2.1 INTRODUCTION:

2.1.1 Morphological studies:

Cyanobacteria represent one of the major eubacterial groups. They are unique among the prokaryotes in possessing the capacity of oxygenic photosynthesis. In addition, cyanobacteria also have the capacity of fixing atmospheric nitrogen. These qualities make cyanobacteria the most successful and widespread group among the prokaryotes, occupying a wide range of terrestrial and aquatic environments. Cyanobacteria are also characterized by a great morphological diversity, unicellular as well as filamentous species being included with a cell volume ranging over more than five orders of magnitude (Whitton, 2000). They often colonize bare areas of rock and soil as earth fossil evidence points to their presence in geographically diverse regions during the Precambrian (2 to more than 3.5 billion years ago). Cyanobacteria were among the pioneer organisms of the early earth (Brock, 1999). The prominent habitats of cyanobacteria are limnic (Muthukumar et al., 2007) and marine environments (Thajuddin and Subramanian., 1990, 1991, 1992, 1994). The cyanobacteria comprise a large component of marine plankton with global distribution (Gallon et al., 1996). The cyanobacteria also include species that run through the entire range of water types, from polysaprobic zones to katharobic waters (Van Landingham, 1982). This widespread distribution reflects a large variety of species, covering a broad spectrum of physiological properties and tolerance to environmental stress (Tandeau de Marsac and Houmard, 1993). A number of freshwater species are also able to withstand relatively high concentrations of sodium chloride. It appears that many cyanobacteria isolated from coastal environments tolerate saline environments i.e. halo tolerant rather than required salinity (i.e. are halophilic). As frequent colonizers of euryhaline (hyper saline) environments, cyanobacteria are found in salt pans (Thajuddin et al., 2002; Nagasathya and Thajuddin, 2008) and salt marshes, and are capable of growth at combined salt concentrations as high as 3-4 molar mass (Reed et al., 1984).

However, phylogenetic relationships among the cyanobacteria are relatively poorly understood. Traditionally, they have been classified using morphological and ecological characters (Getlter, 1932; Desikachary, 1959). Stainier et al., (1978) and Rippka et al., (1979) argued that because cyanobacteria are prokaryotes, their systematic treatment should not be
based on traditional botanical methods but rather on type cultures deposited in culture collections. Morphology may change depending on environmental conditions and the diversity of strains within a culture may be suppressed by selective culturing conditions (Doers and Parker, 1988). Photosynthetic pigment content, lipid composition, differentiated cell structures and other characters may also be subject to change because of the variable expression of cyanobacterial gene products in culture (Kenyon et al., 1972; Klein et al., 1973). Morphological changes induced by culture conditions and environmental plasticity can be problematic for cyanobacterial taxonomy (Stanier et al., 1971).

2.1.2 Molecular studies:

Phylogenetic studies of cyanobacteria have demonstrated that genetic relationships sometime conflict with the morphological classification (Lyra et al., 2001; Iteman et al., 2000; Gugger and Hoffmann, 2004; Muralitharan and Thajuddin, 2008). The comparison of morphological and genetic data is hindered by the lack of cultures of several cyanobacterial morphospecies and inadequate morphological data of sequenced strains. Moreover, some strains may lose some important features such as gas vesicles (Lehtimäki et al., 2000) or form of colony (Gugger et al., 2002) during long-term laboratory cultivation, which complicates identification. Anagnostidis and Komárek (1989) have estimated that more than 50% of the strains in culture collections are misidentified. Therefore, new isolates should be studied by combined morphological and genetic approaches.

2.1.3 The 16S rRNA gene:

Cyanobacteria possess a ribosomal RNA (rRNA) cistron comprises of three genes; the 16S small subunit (SSU), 23S large subunit (LSU) and the 5S subunit, each separated by an internal transcribed spacer region (ITS). A genetic marker often used in phylogenetic studies is the 16S rRNA gene. Within cyanobacteria, sequence information from this gene is widely regarded as one of the most valid criterion for determining relationships between closely related groups, such as species or genera. It is the basis for systematic assignment in the latest edition of Bergey’s Manual of Systematic Bacteriology and has been useful in distinguishing broad taxonomic groups as well as individual species (Litvaitis, 2002; Casamatta et al., 2005; Rajaniemi et al., 2005; Svenning et al., 2005).

The 16S rRNA gene has several interesting properties which promoted its application in phylogenetic studies: (a) rRNAs are universal molecules. Similar structure in all living being suggests that they may have evolved very early and changed little since their origin. (b) The functional importance in protein translation gives the rRNAs a basic and irreplaceable
role in cellular processes. (c) The secondary structure and nucleotide sequence of rRNA seem much conserved globally. For example, certain parts of the molecule are identical among the three kingdoms: eubacteria, archaebacteria and eukaryotes. On the other hand, there are parts of the molecules which evolved more or less rapidly, and these may be useful to compare organisms which are more or less closely related. (d) rRNAs are abundant in cells, especially when growth rate is rapid; they are generally easy to extract, identify and use in partial 16S rRNA gene sequencing technology. (e) The 16S rRNA gene is a long molecule, containing about 1500 nucleotides. This allows for combination of a large number of characters for a satisfactory statistical evaluation of the results. (f) Apparently, there has been no lateral transfer of the genes coding for the rRNA; thus, the determined sequences are truly reflecting the evolution of the studied organisms. (g) According to Woese (1987), 16S rRNA gene is a good “molecular chronometer” i.e. a good measure of the overall rate of evolutionary change in a line of descent. Moreover, the 16S rRNA gene sequences are relatively easy to align, and a large volume data base accumulated (currently over 6000 cyanobacterial sequences), allowing comparisons between strains (Ludwig and Klenk, 2001).

Nucleotide sequence signatures at particular positions on the 16S rRNA gene may discriminate cyanobacterial taxa down to the strain level. Sequence dissimilarities of only one nucleotide have been used to detect a single species in mixed bacterial populations (De Long et al., 1989; Bej et al., 1990). These sequence ‘signatures’ may be detected by specific oligonucleotide probes, various DNA amplification protocols.

In the present study, chapter one deals with the isolation, identification and molecular characterization of cyanobacteria from the rice field.
2.2 REVIEW OF LITERATURE:

2.2.1 Isolation and identification:

Algae are the important food links in the aquatic ecosystems. Being autotrophic and members of the first tropical level, their major role in waters is to capture solar energy to drive the ecosystem. The magnitude of primary production is the leading factor in deciding the tropic structure of water bodies. Several species of algae have been found in tropical condition such as those in India. It provides favourable environment for luxuriant growth of these organisms in natural ecosystem such as different water bodies.

Cyanobacteria are common in eutrophic nature. Being favoured by stable and nutrient enriched water, they may constitute an important part of phytoplankton communities. Growth of the phytoplankton was not nutrient limited instead irradiance and temperature were more important. The Indian subcontinent studies are limited to phytoplankton of large rivers and streams even though their spatial and temporal variations have been studied (Nantiyal et al., 1997).

Wilson et al., (1992) described five marine cyanophages propagated on Synechococcus sp. strain VWH7803 were isolated from three different oceanographic provinces during the months of August and September 1992: coastal water from the Sargasso Sea, Bermuda; Woods Hole harbor, Woods Hole, Mass; and coastal water from the English Channel, off Plymouth Sound, United Kingdom. The five cyanophage isolates were found to belong to two families, Myoviridae and Styloviridae, on the basis of their morphology observed in the transmission electron microscope. DNA purified from each of the cyanophage isolates was restricted with a selection of restriction endonucleases, and three distinguishably different patterns were observed. DNA isolated from Myoviridae isolates from Bermuda and the English Channel had highly related restriction patterns, as did DNA isolated from Styloviridae isolates from Bermuda and the English Channel. DNA isolated from the Myoviridae isolate from Woods Hole had a unique restriction pattern. The genome size for each of the Myoviridae isolates was ca. 80 to 85 kb, and it was ca. 90 to 100 kb for each of the Styloviridae isolates. Southern blotting analysis revealed that there was a limited degree of homology among all cyanophage DNAs probed, but clear differences were observed between cyanophage DNA from the Myoviridae and that from the Styloviridae isolates. Polypeptide analysis revealed a clear difference between Myoviridae and Styloviridae polypeptide profiles, although the major, presumably structural, protein in each case was 53 to 54 kDa.
Cyanobacteria are a highly diverse group in relation to form, function, and habitat (Neilan et al., 1995). Current cyanobacterial systematics relies on the observation of minor and plastic morphological characters. Accurate and reliable delineation of toxic and bloom-forming strains of cyanobacteria has not been possible by traditional methods. They designed general primers to the phycocyanin operon (cpc gene) and developed a PCR which allows the amplification of a region of this gene, including a variable intergenic spacer sequence. Because of the specificity of this PCR for cyanobacterial isolates, the assay is appropriate for the rapid and reliable identification of strains in freshwater samples. Successive restriction endonuclease digestion of this amplification product, with a total of nine enzymes, yielded many identifying DNA profiles specific to the various taxonomic levels of cyanobacteria. The restriction enzyme profiles for MspI, RsaI, and TaqI were conserved for strains within each of the eight genera (40 strains) studied and clearly discriminated among these genera. Intragenic delineation of strains was revealed by the enzymes AluI, CfoI, and HaeIII for members of the genus Microcystis, while strains of genus Anabaena were differentiated by the digestion patterns provided by AluI, CfoI, and ScrFI. Phenetic and cladistic analyses of the data were used to infer the genetic relatedness and evolution of toxic and bloom-forming cyanobacteria.

2.2.2 Cyanobacterial taxonomy:

Komarek (2006) stated the application of modern ecological, ultra structural and molecular methods, aided by the cultivation of numerous cyanobacterial morphotypes, has substantially changed our knowledge of these organisms. It has led to major advances in cyanobacterial taxonomy and criteria for their phylogenetic classification. Molecular data provide basic criteria for cyanobacterial taxonomy; however, a correct phylogenetic system cannot be constructed without combining genetic data with knowledge from the previous 150 years research of cyanobacterial diversity. Thus, studies of morphological variation in nature, and modern morphological, ultrastructural, ecophysiological and biochemical characters need to be combined in a “polyphasic” approach. Taxonomic concepts for generic and infrageneric ranks are re-evaluated in light of combined phenotypic and molecular criteria. Despite their usefulness in experimental studies, the limitations of using strains from culture collections for systematic and nomenclatural purposes are highlighted. The need for a continual revision of strain identification and proper nomenclatural practice associated with either the bacteriological or botanical codes is emphasized. Recent advances in taxonomy are
highlighted in the context of prospects for understanding cyanobacterial diversity from natural habitats, and the evolutionary and adaptational processes that cyanobacteria undergo.

Abed et al., (2002) described a new genus of moderately halophilic, moderately halotolerant and moderately thermophilic cyanobacteria with very thin trichomes. The four strains included in this genus were isolated from benthic microbial mats in a man-made hypersaline pond. Trichomes were around 1 µm thick, with small constrictions at the cross-walls and diffusent colorless sheaths. Thylakoids were parallel to the cell wall, but thylakoids and nucleoid were often excentrically arranged within the cytoplasm with respect to the main trichome axis. Strains grew at between 3.2 and 12–15% (w/v) salinity with optima between 3.2 and 12%. They showed lower temperature limits around 20°C and upper limits between 45 and 50 °C, with optima between 28 and 45–50 °C. Carotenoid and mycosporine amino-acid complements were identical among strains. Phylogenetic analyses based on 16S rRNA gene sequence showed that all strains were closely related (99% or higher similarity) and distantly related to other cyanobacteria (91% or lower similarity) and with all these data a new genus and species was proposed (Halomicronema excentricum).

2.2.3 Molecular characterization:

Lachance (1981) investigated the genetic relatedness of 45 strains of heterocyst-forming cyanobacteria assigned to eight genera by Rippka et al., (1979) and of 19 undescribed strains of the same group by in vitro reassociation of radioiodinated deoxyribonucleic acids. The members of the genera Nodularia, Cylindrospermum, Chlorogloeopsis, and Fischerella formed discrete clusters (intragenic values of relative binding, more than 55%) and showed intergeneric relatedness of less than 40%, results consistent with the classification proposed by Rippka et al., (1979). The genus Nostoc was heterogeneous; four strains previously assigned to Anabaena appeared to belong to Nostoc. The genus Calothrix comprised four clusters with various degrees of internal homogeneity and two strains which showed low relatedness to any others. The general relatedness (i.e., relative binding) of heterocyst formers to various non-heterocystous cyanobacteria was on the order of 10 to 20%.

Sivonen et al., (1990) demonstrated that a strain of the filamentous cyanobacterium Nostoc sp. isolated from a lake in Finland was found to produce at least nine hepatotoxic peptides with chemical and toxicological properties similar to those of the hepatotoxic hepta- and pentapeptides produced by other cyanobacteria. Toxins were isolated and purified by high performance liquid chromatography. Amounts available for five of the purified toxins
(P6, P14, P15, P16, and P18) were adequate for high-performance liquid chromatography amino acid analysis and determination of molecular weight by fast-atom bombardment-mass spectrometry (FAB-MS). Quantities of three toxins (P14, P15, and P16) were analyzed by high-resolution FAB-MS, FAB-MS/MS, and $^1$H - nuclear magnetic resonance. Analysis showed that the toxins are new types of microcystin-LR homologs. Microcystin-LR contains equimolar amounts of D-alanine, L-leucine, D-erythro-4-methylaspartic acid, L-arginine, ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid), D-glutamic acid, and N-methyldehydroalanine (molecular weight [MW], 994). Nostoc sp. strain 152 was found to produce the following microcystin-LR homologs: (i) P6 contains an extra methylene group most probably due to the presence of N-methyldehydrobutyryne instead of N-methyldehydroalanine (MW, 1,008); (ii) P14 is O acetyl-O-demethyl ADDA-microcystin-LR (MW, 1,022); (iii) P15 is 3-demethyl-O-acetylADDA-homoarginine-microcystin-LR (MW, 1,036); (iv) P16 is 3-demethyl-O-acetyl O-demethylADDA-microcystin-LR (MW, 1,008); (v) P18 is 3- demethyl-O-acetyl-O-demethylADDA-homoarginine-microcystin-LR (MW, 1,022). The toxicities of the new microcystin homologs were not significantly different from those of microcystin-LR or demethylmicrocystin-LR.

Manen et al., (2002) investigated the genetic diversity of the genus Arthrospira and to compare it with other cyanobacteria, sequences of 670 nucleotides from the phycocyanin operon were determined for 23 natural, cultivated or commercial strains of Arthrospira and compared with sequences from 20 other non-Arthrospira cyanobacterial strains. The sequenced DNA fragment comprises the last 255 nt of cpcB, the cpcB–cpcA spacer and the first 304 nt of cpcA. The resulting phylogenetic tree confirms that the genus Arthrospira is not related to Spirulina. So far, cpcB–cpcA data suggest that the closest relative of Arthrospira is Planktothrix. Based on this locus, the genus Arthrospira consists of three genetically clustered lineages. However, the distribution of nucleotide substitutions indicates that these three lineages are not the result of a simple cladogenesis characterized by the accumulation of independent substitutions. Instead, the observed clustering is the result of horizontal transfers of blocks of sequences. Analysis of the distribution of substitutions in the sequenced fragment indicates a point of intragenic recombination close to the stop codon of cpcB. The capacity of exchange of genetic material among strains probably explains why morphology and geographical origin do not correlate with the cpcB–cpcA clusters. Nevertheless, this study shows for the first time that the genus Arthrospira, represented here by cultivated and wild specimens, is clearly monophyletic. Moreover, the cpcB–cpcA DNA
fragment, comprising both highly and moderately variable regions, allows (1) a strict differentiation of the taxon Arthrospira from other cyanobacteria (using the coding regions only) and (2) the study of relationships inside Arthrospira (using both the coding and non-coding regions).

Ki et al., (2000) investigated the genetic diversity of Nodularia strains from the Baltic Sea and from Australian waters, together with the proposed type strain of Nodularia spumigena. The Nodularia strains were characterized by using a polyphasic approach, including RFLP of PCR-amplified 16S rRNA genes, 16S rRNA gene sequencing, Southern blotting of total DNA, repetitive extragenic palindromic- and enterobacterial repetitive intergenic consensus-PCR, ribotyping and phenotypic tests. With genotypic methods, the Nodularia strains were grouped into two clusters. The genetic groupings were supported by one phenotypic property: the ability to produce nodularin. In contrast, the cell sizes of the strains were not different in the two genetic clusters. 16S rRNA gene sequences indicated that all the Nodularia strains were closely related, despite their different origins. According to this study, two genotypes of Nodularia exist in the Baltic Sea. On the basis of the taxonomic definitions of Komarek et al. (1993), the non-toxic type without gas vesicles fits the description of N. sphaerocarpa, whereas the toxic type with gas vesicles resembles the species N. spumigena and N. baltica.

Neilan et al., (2002) described the rapid and sensitive methods for the detection and genetic characterization of cyanobacteria have been developed based on DNA amplification techniques. Work describes the molecular methods that have been used to characterize cyanobacteria and their use as tools to identify toxin-producing strains. Different species and strains were compared using restriction fragment length polymorphism (RFLP) of amplified fragments of the phycocyanin gene and the 16S-23S rRNA internal transcribed spacer.

Kondo et al., (2002) stated that DNA base composition and DNA–DNA hybridization among the cyanobacterial genus Microcystis were determined using nine axenic Microcystis strains, including the three ‘morphological’ species of M. aeruginosa, M. viridis and M. wesenbergii. These Microcystis spp. showed a similar DNA base composition (42<1–42<8 mol% GMC) and demonstrated more than 70% DNA relatedness, confirming their synonymy based on bacterial criteria.

Lyra et al., (2001) described the toxic and non-toxic cyanobacterial strains from Anabaena, Aphanizomenon, Calothrix, Cylindrospermum, Nostoc, Microcystis, Planktothrix
(Oscillatoria agardhii), Oscillatoria and Synechococcus genera were examined by RFLP of PCR-amplified 16S rRNA genes and 16S rRNA gene sequencing. With both methods, high 16S rRNA gene similarity was found among planktic, anatoxin-aproducing Anabaena and non-toxic Aphanizomenon, microcystin-producing and non-toxic Microcystis, and microcystin-producing and non-toxic Planktothrix strains of different geographical origins. The respective sequence similarities were 99<9–100%, 94<2–99<9% and 99<3–100%. Thus the morphological characteristics (e.g. Anabaena and Aphanizomenon), the physiological (toxicity) characteristics or the geographical origins did not reflect the level of 16S rRNA gene relatedness of the closely related strains studied. In addition, cyanobacterial strains were fingerprinted with repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC)-PCR. All the strains except two identical pairs of Microcystis strains had different band profiles. The overall grouping of the trees from the 16S rRNA gene and the REP- and ERIC-PCR analyses was similar. Based on the 16S rRNA gene sequence analysis, four major clades were formed. (i) The clade containing filamentous heterocystous cyanobacteria was divided into three discrete groups of Anabaena/Aphanizomenon, Anabaena/Cylindrospermum/ Nodularia/Nostoc and Calothrix strains. The three other clades contained (ii) filamentous non-heterocystous Planktothrix, (iii) unicellular non-heterocystous Microcystis and (iv) Synechococcus strains.

Houmard et al., (1990) explained that the cyanobacteria harvest light energy through multimolecular structures, the phycobilisomes, regularly arrayed at the surface of the photosynthetic membranes. Phycobilisomes consist of a central core from which rods radiate. A large polypeptide (LCM, 75-120 kDa) is postulated to act both as terminal energy acceptor and as a linker polypeptide that stabilizes the phycobilisome architecture. We report here the characterization of the gene (apcE) that encodes this LCM polypeptide in Calothrix sp. PCC 7601. It is located upstream from the genes encoding the major components of the phycobilisome core (allophycocyanin) and is part of the same operon. The deduced amino acid sequence shows that the N-terminal region of LCM shares homology with the other phycobiliprotein subunits and thus constitutes the chromoprotein domain. The other part of the molecule is made up of four repeated domains that are highly homologous to the N-terminal regions of the phycocyanin rod linker polypeptides. The predicted secondary structure of the different domains of the LCM is discussed in relation to the different roles and properties of this large molecule.
Nubel et al., (1997) developed and tested a set of oligonucleotide primers for the specific amplification of 16S rRNA gene segments from cyanobacteria and plastids by PCR. PCR products were recovered from all cultures of cyanobacteria and diatoms that were checked but not from other bacteria and archaea. Gene segments selectively retrieved from cyanobacteria and diatoms in unialgal but nonaxenic cultures and from cyanobionts in lichens could be directly sequenced. In the context of growing sequence databases, this procedure allows rapid and phylogenetically meaningful identification without pure cultures or molecular cloning. We demonstrate the use of this specific PCR in combination with denaturing gradient gel electrophoresis to probe the diversity of oxygenic phototrophic microorganisms in cultures, lichens, and complex microbial communities.

Neilan et al., (1997) stated a primary-structure analysis of the 16s rRNA gene was performed with 10 strains representing five described and one unidentified species of the genus Microcystis. The phylogenies determined illustrate the evolutionary affiliations among Microcystis strains, other cyanobacteria, and related plastids and bacteria. A cluster of 10 strains that included hepatotoxic isolates identified as Microcystis aeruginosa formed a monophyletic group. However, the genus Microcystis appeared to be polyphyletic and contained two strains that clustered with unicellular cyanobacteria belonging to the genus Synechococcus. The clustering of related Microcystis strains, including strains involved in the production of the cyclic peptide toxin microcystin, was consistent with cell morphology, gas vacuolation, and the low G+C contents of the genomes. The Microcystis lineage was also distinct from the lineage containing the unicellular genus Synechocystis and the filamentous, heterocyst forming genus Nostoc. The secondary structure of a Microcystis 16s rRNA molecule was determined, and genus-specific sequence signatures were used to design primers that permitted identification of the potentially toxic cyanobacteria belonging to the genus Microcystis via DNA amplification.

Sivonen et al., (1992) described that Hepatotoxins (microcystins) from seven freshwater Anabaena strains originating from three different Finnish lakes and one lake in Norway were isolated by high-performance liquid chromatography and characterized by amino acid analysis and fast atom bombardment mass spectrometry. All strains produced three to seven different microcystins. A total of 17 different compounds were isolated, of which 8 were known microcystins. The known compounds identified from six strains were MCYST (microcystin)-LR, [D-Asp3] JMCYST-LR, [Dha7] MCYST-LR, [DAsp3, Dha7] MCYST-LR, MCYST-RR, [D_Asp3] MCYST-RR, [Dha71] MCYST-RR, and [D-Asp3,
Dha71] MCYST-RR. With the exception of MCYST-LR and [D-Asp3] MCYST-LR, this is the first time that isolation of these toxins from Anabaena strains has been reported. Three of the strains produced one to three toxins as minor components which could not be identified. Anabaena sp. strain 66 produced four unidentified toxins. The other Anabaena strains always contained both MCYST-LR and MCYST-RR and/or their demethyl variants. Quantitative differences between toxins within and between strains were detected; at times MCYST-LR and at other times MCYST-RR or demethyl derivatives thereof were the most abundant toxins found in a strain.

Barker et al., (1999) explained the filamentous diazotrophic cyanobacterium Nodularia forms water blooms each year in the Baltic Sea. Filaments isolated from such water blooms vary in their trichome width, degree of coiling, and properties of their gas vesicles; previously, these characters have been used to classify individuals to species level. To test the validity of such a phenotypic classification, they determined the nucleotide sequences for a region of the phycocyanin locus that includes a noncoding intergenic spacer (PC-IGS), the IGS between two adjacent copies of the gvpA gene (which encodes the main structural gas vesicle protein) and the rDNA internal transcribed spacer (rDNA-ITS), for 13 clonal Nodularia isolates from the Baltic Sea during August 1994. The complete 16S-rDNA sequence was determined for three isolates and was found to be identical in each of them. Molecular sequences for noncoding regions of the genome were used to assign isolates to three groups on the basis of PC-IGS, two groups on the basis of gvpA-IGS, and three groups on the basis of rRNA gene ITS. No consistent correlation was found between genotype and any of the phenotypic features examined, and no link was found between any of these features themselves, indicating that these characters are not useful for placing Nodularia isolates into meaningful taxonomic groups. The PC-IGS, gvpAIGS, and rDNA-ITS genotypic groupings were not congruent. This might indicate that gene flow occurs between individuals in Nodularia populations.

Litvaitis et al., (2001) demonstrated the Parsimony and Neighbor-joining analyses of 16S rRNA gene sequences of 68 species and strains of cyanobacteria and prochlorophytes supported a monophyletic Nostocales, a monophyletic Stigonematales, three independent lineages of prochlorophytes within the cyanobacteria, and a paraphyletic Chroococcales (p<0.0001) and Oscillatoriales (p = 0.0147). Within the Oscillatoriales, the genus Oscillatoria formed an unnatural taxon (p<0.0001) and needs major revisions. Using constraint analysis, the genus Microcystis was found to be monophyletic and the paraphyletic
positions of *Microcystis elabens* and *M. holsatica* were probably due to long-branch attraction. Further, a separation of Chroococcales based on varying levels of polyunsaturated fatty acids was more consistent with nucleotide-based phylogenies than with existing morphological groupings. It was proposed that Chroococcales be redefined to exclude the genus *Microcystis*, and that a new order be erected for *Microcystis*. Finally, it was more parsimonious to assume a common cyanobacterial/prochlorophyte ancestor, than to evoke de novo synthesis of chl*b* in each prochlorophyte lineage plus in the lineage leading to green chloroplasts. This common ancestor was proposed to have contained both chlorophyll *a* and *b* plus phycobilins. Subsequent multiple losses of chl*b* in cyanobacteria and the loss of chl*a* and phycobilins in prochlorophytes, led to the observed pigment distribution. It was therefore, recommended that Prochlorales be reclassified as cyanobacteria.

Suda et al., (2002) explained a polyphasic approach to clarify the taxonomy of the water bloom-forming oscillatorioid cyanobacteria. Seventy-five strains of oscillatorioid cyanobacteria were characterized by 16S rDNA sequence analysis, DNA base composition, DNA–DNA hybridization, fatty acid composition, phycobilin pigment composition, complementary chromatic adaptation, morphological characters, growth temperature and salinity tolerance. Phylogenetic analysis based on 16S rDNA sequences divided the strains into six groups, all of which were clearly separated from the type species of the genus *Oscillatoria, Oscillatoria princeps* Gomont NIVA CYA 150. Therefore, these strains should be classified into genera other than *Oscillatoria*. Groups I–III were closely related to one another and groups IV–VI were distinct from one another and from groups I to III. Group I was further divided into two subgroups, group I-pc, which includes strains containing only phycocyanin (PC), and group I-pe, which includes strains containing large amounts of phycoerythrin (PE) in addition to PC. This phenotypic distinction was supported by DNA–DNA hybridization studies. Based on the properties examined herein and data from traditional, botanical taxonomic studies, the groups and subgroups were classified into single species and we propose either emended or new taxonomic descriptions for *Planktothrix agardhii* (type strain NIES 204T), *Planktothrix rubescens* (type strain CCAP 1459/22T), *Planktothrix pseudagardhii* sp. nov. (type strain T1-8-4T), *Planktothrix mougeotii* (type strain TR1-5T), *Planktothricoides raciborskii* gen. nov., comb. nov. (type strain NIES 207T), *Tychonema bourrellyi* (type strain CCAP 1459/11BT) and *Limnothrix redekei* (type strain NIVA CYA 277/1T).
Salomon et al., (2003) identified that the colonial and filamentous cyanobacteria frequently have bacteria associated with their extracellular mucus zone or more tightly attached to their cells surface. The toxin-producing cyanobacterium Nodularia spumigena is an important component of the Baltic Sea plankton community, and its filaments were likely to provide a microenvironment suitable for the development of a particular bacteria flora. About 13 bacterial strains associated with filaments of N. spumigena from the Baltic Sea were isolated and identified by sequencing the 16S rRNA gene. Different bacterial lineages were found associated with the cyanobacterial filaments, including the alpha, beta, and gamma subdivisions of the class Proteobacter and the division Firmicutes (Gram-positive bacteria). Several 16S rRNA gene sequences were not closely related to previously reported sequences of cultured bacteria from the Baltic Sea or to any other reported sequence. Conversely, sequences related to the gamma Proteobacter genus Shewanella, a group previously described in the Baltic Sea, were found among the isolates. The bacterial isolates were grown and added to cultures of exponentially growing N. spumigena. Five isolates, related to the alpha and gamma Proteobacter and Firmicutes, affected negatively the cyanobacterial growth, leading to a lower biomass yield up to 38% relative to controls with no bacteria addition. Five gamma Proteobacter-related strains had no effect on the cyanobacterial growth, while three strains related to Shewanella baltica had a positive effect. Although none of the bacterial isolates showed strong algicidal effect, the observed stimulatory and retarding effects on N. spumigena growth under culture conditions denotes the importance of the associated bacterial community for the dynamics of these cyanobacterial populations in nature. Moreover, several new taxa recovered in this study probably belong to species not yet described.

Sanchez et al., (2005) reported in the recent study have shown that the cyanobacterium Microcoleus chthonoplastes forms a consortium with heterotrophic bacteria present within the cyanobacterial sheath. They showed that this consortium was able to grow in the presence of crude oil, degrading aliphatic heterocyclic organo-sulfur compounds as well as alkylated monocyclic and polycyclic aromatic hydrocarbons. They characterized the oil-degrading consortium through the analysis of the 16S rRNA gene sequences. They also performed the study in cultures of Microcoleus grown in mineral medium and in cultures of the cyanobacterium grown in mineral medium supplemented with crude oil. The results indicate that most of the clones found in the polluted culture correspond to well-known oil-degrading and nitrogen-fixing microorganisms, and belong to different phylogenetic groups,
such as the Alpha, Beta, and Gamma subclasses of Proteobacteria, and the Cytophaga/Flavobacteria/Bacteroides group. The control was dominated by one predominant organism (88% of the clones) closely affiliated to *Pseudoxanthomonas mexicana* (similarity of 99.8%). The presence of organisms closely related to well-known nitrogen fixers such as *Rhizobium* and *Agrobacterium* suggests that at least some of the cyanobacteria-associated heterotrophic bacteria are responsible for nitrogen fixation and degradation of hydrocarbon compounds inside the polysaccharidic sheath, whereas *Microcoleus* provides a habitat and a source of oxygen and organic matter.

Ouellette *et al.*, (2006) studied on algae blooms, which include the toxic cyanobacterium *Microcystis*, have reoccurred in the Laurentian Great Lakes, most commonly in the western basin of Lake Erie. Whereas the western basin is the most impacted by toxic *Microcystis* in Lake Erie, there has historically been little effort focused on identifying the spatial distribution of *Microcystis* throughout this lake. To address this lack of knowledge, we have employed a polymerase-chain-reaction-based detection of genes required for synthesis of the toxin microcystin (mcyD and mcyB), as well as 16S rDNA fragments specific to either all *Microcystis* or all cyanobacteria. Using a multiplex approach, we tested 21 samples from 13 field stations and found that toxigenic *Microcystis* were present in the western and eastern basins in the summers of 1999, 2000, and 2002 and the central basin in 1999 and 2002. This is the most extensive distribution of *Microcystis* reported in Lake Erie. Clone libraries (16S rDNA) of these cyanobacterial communities were generated from 7 of the 13 field stations (representing all three basins) to partially characterize this microbial community. These libraries were shown to be dominated by sequences assigned to the *Synechococcus* and *Cyanobium* phylogenetic cluster, indicating the importance of picoplankton in this large lake system.
2.3 MATERIALS AND METHODS:

2.3.1 Study area and sampling:

The Tiruchirappalli district is located in the geographic centre of the state Tamil Nadu of India (Plate 1). The district is bounded on the north by Perambalur District, on the east by Thanjavur District, on the west by Karur and south by Pudukkotai District. The district stretches between the Latitude at 10°41’N and Longitude at 78°44’15’’ E. There are several natural and artificial fresh water bodies distributed more frequently in and around the agricultural land of Tiruchirappalli city. Visible and planktonic samples were collected from three agriculture fields (Mathur, Sooriyur, and Kallanai) by dipping sterilized sampling bottle to 0.5-1 ft. Opened to collect water sample and recapped. Samples were transported in a box containing ice and were preserved at 4°C. Single filament isolation of the field samples was spotted (10 to 30 µl) onto solid media immediately upon arrival in the laboratory.

2.3.2 Media and culture condition:

Different plating techniques as spread and streak method were carried out to purify the culture. A single colony formed on the surface of the agar plate was picked up and transferred to new plate. After several transfers, the single colony was inoculated into the liquid medium. BG-11(N⁻) or (N⁺) medium was used for cultivation of cyanobacteria (Rippka et al., 1979). Ten ml of water sample were inoculated in 50 ml sterilized standard BG-11 medium with and without nitrate nitrogen in 100 ml Erlenmeyer flasks in triplicates for the diversity studies of total and diazotrophic morphotypes. The flasks were shaken well and incubated in growth room. Culturing was carried out with proper light (50 – 75 µE m⁻² S⁻¹) and incubation temperature (24°C).

2.3.3 Morphological studies:

Pure culture was observed under microscope. The cell shape and size were observed, measured by micrometry and documented as microphotograph. Identification of specimens was carried out using the taxonomic publications of Geitler, 1932 and Desikachary 1959).

2.3.4 Axenization:

The cultures were axenized by repeated plating technique followed by combined antibiotic treatment. The antibiotic stock contained Penicillin G (Sodium salt), Streptomycin sulphate and Chloramphenicol in the ratio of 10:5:1.
2.3.5 Preparation of antibiotic stock:

Penicillin (100 mg) and Streptomycin (50 mg) were dissolved in 8 ml of sterile distilled water. Chloramphenicol (10 mg) was dissolved separately in 1 ml of 95% ethanol, mixed to penicillin-streptomycin solution and the solution was made up to 10 ml with sterile distilled water. The antibiotic stock solution was then filter sterilized using Millipore membrane filter (0.22 µm).

Different concentrations of antibiotic mixture were prepared by dissolving in 50 ml of BG 11 N⁺, N⁻ medium as follows.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Antibiotic mixture (µl in 50 ml of BG 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125</td>
</tr>
<tr>
<td>Penicillin (ppm)</td>
<td>25</td>
</tr>
<tr>
<td>Streptomycin (ppm)</td>
<td>12.5</td>
</tr>
<tr>
<td>Chloramphenicol (ppm)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Cells were inoculated into BG 11 medium amended with different concentration of antibiotics and allowed to grow under normal growth conditions. After 24 and 48 hr, the cells were thoroughly washed with sterile distilled water and transferred aseptically to fresh growth medium without antibiotics. Cells were allowed to grow for 15 to 20 days and the purity of culture was checked by inoculating a small aliquot from each flask to Nutrient broth and incubated in dark at 37°C for a period of 48 to 72 hr.

High concentrations of antibiotics were toxic to cyanobacterial cells. But, all the tested cyanobacterial strains could withstand the antibiotics and grew in medium containing 200 ppm of Penicillin, 100 ppm of Streptomycin and 20 ppm of Chloramphenicol and above this concentration the growth was inhibited. The cells of this particular concentration were selected and subcultured. During the study period, the cultures were periodically checked for bacterial contamination.

2.3.6 DNA extraction:

Total genomic DNA was extracted by a modification of a method by Smoker and Barnum (1998). A 1 ml aliquot of mid-to late log phase culture was pelleted by centrifugation, the medium was decanted, and the pellet was resuspended in 500µl of 50mM

[Rest of the text continues here.]
Tris- HCl (pH 8.0)-5mM EDTA (pH 8.0)- 50mM NaCl. Lysozyme was added to obtain a final concentration of 1 mg/ml, and solution was incubated at 55°C for 30 min. After the addition of 10 µl of proteinase K (10 mg/ml) and 20 µl of 10% sodium dodecyl sulfate, the mixture was incubated at 55°C for 10 min or until the solution cleared (complete cell lysis). The solution was chilled on ice and extracted with an equal volume phenol-chloroform-isoamylalcohol (25:24:1, by vol.; Sigma). The organic extraction was repeated, and the supernatant was added to an equal volume of 4M ammonium acetate. Total genomic DNA was precipitated by the addition of 2 volumes of isopropanol followed by centrifugation for 10 min at room temperature. The pellet was washed with 70% ethanol, dissolved in TE buffer (10mM Tris- HCl, 1mM EDTA, pH 8.0) and stored at -20°C and further diluted in TE buffer prior to use in PCR. DNA concentration were estimated directly from ethidium bromide florescence in agarose gel (0.8 % in 1X TAE buffer) against standard quantities of 1 Kb λ bacteriophage DNA, by using a gel documentation system and associated software.

2.3.7 16S rRNA gene amplification:

Fragments of the 16S rRNA gene were amplified by the method of Wilmotte et al. (1993); Nelissen et al. (1994). The amplifications were performed with DNA thermal cycler (Verti® – 96 well thermal cycler, Applied Biosystems, USA). The PCR conditions for the arbitrary primer were as specified by Nubel et al., (1997). The primers were synthesized by XDT Technologies (Germany). The PCR cycle for primer was: Initial denaturation at 94°C for 6 minutes, 35 cycles of cyclic denaturation at 94°C for 1 minute, cyclic annealing at 56°C for 1 minute, cyclic extension at 72°C for 1 minutes and finally the final extension at 72°C for 7 minutes. After the reaction was completed, 10-µl of amplified DNA was separated on 1.2% low melting agarose (Sigma, USA), stained with ethidium bromide and recorded using a CCD camera in Alpha Imager (Alpha Innotech, USA). A ready to use DNA size standard supplied with DNAzyme™ II DNA polymerase kit (Finzymes, Espoo, Finland) was included in the gel.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYA106F</td>
<td>5′-CGG ACG GGT GAG TAA CGC GTG A -3′</td>
<td>Nubel et al., 1997</td>
</tr>
<tr>
<td>CYA781R(a)</td>
<td>5′-GAC TAC TGG GGT ATC TAA TCC CAT T -3′</td>
<td></td>
</tr>
</tbody>
</table>
2.3.8 Sequencing and phylogenetic analysis of 16S rRNA gene

The amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen, GmBh, Germany) as recommended by the manufacturer. The sequences of the PCR products were determined by using the Big Dye Terminator Cycle Sequencing V2.0 kit on an ABI 310 automatic DNA sequence (Applied Biosystems, CA, USA) according to the manufacturer’s instructions. The 16S rRNA gene sequences determined for the freshwater cyanobacterial strains were deposited in the GenBank database. Additionally, a BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) was carried out against GenBank database before submission. The 16S rDNA sequences reported in this study was multiple-aligned using CLUSTAL W, Version 1.7 (Thompson et al., 1994) with a selection of cyanobacterial reference sequences obtained from GenBank (NCBI). The alignment was corrected manually and converted to a distance matrix. The distance matrix was converted to a phylogenetic tree using the Neighbour-joining (NJ) algorithm (Saitou and Nei, 1987) of MEGA 5.05; Tamura et al., 2011, with multiple substitutions corrected and positions with gaps excluded.
2.4 RESULTS AND DISCUSSION:

2.4.1 Strain Isolation:

Totally 43 species belonging to 22 genera of cyanobacteria were recorded in all three sites. Four genera of cyanobacteria viz., Oscillatoria, Phormidium, Nostoc, Scytonema, Dolichospermum were found in all three sites. Maximum 33 species of 18 genera were recorded from Kallanai followed by 24 species belonging from 13 genera, 26 species from 14 genera in Sooriyur and Mathur respectively (Table 1). As per the diversity and abundance of cyanobacteria the member of family Oscillatoriaceae were dominant in all three sites. Cyanobacterial taxa belonging to different genera and unique characteristics has been isolated. The organisms which includes were as follows,

1. Chroococcus minutus NTMS09 (Plate 2a)
   
   Class : Cyanophyceae  
   Order : Chroococcales  
   Family : Chroococcaceae  
   Genus : Chroococcus  
   Species : minutus  

   **Chroococcus minutus** (Kutz.) Nag

   Cells spherical or oblong, single or in groups of 2-4, light blue green, with sheath 6-15 µm, and without sheath 4-10 µm, colonies 10-13×15-20 µm, sheath not lamellated, colorless.

2. Chroococcus turgidus NTMS12 (Plate 2b)

   Class : Cyanophyceae  
   Order : Chroococcales  
   Family : Chroococcaceae  
   Genus : Chroococcus  
   Species : turgidus  

   **Chroococcus turgidus** (Kutz.) Naegeli

   A free floating colony of 2-4 ovoid or hemispherical cells in closed by a very wide hyaline and lamellate colonial sheath, cells bright blue-green contents sometimes coarsely granular in closed by individual sheaths, 8-32 µm in diameter without sheath, 15-50 µm wide including sheath.

3. Chlorogloea fritschii NTMS06 (Plate 2c)

   Class : Cyanophyceae
**Chlorogloea fritschii** (Mitra)

Thallus a deep blue green crust of indefinite size, composed of rounded or irregular packets, cells arranged in vertical and horizontal rows, rounded or angular, without evident mucilage envelopes, with pale blue green, granular contents, usually 6-8 µm diam. (4-12), single or in groups of two or more cells separating for propagation, endospores naked, spherical, 4-9 µm diam., formed singly within the cells and liberated by the rupture of the membrane, on germination forming a uniseriate filaments of 3-12µm broad cells, divided in three directions to produce colonies.

4. **Microcystis aeruginosa** NTMS11 (Plate 2d)
   - **Class**: Cyanophyceae
   - **Order**: Chroococcales
   - **Family**: Chroococcaceae
   - **Genus**: Microcystis
   - **Species**: aeruginosa

**Microcystis aeruginosa** (Kutz.)

Colonies when young round or slightly longer than broad, solid, when old becoming clathrate, with distinct hyaline colonial mucilage, cells 3-7 µm in diameter, spherical, generally with gas vacuoles.

5. **Oscillatoria acuminata** NTMS04 (Plate 2e)
   - **Class**: Cyanophyceae
   - **Order**: Nostocales
   - **Family**: Oscillatoriaceae
   - **Genus**: Oscillatoria
   - **Species**: acuminata

**Oscillatoria acuminata** Gomont

Thallus blue green, trichome more or less straight, not constricted or slightly constricted at the cross wall, 3-5 µm broad, at the ends briefly tapering sharply pointed, bent, cells longer than broad, rarely sub quadrate, 5.5-8 µm long, sometimes granulated at the cross walls, end cell mucronata, without calyptra.
6. *Lyngbya martensiana* NTMS10 (Plate 2f)

Class : Cyanophyceae  
Order : Nostocales  
Family : Oscillatoriaceae  
Genus : *Lyngbya*  
Species : *martensiana*

*Lyngbya martensiana* menegh. ex Gomont

Thallus caespitose, blue green, when dried violet, filaments long more or less flexible, sheath colourless, thick, not coloured violet with chlor zinc iodine outside rough, trichome 6-10 µm broad, not constricted at the cross walls, cross alls sometimes granulated, apices not attenuated, pale blue green, cells 1/2- 1/4 times as long as broad, 1.75-3.3 µm in length, end cell rotund, without calyptra.

7. *Dolichospermum flos-aquae* NTMS07 (Plate 3a)

Class : Cyanophyceae  
Order : Nostocales  
Family : Nostocaceae  
Genus : *Anabaena*  
Species : *flos-aquae*

*Anabaena flos-aquae* (lyngb.) Breb.ex born.et flah. (*Dolichospermum flos-aquae*)

Thallus frothy, gelatinous, lubricious, free floating, bluish in colour, trichomes circinate, 4-8 µm broad, without sheath, cells ellipsoidal, seldom spherical, as long as broad or longer, 6-8 µm, mostly with gas vacuoles, heterocysts ellipsoidal, 4-9 µm broad and 6-10 µm long, spore prominently bent, on the outside convex, on the inside straight, 7-13 µm broad, mostly 9 µm, 20-35 µm rarely 50 µm long, single near the heterocyst or seldom away from it, epispore smooth, colourless or yellowish, often surrounded by a wide gelatinous sheath.

8. *Nostoc commune* NTMS13 (Plate 3b)

Class : Cyanophyceae  
Order : Nostocales  
Family : Nostocaceae  
Genus : *Nostoc*  
Species : *commune*

*Nostoc commune* voucher ex born .et flah.
Thallus firm, gelatinous, at first globose, later flattened, expanding, undulated, membranous or leathery, sometimes irregularly torn, often perforated, many centimeters diam, blue green, olivaceous or brown, filaments flexuous, entangled, sheath mostly distinct only at the periphery, thick, yellowish brown, often lamellated, inside the thallus more or less distinct, but hyaline shorter or a little longer than broad, 5 µm long, heterocysts nearly spherical, about 7 µm broad, spore only once observed, as big as the vegetative cells epispore smooth colourless.

9. *Nostoc ellipsosporum* NTMS01 (Plate 3c)
   Class : Cyanophyceae
   Order : Nostocales
   Family : Nostocaceae
   Genus : *Nostoc*
   Species : *ellipsosporum*

*Nostoc ellipsosporum* (Desm.) Rabenth.ex Born. Et Flah

Thallus gelatinous, irregularly expanded, attached by the lower surface, reddish brown, filaments flexuous, loosely entangled, trichome about 4 µm broad, light blue green or olivaceous, cells cylindrical, 6-14 µm, heterocysts sub spherical, or oblong, 6-7 µm broad, 6-14 µm long, spores ellipsoidal to oblong cylindrical, 6-8 µm broad, 14-19 µm long, epispore smooth, hyaline or brownish.

10. *Calothrix fusca* NTMS 08 (Plate 3d)
   Class : Cyanophyceae
   Order : Nostocales
   Family : Rivulariaceae
   Genus : *Calothrix*
   Species : *fusca*

*Calothrix fusca* (Kutz) Bornet et Flahault

Filaments single, seldom gregarious, in the gelatinous thallus of other algae, 200-300 µm high, 10-12 µm broad, bent at the base and inflated, up to 15 µm broad, at the base, sheath broad, colourless, at the apices diffusent, trichome 7-8 µm broad, ending in a long thin hair, cells often discoid shorter than broad, heterocysts basal, hemispherical, single or double, smaller than the basal cell of the trichome.

11. *Mastigocladus laminosus* NTMS03 (Plate 3e)
   Class : Cyanophyceae
Order: Stigonematales
Family: Mastigocladaceae
Genus: Mastigocladus
Species: laminosus

*Mastigocladus laminosus* Cohn.

Thallus membranous, carnous, spongy, firm often with calcium carbonate, nearly hard layered, blackish, blue or olive green, filaments densely entangle, 4-6 µm broad, curved, with distinct sheath, when older torulose, indistinct diffluent, side branches about 3 µm broad, erect, cells in the main filament barrel shaped to short cylindrical, those of side branches long cylindrical, heterocysts intercalary spherical or ellipsoidal, broader than vegetative cells, up to 6.5 µm broad, single or two together.

12. *Scytonema hofmanni* NTMS05 (Plate 3f)

Class: Cyanophyceae
Order: Nostocales
Family: scytonemataceae
Genus: Scytonema
Species: hofmanni

*Scytonema hofmanni* Ag. ex Born.et Flah.

Stratum cushion like, broadly expanded, 1-3 mm high, blackish blue green, sometimes impregnated with calcium carbonate, amethyst green or bluish gray, filaments 7-12 µm broad, rarely up to 15 µm broad, aggregated in vertical fascicles, false branches aggregated, sheath firm, membranaceous, trichome 5-10 µm broad, olive to blue green, cells unequal in length, heterocysts oblong.

Similar work of Muthukumar et al., (2008) recorded cyanobacterial population in Thanjavur, Tamilnadu, India. Totally 39 species of 20 genera of cyanobacteria were recorded in five different ponds, where a massive bloom of *Microcystis aeruginosa* was recorded, which had a significant effect in reducing the other cyanobacterial population. As many as five species namely *Aphanothece microscopica*, *Synechocystis aquatilis*, *Merismopedia glauca*, *Oscillatoria limnetica* and *O. subbrevis* were common in all the ponds.

Similar work of Taton et al., (2006) explained the five samples from four lakes spanning a range of different ecological environments in Larsemann Hills, Vestfold Hills and Rauer Islands to evaluate the influence of lake characteristics on the cyanobacterial diversity. Relatively, Wilson et al., (1993) demonstrated among the 64 cyanobacterial bloom samples
collected, *Microcystis* as well as *Planktothrix* were the most frequently encountered dominant bloom formers, followed by *Anabaena*, *Woronichinia*, and *Aphanizomenon*. *Microcystis* were found in 53% of the analysed blooms and their presence was mainly assigned to *Microcystis* dominance.

Of the effluents studied by Vijayakumar *et al.*, (2007) Heterocystous cyanobacteria such as *Anabaena beckii*, *A. fertilissima*, *Nostoc calcicola* and *Westiellopsis prolifica* were recorded in dye effluent; on the other hand paper mill effluent recorded only *N. calcicola* and *A. fertilissima*. Similarly, sugar mill effluent was represented only with *N. calcicola* and *A. beckii*. In total 26 species of cyanobacteria were recorded in common to all the effluents analysed. Of them, *Oscillatoria* with 13 species was the dominant genus, which was followed by *Phormidium* (8 species), *Lyngbya* (2 species), *Microcystis* (2 species) and *Synechococcus* with single species.

**2.4.2 DNA extraction:**

Total genomic DNA was extracted from the isolated species. The DNA concentration were estimated directly from ethidium bromide florescence in agarose gel (0.8 % in 1X TAE buffer) against standard quantities of 1 Kb λ bacteriophage DNA, by using a gel documentation system and associated software. The band formed for all the species were at 1000Kb on comparing to the marker DNA. Nucleic acids were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, vol), and the residual phenol was removed once with chloroform/ isoamyl alcohol (24:1, vol). Nucleic acids were purified, desalted, and concentrated with a Centricon-100 concentrator (Millipore). DNA integrity was checked by agarose gel electrophoresis, and quantified using a low DNA mass ladder as a standard (Invitrogen).

**2.4.3 16S rRNA gene amplification:**

The result of 16S rRNA gene amplified product in Fig. 1 shows the product obtained after running PCR in suitable condition with appropriate primer, were subjected to detection for its presence on agarose gel electrophoresis. The amplified product was sequenced. In the same way, Sanchez characterized the oil-degrading consortium through the analysis of the 16S rRNA gene sequences. Similarly, Taton *et al.*, (2006) amplified Seventeen morphospecies and 28 16S rRNA gene-based operational taxonomic units belonging to the Oscillatoriales, Nostocales and Chroococcales were identified.

Relative work of Christina Lyra *et al.*, (2001) compared the molecular characterization of cyanobacterial strains *Anabaena*, *Aphanizomenon*, *Calothrix*,...
Cylindrospermum, Nostoc, Microcystis, Planktothrix (Oscillatoria agardhii), Oscillatoria and Synechococcus genera by RFLP of PCR-amplified 16S rRNA genes and 16S rRNA gene sequencing.

Similarly, Ouellette et al., (2006) demonstrated that PCR was conducted using cyanobacterial 16S rDNA-targeted primers. The forward and reverse primers were labelled with 6-carboxyfluorescein and hexachlorofluorescein, respectively (Sigma-Genosys). PCR amplicons were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia).

2.4.4 Sequence submission:

The 16S rRNA gene sequences determined for the freshwater cyanobacterial strains were deposited in the GenBank database. Additionally, a BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) was carried out against GenBank database before submission. the sequences were submitted and got its accession number as follows,

- Oscillatoria acuminata NTMS02 : HQ219677 (Plate 4)
- Mastigocladus laminosus NTMS03 : HQ219678 (Plate 5)
- Oscillatoria sp. NTMS04 : HQ219679 (Plate 6)
- Scytonema sp. NTMS05 : HQ219680 (Plate 7)
- Chlorogloeopsis fritschii NTMS06 : HQ219681 (Plate 8)
- Dolichospermum flos-aquae NTMS07 : HQ219682 (Plate 9)
- Calothrix sp. NTMS08 : HQ219683 (Plate 10)
- Chroococcus sp. NTMS09 : HQ219684 (Plate 11)
- Lyngbya sp. NTMS10 : HQ219685 (Plate 12)
- Microcystis aeruginosa NTMS11 : HQ537431 (Plate 13)

Relatively rRNA gene ITS of cyanobacteria were sequenced by Barker et al., (1999) explained the nucleotide sequence of the 16S-rDNA was determined for three independent Nodularia isolates (BC Nod-9402, 29408, and 29427), one from each of the groupings identified using the rRNA gene ITS locus.

2.4.5 Phylogenetic analysis of fresh water cyanobacteria:

The 16S rRNA gene sequences of the taxa examined and the sequences of reference organisms obtained from databases were multiple aligned using CLUSTAL W. Jukes-Cantor distances, generated by pairwise comparisons of the isolates, were used to create a phylogenetic tree by Neighbor-joining analysis. Constructed phylogenetic tree (Figure-2) revealed that, the sequences of taxa examined matches to the genus already existing sequences in NCBI. The 16S rRNA gene sequences formed three groups in the phylogenetic
tree as unicellular, heterocyst and nonheterocyst. The taxa whose 16S rRNA gene sequences
were determined in this study, were indicated in colour letters with their accession numbers.
Salomon et al., (2005) also constructed phylogenetic tree based on partial 16S rRNA gene
sequences of Nodularia spumigena-associated bacteria.