Homology studies and phylogenetic analysis of nitrate reductase

The *Arthrospira* nitrate reductase (*narB*) gene sequence obtained in this study was used as a query for a BLASTn search against the cyanobacterial database in Genbank (taxid: 1117). The first hit with the maximum score of 973 was *Trichodesmium erythraeum* IMS101 (complete genome), with an identity of 71 %, at an E Value of 0.0 and a query coverage of 91 %. In *Osillatoria chalybea*, which belongs to the same group Oscillatoriales, the sequence showed 69 % maximum identity at an E value of 0.0 with query coverage of 96 % for the ORF of the *narB* gene. Fig. 50 shows the phylograms of the *narB* gene. The translated NR protein sequence had 733 amino acids (Table 10) and CD-search identified the three known domains of nitrate reductase from other organisms: *MopB_CT_Nitrate-R-NapA-like*, *MopB_Nitrate-R-NapA-like* and *molybdopterin dinucleotide-binding region*, *molybdopterin oxidoreductase Fe4S4* region of PFAM (Fig. 51).

3.6.8. Identification of Nitrite reductase from *Arthrospira platensis* PCC 7345

The gene encoding nitrite reductase (*nirA*) from the cyanobacterium *Arthrospira platensis* PCC 7345 has been identified and sequenced. This gene comprises of 1656 nucleotides shown in Appendix A.2. From the organization of the flanking sequences on the clone (Fig. 53), it is clear that the gene is not arranged in the form of an operon as is known in other cyanobacteria like *Synechocystis* sp. strain PCC 6803 (Frias et al., 1997) and *Trichodesmium* sp. (Wang et al., 2000). Further the gene is flanked on 5’ side by a gene encoding DUF 80 protein and on the 3’ side by a hypothetical protein encoding gene of 145 nucleotides in length.
<table>
<thead>
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<th>Organism</th>
<th>Accession number</th>
<th>Length of sequence (nucleotides)</th>
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<td>1551</td>
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<td>1542</td>
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<td>CP000806</td>
<td>1545</td>
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<tr>
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<td>AP009552</td>
<td>1542</td>
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<tr>
<td><em>Microcystis aeruginosa</em> PCC 7806</td>
<td>AM778958</td>
<td>1542</td>
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<tr>
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<td>1584</td>
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<td>Ava_4539</td>
<td>1611</td>
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<td>SYNPPC7002_A1827</td>
<td>1560</td>
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<td><em>Trichodesmium erythraeum</em> IMS101</td>
<td>Tery_1068</td>
<td>1533</td>
</tr>
<tr>
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<td>alr0607</td>
<td>1611</td>
</tr>
<tr>
<td><em>Arthrosira maxima</em></td>
<td></td>
<td>1685</td>
</tr>
<tr>
<td><em>Arthrosira platensis</em> PCC 7345</td>
<td></td>
<td>1656</td>
</tr>
</tbody>
</table>

Table 11: A comparative table showing Ferredoxin - Nitrite reductase (*nirA*) gene from cyanobacteria along with their accession numbers and size with the *nirA* gene from *Arthrosira platensis* PCC 7345.

**Homology studies and Phylogenetic analysis of nitrite reductases**

The nitrite reductase gene sequence was compared with the genes from other cyanobacteria available in NCBI database (Table 11). In BLASTn similarity search, the first hit with the maximum score of 673/926 was *Cyanothecae* sp. PCC 7424 (complete genome), with a maximum identity of 100 %, at an E Value of 0.0 and the query coverage was 91 %. In, *Trichodesmium erythraeum* IMS101 (complete genome), which belongs to the same group Oscillatoriales, the sequence showed 91 % maximum identity at an E value of 5e-64 with query coverage of 89 %. The gene sequence showed similarity to the plants NIR gene mainly in the conserved domain region belonging to family pfam03460, NIR_SIR_ferr, Nitrite/Sulfite reductase ferredoxin-like half domain. The query length was maximum (13 %) for *Spinacea*
Fig. 53: A schematic map of the genes encoding nitrite reductase (*nirA*) in a 2.86 kb genomic clone of *Arthrosira platensis* PCC 7345. The numbers on the top denote the sizes of the ORF of *nirA*, and the numbers below indicate the size of the genomic clone. It contains NiR (ferredoxin nitrite reductase) of 1650 nucleotides, DUF 820 (domain of unknown function protein) of 328 nucleotides, U1 (unknown hypothetical protein) of 145 nucleotides, U2 (unknown hypothetical protein).

Fig. 54: A phylogram of nitrite reductase (*nirA*) gene of *Arthrosira platensis* PCC 7345. The *nirA* gene sequence was used as an input for the BLASTn program and the input generated is shown as phylogram. The entry highlighted in yellow represent the query sequence.
**oleracea** Fd-nitrite reductase gene with accession number X17031.1. It suggests that **Arthospira platensis** bears an active site much alike that present in those reductases. The NiR protein when translated had 552 amino acids (Appendix A.2), the phylograms is shown in Fig. 54 and in CD-Search identified two domains; Nitrite/Sulfite reductase ferredoxin like half domain and nitrite and sulfite reductase 4Fe-4S region.

### 3.6.9. Identification of Glutamine synthetase gene

A clone of appx. 3 kb was obtained after the secondary screening which showed similarity to glutamine synthetase gene after sequence analysis. It had glutamine synthetase gene (**glnA**) of size 1455 nucleotides having an EcoRI site at 938 base pairs as confirmed by restriction digestion (Fig. 55) and the complete sequence obtained from clone is shown in Appendix A.2. In addition to the **glnA** gene it had an unknown region 24 bases, 173 bases showing similarity to mod4 gene and partial allophyocyanin B-18 subunit gene sequence (Fig. 55).

#### Homology studies and Phylogenetic analysis of Glutamine synthetase

When the glutamine synthetase (**glnA**) gene sequence containing 1455 nucleotides was used as an input to search for the similarity in the cyanobacteria database (taxid: 1117), the first hit with the maximum score of 881/916 was *Microcystis aeruginosa* NIES-843 (complete genome), with a maximum identity of 95 %, at an E Value of 0.0 and the query coverage was 98%. The maximum identity of 100% was obtained with
Fig. 55: A schematic map of the genes encoding glutamine synthetase (*glnA*) in a 3 kb genomic clone of *Arthrospira platensis* PCC 7345. The numbers on the top denote the sizes of the ORF of *glnA*, and the numbers below indicate the size of the genomic clone. It contains GS (glutamine synthetase/glutamate ammonia ligase) of 1455 nucleotides, Mod 1 (mod4 gene) of 173 nucleotides, allo P (allophycocyanin B-18 subunit gene), U (unknown hypothetical protein).

Fig. 56: A phylogram of glutamine synthetase (*glnA*) gene of *Arthrospira platensis* PCC7345. The *glnA* gene sequence was used as an input for the BLASTn program and the input generated is shown as phylogram. The entry highlighted in yellow represent the query sequence.
Anabena variabilis ATCC 29413 and Nostoc sp. PCC 7120 complete genomes at a similar E Value of 0.0 and the query coverage was 98 %. In, Trichodesmium erythraeum IMS101 (complete genome), which belongs to the same group Oscillatoriales, the sequence showed 91 % maximum identity at an E value of 0.0 with query coverage of 99 %. The GS gene sequence when translated had 485 amino acids (Appendix A.2) and phylograms is shown in Fig. 56. In CD-Search it showed two domain hits; Glutamine synthetase, catalytic domain; pfam00120 and Glutamine synthetase, beta-Grasp domain; pfam03951.

3.6.10. Identification of Nitrate/nitrite transporters

There are two types of nitrate/ nitrite transporters known in cyanobacteria. One is nrtP which is found mainly in the marine cyanobacteria and other is nrtABCD type of transporters that are typical of the fresh water cyanobacteria. In Arthrospira platensis PCC 7345 both these type of transporters are found as is evident from the PCR cloning, sequencing and similarity studies done using BLASTn.

A) Identification of nrtP

The nrtP type of nitrate/nitrite transporter was found to be closely associated with the nitrate reductase gene in Arthrospira platensis PCC 7345. The gene has 1513 nucleotides and was found at the 5′end of the nitrate reductase (narB) gene as seen in (Fig. 49) and the sequence in the complete clone is in Appendix A.2.
Homology studies and Phylogenetic analysis of nrtP gene

The nrtP gene sequence containing 1455 nucleotides was used as an input to search for the similarity in the cyanobacteria database (taxid: 1117). The first hit with the maximum score of 2221/2221 was Arthrospira maxima strain FACHB438 nrtP gene with a maximum identity of 93 %, at an E Value of 0.0 and the query coverage was 100 %. The maximum identity of 100 % was obtained with Cyanothecae sp. PCC 7424 (complete genome) at an E Value of 0.0 and the query coverage was 86 %. In Trichodesmium erythraeum IMS101 (complete genome), which belongs to the same group Oscillatoriales, the sequence showed 87 % maximum identity at an E value of 0.0 with query coverage of 97 %. The phylograms is shown in Fig. 52, and the translated protein had 505 amino acids (Appendix A.2). In CD-Search it showed two domain hits, nitrite extrusion protein (nitrite facilitator) and major facilitator superfamily (MFS).

B) Identification of nrtABCD

A single clone containing the complete nrtABCD gene sequence or overlapping clones could not be obtained in this study. As shown by dotted lines in the Fig. 57, there were some missing sequences of unknown length, making the location and orientation of the genes in the map somewhat tentative. Moreover, the gene sequences representing the complete ORFs were not available for nitrate/nitrite transporter genes in Arthrospira platensis PCC 7345. In all, three phagemids clones were identified to contain partial nrtABCD genes. By aligning the sequences from these three clones,
partial \textit{nrt}A, partial \textit{nrt}C and partial \textit{nrt}D were obtained. The \textit{nrt}A sequence was 1234 nucleotides in length and it was incomplete at the 3'end. While, \textit{nrt}C sequence with 1473 nucleotides was incomplete at both the ends, \textit{nrt}D with 861 nucleotides was incomplete at 5'end only. The sequences are given in Appendix A.2.

\textbf{Homology studies and Phylogenetic analysis of \textit{nrt}ABCD}

The \textit{nrt}A, \textit{nrt}C and \textit{nrt}D were checked for the homology with the genes reported for other cyanobacteria and also the whole nucleotide database collection. Since the genes were partial, the analysis is also incomplete and is based on the regions that were available. The \textit{nrt}A gene sequence of 1234 nucleotides showed maximum identity of 100\% with a query coverage of 90\% and e value of 4e-83 with \textit{Cyanothecae} sp. PCC 7425 (complete genome). The \textit{nrt}C gene sequence of 1473 nucleotides showed maximum identity of 92\% with a query coverage of 93\% and e value of 1e-121 with \textit{Cyanothecae} sp. PCC 7425 (complete genome). The \textit{nrt}D gene sequence of 861 nucleotides showed maximum identity of 95\% with a query coverage of 85\% and e value of 8e-96 with \textit{Cyanothecae} sp. PCC 7425 (complete genome). As the gene sequences are partial, CD search analysis was not attempted.

\textbf{3.6.11. Identification of Glutamate synthase gene}

All the 19 clones obtained after secondary screening were checked for the presence of glutamate synthase (\textit{gls}F) gene as described above for the other genes. A single phagemid clone containing the complete glutamate synthase gene could not be
Fig. 57: A schematic representation of \textit{nrtABCD} in \textit{Arthrosira platensis} PCC 7345. After sequence comparison and similarity alignment, it contains a hypothetical protein of 621 nucleotides, \textit{nrtA} (substrate binding protein) of 1240 nucleotides in length, \textit{nrtC} (ATP binding subunit) of 1473 nucleotides in length and \textit{nrtD} (ATP binding subunit) of 861 nucleotides in length of nitrate/nitrite transporter family. The dotted line represents the gap that exists between the different genes (which could not be completed in this study). The positions of the first and last bases of coding region is also denoted, block arrows denote the ORFs with their direction of transcription.

Fig. 58: A schematic map of glutamate synthase (glsF) gene in \textit{Arthrosira platensis} PCC7345 as obtained after alignment of two clones of 1996 and 3952 bases respectively in size. The positions of the first and last bases of coding region are also denoted. Fd GOGAT (ferredoxin - glutamate synthase) of 4601 nucleotides and a hypothetical protein of 1166 nucleotides.
identified. A complete GOGAT gene sequence was obtained after aligning the sequences obtained from two different clones containing the partial gene. The first clone had an insert of 2 kb which was having partial GOGAT gene (5'end) having 900 nucleotides (Appendix A.2). Another clone was identified which had only the GOGAT gene sequence of 4 kb, but it was incomplete at both ends (Appendix A.2). When the sequences were aligned using NCBI BLAST, the glutamate synthase gene of 4607 nucleotides in length was obtained which was having an incomplete 3'end. The whole sequence is given in the Appendix A.2 and the graphical representation is given in Fig. 58.

**Homology studies and Phylogenetic analysis of glutamate synthase**

The GOGAT gene sequence containing 4607 nucleotides was used as an input to search for the similarity in the cyanobacteria database (taxid: 1117). The first hit with the maximum score of 975/1173 was *Cyanothecae* sp. PCC 7424 (complete genome) with a maximum identity of 100 %, at an E Value of 0.0 and the query coverage was 88 %. The maximum identity of 100 % was also obtained with at an E Value of 0.0 and the query coverage was 78 % for *Trichodesmium erythraeum* IMS101 (complete genome), which belongs to the same group Oscillatoriales. As the gene sequences are partial, CD search analysis was not attempted.

In summary, the cloning of the genes involved in nitrate transport and assimilation in *Arthrosira platensis* PCC 7345 has been accomplished by a combination of PCR cloning and genomic library screening. Even though
complete sequences spanning the complete ORFs could not be obtained in all the cases, full length gene sequences of *narB*, *nirA*, *nrtP* and *glnA* have been obtained by this approach, along with their flanking regions. Although *nrtABCD* and *glsF* gene sequences are incomplete, the missing portions are in the middle and the flanking sequences can be used to obtain their full length sequences by PCR cloning approach.
Section 3.7

Transcriptional analysis of Nitrate assimilatory genes

Arthrospira platensis has been well known for its nutritional and biotechnological importance and has received considerable attention for its unique ability to survive in highly alkaline and saline conditions (Vonshak, 1997). However, genetics and molecular biology studies in Arthrospira platensis are scant, and this is particularly true for the genes and enzymes of nitrate assimilator pathway, inspite of the fact that the molecular basis for high protein content in Spirulina must be rooted in nitrate assimilation and N use efficiency in this organism. The biochemical studies (Chapter 2, section 2) on the enzymes of the N-assimilatory pathway from this organism using nitrate and its downstream metabolites (nitrite, ammonium and glutamine) revealed that NR and NiR are nitrate inducible. Cloning and sequencing of the genes of nitrate uptake and assimilation in this study (Chapter 2 section 5) has made it possible to do transcriptional studies for the first time. Primers were designed on the basis of the partial sequences of the NR, NiR, NRT, GS and GOGAT genes obtained by PCR cloning approach (Chapter 2, section 5) and used for RT-PCR analysis of their transcripts in the presence and absence of nitrate and its downstream metabolites. The present section describes the results of these studies.
3.7.1. RNA isolation and analysis

Due to the lack of suitable protocols specific to *Arthospira platensis* for the isolation of high quality RNA with high yields, efforts were made in the present study to optimize an RNA isolation protocol for *Arthospira platensis* by modifying methods based on hot phenol and LiCl, as described in Materials and Methods (Pathak and Lochab, 2010). The analysis of total RNA using Agilent bioanalyzer 2100 revealed that it had RNA integrity values (RIN) values >5, as per the recommended standards (Fleige et al, 2006). As is evident from Fig. 59, the bioanalyzer electropherograms indicate intact 23S and 16S peaks and total area of peaks in accordance with the prescribed standards (Schroeder et al, 2006). The total RNA yields by this method were routinely higher (1 mg/g cell pellet by wet wt.) than most published protocols (and had $A_{260}/A_{280}$ ratios of 1.8-2.0 and RIN values >5 (Table 12), indicating good quality of RNA. Agarose gel electrophoresis further confirmed the RNA quality and the lack of DNA contamination (Fig. 60).

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<th>Parameter</th>
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<th>Value (sample 2)</th>
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<tr>
<td>DNA contamination</td>
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<td>Invisible on agarose gel</td>
</tr>
</tbody>
</table>

Table 12: Yield and quality parameters of total RNA isolated by the optimized method. Sample 1 and 2 represents RNA samples isolated from two different isolation from *A. platensis* cultures.
Fig. 59: An electropherogram summary of the RNA samples from *Arthrosira platensis* isolated using LiCl method. The 16S and 23S peaks were clearly visible in both the samples. The RNA integrity number (RIN values) of 6.9 and 7.9 respectively for the samples 1 and 2.

![Electropherogram](image)

<table>
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<tr>
<th>Name</th>
<th>Start Time [s]</th>
<th>End Time [s]</th>
<th>Area</th>
<th>% of total Area</th>
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<tr>
<td>23S</td>
<td>44.41</td>
<td>55.74</td>
<td>30.6</td>
<td>118</td>
</tr>
</tbody>
</table>

Overall Results for sample 3: 0.75 100L
- RNA Area: 264.9
- RNA Concentration: 1,894 ng/μl
- RNA Ratio (23S / 16S): 5.6
- RNA Integrity Number (RIN): 6.9 (8.02.07)

Overall Results for sample 4: 8.25 100L
- RNA Area: 223.9
- RNA Concentration: 1,595 ng/μl
- RNA Ratio (23S / 16S): 9.9
- RNA Integrity Number (RIN): 7.9 (8.02.07)

Fragment table for sample 4: 8.25 100L

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<th>End Time [s]</th>
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<th>% of total Area</th>
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<td>23S</td>
<td>43.82</td>
<td>54.93</td>
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</table>

Fig. 60: A denaturing agarose gel (1.2 %) isolated from *Arthrosira platensis* isolated using LiCl method. Distinct RNA bands are seen in lanes 1 and 2 representing 16S and 23S.

![Agarose Gel](image)
3.7.2. RT-PCR analyses of NR, NiR, NRT and GS transcript levels

The effect of different N-sources on the transcript levels of NR, NiR, NRT and GS was studied using RT-PCR as described under materials and methods. Since inorganic sources of N are essential for the survival and growth of *A. platensis*, control conditions without N source can only be obtained by recovering the cells from normally grown cultures and washing and resuspending them in N-free media, before introducing a suitable N source for analysis of its effect.

Exponentially growing cultures were harvested, washed twice with N-free medium and resuspended in nitrate free medium for 2 hours. Culture sample was recovered for RNA isolation before reintroducing a source of N either as nitrate (KNO$_3$, 20 mM), or nitrite (KNO$_2$) or ammonium (NH$_4$Cl) or glutamine, at a final concentration of 4 mM each, either individually or in suitable combinations. After incubation for a further 2 h, cells were harvested and total RNA was isolated and used for RT-PCR analysis using gene specific primers for NR, NiR, NRT and GS as described in materials and methods. 16SrRNA was used as an internal housekeeping control for all the conditions. The results of these experiments are described below.

3.7.3. Effect of nitrate removal on NR, NiR, NRT and GS transcript levels

As is evident from Fig. 61, a drastic decrease in the NR transcript level was observed upon transfer from normal to N-free medium for 6 h (C), which is restored to the
initial level upon reintroduction of 20 mM nitrate (CN), clearly indicating the inducibility of NR gene expression by nitrate. The same trend was observed for NiR and NRT (Fig. 61) transcripts, indicating that they are similarly regulated by nitrate induction. However, transcript levels for GS (Fig. 61) did not show such drastic increase or decrease in the presence or absence of nitrate, regardless of the cycle number of the RT-PCR, indicating that GS may be regulated differently.
Fig. 61: Effect of nitrate on the transcript levels of NR, NRT, NiR and GS in *A. platensis*. N denotes normal cultures growing in presence of nitrate (20 mM), C denotes nitrate-free culture and CN denotes reintroduction of nitrate in a nitrate-free culture. 16S rRNA (housekeeping control), NR (nitrate reductase), NRT (nrtP-type nitrate/nitrite transporter), NiR (nitrite reductase), GS (Glutamine synthetase).

Fig. 62: Effect of nitrite on the transcript levels of NR, NRT, NiR and GS in *A. platensis*. C denotes nitrate-free culture and N denotes reintroduction of nitrate in a nitrate-free culture, K denotes addition of nitrite to nitrate-free culture, KN denotes addition of nitrite and nitrate to nitrate-free cultures, rRNA (16S rRNA housekeeping control), NR (nitrate reductase), NRT (nrtP-type nitrate/nitrite transporter), NiR (nitrite reductase), GS (Glutamine synthetase).
3.7.4. Effect of nitrite on transcript levels of Nitrate assimilatory genes

As seen in Fig. 62, when nitrite was introduced in the cultures transferred from normal to N-free medium for 6 h, an increase NR, NiR, NRT and GS transcript levels was observed, which is almost equal to the transcript levels observed after addition of nitrate alone or with nitrite in N-free cultures for NRT and NiR. Nitrite seems to be having an inhibitory effect on the recovery of transcript levels in NR and GS to the original levels.

3.7.5. Effect of ammonium on transcript levels of Nitrate assimilatory genes

As seen in Fig. 63 when cultures growing in normal media were transferred to N-free medium for 6 hours were supplemented with nitrate and ammonium together, the recovery of the expression levels of NR, NiR and NRT was observed, although this is attributed to the presence of 20 mM nitrate rather than ammonia, since ammonia alone did not show any effect on NR, NiR and NRT mRNA levels. However, in case of GS, ammonia independently as well as in the presence of nitrate restored mRNA levels indicating, the regulatory role of ammonia as an inducer for GS gene.
Fig. 63: Effect of ammonia on the transcript levels of NR, NRT, NiR and GS in *A. platensis*. C denotes nitrate-free culture and N denotes reintroduction of nitrate in a nitrate-free culture, A denotes addition of ammonia to nitrate-free culture, AN denotes addition of ammonia and nitrate to nitrate-free cultures, rRNA (16S rRNA housekeeping control), NR (nitrate reductase), NRT (nrtP-type nitrate/nitrite transporter), NiR (nitrite reductase), GS (Glutamine synthetase)

Fig. 64: Effect of glutamine on the transcript levels of NR, NRT, NiR in *A. platensis*. C denotes nitrate-free culture and N denotes reintroduction of nitrate in a nitrate-free culture, G denotes addition of glutamine to nitrate-free culture, GN denotes addition of glutamine and nitrate to nitrate-free cultures, rRNA (16S rRNA housekeeping control), NR (nitrate reductase), NRT (nrtP-type nitrate/nitrite transporter), NiR (nitrite reductase)
3.7.6. Effect of glutamine on transcript levels of nitrate assimilatory genes

As seen in fig. 64, the recovery of the expression levels of NR, NiR and NRT was observed, when the cultures grown as described above were supplied with nitrate and glutamine together, although this can also be attributed to the presence of nitrate rather than glutamine, since glutamine alone did not show any effect on NR, NiR and NRT mRNA levels as was observed for ammonia. Attempts were made to study the effect of glutamine treatment on GS mRNA levels; however these did not yield any conclusive expression pattern.

In conclusion, the current study reports the induction of NR, NiR, NRT genes by nitrate at the mRNA levels in *Arthospira platensis* PCC 7345 which supports the biochemical data earlier observed for NR and NiR (chapter 2 section 2).