable glutamine synthetase during SDS/PAGE of extracts of *Gloeotheca*, nor have we observed any immunological reaction between this protein and antibodies to ADP-ribose (J. Cheng, H. Hilz and J.R. Gallon, unpublished results).

In dense suspensions of *Synechocystis* BO 8402, which fixes N_2 only when incubated under anoxic or micro-oxic conditions, the presumed modified (higher M_r) form of the Fe-protein is not present in the absence of O_2 , but appears when O_2 is introduced into the atmosphere above cultures [34]. Under these conditions, nitrogenase activity is inhibited, so 'modified' Fe-protein may be catalytically inactive. On return to anoxic incubation conditions, nitrogenase activity recovers, accompanied by the re-appearance of the unmodified form of the Fe-protein. Though capable of aerobic N_2 fixation, *Gloeotheca* and *Trichodesmium* behave similarly. Both forms of the Fe-protein can be seen in natural populations of *Trichodesmium* and in aerobically grown cultures of *Gloeotheca*. However, exposure to O_2 at concentrations greater than those in air inhibited N_2 fixation and markedly stimulated the appearance of the 'modified' form of the Fe-protein in both *Gloeotheca* [8,60] and *Trichodesmium* [11,132]. On the other hand, nitrogenase activity can be detected in cultures of *Gloeotheca* grown in the absence of atmospheric O_2 even when they apparently contain only 'modified' Fe-protein, implying that this form retains catalytic activity under these conditions [60]. In *Gloeotheca* therefore, modification of the Fe-protein may render it extremely sensitive to

inactivation by O_2 , rather than catalytically inactive *per se*.

Plectonema boryanum differs from these other non-heterocystous cyanobacteria in that no modification of the Fe-protein of its nitrogenase could be observed, even following exposure to O_2 . Such treatment simply resulted in the complete disappearance of nitrogenase from the cells [126].

There is no evidence that the *nif* genes in non-heterocystous cyanobacteria differ significantly from those in other diazotrophs. DNA gene probes from *Anabaena* PCC 7120, containing the nitrogenase structural genes *nifH*, *D*, and *K* hybridize specifically with DNA from *Cyanothece* PCC 7424 [24], *Gloeotheca* [146,147], *Lyngbya* [147], *Plectonema* [24,147–150], *Pseudanabaena* [24,151,152], *Oscillatoria* [151], *Synechococcus* [24] and *Trichodesmium* [153]. Similarly *nif* gene probes from *K. pneumoniae* hybridize to genomic DNA from *Pseudanabaena* PCC 7409 [152]. In addition, *nif* probes from *Anabaena* 7120 hybridize with, presumed, nitrogenase-specific mRNA in *Synechococcus* RF-1 [154,155]. The *nifH* genes from *Trichodesmium thiebautii* [156] and *Trichodesmium* sp. strain NIBB 1067 [125] have been cloned and partially sequenced. A comparison of the nucleotide sequence, and the deduced amino acid sequence, showed 98% similarity between the two *Trichodesmium* spp. and 80–87% similarity with heterocystous strains *Anabaena* 7120 and *Anabaena flos-aquae*. These findings have been extended with the first report of the complete sequence of *nifH* from natural populations of *T. thiebautii* [157], from which a strong similarity to 23 known *nifH* sequences was confirmed. Partial sequences are also available for *nifH* from *Gloeotheca* sp. CCAP 1430/3 (=PCC 6909), *Plectonema boryanum* UTEX 594 (=PCC 73110) and *Lyngbya lagerheimii* UTEX 1930 [147]. The degree of similarity among *nifH* sequences from the various non-heterocystous cyanobacteria was 70–81%, rather lower than that among the heterocystous *Anabaena* and *Nostoc* strains tested (84–92%). However, the fragments cross-hybridized with each other to a varying extent. The nucleotide and encoded amino acid sequences for *nifU*, *nifH* and *nifD* genes in *Plectonema boryanum* show 70%, 84% and 76% homology with *Anabaena* 7120 *nifU*, *H* and *D* genes, respectively [158]. Furthermore, upstream from both *nifH* and *nifD* (which has been

partially sequenced) in *T. thiebautii* were sequences resembling consensus *nif*-like promoters from other diazotrophs [157]. These findings are consistent with the view that among all diazotrophs, nitrogenase and the *nif* structural genes are highly conserved.

The arrangement of *nif* structural genes has been studied in the unicellular cyanobacteria *Gloeotheca* PCC 6909, *Synechococcus* strains PCC 7335 and 7425 and *Cyanothece* PCC 7424, as well as in the filamentous forms *Pseudanabaena* strains PCC 7403 and 7409, *Oscillatoria tenuis* UTEX 1566, *Trichodesmium* strain NIBB 1067 and *P. boryanum* strains PCC 6306, 73110 and IAM-M 101. In all N_2 -fixing non-heterocystous cyanobacteria, whether or not they can fix N_2 under oxic conditions, the *nif* structural genes are contiguous and arranged in the order *nifHDK* [24,146,148–153,157,158]. Such contiguous arrangement of *nif* structural genes resembles that in bacteria and in heterocysts of cyanobacteria but contrasts with the situation in vegetative cells of the heterocystous cyanobacterium *Anabaena* PCC 7120, in which *nifK* is separated by 11 kb from *nifDH*. In *Anabaena* 7120 and certain other heterocystous cyanobacteria, excision of this element occurs during heterocyst differentiation but, so far, there is no evidence for any similar genomic rearrangement in non-heterocystous cyanobacteria [74,159,160].

Genes resembling *nifH* and *nifDK* (sequence similarity 34% and 15–20%, respectively) have been implicated in the later stages of chlorophyll biosynthesis in *Plectonema boryanum* [158,161,162]. The *nifH*-like gene (*frxC*) encodes a protein that is involved in the light-independent reduction of protochlorophyllide to chlorophyllide, a process that may also involve the product of the *nifDK*-like gene (ORF 467). It is possible that the FrxC protein may donate electrons to the protein encoded by ORF 467, which in turn reduces protochlorophyllide to chlorophyllide [162]. If so, this resembles the catalytic function of the Fe- and MoFe-proteins of nitrogenase: the Fe-protein functions as an electron donor to the MoFe-protein which then catalyses N_2 reduction (see, for example, [163–167]; Fig. 3). The two systems may therefore resemble each other functionally as well as structurally, and may share a common ancestry.

5.2. Derepression of nitrogenase

In diazotrophs, nitrogenase synthesis is derepressed under conditions of nitrogen limitation. N_2 is not an inducer of nitrogenase synthesis, though sustained derepression of nitrogenase depends on active N_2 fixation in order to maintain general nitrogen status and thereby allow protein synthesis to continue [168]. In cyanobacteria that can fix N_2 aerobically, nitrogenase synthesis occurs in the presence of air. In contrast, those cyanobacteria that fix N_2 only under anoxic or micro-oxic conditions require both nitrogen stress and the presence of no more than trace amounts of O_2 for nitrogenase derepression and activity [3,18,35,126,127,169–171]. Apparently, these strains possess inadequate mechanisms to remove atmospheric and endogenously evolved O_2 . Complete absence of O_2 is generally not required but an equilibrium between ingress of atmospheric O_2 , photosynthetic O_2 evolution and respiratory O_2 consumption is necessary. Among these cyanobacteria, detailed studies have largely been restricted to *Plectonema boryanum* in which nitrogenase synthesis has been derepressed under photoautotrophic as well as under photoheterotrophic conditions [3,172,173]. In this cyanobacterium nitrogenase synthesis can be derepressed by sparging with Ar, Ar/ CO_2 , or N_2/CO_2 at the low light intensity of 500 lx (which, for fluorescent lights, corresponds approximately to 6 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ over the range 400–700 nm). However, at higher light intensities addition of DCMU (5 μM) or sodium sulphide (2 mM) is also necessary in order to inhibit photosynthetic generation of O_2 . The duration of exposure to anoxic or micro-oxic conditions necessary for nitrogenase derepression depends on the nitrogen status of the cells. When nitrate-grown cultures of *P. boryanum* are transferred to nitrogen-free medium under anoxic or micro-oxic conditions, nitrogenase derepression takes 24–30 h [3,126]. However, 24 h aerobic incubation in nitrogen-free medium, prior to transfer to anoxic or micro-oxic conditions results in derepression of nitrogenase within 2–3 h [18,126,127,169,170]. Sparging with Ar, Ar/ CO_2 , or N_2/CO_2 need not be continuous. Nitrogenase derepression has also been reported in this cyanobacterium under conditions of periodic sparging [3,126].

The molecular mechanism by which ammonium

represses the synthesis of nitrogenase (and other proteins related to the use of nitrogen sources other than ammonium) is well documented in enteric bacteria (see, for example, [8,174]). The P_{II} protein responds to intracellular nitrogen status and interacts with the two-component NtrB/NtrC system in such a way that the protein NtrC becomes phosphorylated under conditions of nitrogen limitation. Phosphorylated NtrC is required for transcription of, among others, the *nif* genes. Transcription also requires a protein RpoN (NtrA) that functions as a sigma factor to RNA polymerase and allows it to recognise Ntr-regulated promoters. Repression of nitrogenase synthesis by ammonium can be explained as follows: in the presence of ammonium, cells become nitrogen-replete, NtrC is not phosphorylated so the *nif* genes are not, therefore, expressed. Other sources of inorganic nitrogen such as nitrate, which is reduced to ammonium by the action of nitrate and nitrite reductases, similarly repress nitrogenase synthesis.

A P_{II} kinase/ P_{II} protein pair has been found in the non-diazotrophic unicellular cyanobacterium *Synechococcus* PCC 7942 [175], in which it functions as a signal sensor/transmitter of the illumination regime and intracellular nitrogen status [176]. However, despite intensive study, no genes corresponding to the *ntr* genes have been found in cyanobacteria. On the other hand, a novel regulatory gene, *ntcA* (previously known as *bifA*), has been identified in both non-heterocystous and heterocystous cyanobacteria [177–182]. The product of this gene, NtcA, is a DNA-binding protein similar to the cAMP-binding catabolite gene activator protein (CAP: also known as the cyclic AMP receptor protein, Crp) that acts as a positive activator of the *E. coli lac* operon (see [183] and references therein). NtcA binds to a palindromic DNA sequence GTAN₈TAC, situated at varying distances upstream from the transcription start site in the promoters of NtcA-regulated genes [182]. Strains of the heterocystous cyanobacterium *Anabaena* PCC 7120 carrying mutations in *ntcA* were unable to use either nitrate or N₂ as a nitrogen source for growth [184,185], hence NtcA may regulate nitrogenase synthesis in cyanobacteria. Several NtcA-regulated genes in cyanobacteria have been implicated in N₂ fixation and heterocyst differentiation. These include at least some *nif* genes, *glnA* (encoding glutamine synthetase), *xisA* (which encodes

an excisase that is involved in the gene rearrangements that accompany heterocyst differentiation) and *glnB* (which encodes cyanoglobin: M. Potts, personal communication; also see Section 6.4, below). However, NtcA may also regulate genes that respond in a more general way to the presence of nitrogenous compounds. Such genes include *rbcL* (encoding the CO₂-fixing enzyme, Rubisco) and the *gor* genes (which encode glutathione reductase, an enzyme that may have a role in protecting nitrogenase against oxidative stress: [182]; see Section 6.4. However, exactly how NtcA responds to intracellular nitrogen status, and whether P_{II} is involved with NtcA, is not yet known.

5.3. Subcellular location

Following the original proposal that the heterocyst was the site of aerobic N₂ fixation in heterocystous cyanobacteria [186], there was some argument about whether vegetative cells also contained active nitrogenase under anoxic or micro-oxic conditions (for a review, see [187]). For example, the ability of certain non-heterocystous, filamentous cyanobacteria to fix N₂ under these conditions demonstrated that possession of heterocysts was not an essential prerequisite for N₂ fixation. However, the immunocytochemical demonstration that nitrogenase was confined to heterocysts of *Anabaena cylindrica* [188] and other free-living and symbiotic cyanobacteria ([189]; see Plate 4d) incubated under either oxic or anoxic conditions, led to the general belief that the heterocyst was always the sole site of N₂ fixation. Studies on the expression of *nifHDK* in individual cells of *Anabaena* 7120 under both oxic and anoxic conditions [190] supported this view. However, the recent discovery that *Anabaena variabilis* possesses two molybdenum nitrogenases, one (Nif-1) that functions either under oxic or anoxic conditions but is confined to heterocysts and one (Nif-2) that functions only under anoxic conditions but is present in both vegetative cells and heterocysts [191,192], has reopened the whole debate. The situation varies among different strains of heterocystous cyanobacteria: *Anabaena* 7120, for example, possesses a heterocyst-specific Nif-1 system but no functional vegetative cell Nif-2 nitrogenase [192]. Nevertheless, in all heterocystous cyanobacteria incubated aerobically, nitrogenase activity is ap-

parently confined to heterocysts while oxygenic photosynthesis occurs in the undifferentiated vegetative cells [7,8,188,189,193].

In contrast, non-heterocystous cyanobacteria express nitrogenase in what appear to be O_2 -evolving vegetative cells. No obviously differentiated cells have been reported in either unicellular or filamentous forms [20]. Meeks et al. (1978) proposed that nitrogenase may be located in only certain cells within individual filaments of cultures of *P. boryanum* grown under anoxic conditions or within the characteristically ensheathed aggregates of aerobically grown *Gloeotheca* [194]. However, subsequent fluorescence studies by Giani and Krumbein (1986) failed to identify any unusual cells within N_2 -fixing filaments of *P. boryanum* PCC 73110 [170]: the same is true of *Gloeotheca* [187]. On the other hand, Giani and Krumbein (1986) suggested that nitrogenase may be subjected to intracellular separation from the photosynthetic apparatus in *P. boryanum* [170]. That this may also be the case in *Gloeotheca* is consistent with, though not proved by, the findings that photosynthesis and N_2 fixation are not closely linked in this organism [187]. For example, atmospheric O_2 is needed to sustain maximum rates of nitrogenase activity even in illuminated cultures of *Gloeotheca* [195], probably because, except at high light intensities, ATP for N_2 fixation is generated by respiratory rather than photosynthetic activity ([196]; see Section 6.3).

Immunochemical studies have recently been conducted on *Plectonema boryanum* PCC 73110 [126], *Oscillatoria limosa* ([124]; Plate 4b), *Lyngbya aestuarii* (which may be the same organism as *O. limosa*: see Section 6.2.2 [37] and *Gloeotheca* PCC 6909 (Plate 4a) for localization of the Fe-protein of nitrogenase and on *P. boryanum* PCC 6306 [123] for localization of the Mo-Fe protein. These studies have shown that in *Plectonema*, *Oscillatoria*, *Lyngbya* and *Gloeotheca*, nitrogenase is expressed in all the cells and within each cell the antigen is distributed uniformly throughout the cell without any preferential association with any subcellular structures. Although these findings tell us nothing about the cellular distribution of nitrogenase activity, they nevertheless argue against the possibility of intercellular or intracellular compartmentation of nitrogenase in these cyanobacteria.

The situation in the marine non-heterocystous cyanobacterium *Trichodesmium* is more complex (Plate 4c). *Trichodesmium* spp. often grow in distinct colonial aggregates and occur in highly oxygenated waters. Early work suggested that N_2 fixation might be confined to the central regions of these colonies [197]. Individual filaments (trichomes) passing through this region were claimed to possess 10–20 lightly pigmented cells that did not fix CO_2 and, like heterocysts, contained a reducing environment [198]. It was therefore proposed that the central region of colonies of *Trichodesmium* might be the preferred site of aerobic N_2 fixation. In this way, *Trichodesmium*, like heterocystous cyanobacteria, might effect a spatial separation between the O_2 -sensitive process of N_2 fixation and oxygenic photosynthesis, though without any obvious cellular differentiation. However, a more detailed reinvestigation of this possibility by Carpenter et al. (1990) showed that the central region of colonies of *T. thiebautii* might well be photosynthetically active [199]. Furthermore, Paerl et al. (1989) found that almost all cells in colonies of *Trichodesmium* contained immunologically identifiable nitrogenase [122]. Even though subsequent detailed immunological studies have clearly demonstrated that relatively few individual cells in colonies of *Trichodesmium* spp. contained nitrogenase, those that did were always randomly distributed within the aggregate of trichomes rather than being confined to a specific central region [107]. A recent survey of a number of colonial *Trichodesmium* species has shown that, on average, only 14% of individual cells in a colony contained nitrogenase, and this was irrespective of sampling location, season or time of day [52]. This means that, in contrast to all other non-heterocystous cyanobacteria tested, a minority of cells in *Trichodesmium* is engaged in N_2 fixation at any one time. It also suggests that differential gene expression occurs in *Trichodesmium*. However, it remains unknown whether nitrogenase is present in all the cells of a few trichomes or in a few cells of rather more, and perhaps all, individual trichomes in a colony of *Trichodesmium*. The latter hypothesis is, however, gaining support because both natural populations of *T. contortum* and the recently established laboratory culture of *Trichodesmium* [61] possess nitrogenase-containing and nitrogenase-free cells within individual trichomes ([51,52]; C. Fredriksson,

H.W. Paerl and B. Bergman, unpublished data). The nitrogenase-containing cells may correspond to a subtly differentiated region of 7–15 rather short cells that appear brighter in the light microscope but which otherwise are structurally indistinguishable from other cells in the trichome [51]. For instance, there is no thickening of the cell walls as is the case with the clearly differentiated heterocysts of heterocystous cyanobacteria.

Whatever the distribution of nitrogenase among individual cells in a colony of *Trichodesmium*, there is an even distribution of the Fe-protein of nitrogenase within the nitrogenase-containing cells themselves ([10,52]; Plate 4c).

The brightness of certain regions within trichomes of *Trichodesmium* was not due to loss of pigments, as suggested previously. Cells that contained nitrogenase also contained the accessory photosynthetic pigments phycocyanin and phycoerythrin, along with the photosynthetic CO₂-fixing enzyme, Rubisco. These cells therefore appear to have the capacity for both photosynthesis and N₂ fixation [200]. This does not, of course, prove that photosynthesis and N₂ fixation are both active within these cells, and Paerl et al. (1995) have shown that certain regions within trichomes of both natural and laboratory populations of *Trichodesmium* showed no ability to fix CO₂ [201]. It remains possible therefore that the limited number of cells that contain nitrogenase within a colony of *Trichodesmium* are also photosynthetically inactive. If so, this would contrast markedly with the situation in *Plectonema*, *Oscillatoria*, *Lyngbya*, and *Gloeotheca*, but would resemble that in heterocystous cyanobacteria.

5.4. Intracellular concentrations of nitrogenase

A comparison of the intracellular concentration of the Fe-protein of nitrogenase in heterocysts of *Anabaena cylindrica* with that in vegetative cells of *Oscillatoria limosa*, based on measurements of the density of immunogold labelling per unit cell area under conditions where only the presence of antigen limited the degree of labelling, suggested that there were similar levels of nitrogenase antigen in both cell types [124]. Similar intensities of labelling have also been found in *Plectonema boryanum* [126], *Trichodesmium* [107,122] and *Gloeotheca* PCC 6909 (Plate 4a). Ex-

cept in *Trichodesmium*, nitrogenase was present in all of the cells of non-heterocystous cyanobacteria but only in the heterocysts of heterocystous cyanobacteria. Since heterocysts form approximately 5–10% of the total cell population, it appears that a culture of a non-heterocystous cyanobacterium contains 10–20 times the amount of nitrogenase present in an equivalent culture of a heterocystous cyanobacterium. If nitrogenase-containing cells represent 14% of the total population of cells in cultures of *Trichodesmium* [52], then other non-heterocystous cyanobacteria would contain about six times the amount of nitrogenase of an equivalent culture of this organism. However, the maximum rates of N₂ fixation reported in the literature for non-heterocystous cyanobacteria are either identical to, or only marginally higher, than the rates reported for heterocystous cyanobacteria (see Table 3). Antibodies raised against nitrogenase do not differentiate between active and inactive enzyme, so it is possible that most of the nitrogenase detected by immunolabelling in non-heterocystous cyanobacteria is inactive. However, whether or not this is actually the case, it seems that heterocysts, and the nitrogenase-containing cells of *Trichodesmium*, are much more efficient than the vegetative cells of non-heterocystous cyanobacteria in carrying out N₂ fixation.

Why this should be is not clear, but it is likely that heterocysts and, possibly, the N₂-fixing cells of *Trichodesmium*, provide a better O₂-depleted environment for nitrogenase than the vegetative cells of most non-heterocystous cyanobacteria. Consistent with this, many non-heterocystous cyanobacteria cannot sustain an active nitrogenase under the double stress of oxygenic photosynthesis and aerobiosis, so they fix N₂ only when incubated under micro-oxic or anoxic conditions, with photosynthetic O₂ production inhibited by DCMU. Even in those strains that can fix N₂ aerobically, inhibition of photosynthesis by DCMU can often lead to a transient stimulation of nitrogenase activity. This has been observed in *Synechococcus* (*Cyanothece*) RF-1 [202], *Trichodesmium* [12], *Oscillatoria limosa* [42], *Oscillatoria* UCSB8 [46] and UCSB25 (Gallon, unpublished), but not in *Synechococcus* (*Cyanothece*) BG 43522 [26]. The situation in *Gloeotheca* is less clear, there are some reports that N₂ fixation is not stimulated by addition of DCMU [12,187,203] and others where

a clear stimulation was seen [204]. However, the extent to which DCMU might stimulate N_2 fixation would depend upon the extent to which photosynthetic O_2 evolution was coexisting with N_2 fixation in individual cells of *Gloeotheca*. This can vary (see Section 6.2.1).

Another factor that might explain the apparently poor efficiency of N_2 fixation in vegetative cells of non-heterocystous cyanobacteria relative to that in heterocysts might be competition for ATP and reductant. Heterocysts do not grow or divide and their metabolism is primarily directed to supporting N_2 fixation. Their metabolism depends upon the supply of carbohydrate from the two adjacent vegetative cells [205] but the ATP and reductant generated by catabolism of this carbohydrate can be largely directed towards N_2 fixation. A similar argument could be made for the N_2 -fixing cells of *Trichodesmium*, which may not photosynthesize [68], even though they contain phycobiliproteins and Rubisco [51,200]. In contrast, as well as supporting N_2 fixation, the vegetative cells of non-heterocystous cyanobacteria have to generate photosynthate (by CO_2 fixation, a process that also requires ATP and reductant), to scavenge additional O_2 generated during photosynthesis (which can consume reductant) and to provide energy for cell growth and multiplication. It would be of interest, though difficult, to make a comparative study of the abilities of heterocysts, N_2 -fixing cells of *Trichodesmium* and other N_2 -fixing non-heterocystous cyanobacteria to supply reductant and ATP for N_2 fixation and also to scavenge O_2 .

6. Nitrogenase and metabolism

6.1. Adaptation to diazotrophy

When given N_2 as sole nitrogen source, heterocystous cyanobacteria undergo profound morphological and physiological changes, culminating in the development of heterocysts [160,206]. In addition to the appearance of nitrogenase activity, development of heterocysts from vegetative cells results in increased intracellular concentrations of glutamine synthetase and increased respiratory activity, but loss of Rubisco, nitrate reductase and photosynthetic O_2 evolu-

tion [131,205,207–209]. Often, but not always, the accessory photosynthetic phycobiliprotein pigments are lost during heterocyst differentiation. An equivalent study of the metabolic changes that occur in non-heterocystous cyanobacteria during transfer from growth on, say, ammonium to growth on N_2 is lacking. However, there are a number of reports that one or more of the metabolic changes observed during heterocyst differentiation also occur in non-heterocystous cyanobacteria when they adapt to growth under N_2 -fixing conditions. These include a decline in photosynthetic activity (O_2 evolution; CO_2 fixation), an increase in respiratory activity (O_2 uptake; amounts and activity of cytochrome oxidase) and a disappearance of nitrate reductase [20,46,68,126,209–212]. An increase in intracellular glutamine synthetase has also been noted in *Plectonema boryanum* [126] and in the nitrogenase-containing cells of *Trichodesmium* [213], but this increase was much smaller than that observed in heterocystous cyanobacteria during heterocyst differentiation. Unlike heterocysts, the N_2 -fixing vegetative cells of non-heterocystous cyanobacteria retain Rubisco and phycobiliproteins.

It should be emphasized that the metabolic changes that occur during heterocyst differentiation are permanent, whilst those noted during development of N_2 fixation in non-heterocystous cyanobacteria can be reversed following resupply of a nitrogen source such as ammonium. Heterocysts are terminally differentiated cells, specialized in N_2 fixation but lacking the ability either to grow or to divide. They do not dedifferentiate into vegetative cells following addition of ammonium, though further differentiation of heterocysts is blocked by such treatment and the proportion of preexisting heterocysts declines as a result of continued division of vegetative cells. In contrast, N_2 -fixing vegetative cells of non-heterocystous cyanobacteria continue to grow and divide and rapidly revert to non-diazotrophic metabolism following addition of ammonium. Nitrogenase-containing cells of *Trichodesmium* may divide (C. Fredriksson and B. Bergman, unpublished), which indicates that these cells are not terminally differentiated. Indeed, it is possible that these cells may undergo a natural regression during which they lose the ability to fix N_2 (see, for example, [11]), implying that expression of the *nif* genes may

be regulated by the cell cycle. Such behaviour would explain the observation that the proportion of nitrogenase-containing cells is remarkably constant (less than 20%) in all colonies and free-living trichomes of *Trichodesmium* examined.

Nitrate has a similar effect to ammonium in repressing nitrogenase synthesis in cyanobacteria (see, for example, [180]). However, it is often more difficult completely to eliminate nitrogenase activity in cultures growing on nitrate as nitrogen source than in cultures growing on ammonium [187]. Nevertheless, most studies compare cultures grown in nitrogen-free medium (N_2 -fixing) with cultures grown in medium containing nitrate. That the latter are not fixing N_2 cannot be taken for granted and should always be carefully checked. It is usually much safer to compare cultures grown in the absence and presence of ammonium. In a detailed study of the interactions between different nitrogen sources in *Gloeotheca*, Cheng et al. (1995) showed that the ability to assimilate nitrate was present in N_2 -fixing cultures but not in ammonium-grown cultures [214]. In this respect, *Gloeotheca* differs from other N_2 -fixing cyanobacteria [180], including *Plectonema boryanum* [126], in that the presence of nitrate is not strictly required in order to induce its own assimilation system.

6.2. Patterns of diazotrophic growth

6.2.1. Laboratory cultures

In non-heterocystous cyanobacteria, as in heterocystous forms, ammonium derived from N_2 fixation is assimilated by the glutamine synthetase-glutamate synthase (GOGAT) pathway [193,194,215]. A number of non-heterocystous cyanobacteria that can fix N_2 aerobically can be grown in the laboratory with N_2 as the sole nitrogen source. These strains can be cultivated either under continuous illumination or under a regime of alternating light and darkness (see, for example, [6,8,20]). In many cases, N_2 fixation and photosynthesis in these strains show opposing cyclic fluctuations, but growth seems to be continuous except in *Synechococcus* (*Cyanothece*) strains BG 43511 and 43522. In these cyanobacteria cell division exhibits a cyclic pattern, coinciding with the peak of oxygenic photosynthesis [23,26]. On the other hand, fluctuations in N_2 fixation and photo-

synthesis are also seen in non-growing cultures of *Synechococcus* (*Cyanothece*) BG 43511 [216].

Although many non-heterocystous cyanobacteria have been shown to fix N_2 under micro-oxic or anoxic conditions, it is not certain how many of them can actually grow diazotrophically. Laboratory studies have been largely restricted to a single organism, *Plectonema boryanum*. Rogerson (1980) showed that this cyanobacterium could grow photoheterotrophically on N_2 when illuminated continuously under anoxic conditions in the presence of exogenously supplied fructose and a constant concentration of sulphide (which inhibited photosynthetic O_2 evolution but acted as an electron donor to anoxygenic photosynthesis) [172]. Subsequently, Pearson and Howsley (1980) reported concomitant photoautotrophic growth and N_2 fixation in cultures of the same strain grown under micro-oxic conditions [217]. Giani and Krumbein (1986) [170] could not reproduce these results under the high light intensities used by Pearson and Howsley (1980) [217] but found that, when continuously sparged with O_2 -depleted air at light intensities below 500 lx (approximately $6 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), *P. boryanum* grew exponentially under photoautotrophic conditions using N_2 as sole nitrogen source. Furthermore, growth rates were comparable to those in cultures using nitrate as nitrogen source. Moreover, when *P. boryanum* was grown in nitrogen-free medium and sparged with N_2/CO_2 (95:5 v/v) for 15 min at 6 h intervals rather than continuously, it exhibited a cyclic growth pattern rather like that of *Synechococcus* BG 43511 [126,218,219]. Such cultures showed periodic peaks of nitrogenase activity which coincided with troughs in the rate of photosynthetic O_2 evolution [126,218]. Cell growth, measured as an increase in the protein content of the culture, followed each peak of N_2 fixation. This pattern may have its origin in periodic anoxic conditions created by fluctuations in oxygenic photosynthesis, and it has been shown that a stimulation of Photosystem I coupled with a corresponding inhibition of the O_2 -evolving Photosystem II accompanies rapid diazotrophic growth of *P. boryanum* [220]. Significantly, these findings suggest that *P. boryanum* could grow photoautotrophically using N_2 as a sole nitrogen source in appropriate micro-oxic natural habitats.

When growing under alternating light and dark-

ness, cultures of most non-heterocystous cyanobacteria that can fix N_2 aerobically do so during the dark phase, thereby achieving a temporal separation of N_2 fixation and oxygenic photosynthesis (see [8,31,221]; Fig. 2a). The molecular mechanism underpinning this behaviour may, however, vary from organism to organism. In *Oscillatoria limosa*, for example, the disappearance of nitrogenase activity during the light phase coincides with the appearance of the modified form of the Fe-protein of nitrogenase [222]. Nitrogenase is present in the cells throughout a 24 h cycle of alternating light and darkness, though induction of nitrogenase activity at the end of each light period depends on protein synthesis [223]. In contrast, in *Gloeotheca*, nitrogenase undergoes a diurnal cycle of synthesis and degradation [60], with modification of the Fe-protein relegated to a secondary role. Nitrogenase disappears from cells of *Gloeotheca* at the end of each dark phase and is resynthesized at the end of each light phase [60,210]. Synthesis of nitrogenase is regulated by the availability of ATP, reductant and carbon skeletons, which arise from the breakdown of endogenous glucan reserves that accumulate during each light period [8,210]. Breakdown of the enzyme is catalyzed by the action of a proteolytic system that appears to be highly specific for *Gloeotheca* nitrogenase [224]. *Synechococcus* (*Cyanothece*) RF-1 shows a similar pattern of nitrogenase synthesis and degradation [133]. In this cyanobacterium, 'modified' Fe-protein may be targeted for degradation [133], but there is no evidence that this is so in *Gloeotheca*, in which both forms of the Fe-protein disappear together [60]. However, since degradation is very rapid, especially once cells return to the light phase, it is not possible to determine whether disappearance of 'unmodified' protein is due to direct degradation or to modification followed by degradation of 'modified' Fe-protein. On the other hand, 'unmodified' Fe-protein ($M_r=38\ 500$; Fig. 1a) is susceptible to proteolytic degradation by extracts of *Gloeotheca* [224], whilst both 'modified' and 'unmodified' Fe-protein were similarly degraded in extracts of heterocysts of *Anabaena variabilis* [225].

In *Oscillatoria* strain UCSB8, the pattern of nitrogenase activity seen during photoautotrophic growth under alternating 12 h light and 12 h darkness is also seen in cultures growing photoheterotrophically with

glucose as carbon source [46]. Under these conditions, it is extremely unlikely that the pattern of N_2 fixation is imposed by fluctuations in available carbon, as occurs in *Gloeotheca*. Fluctuations in intracellular nitrogen reserves rather than glucan may therefore regulate nitrogenase activity and, perhaps, synthesis in *Oscillatoria* UCSB8 [46]. In *Synechococcus* (*Cyanothece*) strain BG 43511, the status of both intracellular carbon and intracellular nitrogen reserves may regulate cell cycle-linked and cell cycle-independent fluctuations in N_2 fixation [216].

Not all non-heterocystous cyanobacteria fix N_2 exclusively during the dark phase of a cycle of alternating light and darkness. *Gloeotheca*, for example, grown under anoxic conditions [6,20,60] or with a continuous supply of sulphate [226,227] fixes N_2 during the light phase of such a cycle. *Synechococcus* (*Cyanothece*) sp. BG 43511 behaves similarly [228]. Clearly, the 'normal' (in the sense that it also occurs in natural populations [92]) pattern of N_2 fixation in cultures of *Gloeotheca* grown under alternating light and darkness can be extensively modified by the external environment. However, both natural populations and laboratory cultures of the marine aerotolerant diazotroph, *Trichodesmium* fix N_2 during the light phase of a cycle of alternating light and darkness (for details, see [11,12]; Fig. 2b). Two other marine, aerotolerant diazotrophs may behave similarly. Laboratory cultures of the unicellular cyanobacterium *Synechococcus* (*Cyanothece*) strain SF1 [27] and natural populations of the filamentous organism *Lyngbya majuscula* [36], were unable to fix N_2 in the dark.

In *Trichodesmium*, nitrogenase is confined to a few cells that probably do not photosynthesize and may not evolve O_2 . This may be significant in explaining the lack of temporal separation between photosynthetic O_2 evolution and N_2 fixation in this organism, since heterocystous cyanobacteria, which also perform a spatial separation of N_2 fixation and oxygenic photosynthesis, fix N_2 exclusively during the light phase of a cycle of alternating light and darkness [44]. In at least some laboratory cultures of *Trichodesmium*, immunologically detectable Fe-protein persists throughout an alternating cycle of light and darkness, with 'unmodified' (presumed active) Fe-protein present only during the light phase

[139]. This is the exact opposite of the behaviour seen in *Oscillatoria limosa* [222].

Despite the apparent temporal separation between oxygenic photosynthesis and N_2 fixation observed in cultures of the microaerophilic cyanobacterium *Plectonema boryanum*, there have been no studies on cultures growing under alternating light and darkness. Such cultures cannot grow aerobically on N_2 as a nitrogen source ([40]; J.R. Gallon, unpublished observations) and the report that N_2 fixation in *P. boryanum* requires light [219,220] implies that, when grown in the absence of O_2 under alternating light and darkness, this cyanobacterium would fix N_2 during the period of illumination. In this respect it would resemble cultures of *Gloeotheca* grown under anoxic conditions [20,60].

6.2.2. Natural populations

There have been few field investigations on the pattern of N_2 fixation by non-heterocystous cyanobacteria. In a single study, natural populations of *Gloeotheca*, growing on the wall of a limestone cave, were shown to exhibit a diurnal pattern of N_2 fixation virtually identical to that seen in aerobically incubated laboratory batch cultures [92]. However, in a study of N_2 fixation and photosynthesis in North Sea microbial mats, Villbrandt et al. (1990) showed that diel variations of photosynthesis, N_2 fixation and O_2 concentrations in the mat depended strongly on the type of mat, the season and the prevailing weather conditions [114]. Nevertheless, in all cases, activity was either low or completely absent during daytime. Fluctuations in O_2 concentration were an important factor controlling nitrogenase activity in the mat [114]. Microbial mats that contained low biomass (freshly colonized sediment) showed low rates of respiratory O_2 consumption during the night. The cyanobacterial mat remained oxic throughout the night although the concentration of O_2 was below air saturation. During the day, these mats were often supersaturated with O_2 , even though the total rate of photosynthesis in the mat was low. On the other hand, the specific rate of photosynthesis (based on the chlorophyll *a* content) was high. Such conditions are not very suitable for non-heterocystous N_2 -fixing cyanobacteria which possess a limited capacity for protecting nitrogenase from inhibition by O_2 . On the other hand, during the night,

when photosynthetic O_2 evolution had ceased and the prevailing concentration of O_2 had declined, N_2 fixation could take place, supported by aerobic respiration. These early stages of microbial mat development often showed a characteristic diel variation of N_2 fixation similar to that seen in laboratory cultures, with nitrogenase activity confined exclusively to the dark period.

In more mature mats, an interesting observation was that nitrogenase activity peaked at sunrise. This may be explained by the combination of low light intensity (and a resulting low rate of photosynthetic O_2 evolution) coupled with a low endogenous concentration of O_2 . Nevertheless, irradiation provided enough energy to support nitrogenase activity. So far, however, this pattern of behaviour has not been confirmed in laboratory cultures. Culture experiments in which sunrise and sunset are modelled are rare and have not been used for studies of N_2 fixation in cyanobacteria. The importance of such experiments has been stressed by Stal and Heyer (1987) [229]. These authors cultured the non-heterocystous cyanobacterium *O. limosa* diazotrophically under an alternating light/dark cycle, applying anoxic conditions during the dark period. This regime was chosen to model the situation in an established microbial mat. In contrast with mats in an early stage of development, established mats contained high biomass and rapidly turned anoxic when photosynthesis ceased. Cultures of *O. limosa*, grown under a light/dark cycle and given anoxic conditions during the dark period, showed nitrogenase activity mainly at the transitions from dark to light and from light to dark. The former peak (sunrise) was the more pronounced. At the transition from light to dark (sunset), nitrogenase was induced but after a short time activity strongly declined because of the absence of O_2 . *O. limosa* is capable of fermentation of its storage carbohydrate [230] but the energy that is gained from this metabolism is not sufficient to cover the demands of nitrogenase activity. When illumination commenced, a huge peak of nitrogenase activity was seen. N_2 fixation was driven by light energy in an, initially, low O_2 environment. However, within a short time nitrogenase was inactivated by photosynthetically evolved O_2 . This type of diel pattern of N_2 fixation mimics that in established North Sea microbial mats [88,114]. Similar results were ob-

tained with cultures of another strain of *Oscillatoria*, UCSB8, [92], even though in its natural salt marsh habitat this organism fixed N_2 exclusively at night.

Natural populations of the planktic, marine cyanobacterium *Trichodesmium* fix N_2 exclusively during the day. This pattern reflects the synthesis of nitrogenase each morning and degradation each evening [52,65], though superimposed upon this is the modification of the Fe-protein of nitrogenase. At the peak of nitrogenase activity, around noon, the Fe-protein is almost exclusively in its 'unmodified' form. 'Modified' Fe-protein appears subsequently as nitrogenase activity declines during the later part of the day [132].

It is tempting to interpret current data on N_2 fixation by natural populations of non-heterocystous cyanobacteria solely in terms of the temporal separation of N_2 fixation and oxygenic photosynthesis. After all, in many systems, N_2 fixation is confined to the night, whilst cultures of most non-heterocystous cyanobacteria grown in the laboratory under alternating light/dark cycles show the same behaviour. This does not mean, however, that a temporal separation between N_2 fixation and photosynthetic O_2 evolution is the critical mechanism that allows non-heterocystous cyanobacteria to fix N_2 . Several observations do not fit this model. Firstly, in certain microbial mats, diel variations of N_2 fixation are less pronounced and they show considerable activity during daytime illumination [89,93]. Secondly, natural populations of *Trichodesmium*, and possibly some other non-heterocystous cyanobacteria, fix N_2 exclusively during the day [65]. Thirdly, laboratory cultures of *Gloeotheca* can be grown under conditions where they fix N_2 predominantly during the light period of an alternating light/dark cycle [20,60,226,227]. It should also not be forgotten that cultures of non-heterocystous cyanobacteria can fix N_2 in the laboratory under continuous illumination.

Among those microbial mats that do not show the distinct daily variations of N_2 fixation that characterize North sea microbial mats [114] are the mats of Shackleford and Bird Shoal Banks, North Carolina, USA, [89,231] and of Guerrero Negro, Baja California, Mexico (L.J. Stal, unpublished observations). These mats show considerable nitrogenase activity during the daytime. The North Sea, North Carolina and Baja California mats are all built by the same

morphotype of cyanobacterium (*Oscillatoria limosa* / *Lyngbya aestuarii*). This cyanobacterium has been isolated from all of these mats and has been shown to be capable of diazotrophic growth under fully photoautotrophic conditions [37,42]. The morphologies of *O. limosa* and *L. aestuarii* are very similar and they probably represent a single species. This idea is also supported by the fact that the behaviour of nitrogenase in all of these isolates is the same. They are capable of diazotrophic growth under photoautotrophic conditions in continuous light. Nevertheless, when alternating light/dark cycles are applied, nitrogenase activity is confined to the dark period [37,223]. Such behaviour is in contradiction with the field situation in the mats of North Carolina and Baja California, where the differences between day and nighttime nitrogenase activity are less pronounced. The reason for this discrepancy is not clear but several explanations are possible. For example, it cannot be excluded that heterotrophic bacteria contribute to the observed nitrogenase activity in the natural environment. In support of this, Pearl and Prufert (1987) suggested that highly reduced microzones are the sites of N_2 fixation [232], whilst stimulation of nitrogenase activity by addition of organic compounds hints of an involvement of chemotrophic bacteria in this process. However, a systematic study (B. Bebout et al., personal communication) on mats of Bird Shoal, North Carolina, has convincingly demonstrated that cyanobacteria were the predominant N_2 -fixing agents. This has confirmed the earlier experiments of Stal et al. (1984) [88]. In these mats maximum specific activities of nitrogenase corresponded well with maximum rates observed in cultures of *O. limosa* [114].

A spatial separation of N_2 fixation and oxygenic photosynthesis within the microbial mat would explain the presence of activity during the daytime. Light is strongly attenuated in the mat but the extent of attenuation is wavelength dependent [81,233,234]. Far red light penetrates the mat better than the rest of the visible spectrum. This would result in a higher activity of the anoxygenic Photosystem I relative to that of the oxygenic Photosystem II and, therefore, to anoxic or virtually anoxic conditions. In culture, *O. limosa* shows high nitrogenase activities when irradiated with far red light of 700 nm under anoxic conditions [235]. Furthermore, specific activities of

nitrogenase in North Sea microbial mats (on the basis of chlorophyll *a* content) increased with depth [88]. Similar observations were made by Pearson et al. (1979) for a mat of *Microcoleus* (*Symploca*) *chthonoplastes* [38]. One possibility is that the cyanobacteria in the deeper layers carry out anoxygenic photosynthesis [236]. However, this capacity is by no means universal among cyanobacteria and it is uncertain whether cyanobacteria capable of anoxygenic photosynthesis actually occur in these microbial mats. For instance, *M. chthonoplastes* is capable of anoxygenic photosynthesis but has a requirement for O₂ [237]. *O. limosa* resists considerable levels of sulphide, and is even able to oxidize this compound, but cannot perform anoxygenic photosynthesis [238]. In addition, in many coastal microbial mats with high nitrogenase activity, the concentration of free sulphide is usually low. Moreover, cyanobacteria are most likely unable to compete effectively for sulphide with anoxygenic phototrophs [239]. The other possibility is that the cyanobacteria move up and down the mat according to their nutritional status. It is well documented that cyanobacteria may move up and down in a microbial mat as a result of phototactic and/or chemotactic responses [240] but whether such movements could be controlled by the nitrogen status of the organisms needs to be demonstrated.

In addition to spatial separation of O₂ evolution and N₂ fixation in different regions within cyanobacterial mats, there is evidence that *Lyngbya aestuarii* may confine active nitrogenase to photosynthetically inactive terminal regions of individual filaments of this cyanobacterium [37].

6.3. ATP and reductant

N₂ fixation requires ATP and a powerful reductant. Although the supply of ATP and reductant to nitrogenase has not specifically been studied in non-heterocystous cyanobacteria except for *Gloeothoece* [20,187,196], certain general conclusions can be drawn from the conditions under which these organisms fix N₂. Among strains that fix N₂ only under anoxic or micro-oxic conditions, sustained N₂ fixation and diazotrophic growth have been reported only in *Plectonema boryanum* [126,170,172,217]. At low light intensities this cyanobacterium simulta-

neously performs photosynthesis and N₂ fixation [170] and, under these conditions, reductant and/or ATP are probably provided directly by photosynthesis. However, at higher light intensities, photosynthetic O₂ evolution, and therefore non-cyclic generation of ATP and reductant, was low during the period of maximum N₂ fixation [169]. Supply of ATP and reductant under these conditions may therefore come, at least in part, from catabolism of endogenous carbon reserves. *P. boryanum* can also use exogenously supplied carbon sources, catabolism of which may supply ATP and reductant for nitrogenase activity [172,173]. Addition of glycollate to cultures of *P. boryanum* doubles nitrogenase activity [220]. The catabolic pathway of endogenous or exogenously supplied sugars is, however, uncertain. It is also not known whether Photosystem I-mediated cyclic photophosphorylation can occur during the period of active N₂ fixation. This process could provide at least some of the ATP, though none of the reductant, needed for N₂ fixation. As in heterocystous cyanobacteria [241], the proton motive force may be involved in supporting nitrogenase activity in *P. boryanum*, possibly by facilitating the thermodynamically unfavourable reduction of ferredoxin (needed for N₂ fixation) at the expense of NADPH [242]. Alternatively, NADH could reduce ferredoxin in a Photosystem I-mediated process [243,244]. *P. boryanum* contains a ferredoxin (FdxH) that is structurally very similar to a ferredoxin found in heterocysts [245]. This ferredoxin is expressed only under anoxic, nitrogenase-inducing conditions and is an effective electron donor to nitrogenase.

In strains of aerotolerant N₂-fixing cyanobacteria that simultaneously photosynthesize and fix N₂, photosynthesis may, in theory, directly supply ATP and reductant for N₂ fixation, assuming that these processes occur within a single cell type. However, it remains to be demonstrated whether the twin criteria of simultaneous activity and a single location for both photosynthesis and N₂ fixation can be met. Even under continuous illumination, many non-heterocystous cyanobacteria temporally separate maximum rates of N₂ fixation from maximum rates of photosynthetic O₂ evolution. It must, however, be remembered that a low rate of measured O₂ production does not necessarily mean that photosynthetic electron transport is low: photoevolved O₂ could be

reassimilated by respiratory activity and there is evidence that, in many non-heterocystous cyanobacteria, fluctuations in respiratory activity parallel those in N_2 fixation. Moreover, cyclic electron flow around Photosystem I would generate ATP that could be used for N_2 fixation without concomitant O_2 production, whilst an input of electrons from a donor other than water could allow photosynthetic production of both reductant and ATP with no O_2 evolution. However, attempts to demonstrate a direct role for photosynthesis in the provision of ATP or reductant in *Gloeotheca* growing under continuous illumination proved unsuccessful and it was concluded that N_2 fixation, among other anabolic processes, was sustained by non-specific pools of ATP and reductant [187]. These pools could be replenished in the dark by catabolism of, in particular, glucan but, in cultures growing under constant illumination, also by photosynthesis. A much less direct role for photosynthesis in providing ATP and reductant for N_2 fixation was therefore envisaged. Maryan et al. (1986), on the basis of the effects of added KCN on N_2 fixation, respiration and intracellular ATP and ADP in illuminated cultures of *Gloeotheca*, concluded that respiratory metabolism was the main source of energy for N_2 fixation even in the light though, at high light intensities, cyclic photophosphorylation may also provide ATP ([196]; also see [246]).

In non-heterocystous cyanobacteria such as *Trichodesmium*, *Lyngbya majuscula* and *Synechococcus* (*Cyanothece*) SF1, which may fix N_2 during the light phase of a cycle of alternating light and darkness, photosynthesis could directly provide ATP and reductant for N_2 fixation. Nothing is known about the spatial distribution of photosynthesis and N_2 fixation in either *Synechococcus* SF1 or *Lyngbya majuscula*. However, in *Trichodesmium* it is clear that components of the photosynthetic apparatus (phycobiliproteins and Rubisco) coexist with nitrogenase in the same cells [51,200]. This does not, of course, mean that the entire photosynthetic system is present and/or functional in these cells and the observation that the putative N_2 -fixing cells have a reducing environment and a low rate of CO_2 fixation [68,197,198] supports, at first sight, the view that they do not produce O_2 . However, on a colony basis, *Trichodesmium* exhibits a high rate of respiration

which fluctuates in parallel with the diurnal cycle of N_2 fixation [50,247–249]. This compensates any photosynthetic O_2 evolution to the extent that N_2 -fixing colonies receiving less than 150–200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ would exhibit net O_2 consumption rather than evolution [249]. Part of this O_2 consumption may be light-dependent: a consequence of the Mehler reaction [248]. O_2 scavenging through respiration may be particularly efficient in N_2 -fixing cells, which contain higher concentrations of cytochrome oxidase than do cells lacking nitrogenase [212]. It cannot be excluded, therefore, that N_2 fixation and a cryptic oxygenic photosynthesis coexist in a single cell type in *Trichodesmium* and, as a consequence, that photosynthesis may directly supply ATP and reductant for N_2 fixation. On the other hand, since DCMU stimulated N_2 fixation when added to *Trichodesmium* [12], it seems likely not only that some photosynthetically produced O_2 was inhibiting nitrogenase but also that alternative sources of reductant are available in N_2 -fixing cells of *Trichodesmium*.

Cultures of *Gloeotheca*, fixing N_2 during the dark phase of a cycle of alternating light and darkness, generate the necessary ATP and reductant through glucan catabolism [210]. Similarly, in *Cyanothece* spp, degradation of carbohydrate (probably glycogen) granules supports N_2 fixation in cultures growing under alternating light and darkness [221]. In *Synechococcus* (*Cyanothece*) strain BG 43511, degradation of carbohydrate coincided with the period of N_2 fixation [228]. Moreover, addition of glucose or pyruvate could restore nitrogenase activity to cultures whose intracellular carbon reserves were expended [250]. Glucan degradation probably occurs via the oxidative pentose phosphate pathway in these cyanobacteria, as is certainly the case in photoheterotrophically growing cultures of *Oscillatoria* strain UCSB8 [46]. As in heterocystous cyanobacteria (see for example, [244,251]), NADPH generated by the initial reactions of the oxidative pentose phosphate pathway (catalysed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and/or the Entner-Doudoroff pathway (glucose-6-phosphate dehydrogenase) may supply reductant to nitrogenase. This would occur *via* reduction of ferredoxin, the immediate electron donor to nitrogenase. Alternatively, non-photosynthetic sources such as

NADPH-dependent isocitrate dehydrogenase [252] and pyruvate:Fd oxidoreductase [253] could also generate reduced ferredoxin in *Gloeotheca*. The most abundant ferredoxin in *Gloeotheca* has a mid point redox potential (E_m) close to -340 mV, a value considerably less negative than that of other ferredoxins (-390 to -425 mV; see [20]). Such a ferredoxin would be comparatively easier to reduce by NADPH ($E_m = -320$ mV), but electron donation to nitrogenase ($E_m = -460$ mV) would be thermodynamically unfavourable [20,164]. However, this step in *Gloeotheca* may be facilitated by the proton motive force, in a manner similar to the reported reduction of ferredoxin by NADPH in *P. boryanum* [242]. The stimulation of nitrogenase activity in *Gloeotheca*, observed in the presence of 2,4-dinitrophenol and shown to be independent of any effect on the intracellular ratio of ADP to ATP [20], may be related to an effect of membrane electrogenesis (probably the membrane potential, $\Delta\psi$) on the provision of reductant for N_2 fixation.

On the other hand, in cultures of *Gloeotheca* that were fixing N_2 only during the light phase of a light/dark cycle (for example when grown either under anoxic conditions [20] or in aerobic continuous culture [226]), photosynthesis may be the direct source of ATP and reductant. However, as with cultures grown under continuous illumination, any such direct link has not been proved, though the observation that the intracellular concentration of glucan did not fluctuate greatly in cultures of *Gloeotheca* growing under alternating light and darkness in continuous culture [226] is consistent with the idea that photosynthesis may directly supply nitrogenase with ATP and reductant under these conditions. On the other hand, *Synechococcus* (*Cyanothece*) strain BG 43511, like *Gloeotheca*, fixes N_2 during the light period when grown under alternating light and darkness in the absence of O_2 . Nevertheless, in cultures grown under these conditions, N_2 fixation coincided with a net degradation of carbohydrate [228].

Only in *Oscillatoria limosa* has N_2 fixation been observed in the dark under strict anoxic conditions. Here, reductant and ATP may be generated by fermentation [229].

6.4. N_2 fixation and O_2

A major factor that has attracted scientists to the study of N_2 fixation in non-heterocystous cyanobacteria has been the apparent paradox of organisms that simultaneously fix N_2 and photoevolve O_2 . In practice, however, it appears unlikely that this situation ever actually occurs: these organisms separate the two incompatible processes of oxygenic photosynthesis and N_2 fixation either in time or in space. Currently, there is no evidence that any known N_2 -fixing non-heterocystous cyanobacterium catalyses oxygenic photosynthesis and N_2 fixation simultaneously within a single cell. That does not mean, however, that this situation does not occur in one or more strain incubated in the laboratory under continuous illumination or in *Trichodesmium*, *Synechococcus* SF1, *Lyngbya majuscula*, *Plectonema boryanum*, or anoxic or sulphur-replete cultures of *Gloeotheca* incubated under alternating light and darkness. However, it has not categorically been demonstrated that this is so.

Nevertheless, it is reasonable to assume that non-heterocystous cyanobacteria possess particularly efficient mechanisms for limiting O_2 damage to nitrogenase. A wide variety of strategies are known by which diazotrophs, including non-heterocystous cyanobacteria, protect nitrogenase from inactivation by O_2 and it is not intended to do more than summarise these here. For more details see the reviews by Fay (1992) [7] and Gallon (1992) [8].

There is probably a gradation of tolerance to O_2 among non-heterocystous N_2 -fixing cyanobacteria. Some strains can fix N_2 aerobically and can therefore protect their nitrogenase from inactivation by both photosynthetically generated O_2 and the 0.21 atm of O_2 present in air. Those that cannot would, by definition, fix N_2 only when the prevailing concentration of O_2 was low, or even zero. However, among these strains, there will almost certainly be variations in the maximum concentration of O_2 to which they can be exposed without inhibiting N_2 fixation completely. For example, van der Oost et al. (1987) showed that, among unicellular cyanobacteria, N_2 fixation by *Synechococcus* (*Cyanothece*) PCC 7425 was more sensitive to O_2 than N_2 fixation in *Cyanothece* PCC 7822 and both were more sensitive than N_2 fixation in the aerotolerant *Gloeotheca* PCC 6909

[203]. Similarly, *Synechococcus* (*Cyanothece*) strain RF-1 is less tolerant of O_2 than *Gloeotheca*, since cultures of this cyanobacterium grow diazotrophically under air only in standing culture [28,254]. However, even in aerotolerant diazotrophic cyanobacteria, the mechanisms that function to protect nitrogenase from inactivation by O_2 may be saturated at concentrations of O_2 greater than those in air.

Mechanisms that non-heterocystous cyanobacteria use in order to minimize the deleterious effects of O_2 on N_2 fixation include behavioural strategies, physical barriers and metabolic strategies.

Behavioural strategies include avoidance of O_2 , a strategy that is practised by those cyanobacteria that fix N_2 under anoxic or micro-oxic conditions. They are found in environments where the prevailing concentration of O_2 is low, as well as in specialized environments like sulphur springs and waterlogged soil. N_2 fixation by non-heterocystous cyanobacteria often occurs in microbial aggregates where anoxic microenvironments occur (see Section 6.2.2). The temporal separation of photosynthesis and N_2 fixation in most non-heterocystous cyanobacteria exposed to alternating light and darkness may also be considered as a behavioural strategy for limiting O_2 inactivation of nitrogenase.

The best known example of a barrier to N_2 fixation is found in heterocystous cyanobacteria. The heterocyst cell wall is relatively impermeable to atmospheric gases [255]. N_2 is four times as abundant as O_2 in the atmosphere, so sufficient N_2 can enter the heterocyst to sustain measured rates of N_2 fixation. However, any O_2 that penetrates this barrier is consumed by respiratory metabolism. In this way, a virtually anoxic environment surrounds nitrogenase. In theory, the same situation may apply in non-heterocystous cyanobacteria that effect a spatial separation between N_2 fixation and oxygenic photosynthesis but, as explained above, the situation is less clear cut in these organisms. Many cyanobacteria are surrounded by extracellular polysaccharide material. This is particularly evident in the extensive sheath that surrounds the unicellular cyanobacterium *Gloeotheca* and the filamentous cyanobacteria *Lyngbya*, *Phormidium* and *Microcoleus* (*Symploca*) *chthonoplastes*. Whether such sheaths act as a barrier to the influx of atmospheric gases is unclear and

sheathless strains of *Gloeotheca* are known that show a normal sensitivity of N_2 fixation to O_2 [58]. However, although such strains clearly lack the laminated sheath that characterizes *Gloeotheca*, they are not completely devoid of extracellular polysaccharide (S.V. Shestakov, L.J. Stal and J.R. Gallon, unpublished observations). Moreover, in any cultures that are simultaneously fixing N_2 and photoevolving O_2 , a surrounding barrier to the diffusion of gases would be as much a problem as an asset. On the other hand, coupled with a temporal separation between N_2 fixation and photosynthesis, an external barrier that limits gas exchange could benefit the process of N_2 fixation in non-heterocystous cyanobacteria, as in heterocystous cyanobacteria.

The N_2 -fixing nodules of legumes contain an O_2 -binding protein, leghaemoglobin, whose function is to deliver bound O_2 to *Rhizobium*-infected tissue in order to sustain oxidative phosphorylation. The concentration of free O_2 in the infected zone of the nodule remains very low (11 nM) thereby avoiding O_2 inactivation of nitrogenase (for more details, see [8] and references cited therein). A similar protein (GlbN, now designated cyanoglobin) has been found in the heterocystous cyanobacterium *Nostoc commune* [256]. Cyanoglobin is encoded by a gene (*glbN*) that is positioned amid some of the *nif* genes and this protein may therefore have an O_2 -scavenging role in N_2 fixation. In support of this, synthesis of cyanoglobin was induced by nitrogen starvation and incubation under micro-oxic conditions. A survey of 41 cyanobacteria (40 of which were diazotrophs), using a *glbN* gene probe and antibodies raised against cyanoglobin, revealed that this protein was present in many, though not all, heterocystous species. For example, it was missing from the eleven strains of *Anabaena* tested. On the other hand, cyanoglobin was not detected in the non-heterocystous cyanobacteria *Synechococcus* PCC 7942, *Synechocystis* BO 8402, *Chroococcidiopsis*, *Oscillatoria* PCC 7515, *Phormidium* strains SAUG B1462-1 and B1442-1 or *Pseudanabaena* PCC 7403, though it was found in *Trichodesmium* (M. Potts et al., personal communication). Attempts to detect cyanoglobin in extracts of aerobically grown *Gloeotheca*, using antisera to the protein from *N. commune*, were also unsuccessful (J.P.H. Reade, L.J. Rogers and J.R. Gallon, unpublished finding). The distribution of cy-

anoglobin is not therefore universal among either heterocystous or non-heterocystous N_2 -fixing cyanobacteria. Moreover, this protein appears not to be obligatory for N_2 fixation (K. Jäger; M. Potts et al., personal communications).

A number of metabolic strategies are employed by non-heterocystous cyanobacteria in order to cope with O_2 . For example, metabolic consumption of O_2 seems of great importance in maintaining a relatively low concentration of intracellular O_2 in a number of cyanobacteria, including *Gloeotheca* [195], *Synechococcus* (*Cyanothece*) spp. [27], *Microcoleus* (*Symploca*) *chthonoplastes* [39] and *Oscillatoria* spp. [46,235,257]. In many cases, the pattern of respiratory activity parallels that of N_2 fixation in cultures grown under alternating light and darkness or under continuous illumination.

When studying O_2 consumption, it is the sum of all the individual O_2 -consuming reactions that is measured. These individual reactions can be diverse, and include substrate level oxidase activity (for example that of glycollate oxidase) or O_2 consumption linked to aerobic electron transport chains. Such chains may be branched, and have a number of different terminal oxidases (such as *a*-, *d*- or *o*-type cytochromes) and also a variety of electron donors (for example, pyridine or flavin nucleotides linked to carbon metabolism, or ferredoxins linked to carbon metabolism or H_2). Both carbon-linked and H_2 -linked respiratory O_2 consumption are known among non-heterocystous cyanobacteria. For details, see [8].

Oxidases may reduce O_2 either to H_2O (water: a four-electron reduction), to H_2O_2 (hydrogen peroxide: a two-electron reduction) or to $O_2^{\cdot-}$ (superoxide: a one-electron reduction). The last two products can, however, give rise to a variety of reactive O_2 -derived radicals and are viewed as very dangerous species. Non-heterocystous cyanobacteria, like other aerobes, therefore possess a battery of enzymes that act to destroy H_2O_2 and $O_2^{\cdot-}$. These have been most extensively studied in *Gloeotheca* [8]. Whilst these enzymes would function to limit O_2 -induced damage of a wide range of biomolecules, not just nitrogenase, it is interesting that, in the heterocystous cyanobacterium *Anabaena* PCC 7120, the gene encoding one of these enzymes, glutathione reductase, contains an NtcA (BifA) binding site in its promoter region

[182], thereby implying that its transcription may be regulated through nitrogen status.

Perhaps the most intriguing possibility to have emerged in recent years is that nitrogenase may participate in protecting itself from inactivation by O_2 . Nitrogenase is a versatile enzyme that can reduce a variety of substrates in addition to N_2 . These substrates include acetylene (C_2H_2), the basis of the widely used acetylene reduction assay for nitrogenase activity, NO_2^- [258], CO_2 [259] and O_2 [260]. When present in vitro at a concentration greater than four times that of O_2 , the reduced form of the Fe-protein of nitrogenase (acting as a complex with two molecules of $MgADP^-$) reduces O_2 to H_2O_2 and H_2O (Reaction 3, Fig. 3b). The second stage of this reaction, reduction of H_2O_2 to H_2O , is very slow and catalase or peroxidases may be required to prevent accumulation of H_2O_2 in vivo. When the concentration of O_2 exceeds that of the Fe-protein, O_2 is reduced to $O_2^{\cdot-}$, which inactivates nitrogenase and other biomolecules (Reaction 2, Fig. 3b). Thus, whether the ability of nitrogenase to consume O_2 could protect itself from inactivation ('autoprotection') depends critically upon the prevailing relative concentrations of O_2 and Fe-protein and also on the presence of H_2O_2 -removing peroxidases. Catalase would be a poor substitute for peroxidases in this context because the breakdown of H_2O_2 catalysed by catalase would regenerate some of the O_2 originally consumed (Fig. 3). The efficiency of 'autoprotection' would also be affected by the relative concentrations of ATP and ADP in the cells since the complex between Fe-protein and $MgATP^{2-}$ was less effective at reducing O_2 to H_2O_2 than was the Fe-protein: $MgADP^-$ complex. Moreover, unlike the Fe-protein: $MgADP^-$ complex, the complex with $MgATP^{2-}$ could also transfer electrons to the MoFe-protein for subsequent reduction of N_2 (Reaction 1, Fig. 3b). The situation is therefore complex but 'autoprotection' may in theory function under appropriate conditions if the nitrogenase-containing cells can keep the Fe-protein in a functionally reduced state. It has, for example, been calculated that the intracellular conditions are such that 'autoprotection' might function in practice in cultures of the aerobic heterotroph *Azotobacter* to limit the damaging effect of O_2 on nitrogenase [260,261].

There is no direct evidence for 'autoprotection' of

N₂ fixation in cyanobacteria. However, theory suggests that it may have a role in the heterocysts of heterocystous cyanobacteria where the prevailing concentration of O₂ is already low. Elhai and Wolk (1991) have presented preliminary data suggesting that the concentration of O₂ in heterocysts of *Anabaena* was about 0.6 µM [262]. To provide a four-fold excess, the Fe-protein would have to be present at a concentration of 2.4 µM, or 0.173 mg ml⁻¹ (assuming an *M_r* of 72 000 for this protein). Heterocysts vary in size but 7 µm diameter appears typical of many *Anabaena* strains [96]. This gives a volume of about 180 µm³ (1.8 × 10⁻¹⁰ ml). A single heterocyst would therefore have to contain 30 fg of Fe-protein in order for the basic conditions of 'autoprotection' to apply. If it is assumed that the protein content of a cyanobacterium with cells of this size is 0.1 mg (10⁶ cells)⁻¹, which is the case in *Gloeotheca* [210], and that the protein content of a heterocyst is similar to that of a vegetative cell, then each heterocyst contains approximately 100 pg of protein. For 'autoprotection' of N₂ fixation to function, the Fe-protein would thus have to constitute 0.03% of the total protein of a heterocyst. Since the Fe-protein is readily detectable in SDS/PAGE gels of heterocyst proteins [263], it seems quite likely that the actual amount of this protein in heterocysts is much greater than 0.03% of the total. In theory, therefore, 'autoprotection' may contribute to protection of nitrogenase from O₂ damage in heterocystous cyanobacteria.

In the case of a non-heterocystous cyanobacterium such as *Gloeotheca*, a similar calculation can be made. Typical *Gloeotheca* cells are 6 µm × 10 µm (Plate 1a). This gives a cell volume of 225 µm³ (2.25 × 10⁻¹⁰ ml), which corresponds reasonably well with that of 2.79 × 10⁻¹⁰ ml actually measured (M. Davey and J.R. Gallon, unpublished) by monitoring the uptake of ³HOH, which readily enters cells, and [U-¹⁴C]sorbitol, which enters the periplasmic space but does not cross the plasmalemma [241]. Assuming that the Fe-protein of nitrogenase constitutes 0.5% of the total protein (which is the most that appears likely by inspection of stained SDS/PAGE gels), then an individual cell of *Gloeotheca* is unlikely to contain more than 0.5 pg of Fe-protein. The subunits of the homodimeric *Gloeotheca* Fe-protein have an *M_r* of 38 500 ('unmodified') or 40 000

('modified'). Taking a median value of 78 500 for the dimer, and assuming a cell volume of 2.5 × 10⁻¹⁰ ml, each cell would contain 6.4 amol of Fe-protein at a concentration of 26 µM. 'Autoprotection' could therefore function in *Gloeotheca*, but only if the intracellular concentration of O₂ was maintained by other processes at 6.5 µM or less (for comparative purposes, it should be noted that air-saturated water has a dissolved O₂ concentration of 258 µM at 25°C). The absolute value of the intracellular concentration of O₂ in *Gloeotheca* is not known though fluctuations in the relative concentration have been measured [264]. These show that intracellular O₂ is extremely low during the maximum phase of N₂ fixation in cultures growing under alternating light and darkness (C. Du, C.J. Restall and J.R. Gallon, unpublished findings). Furthermore, immediately following the maximum of nitrogenase activity (about 6 h into the dark phase), the intracellular ratio of ADP to ATP rises from about 0.6 to as high as 3 [210]. These conditions would also favour the operation of 'autoprotection' at a time when it is most needed, since at this time nitrogenase synthesis has ceased and O₂-inactivated enzyme cannot be replaced by *de novo* synthesis. However, 'autoprotection' requires a source of reductant, and this may well be limited during the latter stages of the dark period because of exhaustion of usable glucan reserves. It is difficult therefore to assess the impact, if any, of 'autoprotection' on N₂ fixation in cultures of *Gloeotheca*.

It should also be stressed that, during the operation of 'autoprotection', electrons delivered to the Fe-protein of nitrogenase are diverted to O₂ and away from the MoFe-protein of nitrogenase (Fig. 3a). N₂ reduction would therefore be proportionally inhibited. However, a temporary loss of N₂ fixation during a transient period of O₂ stress may be preferable to an irreversible inactivation of nitrogenase. A temporary loss of nitrogenase activity during transient O₂ stress has been reported in *Oscillatoria limosa* [235]. Recovery was independent of nitrogenase synthesis. This is consistent with the operation of 'autoprotection' in this organism under conditions when the other O₂-consuming reactions are saturated but the intracellular concentration of O₂ does not exceed a value consistent with the functioning of the Fe-protein as a harmless O₂-consuming enzyme.

In *Gloeotheca*, there are also reports of a synthesis-independent recovery of nitrogenase from transient exposure to 0.8 atm of O₂ [195], though recovery of nitrogenase from exposure to 1 atm of O₂ usually depends upon protein synthesis [195,265]. Indeed, synthesis of nitrogenase, in order to replace O₂-inactivated enzyme is probably an important component of the mechanisms that allow this cyanobacterium to fix N₂ aerobically [8]. Nevertheless, it is possible that 'autoprotection' has a role in the protection of *Gloeotheca* nitrogenase from exposure to 0.8 atm of O₂, though the process may be swamped by exposure to 1 atm of O₂.

In *Azotobacter* spp., transient protection of nitrogenase from exposure to concentrations of O₂ that saturate respiratory consumption (including, presumably, 'autoprotection') is provided by the formation of an O₂-stable, though catalytically inactive, complex with an iron-sulphur protein, FeSII (for more details, see [8,266]). Antisera raised against FeSII failed to react with any *Gloeotheca* protein (J.P.H. Reade, L.J. Rogers and J.R. Gallon, unpublished), so it is assumed that no similar system functions in this cyanobacterium.

In all of the cyanobacteria so far examined, the unidentified modification of the Fe-protein, mentioned above, is stimulated by exposure to elevated O₂. However, in *Gloeotheca*, there is no evidence that this modification either allows the protein to function in cultures exposed to elevated O₂ or to recover activity spontaneously following transient O₂ stress [60].

7. Circadian rhythms

The pattern of N₂ fixation seen in cultures of non-heterocystous cyanobacteria growing under alternating light and darkness constitutes a diurnal cycle, regardless of whether nitrogenase activity is confined to the light or dark phase. However, many cultures grown under constant illumination also exhibit fluctuations in their rate of N₂ fixation. These fluctuations may or may not exhibit a diurnal cycle. For example, successive peaks of nitrogenase activity were separated by about 40 h in *Gloeotheca* strain CCAP 1430/3 (=PCC 6909) [267] and by about 120 h in *Oscillatoria* sp. UCSB8 [46]. On the other hand,

cultures of *Oscillatoria limosa* [223] and *Cyanothece* BH68 [31], grown under continuous illumination, showed fluctuations in nitrogenase activity with peaks separated by about 24 h. Some strains of *Synechococcus* (*Cyanothece*) and *Gloeotheca* apparently show no fluctuations in nitrogenase activity when grown under continuous illumination [19] though, in *Synechococcus* (*Cyanothece*) strains RF-1 and BG 43511, the diurnal pattern imposed by exposure to alternating light and darkness persists when cultures are returned to constant illumination [254,268]. In many cases, the observed fluctuations in nitrogenase activity correspond to reciprocal fluctuations in net photosynthetic O₂ evolution [46,254,257,268].

In *Cyanothece* strains (including those designated as *Synechococcus*), the diurnal pattern of N₂ fixation may be considered as endogenous in the sense that, once imposed, it persists in cultures removed from the stimulus of alternating light and darkness. In the case of *Synechococcus* (*Cyanothece*) strain BG 43511, this circadian rhythm may reflect synchronous cell division, with N₂ fixation (and transcription of the *nif* genes) and oxygenic photosynthesis confined to different phases of the cell cycle [26,228,269,270]. Zehr (1995) has speculated that, in *Trichodesmium* (see Section 6.1, individual trichomes may alternate between phases of oxygenic photosynthesis and N₂ fixation and that this, too, may reflect the confinement of these incompatible activities to different phases of a cycle of cell division. However, whilst such behaviour would be consistent with many of the physiological properties of *Trichodesmium*, it has not yet been directly demonstrated [11]. Moreover, the situation in *Synechococcus* (*Cyanothece*) strain BG 43511, is complicated by the observation that apparently endogenous rhythms in N₂ fixation and photosynthesis are also seen in non-growing cultures [216]. This implies a regulatory system separate from that proposed to be linked to the cell cycle.

Most studies on the endogenous circadian rhythm of N₂ fixation have concentrated on *Synechococcus* (*Cyanothece*) strain RF-1 [9,271–276]. There is, however, good evidence that *Cyanothece* BH 68 may also show endogenous circadian rhythms both in N₂ fixation [31] and in carbohydrate deposition [221].

Until relatively recently, circadian rhythms were considered to be restricted to eukaryotes [277]. It is now, however, accepted that this is not the case

[278]. For example, work with *Synechococcus* strain PCC 7942 and *Synechocystis* strain PCC 6803 [279–281] has shown that cyanobacteria exhibit circadian rhythms in the expression of many genes, one of which, *psbAI* (which encodes the D1 protein, an integral component of the reaction centre of Photosystem II) has been more extensively investigated [279].

Synechocystis PCC 6803 and *Synechococcus* PCC 7942 do not fix N_2 , but *Synechococcus* (*Cyanothece*) RF-1 exhibits circadian rhythms in N_2 fixation [272] as well as in amino acid uptake [282], photosynthesis ([283]; Y.-J. Shei, unpublished, cited in [9]), protein synthesis [284] and intracellular concentrations of ATP (T.-H. Chen, unpublished, cited in [9]). The observed pattern of N_2 fixation conforms to the criteria of an endogenous circadian rhythm in that, once established, it persists in the absence of any apparent external signal, it can be reset by altering the pattern of light and darkness that cultures receive [272] and it responds to a pulse of low temperature [274,284]. The circadian rhythm is exerted at the level of transcription of one or more *nif* gene [155] but *nif* gene expression is not needed for establishment of the rhythm [273].

However, not all non-heterocystous cyanobacteria exhibit endogenous circadian rhythms in N_2 fixation. For example, when grown under alternating 12 h light and 12 h darkness, *Gloeotheca* exhibits a diurnal rhythm in nitrogenase activity. However, this pattern is lost on transfer to continuous illumination [118,267,285]. This suggests that the N_2 fixation rhythm in *Gloeotheca* is more to do with metabolic changes and environmental conditions than with an endogenous circadian clock.

8. Concluding remarks

Twenty years ago, it would have been impossible to write a review about N_2 fixation in non-heterocystous cyanobacteria. The body of experimental data simply was not there. During the last two decades, however, there has been a burgeoning scientific interest in these organisms. The reasons for this are complex, and centre on a number of factors. Firstly, ever since the observation that N_2 fixation was an O_2 -sensitive process, there has been much interest in determining the mechanisms that diazotrophs use in

order to protect nitrogenase from inactivation by O_2 . Since cyanobacteria actually photoevolve O_2 , the mechanisms that they possess should, in theory, be more efficient than those of other diazotrophs. Most non-heterocystous cyanobacteria do not confine nitrogenase to non-photosynthetic cells, so these organisms are particularly suitable for such studies. Secondly, non-heterocystous cyanobacteria are the closest surviving relatives of the free-living ancestor of modern chloroplasts that, according to the endosymbiont theory, was assimilated by the heterotrophic organism from which modern eukaryote algae and higher plants evolved. One of the persistent goals of modern research into N_2 fixation is the transfer of the ability to fix N_2 to the chloroplasts of leaves of non-diazotrophic crop plants, thereby generating strains that do not need a regular supply of expensive, and polluting, nitrogen-based fertilizer. However, in order for this can be achieved, any introduced *nif* genes should be correctly expressed and the nitrogenase produced in this way must integrate properly with chloroplast metabolism in order to be catalytically active. Whilst chloroplasts could readily generate ATP and reductant for N_2 fixation, and carbon skeletons for assimilation of newly fixed nitrogen, they also photoevolve O_2 , so there is an additional requirement that nitrogenase be protected from O_2 damage. All of these problems are already faced, and solved, by free-living non-heterocystous cyanobacteria. Consequently, a thorough understanding of N_2 fixation in non-heterocystous cyanobacteria, including its regulation and integration with other metabolic processes, could greatly assist attempts to generate novel N_2 -fixing crop plants. Thirdly, because H_2 is an obligatory by-product of the nitrogenase-catalysed reduction of N_2 , there has been much recent interest in using N_2 -fixing cyanobacteria to convert solar energy into industrially useful H_2 gas. The fact that most non-heterocystous cyanobacteria would produce H_2 at night suggests that they might be a valuable component of any system designed for continuous H_2 production using natural illumination, which comes as alternating light and darkness. Fourthly, the more recent appreciation that N_2 -fixing non-heterocystous cyanobacteria make a substantial contribution to the global nitrogen cycle, especially in the marine environment, has stimulated interest in cyanobacteria such as *Tri-*

chodesmium. This interest has been intensified by the finding that *Trichodesmium* produces a small proportion of unusual cells that appear to act as heterocyst equivalents, even though they lack the structural modifications that, in heterocysts, contribute to the protection of nitrogenase from inactivation by O_2 .

It is, hopefully, apparent from this review that much has been achieved and that we now have a detailed understanding of some of the metabolic processes related to N_2 fixation in non-heterocystous cyanobacteria. Much, for example, is known about the diverse mechanisms that these organisms use in order to protect nitrogenase from inactivation by O_2 , though it is still not possible to gauge the relative significance of each of these mechanisms in individual cyanobacteria. It almost certainly varies from organism to organism. Rather less is known about some other areas, such as the supply of ATP and reductant to nitrogenase, the regulation of N_2 fixation by fixed nitrogen, and the molecular mechanisms underpinning the clear fluctuations in nitrogenase activity seen in cultures growing under alternating light and darkness. However, steady progress is being made and, hopefully, will continue. Much less is known about the control of *nif* gene transcription by O_2 or about the details of N_2 fixation in natural populations of non-heterocystous cyanobacteria. Perhaps not surprisingly, given their quantitative significance, most available information concerning natural populations comes from studies in the marine environment, both with planktic strains and with mat-forming cyanobacteria on the littoral fringes of the oceans. Nevertheless, there is a clear need for further studies on these systems as well as in other ecosystems.

Finally, there is probably something fundamental in human nature that makes us concentrate on the unusual at the expense of the commonplace. Certainly, N_2 fixation is unusual among non-heterocystous cyanobacteria. Most strains currently known cannot fix N_2 . For example, in a comprehensive list of cyanobacterial cultures available in the collection of the Pasteur Institute in Paris, no more than about 40% of the non-heterocystous strains were reported as fixing N_2 [22]. Moreover, of those non-heterocystous cyanobacteria that can fix N_2 , most do so only under anoxic or micro-oxic conditions. Aerobic N_2 fixation is very unusual among these organisms. De-

spite this, it is the few strains that can fix N_2 aerobically that have received extensive scientific study. Representatives of the more numerous non-heterocystous cyanobacteria that can fix N_2 only when the prevailing concentration of O_2 is low have been comparatively neglected. There is a need to remedy this deficiency. Another, though less dramatic, example of our obsession with things unusual is reflected in the current intensive study of the diel pattern of N_2 fixation in *Trichodesmium*. Whilst it is easy to justify the need to study this organism because of its huge potential for N_2 fixation, it should be remembered that it is extremely unusual, if not unique, among aerotolerant non-heterocystous N_2 -fixing cyanobacteria in that it fixes N_2 during the day. Most non-heterocystous cyanobacteria that fix N_2 aerobically do so at night.

This unusual behaviour of *Trichodesmium* itself highlights the need to look at many organisms, not just a select few. Twenty years ago, for want of direct information, scientists assumed that most of the properties of N_2 fixation in non-heterocystous cyanobacteria would be fundamentally similar to those in heterocystous strains. Now we are aware that much is different between heterocystous and non-heterocystous cyanobacteria. We have also now become aware that different non-heterocystous cyanobacteria exhibit important differences. Whilst this can be taken as a measure of scientific progress, it emphasizes the need to extend our studies to other neglected strains of non-heterocystous cyanobacteria. For example, nothing is known about N_2 fixation in the cyanobacteria of Section II (Table 1), or in those non-heterocystous cyanobacteria that enter into symbiotic associations. Hopefully, this will be remedied. However, it remains to be seen whether the next twenty years of research into N_2 fixation by non-heterocystous cyanobacteria will reveal more differences or some general unifying principles. Whatever the final outcome, the undertaking will certainly be worthwhile.

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