3.1 INTRODUCTION

Studies concerning the ways in which photosynthetic microbes sense and respond to their environment is an important mode to gain insight into their cellular metabolic activities. Nutrient availability is an important factor affecting the cellular activities in cyanobacteria. When growing photoautotrophically, cyanobacteria require only a few nutrients but must adapt to environmental transients, of which changes in light, nitrogen, carbon and sulphur are the most vital factors. This chapter deals with the modification of the cyanobacterial light harvesting apparatus in response to different nitrate concentration.

Nitrogen, in the form of nitrate, ammonia or urea (or combinations of these) is added to most media for commercial aquaculture in high concentration. Changes in N supply are known to strongly influence the growth and biochemical composition of microalgae (Lourenco et al., 2002). Production of proteins is favoured during periods of N sufficiency, with limited carbohydrate synthesis; conversely during periods of reduced N-availability, carbohydrate accumulates and protein production drops (Fernandez-Reiriz et al., 1989) whereas lipids usually increase (Shifrin and Chisholm, 1981). Multimolecular structures, the PBS, primarily harvesting light energy in cyanobacteria, comprise of pigmented, water soluble proteins, the phycobiliproteins, which may constitute ~50% of the soluble proteins of the cyanobacterial cell. Thus, nitrogen availability is a key factor affecting the synthesis and degradation of PBS.

In general, the intracellular reserves for a specific nutrient decline when that nutrient is limiting, but increases when it is in excess, or when levels of another nutrient limit growth. Hence, both the types and numbers of cellular inclusion bodies may provide a gauge for evaluating the nutrient status of the cell. The regulation of storage body accumulation has not been investigated thoroughly, but evidence suggests that

enzyme activities for both synthesis and degradation are always present (Grossman et al., 1994).

PBS, the major light harvesting complexes of the cell, are sometimes considered to be nutrient reserves mainly for nitrogen. PBS are known to be degraded in N and S-deprived cells. There is evidence that PBS content can decline during N-deficiency in some cyanobacteria without affecting their growth rate (Boussiba and Richmond, 1980; Wyman et al., 1985). The use of PB as amino acid storage molecules may be especially important for marine cyanobacteria, since, nitrogen may frequently be limiting in marine environments (Grossman et al., 1994).

Studies concerning environmentally regulated degradation of the PBS have already begun to provide insights into processes involved in the targeting of macromolecular complexes for degradation and the machinery that implements this degradation. This chapter presents the effect of different nitrate concentrations on the degradation and synthesis of the different components of PBS complex in the three selected cyanobacterial species.

3.2 Material and Methods

Materials:

Standard protein molecular weight markers were obtained from Sigma-Aldrich Co., USA. All other reagents used were of analytical grade available from commercial sources and used without further purification.

Methods:

3.2.1 *Quantification of Chl 'a' and Phycobiliproteins*:

Fresh water strain, *Arthrospira indica*, marine strains, *Phormidium tenue* and *Lyngbya limnetica* were cultured in Zarrouk's and ASN-III medium, respectively (section 1.2.1) with different nitrate concentrations. Concentrations of nitrate selected

were, 0 ppm nitrate as control, normal nitrate concentration in medium, twice the normal concentration, four times the normal concentration and six times the normal concentration of the medium. So accordingly, for marine strains the different nitrate concentrations supplied were, 0 ppm, 750 ppm, 1500 ppm, 3000 ppm and 4500 ppm. Similarly for freshwater strain *A. indica* the concentration of nitrate supplied were 0 ppm, 2500 ppm, 5000 ppm, 10000 ppm and 15000 ppm. In the medium sodium nitrate was used as the sole nitrogen source provided. All the experiments were carried out in duplicates and the graphical data represents the mean values. Cultures were harvested every seven days for 5 weeks, by centrifugation at 10000 xg for 30 min at 4°C.

For PB extraction the harvested cell mass was suspended in 100 mM Na-Phosphate buffer (pH 7.0) and the cell mass was disrupted by sonication for 20 s. Repeated cycles of freezing at –195°C in liquid nitrogen and thawing at room temperature were carried out till complete extraction was done. This was then followed by centrifugation at 10000 xg for 30 min at 4°C and the clear supernatant containing PB was collected.

Estimation of PB

Estimation of PC, APC and PE were done as mentioned in section 2.2.1.

For Chl '*a*' extraction the cell mass was suspended in 90% methanol and the extraction was carried out twice at 4°C for an hour, in the dark, followed by centrifugation at 10000 xg for 10 min at 4°C. Cultures were kept under different nitrate conditions in duplicates.

Estimation of Chl 'a'

Estimation of chl '*a*' was done as mentioned in section 1.2.2.

3.2.2 *Spectral Analysis*:

UV-VIS absorption spectroscopy

To study the spectral nature of PB under different nitrate concentrations, the cells were cultured as mentioned in section 3.2.1 and were harvested in the log phase. Extraction was done as mentioned in section 2.2.1 and the spectra recorded in UV-Vis range with respect to phosphate buffer as blank. All UV-Vis absorption spectra were recorded on a CARY 500 Scan UV-Vis, NIR spectrophotometer with 1 cm path length.

FT-IR Spectra

The PB extracted from the cells in the log phase was dialyzed against water at 4°C for 48 hrs and then freeze dried by Virtis Freeze mobile 8EL and the spectras were recorded on a Perkin Elmer Spectrum GX FT-IR spectrophotometer as KBr pellet.

3.2.3 *Gel Electrophoresis*:

The PB extracted from cells grown under different nitrate conditions were electrophoresed by SDS-PAGE (15%) (Sambrook et al., 1989). Samples were heated for about 5 min at 95°C with 2% (w/v) SDS, 10% (v/v) glycerol, 4.5% (v/v) β-mercaptoethanol, 0.25% (w/v) bromophenol blue and 60 mM Tris (pH 6.8) for about 5 min at 95°C. Gels were run at 20±2°C and visualized by silver staining (Wray et al., 1981) and photographic documentation was done. The molecular weight of the separated linkers and subunits were determined by calibrating the gel with molecular weight markers.

3.3 Results

3.3.1 *Growth curves and PB content*:

Apart from their requirements of light and water, the most conspicuous demand, photoautotrophic organisms make on the environment, is for the various mineral elements that are indispensable for life. 'N' is an important mineral element required by cyanobacteria for its various cellular metabolic activities including PB production. The changes in PB production due to deficiency or excess of nitrate are documented here.

A. indica when grown in media, completely lacking in any form of nitrogen, showed a steep downwards growth curve and also the doubling time was as high as at ~17 hrs (Table 3.1). A decreased doubling time was observed in cultures grown with 10000 ppm (~7 hrs) and 15000 ppm nitrate (~8 hrs). In ASN-III media (2500 ppm nitrate) culture showed a doubling time of ~9 hrs. PC seemed to increase considerably in culture grown in media containing 10000 ppm and 15000 ppm nitrate (Fig.3.1a). But APC content seemed to be highest in '5000 ppm' PB extract with a APC/PC ratio of 0.75 and was lowest in media with no nitrate with an APC/PC ratio of 0.37.

Media with no nitrate also had a damaging effect on *L. limnetica* showing an increased doubling time of ~15 hrs, the normal being ~12 hrs. Highest content of PC is observed in '3000 ppm' and '4500 ppm' PB extract. But the highest APC/PC ratio was observed in culture grown in '1500 ppm' nitrate. Highest content of PE was observed in '3000 ppm' PB extract. PE/PC ratio was not altered much, under any of the conditions and was found to have an average value of 0.1 under all 5 conditions. All the three main components of PBS decreased considerably when *L. limnetica* was grown in a medium with no nitrate content.

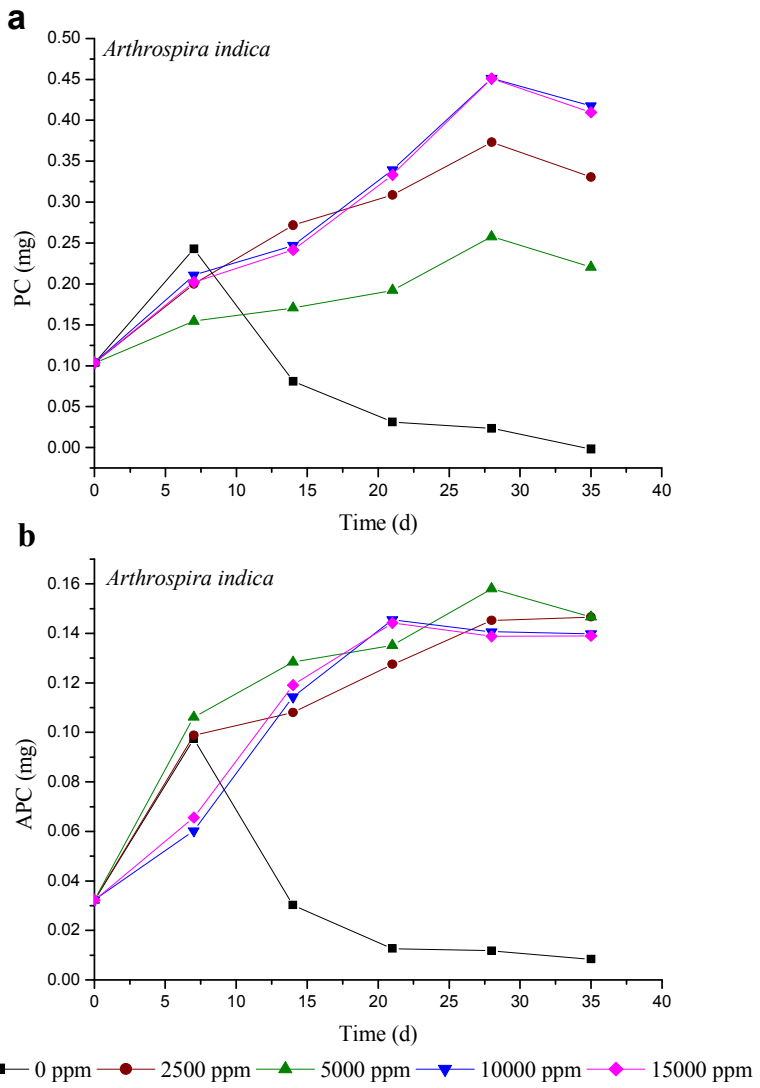

Fig. 3.1(a-b) Variation in PB content under different nitrate concentrations in *A. indica*

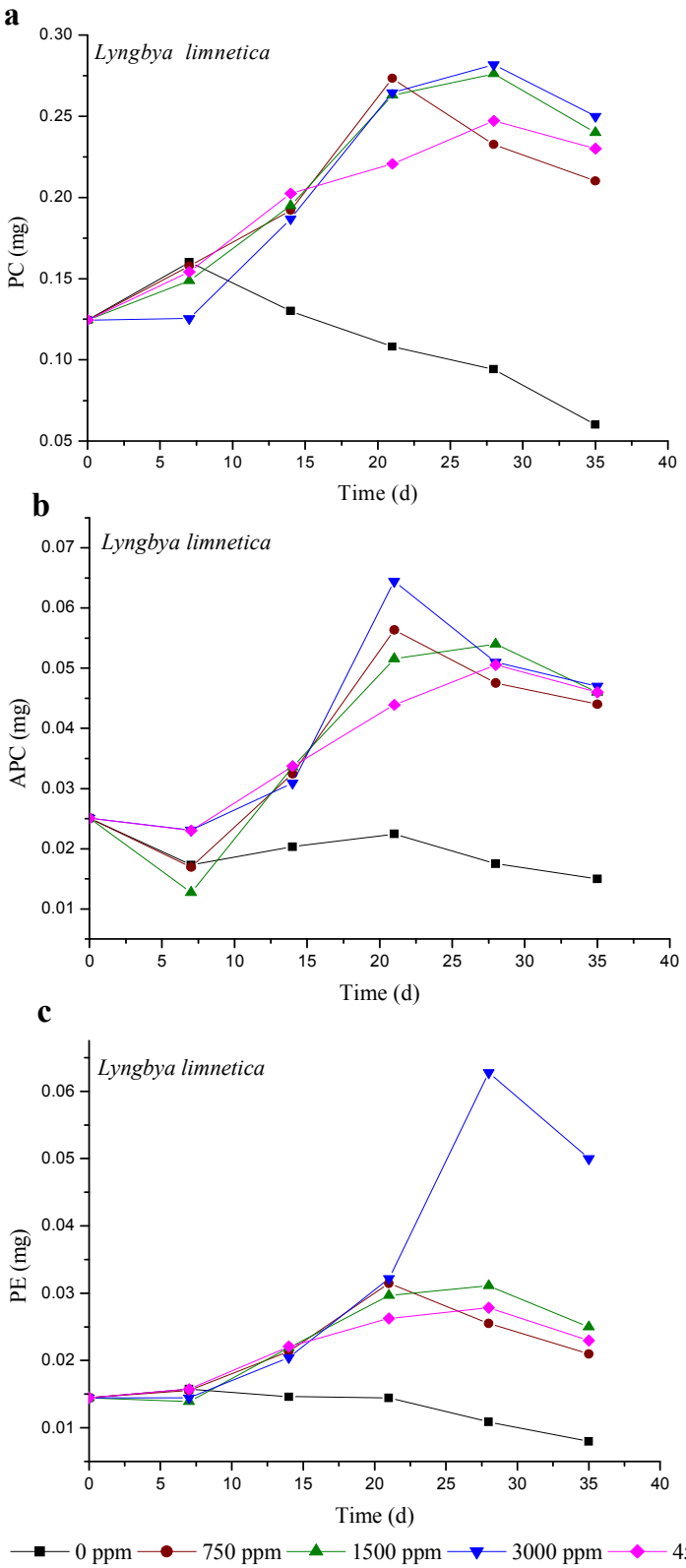

Fig. 3.2(a-c) Variation in PB content under different nitrate concentrations in *L. limnetica*

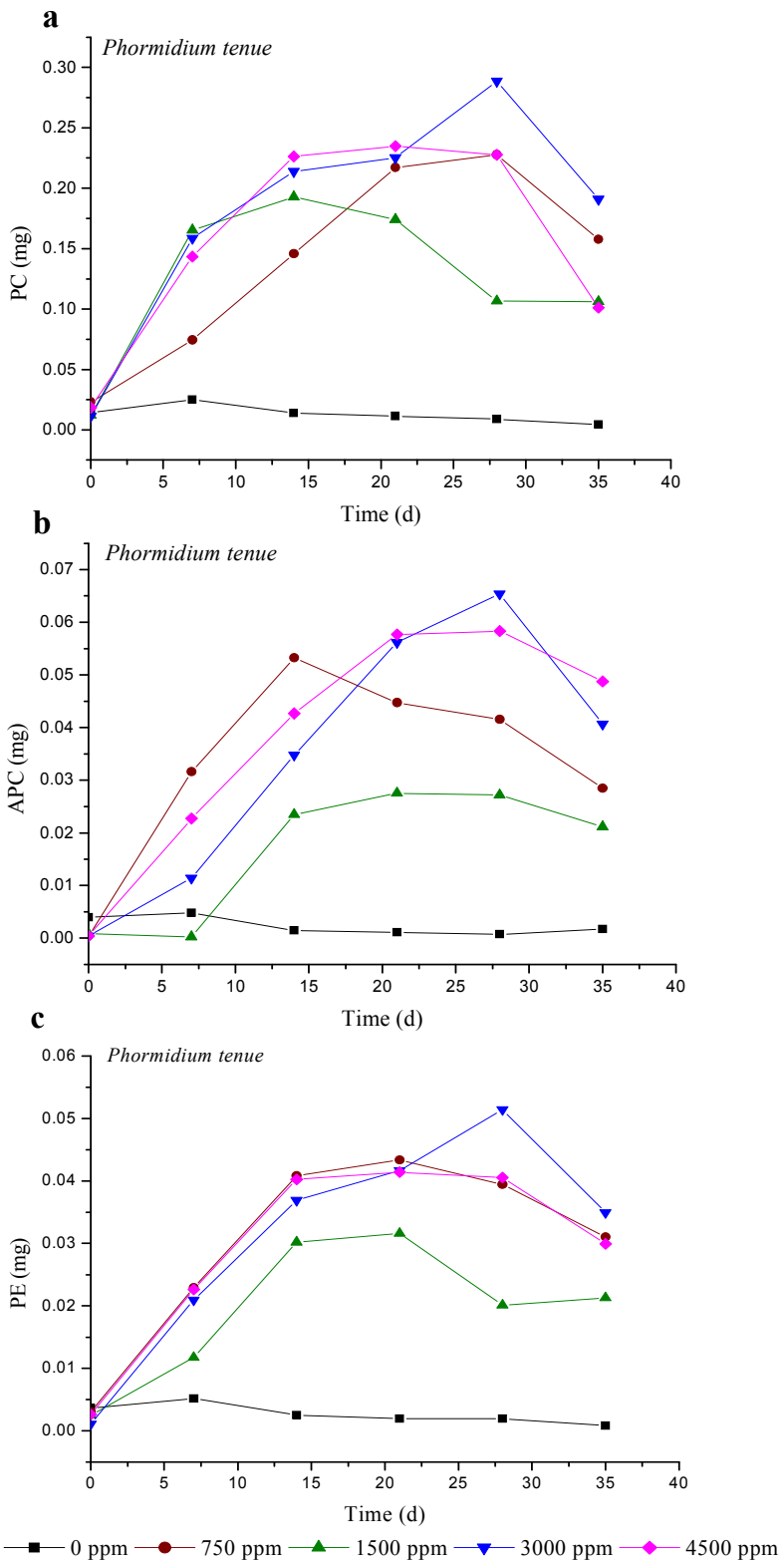

Fig. 3.3(a-c) Variation in PB content under different nitrate concentrations in *P. tenue.*

In *P. tenue* 3000 ppm and 4500 ppm of nitrate was found to be the best for growth showing a doubling time of ~11 hrs and ~10 hrs respectively. Deficiency of nitrate did affect *P. tenue* to increasing the doubling time from ~13 hrs as in normal ASN-III medium to ~19 hrs (Table 3.1). High content of PC is observed in PB extract from cultures grown with 3000 ppm and 4500 ppm nitrate. But PC content is low in 1500 ppm even as compared to that in 750 ppm nitrate (ASN-III medium) as can be seen in Fig.3.3a. Moreover APC/PC ratio is highest in '750 ppm' PB extract. 1500 ppm does not seem to be favorable for PE too (Fig.3.3c). PE/PC ratio is seen to be highest in 750 ppm. PC, APC as well as PE decrease considerably under conditions of nitrate deficiency.

Table 3.1: Doubling Time* of the three strains under different nitrate concentrations#

A. indica	***L. limnetica***	***P. tenue***
~17 hrs (*0 ppm*)	~15 hrs (*0 ppm*)	~19 hrs (*0 ppm*)
~9 hrs (*2500 ppm*)	~12 hrs (*750 ppm*)	~13 hrs (*750 ppm*)
~11 hrs (*5000 ppm*)	~14 hrs (*1500 ppm*)	~15 hrs (*1500 ppm*)
~7 hrs (*10000 ppm*)	~11 hrs (*3000 ppm*)	~11 hrs (*3000 ppm*)
~8 hrs (*15000 ppm*)	~11 hrs (*4500 ppm*)	~10 hrs (*4500 ppm*)

*** The doubling time is expressed as approx. values as decimals have been eliminated from the values displayed.**
 # Nitrate concentrations in the media are shown in the bracket.

3.3.2 *Spectral Analysis*:

Peaks obtained by the absorption spectra of PB can prove to be one of the important criterion towards structural analysis of PBS. Effect of different nitrate concentration on the absorption spectra of PB in the UV-Vis range is documented here.

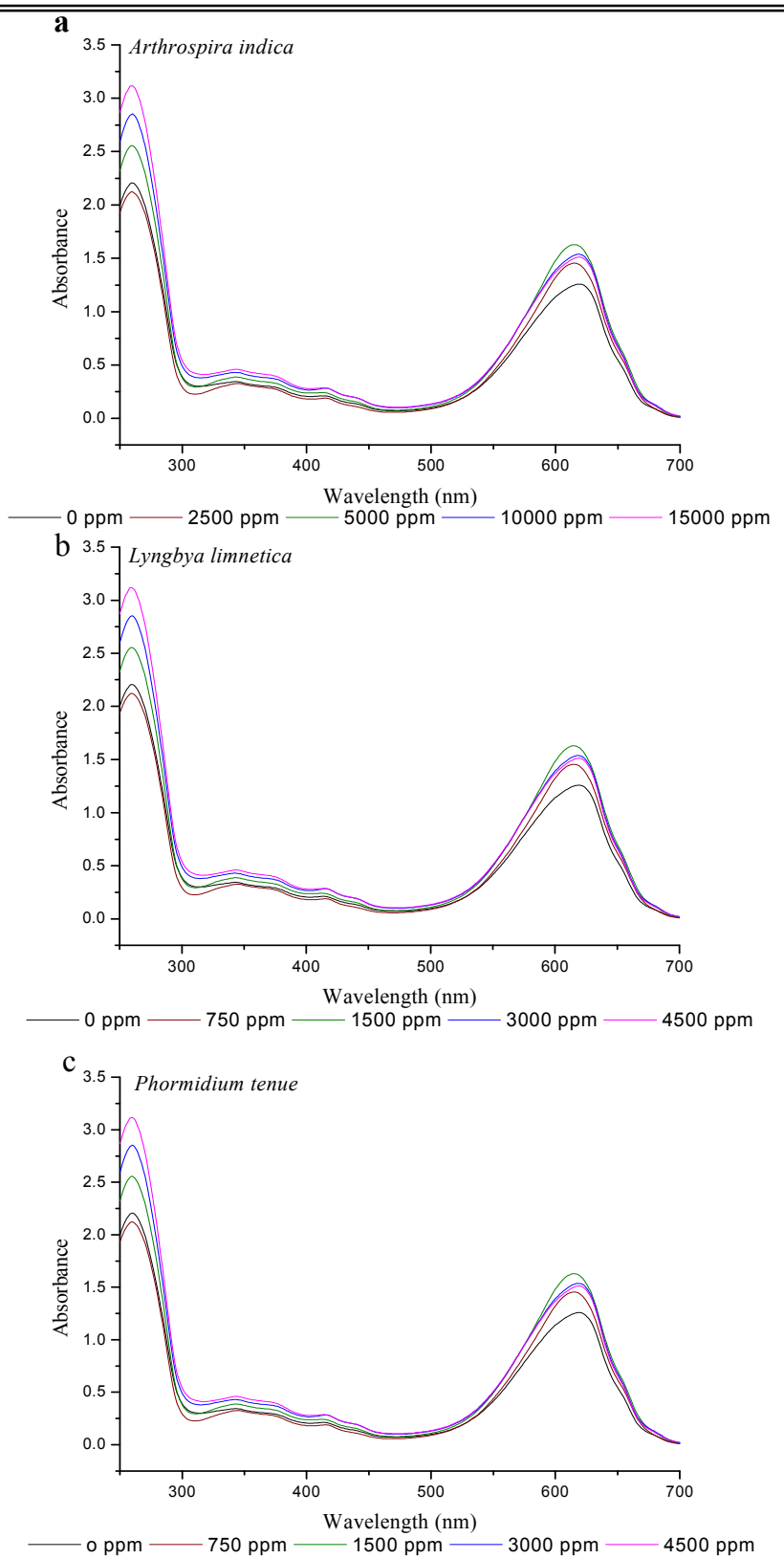

Fig. 3.4(a-c) Absorption spectra of PB extract under different nitrate concentrations.

PC being the major component of PBS, exhibits an intense peak at ~620 nm in almost all the samples of all the three cyanobacterial species (Fig.3.4). A slight shoulder at ~652 nm is notable in all the samples which is characteristic of APC. No significant difference is observed in position of λ_{max} of PB extract of cultures grown in medium with different concentration of nitrate.

PB extract from culture grown under nitrate deficient condition shows a blue shift of ~3 nm in *A. indica* (Table 3.2). The peak of 280 nm is highest in '15000 ppm' PB extract, which signifies that high nitrate content enhances the overall protein production and does not affect only PB production (Fig.3.4a).

Table 3.2 Absorption maxima of PB extract in visible region under different nitrate conditions[#].

A. indica	***L. limnetica***	***P. tenue***
616 nm (*0 ppm*)	615 nm (*0 ppm*)	617 nm (*0 ppm*)
619 nm (*2500 ppm*)	619 nm (*750 ppm*)	619 nm (*750 ppm*)
619 nm (*5000 ppm*)	618 nm (*1500 ppm*)	619 nm (*1500 ppm*)
619 nm (*10000 ppm*)	619 nm (*3000 ppm*)	619 nm (*3000 ppm*)
620 nm (*15000 ppm*)	620 nm (*4500 ppm*)	620 nm (*4500 ppm*)

 # Nitrate concentrations in the media are shown in the bracket

No significant difference was observed in λ_{max} position of PB extract of *L. limnetica* grown with 750 ppm, 1500 ppm, 3000 ppm and 4500 ppm nitrate content (Fig.3.3b). But as in *A. indica*, PB extract from nitrate lacking media shows a ~4 nm blue shift, shifting the λ_{max} to 615 nm which is observed in rest of the samples. A weak absorption peak at ~350 nm is also visible which is also due to PC chromophore. A slight shoulder of PE peak at ~562 nm is also detected under all the conditions.

No prominent differences in λ_{max} position of PB extract spectra of *P. tenue* under varied nitrate content was observed. Though a blue shift of ~2 nm is observed in spectra of PB extract from "no nitrate" conditioned culture (Fig.3.4c).

FT-IR spectra of the different samples showed no major variation. No shifts in the two amide peak positions were observed.

3.3.3 *Protein Profiling*

In addition to phycobiliproteins, PBS are also composed of linker polypeptides, that amount to about 10-20% of the total protein content of PBS (Tandeau de Marsac and Cohen-Bazire, 1977). They mainly serve to connect the rods to the core and the core to the thylakoid. They are also responsible for the aggregation of phycobiliproteins in forming hexamers or trimers. Thus, any change in the PBS structure leads to changes in the linker composition of PBS. Protein profile of PB obtained from cultures grown under different nitrate contents is described below.

Bands of α and β subunits of PC, APC and PE which lie in the range of 14 kD-25 kD were visible even prior to staining but were not very resolved in all the three cultures (Fig.3.5a-c). In all the three selected cyanobacteria the culture grown without nitrate content was the most affected. Protein profile of PB extract of these cultures reveal drastic decrease in the linkers of group II and III but those belonging to group I and IV do not seem to very affected. Even in the other conditions there is no striking difference observed in all the three cultures. The protein profile of rest of the samples appears to be similar.

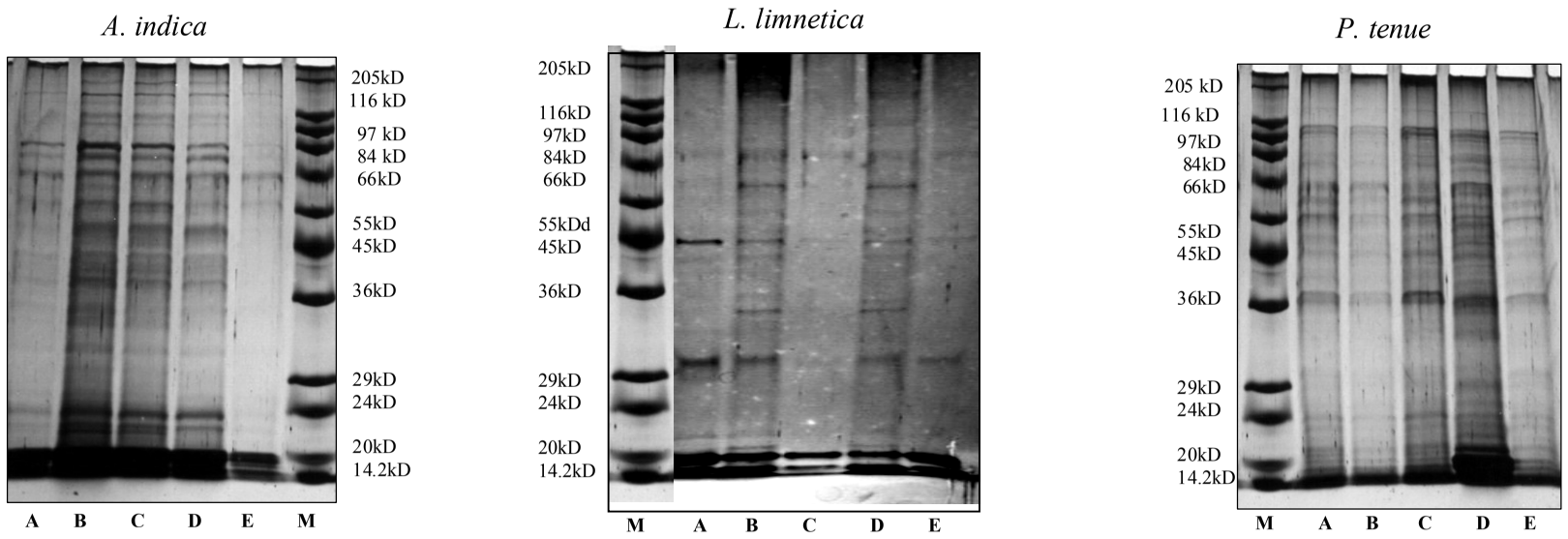

Fig.3.5(a-c) Protein profile of PB extract of cultures grown under different nitrate concentration (in ppm). **(a)** LaneA: control (no nitrate) LaneB: 2500 ppm LaneC:5000 ppm LaneD:10000 ppm LaneE:15000 ppm LaneM: Marker. **(b-c)** LaneA: control (no nitrate) LaneB: 750 ppm LaneC:1500 ppm LaneD:3000 ppm LaneE:4500 ppm LaneM: Marker

3.4 Discussion

Phycobiliproteins, are proteins with chromophore group attached to the apoprotein part. Proteins are chains of different amino acids. All of the 20 amino acids found in nature have a –COOH group and $–NH_2$ group. Nitrogen, thus, is an important factor affecting the composition of any protein. Changes in nitrogen availability are known to strongly influence the growth and biochemical composition of cyanobacteria. Complying with the above fact, complete lack of nitrate in the media does show a steep downward growth curve in all the three studied cyanobacterial strains. The doubling time increases as growth declines. Increase in nitrate composition does, on the other hand seem to enhance growth. The doubling time of all the 3 cyanobacterial strains, *A. indica*, *L. limnetica* and *P. tenue* show a decrease in doubling time with higher nitrate content. This could be due to the fact that high nitrate content would facilitate the optimum protein production which in turn would enhance the metabolic process, consequently decreasing the doubling time. Phycobiliprotein composition is also altered by changes in different nitrate composition. "No nitrate" condition does show a drastic decrease in all the three kinds of Phycobiliprotein in all the three strains. Though as nitrate content increases, PB content is also expected to increase, yet in twice the nitrate concentration, PC in *A. indica* and PC and APC in *P. tenue* showed a lower content as compared to that under normal condition. This proves that this concentration may be a limiting concentration for PB production in these two cyanobacterial strains. But such a limiting effect is not observed in any of the three phycobiliproteins of *L. limnetica*. So these limiting factors may vary from organism to organism. It was observed that, though PC content showed a rise under high nitrate concentrations (3000 and 4500 ppm in ASN-III and 10000 and 15000 ppm in Zarrouk's medium) through out the growth curve, but PE showed a rise in content in '3000 ppm' samples in the later stages of log phase, indicating that

concentration of a particular nutrient and growth stage may have a regulatory effect on the metabolism of the organism.

Absorption spectra is known to be shifted by many factors such as aggregation state (MacColl et al, 1980), covalent bonds between phycobilins and apoprotein (Glazer, 1981, 1984, 1985) or even linkers (Sidler, 1994). Spectra of all the samples displayed a significant peak at ~620 nm. Though APC and PE are also characterized with specific λmax, at ~652 nm and ~562 nm respectively, yet PC being the dominant one, PB extract shows a distinct peak at 620 nm, with only a shoulder at ~652 nm. PE peak is either overlapped by PC peak or a slight shoulder is observed. No significant shifts are observed in the PB extract samples, except the "no nitrate" samples which display blue shift. Different factors are responsible for the changes in spectral pattern. Most influencing among them are the interaction of bilins with the apoprotein part and the interactions involving the bilins and their near surrounding resulting in great modulation of bilin energies. Aggregation of phycobiliproteins and linkers also affects the spectra of phycobiliproteins, which may be one of the main influencing factor responsible for the blue shift. Sidler (1994) has reported that linker next to the core, LRC, red shifts the spectrum of phycocyanin disk to optimize rod to core energy transfer, so in a nitrate deficient environment, definitely the phycobiliprotein and linker content would decrease affecting the energy transfer and in turn creating a blue shift.

Assembly of phycobiliproteins into PBS is dependant on linkers. Thus presence of linkers is a pronounced indicator of the state of PBS in the cell. Under "no nitrate" conditions linkers of group II and III showed a notable reduction on SDS PAGE profile. These two groups are mainly responsible for structural formation of PBS, thus nitrate deficiency initially leads to PBS degradation. Low molecular weight linkers may be the last to be affected as group I which forms the subunits and group II which are also low molecular weight linkers, do not

show decrease in the number. This indicates that, during degradation due to limiting nitrate concentration, PBS structure is first disintegrated and the subunits may be affected during later stages of acute nitrate deficiency.