5.1 Introduction:

Cyanobacteria are O$_2$-evolving photosynthetic prokaryotes, many of which are also capable of autotrophic growth using N$_2$ as the sole nitrogen source (Stewart, 1980; Gallon, 1989). Cellular integration of N$_2$-fixation in cyanobacteria requires strategies for protection of nitrogenase from atmospheric and photosynthetically produced O$_2$, provision of ATP and reductant and efficient assimilation of N$_2$-derived ammonia. In some cyanobacteria this is achieved by development of specialized cells called heterocysts, resulting in spatial separation of photosynthesis (located in vegetative cells) and N$_2$-fixation (located in heterocysts); fixed carbon moved from the vegetative cells to heterocysts and fixed nitrogen from heterocysts to vegetative cells (Stewart, 1980; Wolk, 1982; Bergman et al., 1986). Several other metabolic changes conducive to nitrogenase functioning occur during heterocyst development, including: (1) loss of photosynthetic O$_2$ evolution, phycobiliproteins and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo); (2) increase in respiratory activity and in the activity of enzymes of oxidative pentose phosphate pathways; (3) loss of nitrate
reductase systems; (4) increased levels of glutamine synthetase (GS) necessary for assimilation of N\textsubscript{2}-derived ammonia (Wolk, 1982; Kumar et al., 1985, Rai & Bergman, 1986; Renström-Kellner et al., 1990).

The strategies for O\textsubscript{2} protection during aerobic N\textsubscript{2}-fixation by non-heterocystous cyanobacteria have been studied in detail, with conclusion of a temporal separation of N\textsubscript{2}-fixation and photosynthesis (Gallon, 1992). However, very few studies have been conducted on non-heterocystous cyanobacteria which fix N\textsubscript{2} under microaerobic or anaerobic conditions. The reasons for lack of aerobic N\textsubscript{2}-fixation in such cyanobacteria are not fully understood. Furthermore, virtually no information is available regarding levels of phycoerythrin (PE), GS, nitrate uptake, NR and RuBisCo under diazotrophic growth conditions in these cyanobacteria.

\textit{Plectonema boryanum} PCC 73110 is a filamentous non-heterocystous cyanobacterium which fixes N\textsubscript{2} under microaerobic to anaerobic conditions (Stewart & Lex, 1970). In the present investigation, this strain was used to study the derepression, subcellular localization and regulation of nitrogenase, and changes in GS, RuBisCo, PE, nitrate uptake and NR when nitrate-grown cultures adapted to diazotrophic growth. The data are discussed in relation to the known facts about the above aspects in heterocystous cyanobacteria.
5.2 Methods:

5.2.1 Organisms and growth conditions:

Plectonema boryanum PCC 73110 (ATCC 29407 and UTEX 594) and Gloeothecae PCC 6909 (ATCC 27152 and UTEX 795) were grown in batch cultures in BG-11 medium (Rippka et al., 1979) at 25° C and a photon fluence rate of 20 μmol m^{-2} s^{-1}. Oscillaria limosa (Stal & Bergman, 1990) was grown on artificial sea water medium ASN_{3} (Rippka et al., 1979) at 20° C and a photon fluence rate of 20 μmol m^{-2} s^{-1}. N_{2}-fixing cultures of Gloeothecae and O. limosa were obtained by transferring the cultures to nitrogen-free media ('N_{2}-medium'; BG-11_{O} and NO_{3}⁻-free ASN_{3}, respectively). Derepression of nitrogenase in P. boryanum was achieved as described below.

5.2.2 Nitrogenase derepression:

Aerated batch cultures of P. boryanum grown on BG-11_{O} medium were harvested by centrifugation during the exponential phase. The cells were washed and resuspended in BG-11_{O} medium to a cell density of 200 μg ml^{-1} (3 μg chlorophyll a ml^{-1}). These cultures were subdivided into 20 ml batches and transferred to 100 ml capacity serum stoppered Erlenmeyer flasks. These were sparged with desired gas mixture at specific times and maintained at 20° C and a photon fluence rate of 10 μmol m^{-2} s^{-1}. These flasks were directly used for acetylene reduction assay, each assay lasting 30 min; the cultures were not transferred to another vessel, in order to avoid air contamination.
5.2.3 Enzyme assays:

Nitrogenase, glutamine synthetase, nitrate reductase were done as detailed in the "MATERIALS AND METHODS".

5.2.4 Estimations:

Estimation of chlorophyll, proteins, O₂-exchange, NO₃ uptake, antibodies were done according to earlier description given in the "MATERIALS AND METHODS".

5.2.5 Procedures:

Transmission electron microscopy, immunolabelling and immunoblotting were performed as given in the "MATERIALS AND METHODS".

5.3 Results:

5.3.1 Derepression of nitrogenase:

No nitrogenase activity or protein could be detected in NO₃⁻ or NH₄⁺-grown P. boryanum cultures either under air or with N₂/CO₂ (95:5, v/v) sparging (data not shown). Upon transfer to N₂-medium and periodic sparging with N₂/CO₂, development of acetylene reducing activity started after 30 h (Fig. 5.1a). The activity continues to increase during next 40 h, after which it steadily declined. The pattern and specific activity were similar to those noted by Stewart & Lex (1970). However, when the cells
Fig. 5.1. Nitrogenase derepression in *P. boryanum*. (a) NO$_3^-$-grown cells were transferred to N$_2$-medium as detailed in Methods and maintained at 25°C with a photon fluence rate of 10 µmol m$^{-2}$s$^{-1}$. Starting at zero time and at 6 h intervals they were sparged for 15 min with N$_2$/CO$_2$ (95:5, v/v) at a rate of 1000 ml h$^{-1}$. Nitrogenase activity was measured in 30 min assays under a N$_2$/CO$_2$ gas phase and under the light and temperature conditions mentioned above. In this and other figures nitrogenase activity quoted is the mean of that during the 30 min before the points shown. (b) Same as (a) except that prior to the start of N$_2$/CO$_2$ sparging (zero time) the cultures were nitrogen-starved for 24 h by incubating them in N$_2$-medium with continuous air sparging (2000 ml h$^{-1}$). Symbols in (b): ▲, control; □, plus chloramphenicol (100 µg ml$^{-1}$); ■, plus rifampicin (100 µg ml$^{-1}$). Chloramphenicol and rifampicin were added at the start of N$_2$/CO$_2$ sparging (i.e. after the aerobic nitrogen-starvation period). This and other experiments (Figs. 5.2a, 5.3, 5.4, 5.5) were all done in duplicate. The values presented are means of two measurements from each replicate.
were nitrogen starved for 24 h under aerobic conditions prior to the periodic $N_2/CO_2$ sparging, acetylene-reducing activity developed much faster (within 2 h) and reached a peak by 6-7 h (Fig. 5.1b), with maximal specific activity similar to that in Fig. 5.1(a). To see if the faster development of nitrogenase activity was due to the activation of pre-existing nitrogenase protein developed during the 24 h nitrogen starvation, development of acetylene-reducing activity was followed in cells where chloramphenicol or rifampicin was added at the end of nitrogen starvation and just before the start of $N_2/CO_2$ sparging. As seen in Fig. 5.1(b), acetylene-reducing activity did not appear under such conditions. These results indicate that nitrogenase protein was absent during the aerobic nitrogen starvation period and that the derepression on $N_2/CO_2$ sparging was due to fresh synthesis of nitrogenase. An absence of nitrogenase protein in aerobic cultures was also observed by immunoblotting cell extracts of *P. boryanum* which has been nitrogen starved for 30 h under aerobic conditions (Fig. 5.2b, lane 1). These results show that nitrogenase derepression required both low cellular nitrogen and microaerobic to anaerobic conditions, and that the longer time required for nitrogenase derepression in Fig. 5.1(a) was due to the time required for the depletion of intracellular nitrogen reserves under non-optimal growth conditions.

Reexposure of $N_2$-fixing *P. boryanum* cells to air caused a rapid decline in acetylene-reducing activity, which became
undetectable after 90 min of exposure (Fig. 5.2a). To see whether the decline in nitrogenase activity was due to inactivation of the enzyme or to protein degradation/modification, immunoblots of cell extracts were done using *P. boryanum* cells exposed to air for increasing period of time after the appearance of peak nitrogenase activity (Fig. 4.3b). After 2 h of exposure to air, when nitrogenase activity has become undetectable, nitrogenase Fe-protein was still detectable (lane 3), although the cross-reaction was less intense than that in *P. boryanum* cells under N\(_2\)/CO\(_2\) (lane 2). In both cases, only a single polypeptide of 36 kDa was detected corresponding to nitrogenase Fe-protein. These data indicate that loss of nitrogenase activity on exposure to air was due to nitrogenase inactivation followed by degradation. Such inactivation did not involve modification of Fe-protein to a higher molecular mass form as noted in other cyanobacteria (Ernst *et al.*, 1990; Reich & Böger, 1989; Smith *et al.*, 1987; Stal & Bergman, 1990). To see if the inactivation of nitrogenase was reversible, *P. boryanum* cells were transferred back to N\(_2\)/CO\(_2\) atmosphere after 90 min exposure to air (Fig. 5.2a). Acetylene-reducing activity reappeared after 90 min and reached a peak in 3 h. Such reappearance of nitrogenase activity was sensitive to chloramphenicol and rifampicin indicating fresh nitrogenase synthesis to be necessary. Thus, inactivation of nitrogenase under air concluded to be irreversible.
Fig. 5.2. (a) Loss of nitrogenase activity on exposure to air (○) and its regain upon transfer to a N₂/CO₂ atmosphere (●) in *P. boryanum*. Nitrogenase derepression was achieved as in Fig 5.1(b) and after appearance of the nitrogenase peak, the cells were sparged with air for 5 min at the rate of 2000 ml h⁻¹. Nitrogenase activity was measured at 30 min intervals under aerobic conditions. At the time indicated by the arrow, the gas phase was changed to N₂/CO₂ in the absence (○) and presence (●) of chloramphenicol or rifampicin (both 100 µg ml⁻¹). (b) Detection of nitrogenase Fe-protein by immunoblotting in cell extracts of *P. boryanum*. Lane 1, extract from cells maintained in aerobic N₂-medium for 30 min; lane 2, extract from cells with peak nitrogenase activity; lane 3, extract from cells which were exposed to air for 2 h after appearance of the nitrogenase peak; lane 4, extract of cells which had been exposed to air for 4 h after appearance of nitrogenase activity; lane 5, molecular mass markers.
5.3.2 Growth, N₂ fixation and O₂ evolution:

When nitrogenase was derepressed as in Fig. 5.1(b), and cultures maintained under similar conditions over a prolonged period, repeated peaks of acetylene-reducing activity was observed (Fig. 5.3a). An increase in protein content, which was taken as indicative of growth, followed. A detailed analysis of one such peak of nitrogenase activity and growth phase (Fig. 5.3b) showed that during appearance of nitrogenase the rate of net O₂ evolution declined rapidly, becoming undetectable by the time nitrogenase activity reached its peak. No growth occurred during this period. Net O₂ evolution was detected again after 7 h. This coincided with growth and with a decline in nitrogenase activity. These data indicate that under the conditions used here, *P. boryanum* can grow photoautotrophically using N₂ as sole nitrogen source, in repeated cycles of nitrogen fixation and growth, and under such conditions there is a temporal separation of net O₂ evolution and nitrogenase activity. During the maximal N₂-fixing period, O₂ evolution may be balanced by respiratory O₂ consumption, resulting in the absence of net O₂ exchange.

5.3.3 Effects of NH₄⁺ and NO₃⁻ and darkness on nitrogenase activity:

After nitrogenase derepression as in Fig. 5.1(b), the effects of darkness, NH₄⁺ and NO₃⁻ were studied during the 6 h stable period when maximal nitrogenase activity was expressed. Transfer of N₂-fixing cells into darkness resulted in a rapid
Fig. 5.3. (a) Nitrogenase activity (○) and protein content (□) of P. boryanum. (b) Nitrogenase activity (■), protein content (○) and net O₂ evolution rate (□) during the first phase of N₂ fixation in (a). Culture conditions and other details as in Fig. 5.1 (b) control.
decline in acetylene-reducing activity, which became undetectable within 2 h (Fig. 5.4). This decline was similar to that observed in air. ATP and/or reductant were probably the essential factors supplied by light reactions. Addition of 2 mM-NH$_4$Cl resulted in a slower decline of acetylene-reducing activity, perhaps because NH$_4^+$ may have acted by repressing nitrogenase synthesis rather than inhibiting the activity. NO$_3^-$ did not affect nitrogenase activity during the initial 2 h of incubation, but a slow decline in activity was seen thereafter. The difference in the effects of NO$_3^-$ and NH$_4^+$ may be due to slower rates of nitrate uptake and/or metabolism in N$_2$-fixing cultures (see Table 5.1).

5.3.4 NO$_3^-$ uptake and NR activities:

NO$_3^-$-grown cultures of *P. boryanum* showed NO$_3^-$ uptake and NR activities (Table 5.1) similar to those reported earlier (Ida & Mikami, 1983). In contrast, NO$_3^-$ uptake and NR activities of N$_2$-fixing cultures were only 10% of those in NO$_3^-$-grown cells (activities measured after appearance of peak nitrogenase activity). These activities did not change during the period of nitrogenase decline that also corresponded to net O$_2$ evolution and growth (data not shown). NH$_4^+$-grown cells had no detectable levels of NO$_3^-$ uptake and NR. Essentially similar results were found in the case of non-heterocystous cyanobacteria *Gloeothecae 6909* and *O. limosa*, which fix N$_2$ aerobically (Table 5.1). When N$_2$-fixing cells of *P. boryanum* were transferred to NO$_3^-$ medium, NO$_3^-$ uptake and NR activities increased reaching a maximum within
Fig. 5.4. Effects of air, darkness, NH$_4^+$ and NO$_3^-$ on nitrogenase activity in *P. boryanum*. Nitrogenase activity was derepressed as in Fig. 5.1(b). After the appearance of the nitrogenase peak, the conditions were changed (zero time) and the response of nitrogenase activity was monitored during the next 6 h. ○, Cultures transferred to darkness; ■, NH$_4$Cl added to a final concentration of 2 mM; □, KNO$_3$ added to a final concentration of 10 mM; △, cultures transferred to aerobic atmosphere. The NH$_4$Cl and KNO$_3$ solutions were sparged with N$_2$/CO$_2$ for 30 min before addition. The solutions were also buffered with 10 mM HEPES/NaOH (pH 7.5). One hundred percent activity represents 18 nmol C$_2$H$_4$ formed min$^{-1}$ (mg protein)$^{-1}$.
Table: 5.1. NO$_3^-$ uptake and NR activities in *P. boryanum*, *Gloeothecce 6909* and *O. limosa* grown on N$_2$, NO$_3^-$ or NH$_4^+$:

NO$_3^-$ uptake activities are expressed as nmol NO$_3^-$ taken up min$^{-1}$ (mg protein)$^{-1}$ and NR activities as nmol NO$_2^-$ formed min$^{-1}$ (mg protein)$^{-1}$. NO$_3^-$-medium represents BG-11 in the case of *Gloeothecce 6909* and *P. boryanum*, and ASN$_3$ in the case of *O. limosa*. N$_2$-medium represents the respective nitrogen-free medium (BG-11$_0$ or NO$_3^-$-free ASN$_3$). NH$_4^+$-medium represents N$_2$-media supplemented with NH$_4$Cl (final concentration 2 mM) and buffered with 10 mM-HEPES/NaOH (pH 7.5). The values presented are means ± SE of three measurements from a single experiment done in triplicate. ND, Not detectable.

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>Nitrate uptake</th>
<th>Nitrate reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plectonema</td>
<td>Gloeothecce</td>
</tr>
<tr>
<td>N$_2$-medium</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>NO$_3^-$-medium</td>
<td>7.5 ± 0.3</td>
<td>4.9 ± 0.25</td>
</tr>
<tr>
<td>NH$_4^+$-medium</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>
10-12 h (Fig. 5.5). The increase was sensitive to chloramphenicol. These results imply that the NO$_3^-$ uptake and NR systems in *P. boryanum* are substrate-inducible and NH$_4^+$-repressible.

5.3.5 Nitrogenase localization:

Immunogold localization of nitrogenase Fe-protein in NO$_3^-$-grown cells (Fig. 5.6a) and NH$_4^+$-grown cells (data not shown) showed label intensity similar to background (4-6 gold particles per $\mu$m$^2$ cell area). N$_2$-fixing *P. boryanum* cells showed nitrogenase antigen uniformly distributed throughout the cell without preferential association with any cell structure (Fig. 5.7a). All the cells in all the filaments examined had a similar pattern and intensity of labelling. Cells undergoing division also had nitrogenase label. The density of label was 95 ± 12 gold particles per $\mu$m$^2$ cell area which is comparable to that in the heterocysts (unpublished data. See also Stal & Bergman, 1990). These results confirm the lack of nitrogenase in NO$_3^-$- and NH$_4^+$-grown cells and imply no spatial separation or subcellular compartmentalization of nitrogenase in *P. boryanum*. Similar results have been reported in *P. boryanum* 581 using antibodies against nitrogenase Mo-Fe protein (Smoker *et al.*, 1989).

5.3.6 GS activity and cellular localization of GS antigen:

Potential changes in GS activity and protein concentration during derepression of nitrogenase were examined in NO$_3^-$-grown
Fig. 5.5. Induction of NO$_3^-$ uptake (□) and NR (○) activities on transfer of N$_2$-fixing P. boryanum cells to NO$_3^-$-medium (BG-11) without (■) or with (●) chloramphenicol (100 μg ml$^{-1}$). Nitrogenase was derepressed as in Fig. 5.1(b). After the appearance of the nitrogenase peak, the cells were harvested by centrifugation and resuspended in NO$_3^-$-medium (zero time), then maintained under aerobic growth conditions. At time intervals samples were withdrawn and NO$_3^-$ uptake rate and NR activities measured.
Fig. 5.6. Immunogold localization of nitrogenase (a), GS (b), RuBisCo (c) and PE (d) in nitrate grown cells of *P. boryanum*. Rabbit anti-*E. rubrum* nitrogenase Fe-protein (a), anti-*Anabaena* 7120 GS (b), anti-*S. alba* RuBisCo (c) and anti-*P. percinninum* PE (d) were used as primary antibodies at a dilution of 1:100. Goat anti-rabbit IgG conjugated to 5nm (a,c,d) or 10 nm (b) colloidal gold was used as secondary antibody at a dilution of 1:20. Ch, carboxysomes. Bar = 1 μm (all parts of the figure are at the same magnification).
and \( \text{N}_2 \)-fixing \( P. \text{boryanum} \) cells. GS biosynthetic activity in the two cultures was \( 45 \pm 2.8 \) and \( 56 \pm 3.1 \) nmol product formed min\(^{-1}\) (mg protein\(^{-1}\)), respectively. Immunolabelling experiments showed that the GS antigen was distributed throughout the cell both in \( \text{NO}_3^- \)-grown and \( \text{N}_2 \)-fixing cells (Figs. 5.6b, 5.7b). The relative density of the gold label were \( 22 \pm 2.5 \) and \( 27 \pm 2.6 \) gold particles per \( \mu \text{m}^2 \) cell area respectively. Thus, a 20% increase in GS activity and protein label occurred on nitrogenase derepression. This is in contrast to the report of Nagatani & Haselkorn (1978), who found no increase in GS activity during nitrogenase derepression under an argon atmosphere. The differences may have arisen due to different condition used for nitrogenase derepression, including the fact that we have used \( \text{N}_2/\text{CO}_2 \) (95:5, v/v) for nitrogenase derepression.

5.3.7 RuBisCo localization:

RuBisCo was localized in \( \text{NO}_3^- \)-grown and \( \text{N}_2 \)-fixing cells of \( P. \text{boryanum} \) (Figs. 5.6c, 5.7c). In the latter case, cells were processed for immunolabelling at the beginning of the appearance of nitrogenase, when the nitrogen stress, and therefore the difference in RuBisCo, is likely to be highest. RuBisCo was present in both \( \text{NO}_3^- \)-grown and \( \text{N}_2 \)-fixing cells. In both cases, an intense labelling was found in carboxysomes and a lower intensity in the cytoplasm. The overall level of RuBisCo in \( \text{N}_2 \)-fixing cells was 20% lower than that in \( \text{NO}_3^- \)-grown cells (\( 43 \pm 3.4 \) and \( 54 \pm 4 \) gold particles per cell, respectively). Smoker \textit{et al.} (1990)
Fig. 5.7. Immunogold localization of nitrogenase (a), GS (b), RuBisCo (c) and PE (d) in N\textsubscript{2}-fixing \textit{P. boryanum} cells. Nitrogenase derepression was achieved as in Fig. 5.1 (b). The cells used for immunolabelling were sampled either at the beginning (c,d) or at the peak (a,b) of nitrogenase activity. Other details as in Fig. 5.6.
noted a much higher (over 50%) reduction in RuBisCo levels on nitrogenase derepression in *P. boryanum* 581. However, the cells had been stressed for nitrogen for 40 h in an argon atmosphere lacking *N*₂. These results show that unlike the situation in heterocysts, derepression of nitrogenase in *P. boryanum* does not lead to total loss of RuBisCo.

5.3.8 Localization of PE:

During nitrogenase derepression in *P. boryanum*, a transient decrease in phycocyanin had been noted (Stewart & Lex, 1970; Weare & Benemann, 1974). To see if the PE concentration also change during nitrogenase derepression, immunogold labelling was examined in NO₃⁻-grown and N₂-fixing cells. In the latter case, cells for immunolabelling were taken at the beginning of the appearance of nitrogenase activity, when the differences are likely to be maximal. An intense PE labelling associated with thylakoid membranes was found both in NO₃⁻-grown and N₂-fixing cells (Figs. 5.6d, 5.7d). In both cases, the amount of PE was similar (447 ± 15 and 435 ± 18 gold particles per cells, respectively), indicating little or no degradation of PE in *P. boryanum* under the conditions of nitrogenase derepression used here.

5.4 Discussion:

Under a N₂/CO₂ atmosphere *P. boryanum* synthesized nitrogenase and showed nitrogenase activity in the absence of
combined nitrogen (Figs. 5.1, 5.3). These activities are among the highest reported by other workers using this strain (Stewart & Lex, 1970; Weare & Benemann, 1974; Nagatani & Haselkorn, 1978; Rogerson, 1980; Pearson & Howsley, 1980; Giani & Kumbein, 1986). Since the cultures used were non-synchronous, development of nitrogenase in all the cells (including those undergoing division) and the uniform distribution throughout the cells (Fig. 5.7) argue against the possibility of nitrogenase being expressed during a particular phase of the life cycle or being compartmentalized within the cell (Mitsui et al., 1986; Giani & Kumbein, 1986). Similar patterns of labelling have been found in O. limosa (Stal & Bergman, 1990), P. boryanum UTEX 581 (Smoker et al., 1989) and Gloeothecae PCC 6909 (A. N. Rai & Bergman, unpublished results).

Furthermore, the fact that the nitrogenase occurred in all the cells in P. boryanum with the labelling intensity comparable to that of heterocysts (which form only 5-10% of the total cell population in heterocystous cyanobacteria), means that the total nitrogenase protein in P. boryanum filaments is likely to be 10-20 times higher than that in filaments of heterocystous cyanobacteria. However, nitrogenase activity in P. boryanum was only 2-3 fold higher than that reported in heterocystous cyanobacteria. It is likely that this is due to limited availability of ATP and reductant. Similar arguments may apply in cases of other nonheterocystous cyanobacteria e.g. Gloeothecae and Oscillatoria.
O₂ removal or nitrogen limitation alone did not lead to derepression of nitrogenase. This was indicated by the lack of nitrogenase derepression under aerobic conditions irrespective of the nitrogen status of the cell, and by the fact that even on N₂/CO₂ sparging nitrogenase derepression occurred only under nitrogen-limited conditions (Figs. 5.1, 5.2, 5.6a). These results are consistent with, and provide evidence for, the suggestion that nitrogenase derepression in P. boryanum requires both O₂ removal and nitrogen limitation (Nagatani & Haselkorn, 1978).

Absence of nitrogenase derepression in the presence of combined nitrogen is consistent with earlier observations in cyanobacteria including P. boryanum (Stewart, 1980; Gallon, 1989; Stewart & Lex, 1970). However, the fact that O₂ removal or lowering of O₂ tension was necessary for nitrogenase derepression, despite a temporal separation of net O₂ evolution and nitrogenase activity, and that nitrogenase activity decline sharply on exposure to air or with the onset of net O₂ evolution endogenously (Fig. 5.3), suggests that the O₂-scavenging capacity of P. boryanum is much more limited than in other non-heterocystous cyanobacteria. Indeed, Weare & Benemann (1974) found respiration to be only limited significance in O₂-protection in P. boryanum.

Our studies showing irreversible inactivation of nitrogenase by O₂ in P. boryanum (Fig. 5.2) are consistent with the results of Weare & Benemann (1974). Nitrogenase Fe-protein has been shown to be modified to a higher molecular mass form by O₂ in some heterocystous cyanobacteria, which results in
reversible inactivation of the protein but renders it insensitive to \( \text{O}_2 \) damage (Smith et al., 1987; Reich & Böger 1989; Ernst et al., 1990). A similar modification has also been noted in nonheterocystous cyanobacteria \( \text{O. limosa} \) (Stal & Bergman, 1990; Villbrandt et al., 1992), \( \text{Trichodesmium} \) (Ohki et al., 1991) and \( \text{Synechocystis} \, \text{BO-8402} \) (Brass et al., 1992). The fact that such a modification was not found in \( \text{P. boryanum} \) (Fig. 5.2b) may explain why inactivation of nitrogenase in this strain was irreversible and resulted in degradation of the enzyme.

The temporal separation of \( \text{N}_2 \) fixation and net \( \text{O}_2 \) evolution noted here (Fig. 5.3) also confirms the findings of Weare & Benemann (1974). In addition, the results show that \( \text{P. boryanum} \) can grow photoautotrophically with repeated cycles of \( \text{N}_2 \) fixation and growth. Since nitrogenase was found to be irreversibly inactivated and degraded on exposure to air, and regain of nitrogenase activity required fresh nitrogenase synthesis (Figs. 5.2, 5.4), it is possible that repeated phases of \( \text{N}_2 \) fixation required fresh nitrogenase synthesis and that during the following microaerobic phase nitrogenase was degraded. Giani & Krumbein (1986) have demonstrated \( \text{N}_2 \) fixation and concomitant photoautotrophic growth in \( \text{P. boryanum} \) at lower light intensities at continuous \( \text{N}_2/\text{CO}_2 \) flushing. Thus, depending on the culture conditions, \( \text{P. boryanum} \) seems capable of photoautotrophic growth either with repeated phases of \( \text{N}_2 \) fixation (when sparged with \( \text{N}_2/\text{CO}_2 \) periodically) or concomitantly with continuous \( \text{N}_2 \) fixation when sparged with \( \text{N}_2/\text{CO}_2 \) continuously to remove any net
O₂ evolved, keeping the culture microaerobic). Although, the precise mechanism of temporal separation is not clear, it is possible that this is achieved by changes in rates of photosynthesis, with respiration balancing photosynthetic O₂ evolution during the growth that follows. This may occur due to transient changes in phycobiliprotein levels. Although we found no significant changes in PE levels (Figs. 5.6, 5.7), phycocyanin levels are known to change and repeated degradation and synthesis of phycocyanin has been noted earlier (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).

Mo may be a limiting factor in cyanobacterial cells (Bagchi et al., 1985). Nitrogenase and NR are both molybdoenzymes and require reduced ferredoxin as electron donor (Guerrero & Lara, 1987). Indeed, Nagatani & Haselkorn (1978) have shown that in absence of Mo, nitrogenase proteins synthesized are inactive. Occurrence of NR and nitrogenase in the same cell is likely to lead to competition for Mo and reduced ferredoxin. Heterocystous cyanobacteria avoid such competition for Mo and reduced ferredoxin by spatial separation of nitrogenase and NR (Kumar et al., 1985; Rai & Bergman, 1986). Such spatial separation is not possible in non-heterocystous cyanobacteria since nitrogenase is present in all the cells. A temporal separation of NR and nitrogenase was not found in non-heterocystous cyanobacteria tested (Table 5.1). However, that nitrate uptake and NR were found to be NO₃⁻-inducible, with very low activities in N₂-fixing cultures (Fig. 5.5; Table 5.1) means that these cyanobacteria are
able to efficiently minimize competition between NR and nitrogenase under diazotrophic growth conditions. It is noteworthy that N$_2$-fixing *P. boryanum* cells retain the capacity to develop nitrate uptake and NR (Fig. 5.5), unlike heterocysts, where, these systems are lost (Rai & Bergman, 1986).

As in heterocystous cyanobacteria, the GS-GOGAT pathway has been shown to be the route of primary NH$_4^+$ assimilation in *P. boryanum* (Meeks *et al.*, 1978). An increase in GS activity and protein level has been noted when vegetative cells differentiate into heterocysts (see Wolk, 1982; Bergman *et al.*, 1985). This has been shown to be linked to nitrogenase expression and to be necessary for assimilation of N$_2$-derived NH$_4^+$ (Renstrom-Kellner *et al.*, 1990). Our results showing an increase in GS with induction of nitrogenase (Figs. 5.6, 5.7) are consistent with the above view. The increase (20%) noted in *P. boryanum* is apparently less than that in heterocysts (100%). However, since heterocysts constitute only 5-10% of the total cell population, the overall increase in GS of a N$_2$-fixing culture of heterocystous cyanobacterium would be only 5-10%. Thus, the GS increase in *P. boryanum* is in fact higher than that in heterocystous filaments. This may reflect the fact that nitrogenase activity and therefore, the likely rate of primary ammonia production, is 2-3 fold higher in *P. boryanum*. This findings also explain why the NO$_3^-$ is less inhibitory and acts more slowly than NH$_4^+$. As mentioned above, NO$_3^-$ uptake and NR levels are very low in N$_2$-fixing cultures and availability of NO$_3^-$ requires 3-4 h for
induction of $\text{NO}_3^-$ uptake and NR activity (Table, 5.1; Fig. 5.5), while $\text{NH}_4^+$ assimilation remains active throughout. These effects resemble the effects of $\text{NO}_3^-$ and $\text{NH}_4^+$ on nitrogenase activity and protein in heterocysts (Renstrom-kellner et al., 1990). Thus, during $\text{N}_2$-fixation *P. boryanum* cells functionally resembles heterocysts. However, unlike heterocysts, $\text{N}_2$-fixing *P. boryanum* cells retains PE and RuBisCo (Figs. 5.6, 5.7), they retain the capacity to develop $\text{NO}_3^-$ uptake and NR activity on nitrate availability (Fig. 5.5), and their PC levels change only transiently (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).
**CHAPTER 6**

**SUMMARY**

*Nostoc* ANTH cyanobiont was characterized in its symbiotic, free-living, immobilized and reconstituted states. Studies such as morphological and physiological changes occurring during above mentioned states as well as localization of various enzymes of nitrogen metabolism were performed. For comparative purposes non-heterocystous cyanobacteria *Plectonema boryanum* was studied with regard to nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy. The findings are summarized below:

1. Characterization of symbiotic, free-living, immobilized and reconstituted *Nostoc* ANTH cyanobiont:

   a) Axenic culture of *Anthoceros* was raised from spores. The cyanobiont *Nostoc* was isolated from *Anthoceros* gametophytic thalli and raised in axenic culture. Using these two axenic cultures of the symbionts, *Anthoceros-Nostoc* symbiosis was reconstituted in the laboratory under axenic conditions. Reconstitution was verified by microscopic observations of *Nostoc* colonies in the gametophytes of *Anthoceros*, growth of these *Nostoc* containing *Anthoceros* thalli in combined nitrogen free medium and the nitrogen fixation by these *Anthoceros* thalli. As the symbiosis progressed, heterocyst frequency increased.

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progressively to a maximum of 47% and nitrogen fixation also progressed similarly. Maximum nitrogenase activity was recorded in the middle portion of the thallus, although, the heterocyst frequency was highest in the basal portion of the thallus. This was because of occurrence of double or multiple heterocysts towards the base which although possess nitrogenase protein, get limited fixed carbon from the vegetative cells.

b) The cyanobiont isolated from the Anthoceros thalli is a Nostoc sp. (referred as Nostoc ANTH). It is capable of autotrophic growth using atmospheric $N_2$ and can also utilize other combined nitrogen sources such as ammonia and nitrate for its growth. The heterocyst frequency in the cultured free-living Nostoc ANTH was recorded to be 14% which is much lower than its heterocyst frequency in symbiotic state but definitely higher than other free-living cyanobacteria (5-7%).

c) Rate of nitrogen fixation and heterocyst frequency increased progressively when free-living Nostoc ANTH was supplied with sugars as fixed-carbon sources. Best response was obtained with the provision of glucose as fixed-carbon source. It is known that Anthoceros transfers fixed-carbon to the cyanobiont in symbiosis. Data obtained here using glucose indicated that the fixed-carbon transferred may be in the form of glucose and availability of fixed-carbon may be one of the reasons for high heterocyst frequency and higher nitrogenase activity in symbiotic Nostoc.
d) Immobilization mimics one of the conditions that the cyanobiont faces while undergoing symbiotic association. Immobilization was done in calcium alginate beads which was found to be convenient and harmless. The cyanobiont initially undergoes shock upon immobilization but subsequently recovers fully. Nitrogenase activity showed a drop initially but recovered after a time gap of 144 h and subsequently exceeded its original value in free-living state. The effects of fixed nitrogen sources on heterocyst frequency and nitrogenase activity were milder than in free-living state. Similarly, rate of photosynthetic $O_2$-evolution and respiratory $O_2$-consumption showed severe inhibition upon immobilization. However, immobilized cells did recover fully between 200 h to 300 h. Drop in these two processes may explain the initial drop in nitrogenase activity as ATP and reductants necessary for nitrogenase activity are generated during photosynthesis.

e) *Nostoc* ANTH in immobilized state stayed viable for a long period (upto 5 years) in desiccated condition. These beads when put in nutrient medium swell up and *Nostoc* filaments grow out of the beads into the medium. It is interesting to note that the immobilized beads become the size of mustard seeds upon desiccation and are easily transportable and can be regenerated as and when required.

2. Immuno-electronmicroscopic localization of various enzymes: localization of nitrogenase, Glutamine synthetase (GS),
phycoerythrin (PE), ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCo) and hydrogenase was studied using immunochemical techniques in the cyanobiont and the free-living cultured isolate *Nostoc ANTH* of *Anthoceros punctatus*. In both cases, nitrogenase was located in heterocysts only and was uniformly distributed within the cell. This put rest to the speculation that vegetative cells of cyanobionts in symbiosis may contain nitrogenase. GS was located both in heterocysts and vegetative cells, with a uniform cellular distribution in each cell type. Whereas heterocysts of *Nostoc ANTH* had about two-fold higher label than vegetative cells, labeling in heterocysts and vegetative cells of the cyanobiont was similar. While the GS content of the vegetative cells of the cyanobiont and *Nostoc ANTH* was comparable, the apparent GS content of the cyanobiont heterocysts was 60% less than that in *Nostoc ANTH* heterocysts. With this finding it becomes apparent that in all cyanobionts liberating ammonia, GS levels in heterocysts are repressed and become similar to that in vegetative cells. It seems higher level of GS in heterocysts is essential for assimilation of $\text{N}_2$-derived ammonia. PE and RubisCo were located in vegetative cells only. PE was located on thylakoid membranes and RubisCo in the carboxysomes and cytoplasm. Carboxysomes had much higher levels of RubisCo than that in cytoplasm. In each case pattern and extent of labeling in the cyanobiont and *Nostoc ANTH* was similar. Hydrogenase was located both in vegetative cells and heterocysts of *Nostoc ANTH*; the former having consistently higher label than the latter. A similar pattern and level of hydrogenase labeling
was found in the cyanobiont cells residing in Anthoceros punctatus tissue. In Nostoc ANTH as well as in cyanobiont cells, a higher intensity of hydrogenase labeling was observed along the plasma membranes between vegetative cells. The eukaryotic partner did not show any hydrogenase antigen.

3. Nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous Cyanobacterium Plectonema boryanum PCC 73110: The regulation of nitrogenase derepression, plus the catalytic activity and protein concentration of glutamine synthetase (GS), nitrate reductase (NR), ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) and phycoerythrin (PE) were studied in filamentous non-heterocystous cyanobacterium Plectonema boryanum PCC 73110. Both nitrogen limitation and microaerobic incubation were essential for derepression of nitrogenase. Oxygen caused irreversible inactivation of nitrogenase, as well as repression of its synthesis. A temporal separation of nitrogen fixation and photosynthetic $O_2$-evolution was observed under $N_2/CO_2$ (95:5 v/v) atmosphere. Repeated peaks of nitrogenase and growth were observed. Immunogold localization showed that in $N_2$-fixing cultures, all cells, including those undergoing division, contained nitrogenase, and that the nitrogenase antigen was uniformly distributed throughout the cells without any preferential association with cellular structures. Thus, the earlier speculation about possible subcellular compartmentalization of nitrogenase in non-heterocystous
cyanobacteria seems unfounded. RuBisCo was mainly located in carboxysomes of both $\text{N}_2$-fixing and $\text{NO}_3^-$-grown cells. Both $\text{N}_2$-fixing and $\text{NO}_3^-$-grown cells showed similar levels of PE, which was associated with the thylakoid membranes. GS antigen was distributed throughout the cells and the relative amounts of this enzyme, as well as its activity, were 20% higher in $\text{N}_2$-fixing than $\text{NO}_3^-$-grown cultures. $\text{NO}_3^-$-uptake and NR systems were found to be $\text{NO}_3^-$-inducible, with very low activities in $\text{N}_2$-fixing cultures. The latter may be important in avoiding competition for Mo between nitrogenase and NR. Thus, $\text{P. boryanum}$ differs from heterocystous cyanobacteria in having nitrogenase in all the cells. However, as in heterocysts, GS levels go up on derepression of nitrogenase, although not to the same extent. Furthermore, $\text{NO}_3^-$-uptake and reductase activities are lost under conditions of nitrogenase derepression, as has been noted on heterocyst development.