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ECOLOGICAL AND BIOCHEMICAL STUDIES ON CYANOBACTERIA OF COCHIN ESTUARY AND THEIR APPLICATION AS SOURCE OF ANTIOXIDANTS

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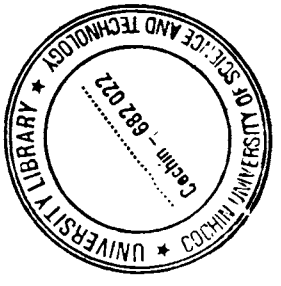

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UNDER THE FACULTY OF MARINE SCIENCES

BY

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CERTIFICATE

This is to certify that the thesis entitled **"ECOLOGICAL AND BIOCHEMICAL STUDIES ON CYANOBACTERIA OF COCHIN ESTUARY AND THEIR APPLICATION AS SOURCE OF ANTIOXIDANTS"** *is an authentic record of research work carried out by* **Ms. Sincy Joseph,** *under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of* **Doctor of Philosophy** *and no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.*

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ABBREVIATIONS

AN medium	:	Allen and Nelson medium
ANOVA	:	Analysis of Variance
BSA	:	Bovine Serum Albumin
CATs	:	Catalases
CD	:	Conjugated dienes
CDNB	:	1- chloro – 2,4 – dinitrobenzene
cm	:	centimetre
d	:	Margalef's richness index
DNA	:	Deoxyribonucleic acid
DTNB	:	5'5"- dithiobis(2-nitrobenzoic acid)
E,A	:	absorbance
EDTA	:	Ethylene diamine tetra acetic acid
EEZ	:	Exclusive Economic Zone
et al.,	:	Co- authors
Fig	:	Figure
g	:	gram
GF/F	:	Glass fibre filter
GPXs	:	Glutathione peroxidases
GSH	:	Glutathione
GSTs	:	Glutathione - S – transferases
H	:	Shannon diversity index
ha	:	hectares
IP	:	inhibition of peroxidation
IU	:	international unit
J'	:	Pielou's evenness index
kg	:	kilogram
kms	:	kilometres
LC	:	lethal concentration
m	:	meter
M	:	molarity
MDA	:	Malondialdehyde
MDS	:	Multidimensional scaling
mg	:	milligram
min	:	minute
ml	:	millilitre
ml/L	:	millilitre per litre
mm	:	millimetre
mM	:	millimoles
mon	:	monsoon

MPN	:	Most Probable Number
N	:	normality
NADH	:	Nicotinamide adenine dinucleotide
NADPH	:	Nicotinamide adenine dinucleotide phosphate (reduced)
NBT	:	Nitroblue tetrazolium
NF medium	:	Nitrogen free medium
nm	:	nanometre
PMS	:	Phenazine methosulphate
ppt	:	parts per thousand
ROS	:	Reactive Oxygen Species
rpm	:	revolution per minute
S	:	Similarity
S- R	:	Sedgwick – Rafter
SOD	:	Superoxide dismutase
SWEM	:	Seawater Enrichment Medium
t	:	tonne
TBARS	:	Thiobarbituric acid reactive substances
Tris	:	Tris (hydroxymethyl) aminomethane
UV	:	Ultra violet
v/w	:	volume per weight
λ	:	Simpson's index
µg/L	:	microgram per litre
µg/ml	:	microgram per millilitre
µM	:	micromoles
µmol/L	:	micromole per litre

CHAPTER 1

Introduction

Cyanobacteria are nature's unique gift to mankind, as they possess several innate properties that make them ideal organisms with potential for multifaceted biotechnological applications. They are large and morphologically diverse group of unique photosynthetic organisms of great importance because of their very long existence for well over 3.5 billion years and cosmopolitan distribution in terrestrial, freshwater and marine habitats. As a basic research tool, they are largely known to provide critical insights into the origin of life, photosynthesis, nitrogen fixation and primary metabolism.

On account of their immense applied biotechnological potentials, they are being explored widely. Recently, cyanobacteria have gained significance as sources of wholesome food materials, fixed atmospheric nitrogen, natural colourants, bioplastics, biofuels, fine chemicals, bioactive substances, common and fine chemicals like lipids, pigments, enzymes, polysaccharides, glycerol and other novel biologically active compounds. Active research in the field of cyanobacterial biotechnology has opened up vast opportunities, whereby, these microbes could be used for eliminating human sufferings.

The importance of cyanobacteria in aquaculture is not surprising as they are the natural food source and feed additive in the commercial rearing of many aquatic animals (Aaronson *et al.*, 1980; De La Noue and De Pauw, 1988). Although several alternatives for algae such as yeasts and microencapsulated feeds exist, (Jones *et al.*, 1987; Nell, 1993; Heras *et al.*, 1994; Nell *et al.*, 1996), live cyanobacteria are still the best and the preferred food source (Benemann *et al.*, 1987).

Cyanobacteria not only act as essential food sources, but also play a role in enhancing the quality of the animal species cultured (Borowitzka, 1997). Recent research in natural products of cyanobacteria has made significant advances in aquaculture and they have been shown to produce a variety of compounds and some of them have been proved to possess biological activity of potential medicinal value (Kumar *et al.*, 2003). They

deserve special attention due to their antioxidant potential as they constitute significant amount of carotenoids, phycocyanin, vitamin A, vitamin C, flavanoids and phenolic compounds (Miki, 1991; Ho, 1992; Miranda *et al.*, 1998; Bhat and Madyasta, 2000) which are able to scavenge highly reactive free radicals that cause tissue damage (Halliwell and Gutteridge, 1985).

General features of cyanobacteria

Cyanobacteria are Gram-negative prokaryotes as they lack internal organelles, a discrete nucleus and the histone proteins associated with eukaryotic chromosomes. Like all eubacteria, their cell wall contains peptidoglycan (Stanier, 1988; Weckesser and Jurgens, 1988; Castenholz and Waterbury, 1989). Studies of metabolic similarities and ribosomal RNA sequence suggest that cyanobacteria form a good monophyletic taxon. The autotrophic cyanobacteria were once classified as 'blue green algae" because of their superficial resemblance to eukaryotic green algae. With the advent of electron microscopy and several other sophisticated instruments and techniques, it was realized that the so called 'blue-green algae' are really Gram-negative bacteria in their cell structure and chemistry, but form a connecting link with algae because of their pigment composition and photosynthetic mechanism. They have an elaborate and highly organized system of internal membranes which function in photosynthesis. Chlorophyll-a and several accessory pigments (phycoerythrin and phycocyanin) are embedded in these photosynthetic lamellae, the analogs of the eukaryotic thylakoid membrane (Rippka *et al.*, 1979). These photosynthetic pigments impart a rainbow of possible colours; yellow, red, violet, green, deep blue and blue-green to the cyanobacteria.

In all, about 150 genera and 2000 species of cyanobacteria have been reported so far (Fott, 1971). Traditional systematics of blue green algae is mainly based on cytomorphological and to a lesser degree, on ecological characteristics (McGuire, 1984; Campbell and Golubic, 1985). Cyanobacteria

may be single-celled or colonial. Depending upon the species and environmental conditions, colonies may form filaments, sheets or even hollow balls.

Cyanobacteria are composed of two broad morphological categories of thallus organization:

i. Nonfilamentous forms or coccoid and palmellate forms,

ii. Filamentous forms with a characteristic trichome.

The trichome usually includes vegetative cells, heterocyst and akinetes. Vegetative cells are the normal, photosynthetic cells formed under favourable conditions. The photosynthetic vegetative cells fix carbon dioxide through the reductive pentose phosphate pathway and provide fixed carbon in the form of carbohydrate. The photosynthetic apparatus of cyanobacteria is very similar to plants producing oxygen through photosystem II. Akinetes or resting spores are very large cells, thick walled and have accumulated food reserves in the form of cyanophycin granules, which may form under unfavourable conditions (Whitton, 1987). Heterocysts are thick-walled cells, differentiated by the strains in conditions of nitrogen starvation, having the ability to fix nitrogen, that is to convert nitrogen gas from the air to ammonia (NH_3), nitrites (NO_2) or nitrates (NO_3), which can then be used for the synthesis of aminoacids, proteins and other nitrogen compounds in the neighbouring cells (Kumar, 1985; Stahl and Krumbein, 1985; Van Baalen, 1987; Gallon and Chaplin, 1989). They protect the nitrogenase complex from oxygen damage since these enzymes are highly sensitive to oxygen.

Cyanobacteria show wide ecological tolerance and they occur in almost every conceivable habitat on earth (Hof and Fremy, 1933; Desikachary, 1959; Van Baalen, 1962; Fogg *et al.*, 1973; Carr and Whitton, 1982; Gallon, 1992; Paerl and Millie, 1996). They are found naturally as planktonic forms in both freshwater and marine ecosystems and also as gelatinous mat like growth on shaded soil, rocks, mud, wood and some living

organisms. Cyanobacteria produce the compounds responsible for 'earthy' odours we detect in soil and some bodies of water.

Several species of cyanobacteria grow in abundant quantities so as to colour the entire body of water and in some cases these are caused by a single species. Such growths are called 'Water blooms'. *Trichodesmium erythraeum* and *Trichodesmium thiebautii* are known to form water blooms for more than a century in the Indian Ocean, Bay of Bengal, and the Arabian Sea.

Cyanobacteria have been tremendously important in shaping the course of evolution and ecological change throughout the earth's history. The oxygen atmosphere that we depend on was generated by numerous cyanobacteria photosynthesizing during the Archaen and proterozoic era.

Study sites

Cochin backwater system is the longest estuarine system on the south west coast of India, which forms a permanent part of Vembanadu Lake. It has all the characteristics of a tropical positive estuary. It includes a system of interconnected lagoons, bays and swamps penetrating the mainland and enclosing many islands in between. It extends between Thanneermukkam bund at the south and Azhikode at the north (9°30'-10°12'N and 76°10'-76°29'E) and extends over an estimated length of ~60kms and an area of ~21,050 ha. The Cochin backwater system is connected to the Arabian Sea through a permanent opening, the Cochin Barmouth, which is about 450m wide. Here, the depth is maintained by dredging as this opening is used for navigational activities. The Barmouth is also responsible for the tidal flux of the Cochin backwater system and is the only source of seawater intrusion into the estuary. Tides are of semidiurnal type, showing substantial range and time. The average tidal range near the mouth of the estuary is ~0.9m.

Two major rivers discharge freshwater into the estuarine system, the Periyar flows into the northern parts and Moovattupuzha into the southern parts of the estuary. Several industrial units are situated on both sides of the river Periyar and on the southern bank of the river Moovattupuzha; the effluents from these factories cause pollution of the estuary. The saline water intrusion to the southern part of the estuary is regulated by Thanneermukkam bund, a salt-water barrier commissioned in 1975, which is opened during monsoon. Cochin is the major port on the south west coast of India

Significance of the study

Cyanobacteria have assumed great significance in biotechnology. The basic and fundamental requirement for initiating cyanobacterial biotechnology is to first enumerate the available natural cyanobacterial wealth and to understand their ecobiological properties. Therefore, it is necessary that a detailed survey of the surrounding habitats are made, to identify the available cyanobacterial species and subsequently to isolate, purify and establish a culture collection which could be used for further studies.

Of the total estimated area of 510 million sq. km of the earth, about 70.68 per cent is occupied by oceans. Of the total photosynthetic productivity of 555.2 billion tons dry weight/ year of the earth only 35.4 per cent is contributed by the oceans. Therefore, the new age into which we are moving is not only the age of the atom, the electron and the space, it is also the new age of the sea. India is bestowed with an Exclusive Economic Zone (EEZ) extending to 2.02 million km^2 (Kesava Das and Desai, 1988). Therefore, It is pertinent to make earnest attempts to study the ocean surrounding the country for the resource potential.

Cyanobacteria, till recently in the oblivion uncared and unrecognized, have shot into fame and popularity. They inhabit various types of marine environments such as oceans, estuarine salt lakes, inland saline lakes, salt

marshes and hypersaline salt-pans (Fogg *et al.*, 1973). Now scientists have started realizing the scope available to harness the marine forms and to make them perform tasks in varied areas (Mitsui, 1980a and 1980b; Venkataraman and Kaushik, 1980; Venkataraman and Becker, 1985; Sivonen *et al.*, 1989; Subramanian, 1991; Prabaharan 1992). Aquatic ecosystem in the south west coast of India is noted for its diversity of habitats. Very often, these environments turn blue-green when the bloom of blue-green algae (cyanobacteria) appear consequent to eutrophication.

So far, there has been no systematic survey of the blue-green algae of Cochin estuary. This work is the first of its kind, surveying a continuous stretch of the estuary. Studies on cyanobacteria of Cochin estuary is expected to provide a wealth of information that is not only academically rewarding, but also very useful in planning mass cultivation strategies oriented towards the biotechnological exploitation of these organisms.

Growth and productivity of cyanobacteria depend on the hydrographic parameters of the particular environment (Gopinathan *et al.*, 2001), and since the hydrographic parameters such as temperature, salinity, light intensity, dissolved oxygen and nutrients vary from place to place and season to season, frequent monitoring of these parameters are highly essential. Moreover, no report supporting the cyanobacterial distribution and abundance in Cochin estuary with respect to physico-chemical parameters have been obtained so far.

Aquaculture, in countries like India, has received a great fillip in recent years and the demand for fish feed has suddenly skyrocketed. It has, therefore, become necessary to look for inexpensive, and yet nutritionally efficient feed. Hence, it is essential to turn towards natural sources such as microalgae, especially cyanobacteria, as these are the biological starting points of energy flow through the most important aquatic food chain. Microalgae are not only important as food source but, together with bacteria,

they regulate the oxygen and CO_2 balance in the aquaculture systems (Pruder, 1983).

Microalgae in general, and cyanobacteria in particular, prove to be the best natural feed for aquaculture and the best supplements in other animal husbandry programmes (Brown and Farmer, 1994). The biochemical composition of the algae plays an important role in their nutritional value and is crucial to the growth and development of the animals cultured. Cyanobacteria are usually non-pathogenic and have high nutritive value, rich in carbohydrates, proteins, lipids, minerals and vitamins (Cannell, 1989).

The untapped potential of cyanobacteria for their scientific exploitation has been realized only in recent years. It needs an extensive screening, which is expected to result in the discovery of better cyanobacterial strains of immense industrial interest. The acquisition of fundamental knowledge of these versatile organisms is necessary for further progress. Evaluation of their physiological as well as biochemical characteristics lead to the selection of more prospective strains. It is, therefore, very essential to prepare an excellent database by determining the biochemical composition of cyanobacteria which can be used as a potential food source.

Antioxidants are usually used as additives in the food industry to prevent lipid peroxidation (Yen and Chang, 2003). Although synthetic antioxidants are widely applied in food processing, they have been reassessed for their possible toxic and carcinogenic components formed during their degradation. On account of these health hazards, natural antioxidants are preferred to synthetic forms. Natural antioxidants are usually more expensive and inferior in effect, however finding out safer, more effective and low-cost natural antioxidants is highly desirable. Microbes have been shown to be a potential source for the production of natural antioxidants (Larson, 1988). Karni *et al.*, 1984 and Sukenik *et al.*, 1993 suggested that cyanobacterial cells have protective antioxidant compounds and possess an

excellent antioxidant defence system. Therefore, screening and selection of cyanobacteria with high antioxidant property and formulation of fish feed by incorporating live cyanobacteria as a source of natural antioxidant compounds would be of great achievement in aquaculture development.

In view of the great biotechnological potential of cyanobacteria and the abundance of their biomass in the marine environment of the world in general, and the Indian subcontinent in particular, an extensive study has been carried out on their qualitative and quantitative distribution along Cochin estuary and on their biochemical composition and antioxidant potential so as to find the possibility of using them as nutritional supplements for application in aquaculture systems. The main objectives of the present study are listed as follows: -

(i) Determination of qualitative and quantitative distribution of cyanobacteria in Cochin estuary

(ii) Assessment of the physico-chemical parameters and nutrients of the study sites

(iii) Evaluation of the effect of physico-chemical parameters on cyanobacterial distribution and abundance

(iv) Isolation, identification and culturing of cyanobacteria.

(v) Estimation of the biochemical composition and productivity of cyanobacteria.

(vi) Testing the efficacy of the selected species of cyanobacteria as sources of antioxidants against ethanol induced lipid peroxidation in Tilapia, *Oreochromis mossambicus* (Peters).

The thesis is presented in seven chapters. Chapter 1 gives a general introduction to the topic. Identification, purification and culturing of cyanobacteria isolated from Cochin estuary and a qualitative analysis of their distribution in the study area are presented in Chapter 2. The third chapter deals with the quantitative distribution of cyanobacteria along Cochin estuary and determination of community structure with respect to cyanobacterial species count. Investigation on the variation of prime hydrographical parameters at the study sites is presented in Chapter 4. Chapter 5 deals with the effect of physico–chemical parameters and nutrients on cyanobacterial growth and distribution in Cochin estuary. The sixth chapter highlights the optimisation of culture conditions for obtaining high cell yield and evaluation of biochemical characteristics of cyanobacteria leading to the selection of more prospective strains in aquaculture. Assessment of the efficacy of whole cell cyanobacteria as source of antioxidants *in vitro* as well as *in vivo* is depicted in chapter 7, which is followed by summary, list of references cited and appendices.

CHAPTER 2

Seasonal and spatial distribution of cyanobacteria in Cochin estuary: Qualitative analysis

2.1 Introduction

Cyanobacteria comprise a unique group of organisms whose distribution around the world is rivalled only by bacteria. About 20 percent of all known cyanobacteria occur in saline environments and a majority of them are truly marine (Desikachary, 1959) and form an important class of marine vegetation (Van Baalen, 1961). They inhabit various types of marine habitats such as oceans, estuaries, saline backwaters, estuarine salt lakes, inland saline lakes, salt marshes and hyper saline salt-pans (Fogg *et al.*, 1973).

There have been a number of sporadic and casual reports on the occurrence of cyanobacteria in the marine environment of India such as planktons of Indian Ocean by Cleve (1901), Cyanobacterial flora from the salt lakes of Calcutta by Biswas (1926) and *Trichodesmium* from Kurusadai Island by Iyengar (1927). But these reports are not comprehensive and are limited to certain areas and species only. There are other reports such as occurrence of ten species of cyanobacteria from seawater near Bombay by Ramachandran (1982), 17 species of seven genera from Madras coast by Anand and Venkatesan (1985) 25 taxa from Madras and South Arcot District by Anand *et al.* (1986), 67 taxa from various saline habitats of West Bengal by Santra *et al.* (1988) and 163 species from southern east coast of India by Thajuddin and Subramanian (1991 and 1992).

Eventhough only a very few studies have been carried out in India, several reports are available on the cyanobacterial flora of saline environments from different parts of the world such as American coast (Zaneveld, 1972), saline waters in Chad area (Oah, 1973), Southern Baffin Island (Moore, 1974), Gulf of Baabo (Pulz, 1975) Coast of Chille (Gonzalez and Para, 1975), marine coastal environments of Sinai Peninsula (Potts, 1980) South eastern coast of United States (Marshal, 1981) and Red sea coast of Saudi Arabia (Khoja, 1987).

There are a number of similar reports with lesser number of species from different parts of the world (Hof and Fremy, 1933; Umezaki, 1961; Stewart and Pugh, 1963; Webber, 1967; Whitton, 1968; Petrov, 1974; Halferin, 1974; John and Lawson, 1977; Ralph, 1977; Sage and Sullivan, 1978; Basson, 1979; McCarthy and Carpenter, 1979; Aleem, 1978 and 1980).

Thajuddin and Subramanian (1990) and Thajuddin (1991) made a modest attempt to understand certain aspects of ecobiology of natural marine cyanobacterial population in Gulf of Mannar region of the Indian coast (southern east coast) especially in Mandapam – Rameswaram – Kurusadai Island region. Very little is known about the floristic wealth of cyanobacteria from south west coast of India. Information on various aspects of algal plankton and their role in the productivity of Cochin estuary is available from the studies of George, 1958; Qasim *et al.*, 1969 and 1972a; Gopinathan, 1972; Joseph and Pillai, 1975; Sreekumar and Joseph, 1995. But only a very few attempts have been made, so far, to understand the cyanobacterial flora of Cochin estuary. Studies on cyanobacteria from the marine, freshwater and estuarine environments of Cochin, their isolation and maintenance were done by Newby, (2002). Ten species of cyanobacteria were reported from Cochin estuary in this study. However, there has been so far, no systematic survey of the blue green algae of the south west coast of India. Therefore, in the present study, a detailed survey of the Cochin estuary has been made to understand the species diversity of the available cyanobacteria.

Blue green algal taxonomy, in recent years, has been a subject that aroused considerable disagreement among phycologists. The fundamental difference in the cellular organization of cyanophyceans and other algae, however, led to the taxonomical treatment of blue green algae as a separate class or division (Fritsch, 1952). Intensive investigation on cultures of blue green algae for the morphological features as well as for their physiological characteristics were suggested, for evolving a better system of classification by designating reference cultures. The bacterial features, which are typically

possessed by cyanobacteria, make a classification based upon the principles of the International Code of Nomenclature of Bacteria (1975) suitable. They are accordingly placed within the group 'eubacteria' in the phylogenetic taxonomy, distinct and apart from the archaeobacteria and eukaryotes.

Cyanobacteria exhibit remarkable physiological, morphological and ecological adaptations to the marine environment (Fogg, 1982). Morphologically and functionally diverse cyanobacterial taxa are found in planktonic and benthic estuarine habitats. The systematics of cyanophyceans should be based upon the information incorporated from both field studies and laboratory techniques. Choice of a suitable medium that would support normal growth of the organism without bringing in morphological changes is of prime importance in the study of the taxonomy of blue green algae in culture. It is very difficult to isolate planktonic cyanobacteria and to obtain them in axenic cultures. The main causes of these difficulties are: (i) these organisms will not usually grow on solidified media; (ii) they prefer high carbonate media; (iii) they will not withstand exposure to the moderately high temperatures (35^0C). Accordingly, a number of alternative methods for selection and culture have been developed to obtain axenic, cloned cultures of these organisms such as serial dilution, subculturing, filtration (Meffert, 1972), centrifugation (SCOR working group, 1974), application of antibiotics or antibacterial agents to remove contaminating heterotrophic bacteria (Carmichael and Gorham, 1974) etc. Considerable success has been achieved with Germanium dioxide in controlling diatom growth, which sometimes outnumber cyanobacteria (Lewin, 1966). Progress in the study of taxonomic problems, will largely be dependent on the use of these methods of culture and their successful modifications.

In the present study, an attempt has been made to determine the distribution and abundance of cyanobacteria in the Cochin estuary and the nearshore waters. Subsequently, they were isolated, purified and maintained as pure cultures in the laboratory with a view to understanding their physiological and biochemical characterisation.

2. 2 Materials and Methods

2.2.1 Sampling sites

Ten stations from Cochin Estuary were selected for the study. The details of the sampling sites are presented in Table 2.1 and Fig 2.1 (PLATE1).

Table 2.1 Details of the sampling sites

Sl.No.	Stations	Position		Depth (m)	Description
		Latitude	Longitude		
1	Eloor	10°5'23"N	76°17' 49"E	5.3	Industrial Belt
2	Varapuzha	10°4' 30"N	76°16' 48"E	3.9	Industrial Belt
3	Vaduthala	10°12' 13"N	76°15' 9"E	1.5	Disposal of domestic wastes
4	Bolghaty	9°58' 52"N	76°15' 50"E	3.5	Inland navigation and other tourism operations
5	Barmouth	9°58' 26"N	76°14' 39"E	4.6	Cochin Harbour entrance
6	Mattanchery Harbour	9°56' 47"N	76°15' 52"E	3.3	The fishing and processing unit operations
7	Thevara	9°55' 35"N	76°17' 53"E	2.1	Sewage outfall
8	Edakochi	9°54' 33"N	76°17' 35"E	1.4	Domestic sewage outfall
9	Kannamali	9°52' 7.5"N	76°15' 47.9"E	---	Seashore area
10	Puduvaippu	9°59' 26.1"N	76°14' 8.4"E	---	Mangrove area

Out of the ten stations, Eloor (Stn.1), Varapuzha (Stn.2), and Vaduthala (Stn.3) recorded salinity near zero and hence considered as freshwater regions whereas the next five stations such as Bolghatty (Stn.4), Barmouth (Stn.5), Mattanchery (Stn.6), Thevara (Stn.7) and Edakochi (Stn.8) are considered as saline regions as they recorded salinity above 30 ppt. The 9th station, Kannamali was selected as the seashore station where salinity was in the range of 25 – 37 ppt throughout the year. Puduvaippu, which was selected as the 10th station of this study was one of the mangrove areas in Cochin. At present, this mangrove vegetation consists primarily of *Avicennia*,

Fig 2.1 Map showing study sites

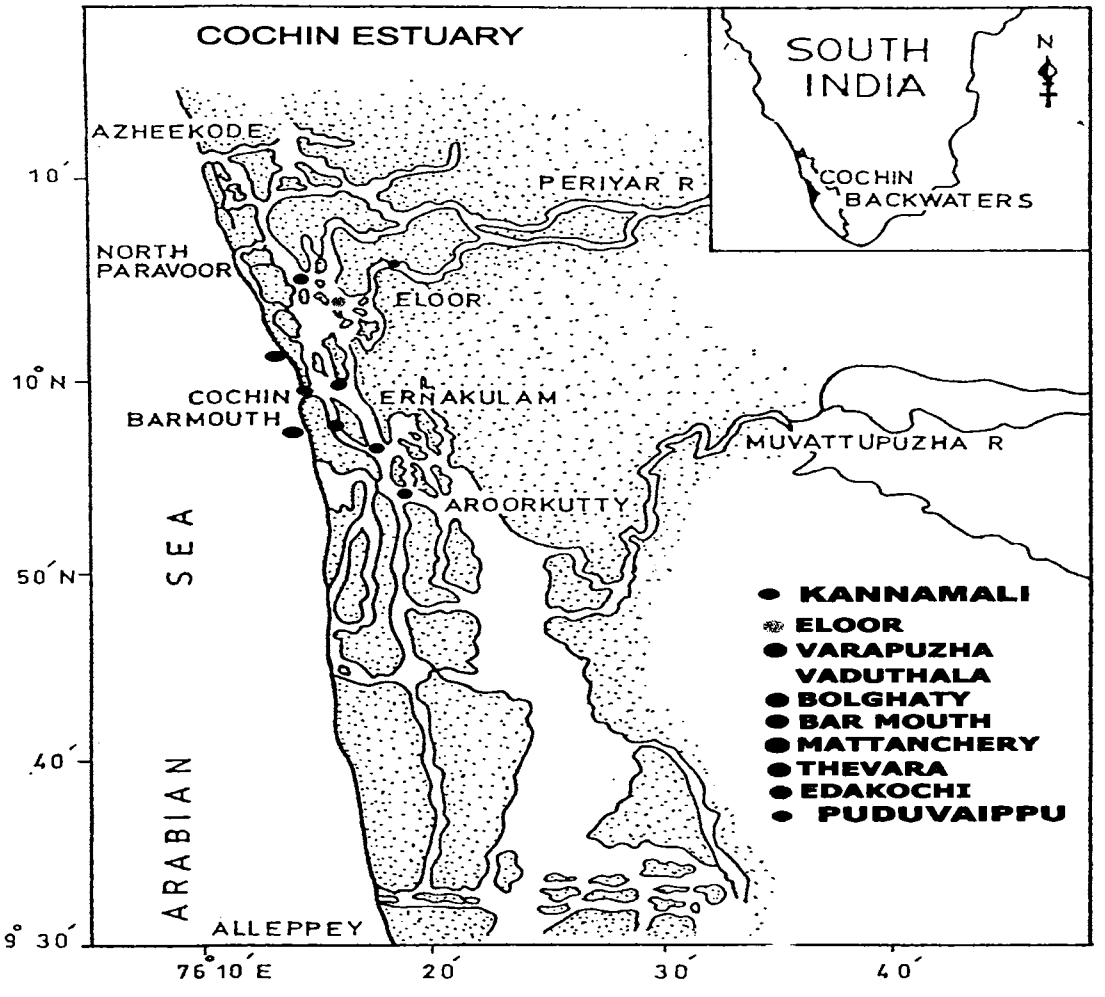


PLATE 1

which is growing gregariously on the western side with *Rhizophora* and *Bruguiera* recording occasional growth.

2.2.2 Sampling Procedures and Processing

In order to evaluate seasonal variation in species diversity and distribution, monthly collection of water and sediment samples from Cochin backwaters and nearshore areas were conducted from April 2002 to March 2003. In the second year of the study (April 2003 to March 2004), sampling was held bimonthly.

Water samples were collected using Niskin water sampler and transferred aseptically to sterile bottles and transported to laboratory. For isolation and culturing of cyanobacteria the samples were inoculated in the enrichment medium, Allen and Nelson (1910). Germanium dioxide was used in the nutrient medium to inhibit the growth of diatoms. The cultures were incubated at 25^0C with an illumination of 2000 lux for 30 days. The cultures were observed under the light microscope (400x magnification) and morphological characteristics of the species were carefully studied.

2.2.3 Preparation of media

Composition of Allen and Nelson (1910) medium

	Ingredients	**Quantity**
Solution - A	KNO_3	2.2 g
	Distilled Water	100 ml
Solution - B	Na_2HPO_4	4 g
	$CaCl_2$	4 g
	$FeCl_3$	2 g
	Conc.HCl	2 ml
	Distilled water	80 ml

Solution A and solution B were prepared separately. 2ml solution A and 1ml solution B were added to 1000ml seawater, pH adjusted to 7 and autoclaved.

2.2.4 Purification of Cyanobacteria

Often initial isolations of cyanobacteria from the natural environment may give rise to mixed cultures. Therefore, it is essential to purify the individual types of cyanobacteria from mixture. Several methods were employed for purification of cyanobacteria depending on the degree of contamination. They are: pipette method, centrifugation or washing method, the method by exploiting the phototactic movement, agar plating method, serial dilution techniques and antibiotic treatment. Addition of Germanium dioxide (GeO_2) was effective to inhibit the growth of diatoms. Filamentous cyanobacteria were difficult to maintain in pure cultures. However, the cultures were made pure by repeated and frequent subculturing in liquid medium.

2.2.5 Identification

Although cyanophyceans are polymorphic and vary in morphology depending on environmental conditions, a morphological approach is fully justified as a taxonomic treatment from practical point of view. (Skulberg *et al*, 1993). They include attributes such as cell width, cell length, cell shape (terminal cell and calyptra), indentation, heterocysts, akinetes (position in trichomes) etc. Cytological data used for the description of the species comprise microscopically visible cellular inclusions. The most prominent structures are glycogen granules, lipid globules, cyanophycin granules and polyphosphate bodies (van den Hoek, 1978). Gas vacuoles give the strongly refractive appearance to the cells of some planktonic cyanophyceans under light microscope.

Identification keys

Several comprehensive manuals and reference books are available to guide proper identification of the cyanophyceans. Identification was done based on their morphology as per the methods of Desikachary, 1959; Fogg, 1975; Burlew, 1976; Kinne, 1976; Staley *et al.*, 1989; Skulberg *et al.*, 1993; Golubic and Browne, 1996. The properties to be tested during the identification are presented in keys or diagnostic tables.

A provisional key to the common genera of cyanophyceans (Skulberg *et al.*, 1993)

I. Unicellular or colonial, reproduction by binary fission

A. Cell shape coccoid or ellipsoid, forming aggregates

1. Cells elongate, dividing lengthwise .... *Coelosphaerium*
2. Cells egg shaped or heart shaped, division in three planes ..... *Gomphosphaeria*
3. Cells coccoid, division in two or three planes ..... *Microcystis*
4. Cells elongate, division in one plane only .... *Synechococcus*
5. Cells coccoid, division in one plane only ..... *Synechocystis*

B. Cells rod shaped or elongate, in short chains

1. Cells short rods with rounded or squarish ends ..... *Pseudanabaena*

II. Multicellular, forming filaments

A. Trichomes with non-differentiated cells, reproduction by fragmentation (hormogonia)

a. Filaments single or in loose masses, sheath usually not present

1. Trichomes more or less straight, end cell distinctly marked ..... *Oscillatoria*
2. Trichomes in bundles (marine) ....*Trichodesmium*

b. Filaments single or in loose masses, sheath present

1. Trichomes many in a sheath .... *Schizothrix*

2. Trichomes single in firm sheath ....*Lyngbya*
3. Trichomes single in mucilaginous sheath .... *Phormidium*

B. Trichomes with heterocysts, reproduction by fragmentation (hormogonia) and akinetes

1. Heterocysts generally terminal on the trichomes, a single akinete adjoining .... *Cylindrospermopsis*
2. Heterocysts generally intercalary, cells and heterocysts cylindrical, end cells elongated, filaments in flake-like colonies ..... *Aphanizomenon*
3. Heterocysts generally intercalary, vegetative cells homogenous, filaments flexuous and contorted, developing in gelatinous colonies ..... *Nostoc*
4. Heterocysts generally intercalary, cells spherical or longer than wide, filaments separate or in tangled masses .... *Anabaena*
5. Heterocysts intercalary, trichomes more than one in a sheath ..... *Hormothamnion*
6. Heterocysts intercalary, cells and heterocysts compressed (discoid) .....*Nodularia*
7. Heterocysts basely, akinetes next to the heterocyst, colonies spherical or hemispherical..... *Gloeotrichia*

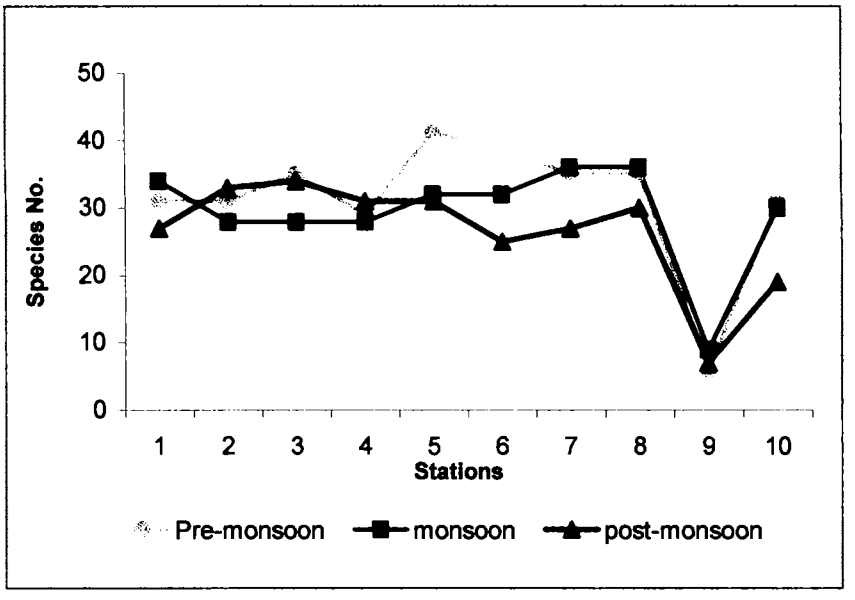
2.3 Results

2.3.1 Distribution pattern

In the present study, a total number of 75 species of cyanobacteria from 24 genera belonging to 7 families and 4 orders of the class cyanophyceae were recorded of which, 31 were unicellular colonial forms, 43 nonheterocystous filamentous forms and two were heterocystous filamentous forms. The photographs of various species of cyanobacteria under 400x

magnification is given in plates 2 to 8. The seasonal and spatial distribution of cyanobacteria in the ten selected stations is given in Table 2.2 and Fig. 2.2.

Fig. 2.2 Seasonal and spatial variation in species



Cyanobacteria from Cochin estuary belonged to the following genera : *Aphanocapsa, Aphanothece, Chroococcus, Coelosphaerium, Dactylococcopsis, Eucapsis, Gloeocapsa, Gloeothece, Microcystis, Synechococcus, Synechocystis, Johannesbaptistia, Chlorogloeoa, Dermocarpa, Myxosarcina, Spirulina, Arthrospira, Oscillatoria, Phormidium, Lyngbya, Anabaena, Pseudanabaena, Plectonema* and *Tolypothrix*. Among these, *Oscillatoria* was represented by maximum number of species; 19 species were observed in this genera followed by *Phormidium*, represented by 13 species and then by *Gloeocapsa,* by 7 species, *Lyngbya,* by 6 species, *Chroococcus,* by 5 species *Aphanocapsa, Aphanothece, Gloeothece, Microcystis, Synechococcus* and *Synechocystis* by 2 species and others by a single species.

Table 2.2. Seasonal and spatial distribution of Cyanobacteria

Sl.no.	Cyanobacteria	Stations									
		1	2	3	4	5	6	7	8	9	10
Phylum : Cyanophyta											
Class : Cyanophyceae Sachs											
Order : Chroococcales Wettsein											
Family : Chroococcaceae Nageli											
1	*Aphanocapsa brunnea* Nag.	*	*	**	**	*	**	**			*
2	*Aphanocapsa littoralis* Hansgirg				*	*		*	*		
3	*Aphanothece castagnei* (Breb.) Rabenh.	**	*	*	*	**	*	*			*
4	*Aphanothece nidulans* Richter,P.			*	*	*	*	*	*		
5	*Chroococcus coharens* (Breb.) Nag.					*			*		
6	*Chroococcus montanus* Hansgirg	*	*	**		**	**	*	*		
7	*Chroococcus turgidus* (Kutz.) Nag.	***	***	***	***	***	***	***	***	*	**
8	*Chroococus tenax* (Kirchn.) Hieron.	**	***	***	***	***	***	**	***	*	**
9	*Chroococcus minutus* (Kutz.) Nag.	**	*		*		**	**			**
10	*Coelosphaerium dubium* Grunow		**	*		**	*	*	*		*
11	*Dactylococcopsis raphidioides* Hansg.	**		**	*	**	*	*	**		*
12	*Eucapsis minuta* Fritsch				*						
13	*Gloeocapsa compacta* Kutz.	***	***	***	***	**	**	***	***		**
14	*Gloeocapsa crepidinum* Thuret	**	***	***	***	***	***	***	***		**
15	*Gloeocapsa dermochroa* Nag.							*		**	
16	*Gloeocapsa gelatinosa* Kutz.		*	***	*	*	*	**	**		*
17	*Gloeocapsa livida* (Carm.) Kutz.	***	***	***	***	***	**	***	***		***
18	*Gloeocapsa quaternata* (Breb.) Kutz.	***	***	**	***	***	**	**	**		*
19	*Gloeocapsa atrata* (Turp.) Kutz.	*	*	*				*			*
20	*Gloeothece rhodochlamys* Skuja	*									
21	*Gloeothece rupestris* (Lyngb.) Bornet	***	***	***	***	***	***	***	***		**
22	*Microcystis orissica* West, W.	**	*		*	**	*	*	**		*
23	*Microcystis stagnalis* Lemm.			**		**		*	**	**	
24	*Synechococcus cedrorum* Sauvageau	**	*	*	***	***	*	**	**		*
25	*Synechococcus elongatus* Nag.	***	***	***	***	***	***	***	***	***	***
26	*Synechocystis aquatilis* Sauv.	***	***	**	***	***	***	*	***		***
27	*Synechocystis salina* wislouch	***	***	***	***	***	***	***	***	*	***
Family : Entophysalidaceae											
28	*Johannesbaptistia pellucida* (Dickie) Taylor et Drouet	*	*				**	***	*	**	
29	*Chlorogloea fritschii* Mitra	*	**	**	**	***	***	**	***		*
Order : Chaemosiphonales											
Family : Dermocarpaceae											
30	*Dermocarpa olivaceae* (Reinsch) Tilden				*		*	*	*		
Order : Pleurocapsales											
Family : Pleurocapsaceae											
31	*Myxosarcina burmensis Skuja*	*	*	*	*	*	**	**	*		*
Order : Nostocales											
Family : Oscillatoriacea											
32	*Spirulina labyrinthiformis (Menegh.) Gomont*										**
33	*Arthrospira tenuis* Bruhl et Biswas	*	*	**				*			*
34	*Oscillatoria accuminata* Gomont		**	**		*	*	*	**		
35	*Oscillatoria angustissima* W. et G.S.West	*	**	**	***	***	*	***	**		**
36	*Oscillatoria acuta* Bruhl et Biswas, orth. Mut. Geitler										*

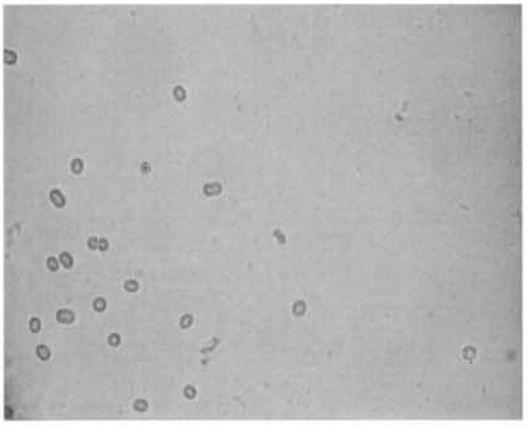
Table 2.2 Cntd.

Sl.no	Cyanobacteria	Stations									
		1	2	3	4	5	6	7	8	9	10
37	*Oscillatoria amoena* (Kutz.) Gomont										*
38	*Oscillatoria cortiana* Meneghini ex Gomont		*	*					*		
39	*Oscillatoria jasorvensis* Vouk.	*				*	*				
40	*Oscillatoria foreaui* Fremy	* *	* *	*	* *	* * *	* * *	* * *	* * *	*	*
41	*Oscillatoria fremyii* De Toni, J.	* * *	* * *	* * *	* * *	* * *	* * *	* * *	* * *	* *	* * *
42	*Oscillatoria kuetzingiana* Nag.		*	*	*						
43	*Oscillatoria laete-virens* (Crouan) Gomont Var. *minimus* Biswas					*	*	*	*		
44	*Oscillatoria limnetica* Lemm.						*				
45	*Oscillatoria minnesotensis* Tilden	*	*	* *	*	*	* *	* *	* *		*
46	*Oscillatoria pseudogeminata* G. Schmid	* * *	* * *	* * *	* * *	* *	* * *	* * *	* * *	*	* * *
47	*Oscillatoria proteus* Skuja					*			*		
48	*Oscillatoria salina* Biswas		* *			*					
49	*Oscillatoria subtillissima Kutz.*	* * *	* * *	* * *	* * *	* * *	* * *	* * *	* * *	* * *	* * *
50	*Oscillatoria tenuis* Ag. ex Gomont					*					
51	*Oscillatoria vizagapatensis* Rao, C.B.	*						*	*		* *
52	*Oscillatoria willei* Gardner em. Drouet	* * *	* * *	* *	* * *	* * *	* * *	* *	* *	*	* * *
53	*Phormidium abronema* Skuja							*			
54	*Phormidium angustissimum* W. et G.S.West.	* *		* *	*	*	* *	*	* *		
55	*Phormidium bohneri* Schmidle	* * *	* * *	* * *	* *	* * *	* *	* *	* *		* *
56	*Phormidium corium* (Ag.) Gomont	* *	*	* *	* * *	* * *	* *		* *		*
57	*Phormidium dimorphum* Lemm.	*	*	*		*	*		*		*
58	*Phormidium foveolarum* (Mont.) Gomont	* *	* *	* *	* *	* * *	*	*	* *		*
59	*Phormidium jadinianum* Gomont						*				* *
60	*Phormidium lucidum* Kutzing ex Gomont			*				*			
61	*Phormidium molle* (Kutz.) Gomont	*	*	*	* *	*	*	*	* *		*
62	*Phormidium mucicola* Hub.-Pestalozzi et Naumann					*	* *	*	*		
63	*Phormidium purpurescens* (Kutz.) Gomont	*	*	* *		*	*	* *	*	*	*
64	*Phormidium tenue (Menegh.) Gomont*	* * *	* * *	* * *	* * *	* * *	* * *	* * *	* *	*	* * *
65	*Phormidium valderianum* (Delp.) Gomont	* * *	* * *	* * *	* * *	* * *	* *	* * *	* * *		* * *
66	*Lyngbya aerugineo-coerulea* (Kutz.) Gomont	* *		*	*	* *			*		
67	*Lyngbya cryptovaginata* Schkorbatow					* *		*			
68	*Lyngbya martensiana* Menegh. ex Gomont	* * *	* * *	* *	* * *	*	* *	* * *	* * *		* *
69	*Lyngbya nordgardhii* Wille		*		*	*	*				* *
70	*Lyngbya putealis* Mont. ex Gomont						*				
71	*Lyngbya semiplena* (C.Ag.) J.Ag. ex Gomont	* * *	* * *	* * *	*	*	*	* *	*		* *
Family : Nostocaceae											
72	*Anabaena khannae* Skuja	* *	*				*	*			
73	*Pseudanabaena schmidlei* Jaag. O *f.robusta* Skuja			*			*		* *		
Family : Scytonemataceae											
74	*Plectonema nostocorum* Bornet ex Gomont						*				
75	*Tolypothrix tenuis* (Kutz.) Johs. Schmidt em.		*	*					*		

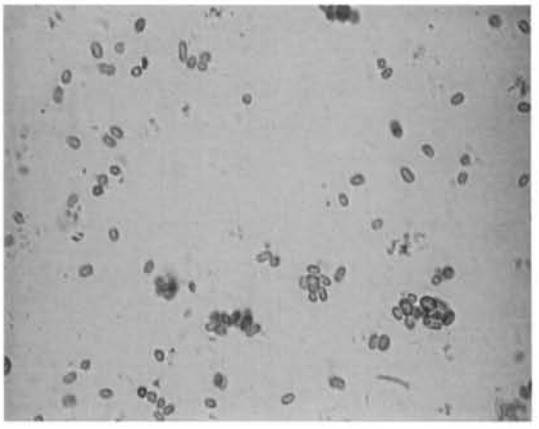
* Presence in premonsoon season

* Presence in monsoon season

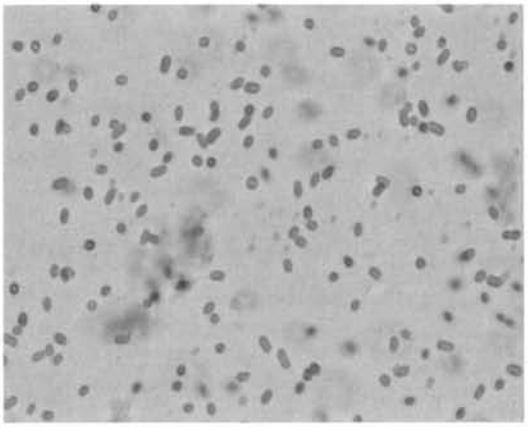
* Presence in postmonsoon season



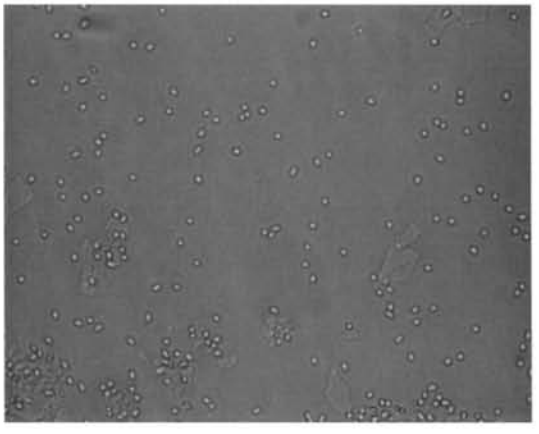
Aphanocapsa littoralis



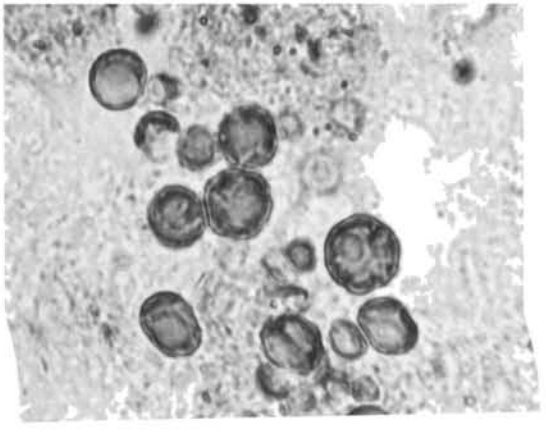
Aphanothece castagnei



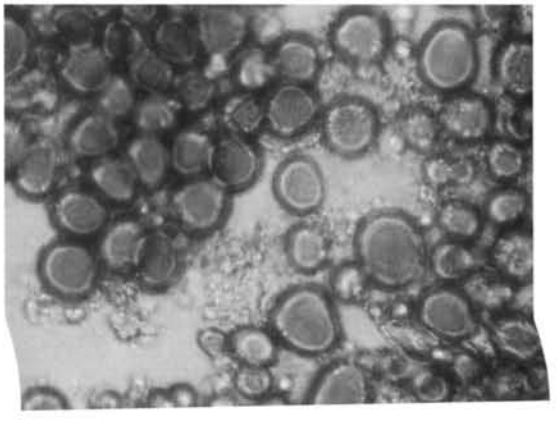
Synechococcus elongatus



Synechocystis salina



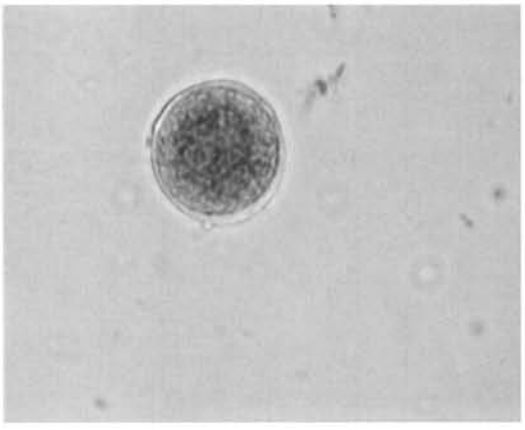
Chroococcus turgidus



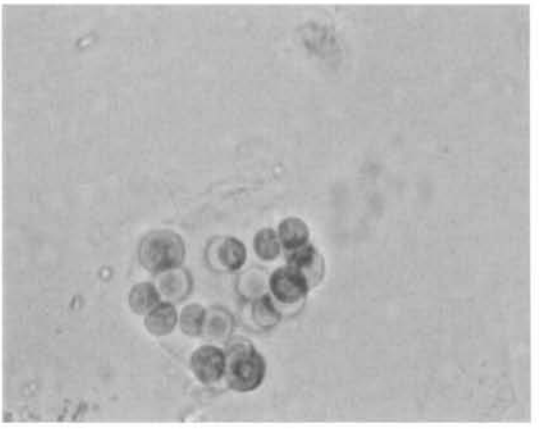
Chroococcus montanus

Light microscopic photographs (400 X) of cyanobacteria isolated from Cochin estuary

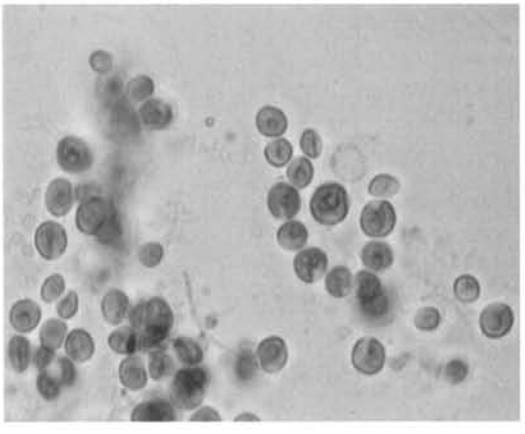
PLATE-2



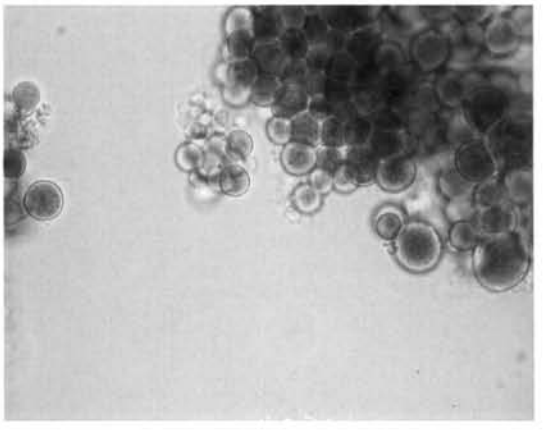
Chroococcus tenax



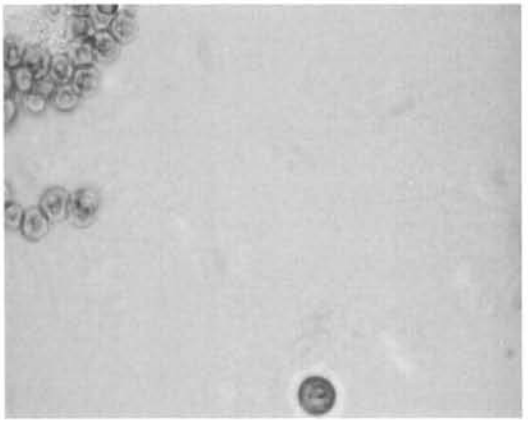
Gloeocapsa crepidinum



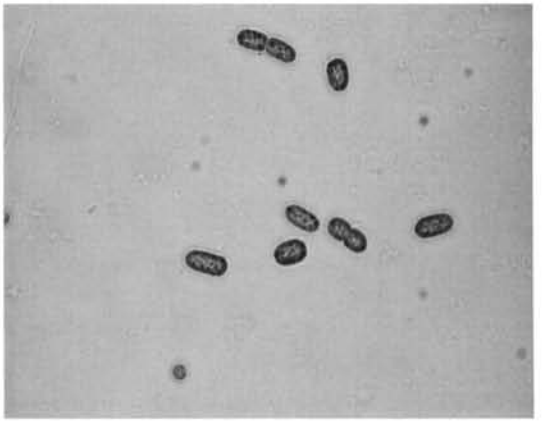
Gloeocapsa livida



Gloeocapsa quaternata



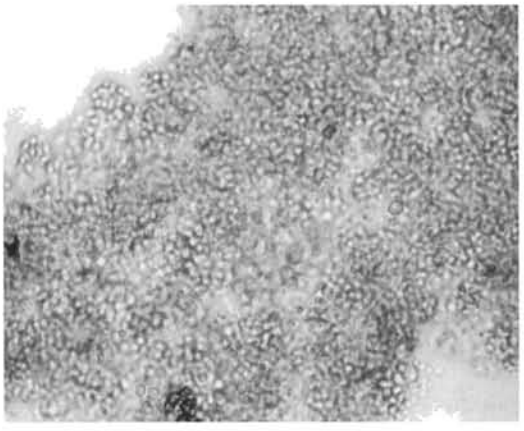
Gloeocapsa gelatinosa



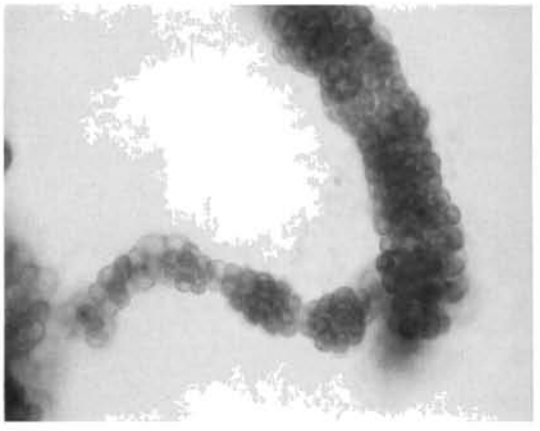
Gloeothece rupestris

Light microscopic photographs (400 X) of cyanobacteria isolated from Cochin estuary

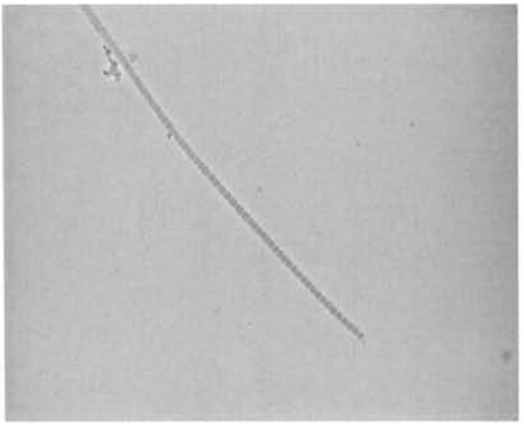
PLATE-3



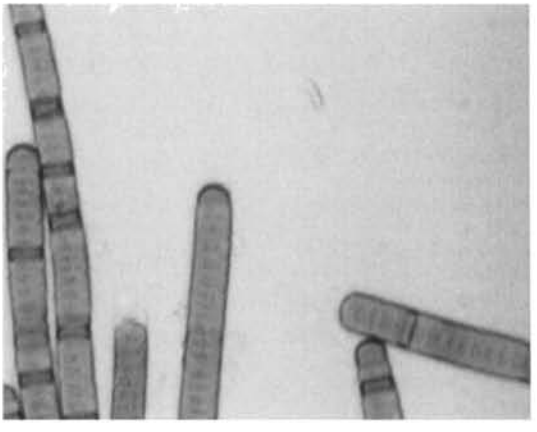
Microcystis stagnalis



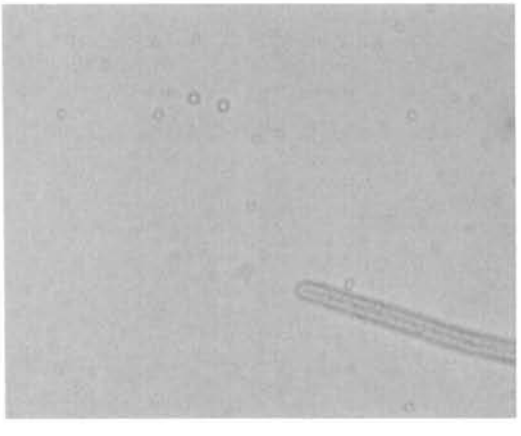
Chlorogloea fritschii



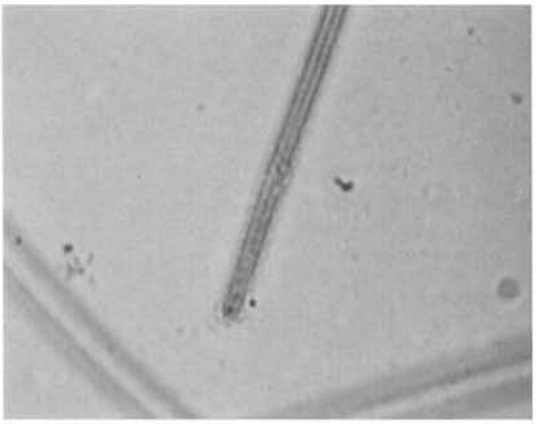
Oscillatoria fremyii



Oscillatoria vizagapatensis



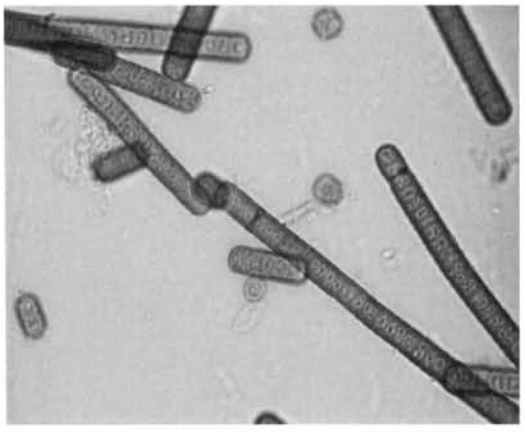
Oscillatoria laete-virens minimus



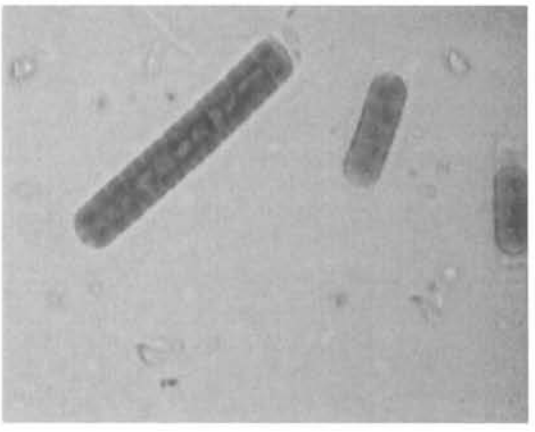
Oscillatoria minnesotensis

Light microscopic photographs (400 X) of cyanobacteria isolated from Cochin estuary

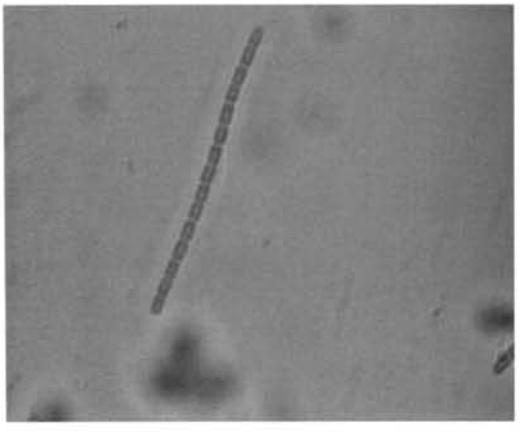
PLATE-4



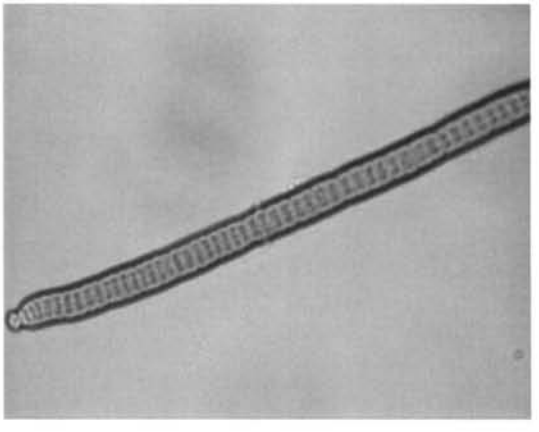
Oscillatoria proteus



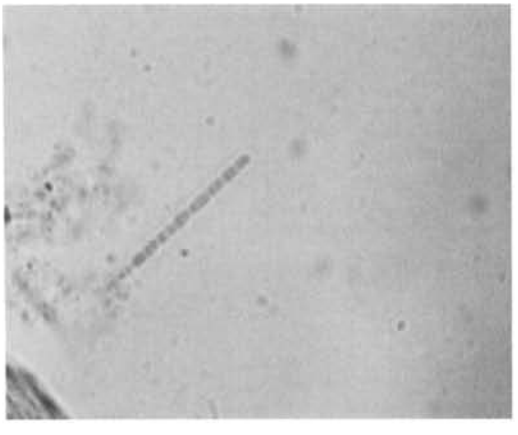
Oscillatoria tenuis



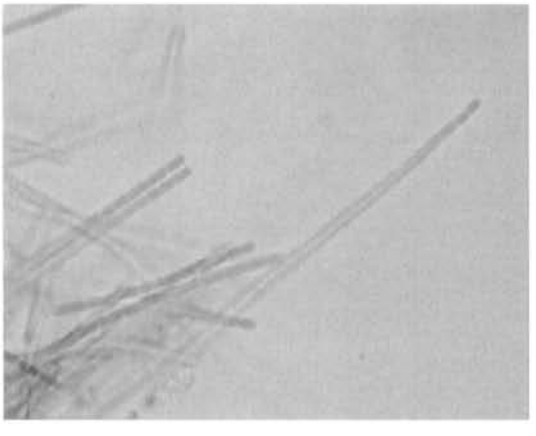
Oscillatoria pseudogeminata



Oscillatoria amoena



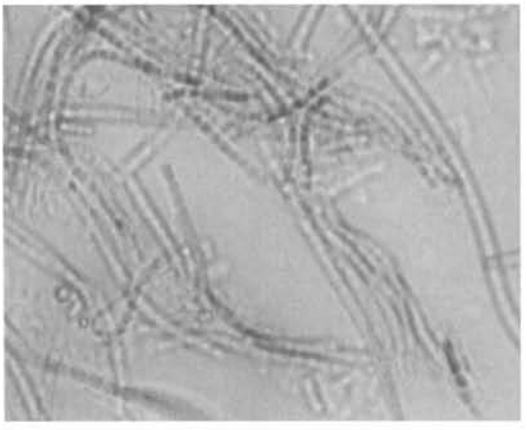
Oscillatoria willei



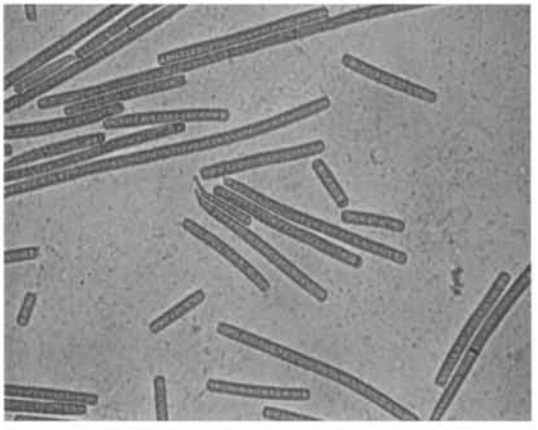
Oscillatoria kuetzingiana

Light microscopic photographs (400 X) of cyanobacteria isolated from Cochin estuary

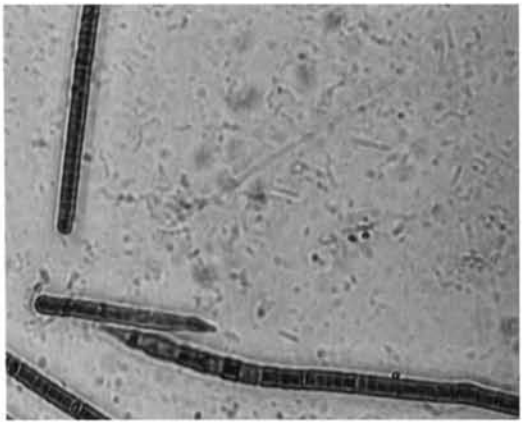
PLATE-5



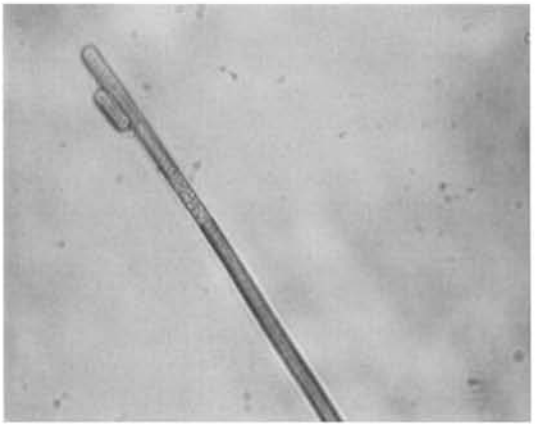
Oscillatoria foreaui



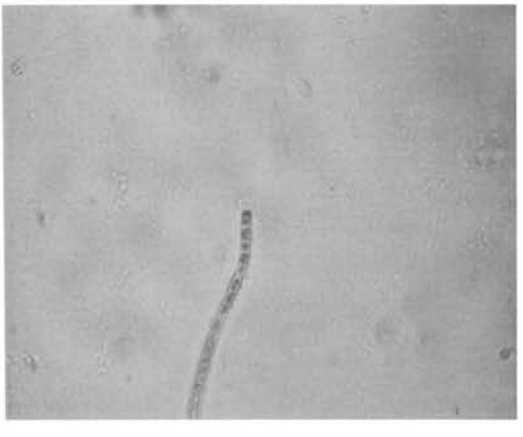
Oscillatoria salina



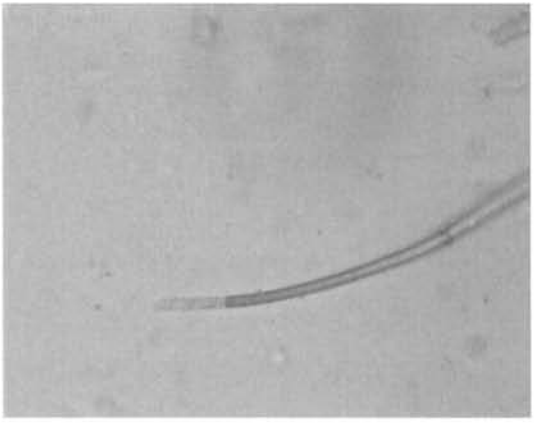
Oscillatoria acuminata



Phormidium lucidum



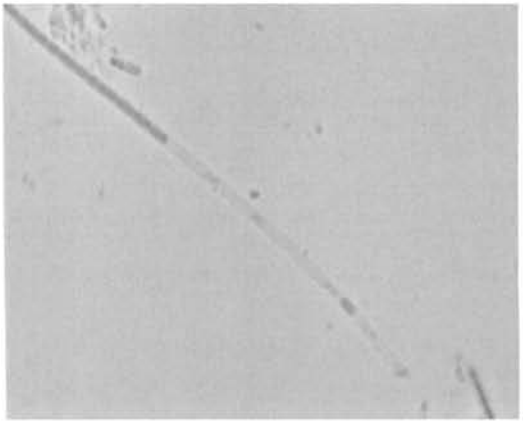
Phormidium bohneri



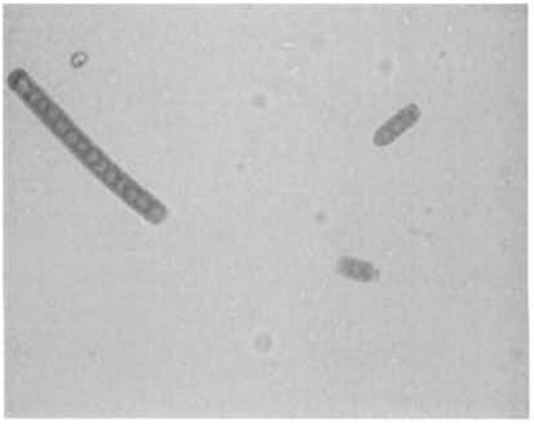
Phormidium angustissimum

Light microscopic photographs (400 X) of cyanobacteria isolated from Cochin estuary

PLATE-6



Phormidium tenue



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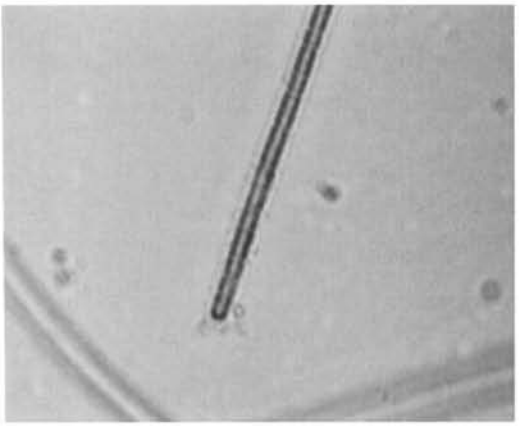
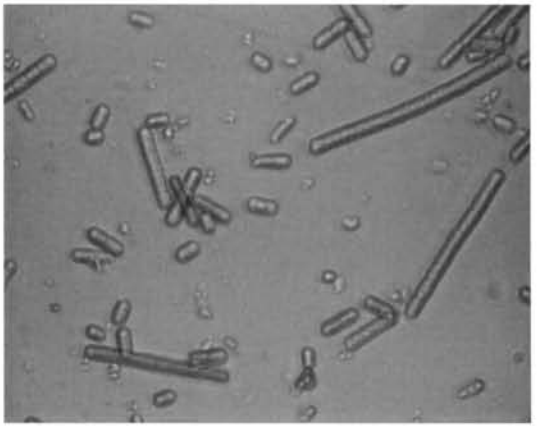


Phormidium mucosum



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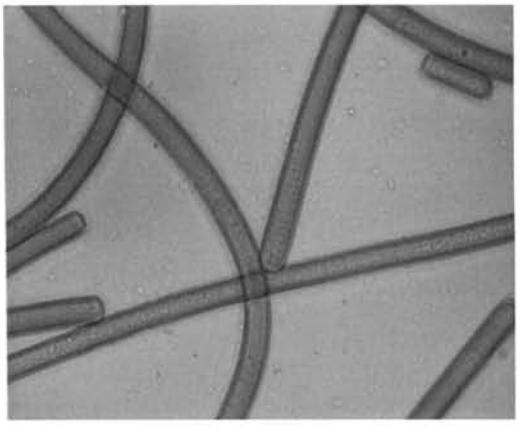
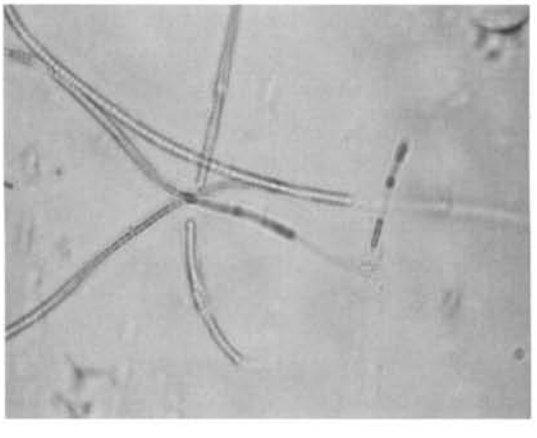


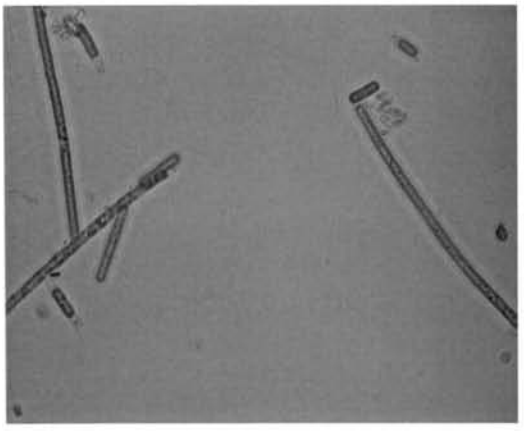
Phormidium abronema



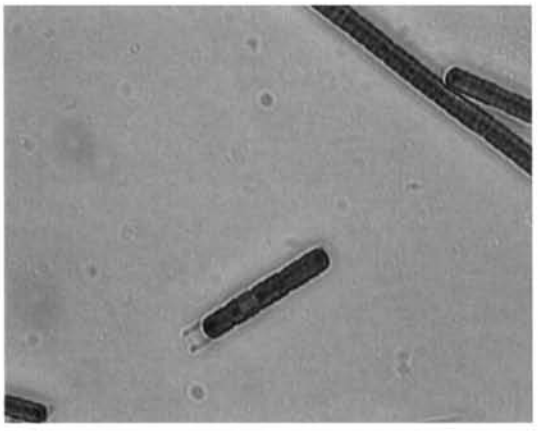
Phormidium foveolarum

Light microscopic photographs (400 X) of cyanobacteria isolated from Cochin estuary

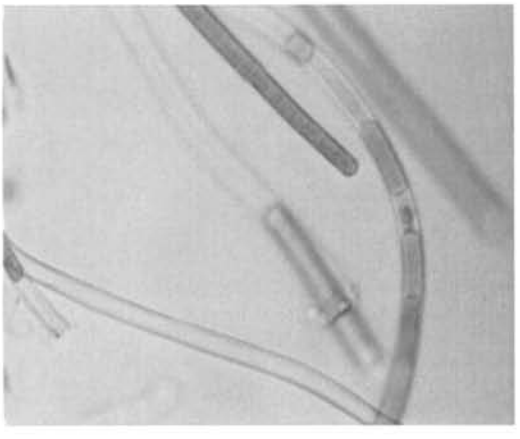
PLATE-7



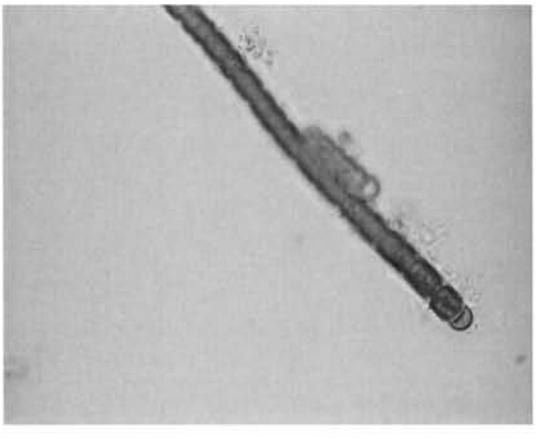
Phormidium corium



Lyngbya martensiana



Lyngbya semiplena



Tolypothrix tenuis

Light microscopic photographs (400 X) of cyanobacteria isolated from Cochin estuary

PLATE-8

Predominant species

The species of cyanobacteria found widely distributed all over the study area are considered as versatile species. *Chroococcus turgidus, C. tenax, Synechococcus elongatus, Synechocystis salina, Oscillatoria foreauii, O. fremyii, O. pseudogeminata, O. subtillissima, O. willei, Phormidium purpurescens and P. tenue* were observed in all the ten stations at least once. Out of these, *Synechocystis salina* and *Oscillatoria subtillissima* were present in all the stations in pre-monsoon; *Synechococcus elongatus, Synechocystis salina* and *Oscillatoria subtillissima* in monsoon and *Synechooccus elongatus* and *Oscillatoria subtillissima* in the post-monsoon period.

Heterocystous forms

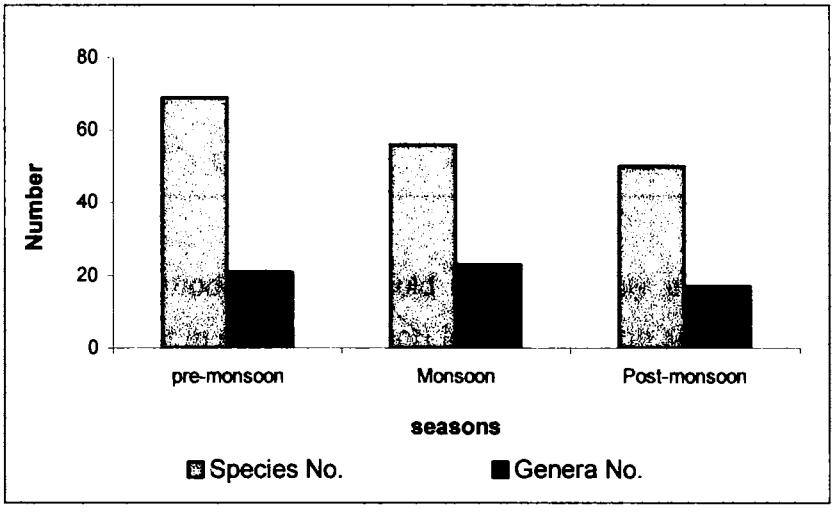
When compared to the unicellular and non-heterocystous filamentous forms, the heterocystous forms were very low in numbers. Out of the total 75 species obtained from the study sites, only two were heterocystous; *Anabaena khannae* from Nostocaceae family and *Tolypothrix tenuis* from Scytonemataceae family.

2.3.1.1 Seasonal distribution

Cyanobacteria showed considerable seasonal variations in their distribution (Table 2.2 and Fig. 2.3). Maximum number of species was found in pre-monsoon season. In all, 69 species of cyanobacteria belonging to 21 genera were reported in the pre-monsoon; 56 species belonging to 23 genera and 50 species from 17 genera in the monsoon and post-monsoon season respectively. *Eucapsis minuta* was found only in the post-monsoon season whereas, *Dermocarpa olivacea, Oscillatoria culcuttensis* and *Lyngbya putealis* only in monsoon and *Chroococcus coharens, Gloeothece rhodochlamys, Oscillatoria acuta, O. cortiana, O. limnetica, O. tenuis and Phormidium abronema* only in pre-monsoon season. *Gloeocapsa dermochroa* and *Anabaena khannae* could not be observed in any of the stations in the pre-monsoon period, whereas, *Aphanocapsa brunnea, Gloeocapsa sp. Oscillatoria accuminata, O. kuetzingiana, Phormidium angustissima,*

Phormidium dimorphum, Lyngbya aerugineo, and *Lyngbya nordgardhii* were absent in the monsoon season and *Aphanocapsa littoralis, Aphanothece castagnei, Aphanothece nidulans, Chroococcus minutus, Myxosarcina burmensis, Dactylococcopsis raphidioides, Spirulina labyrinthiformis, Oscillatoria deflexa, Oscillatoria vizagapatensis, Oscillatoria salina, Oscillatoria schultzii, Phormidium jadinianum, Phormidium lucidum, Pseudanabaena schmidlei* and *Plectonema nostocorum* were completely absent from the study area in the post-monsoon season.

Fig 2.3 Seasonal variation in species and genera

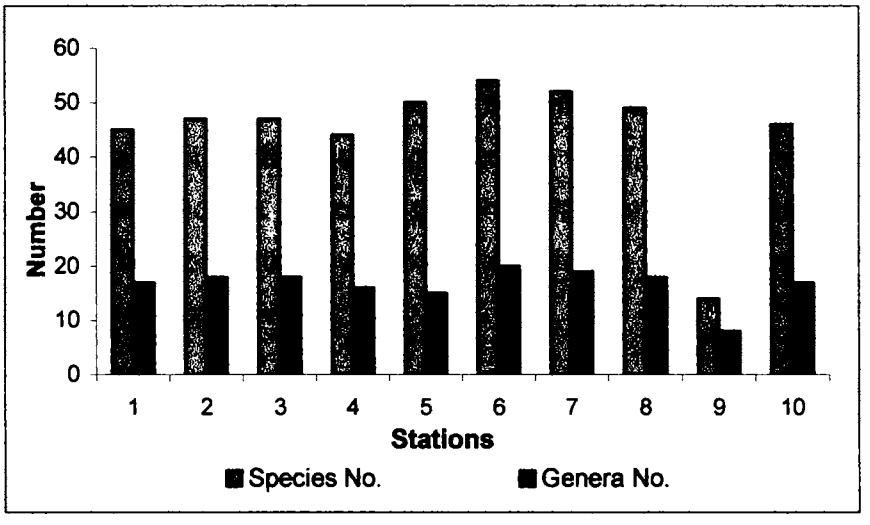


2.3.1.2 Spatial distribution

Among the 10 stations studied, maximum number of species was recorded from station 6 (Mattanchery) and minimum in station 9 (Kannamali) (Table 2.2 and Fig. 2.4). A total number of 45 species belonging to 17 genera were obtained from station 1 (Eloor). Similarly, 47 species of 18 genera from station 2 (Varapuzha) and station 3 (Vaduthala), 44 species of 16 genera from station 4 (Bolghatty), 50 species of 15 genera from station 5 (Barmouth), 54 species of 20 genera from station 6, 52 species of 19 genera from station 7 (Thevara), 49 species of 18 genera from station 8 (Edakochi), 14 species of 8

genera from station 9 (Kannamali) and 46 species of 17 genera from station 10 (Puduvaippu) were observed through out the study period from April 2001 to March 2003.

Fig 2.4 Spatial variation in species and genera



Gloeothece rhodochlamys could be found only in fresh water. The species was present in station 1 (Eloor) in pre-monsoon season. The species obtained only from saline environment were *Aphanocapsa littoralis, Chroococcus coharens, Eucapsis minuta, Gloeocapsa dermochroa, Dermocarpa olivaceae, Oscillatoria laete-virens, Oscillatoria limnetica, Oscillatoria schultzii, Oscillatoria tenuis, Phormidium abronema, Phormidium jadinianum, Phormidium mucicola, Lyngbya cryptovaginata, Lyngbya putealis* and *Tolypothrix tenuis. Spirulina labyrinthiformis, Oscillatoria acuta and Oscillatoria amoena* were observed only in mangrove water samples.

The number of cyanobacterial species present in the seashore region was very less when compared to other stations. Only 14 species from 8 genera were recorded from the seashore station throughout the period. *Synechococcus elongatus, Synechocystis salina, Oscillatoria fremyii and*

Oscillatoria subtillissima were obtained from this site in all the seasons, where as *Gloeocapsa dermochroa, Microcystis stagnalis, Oscillatoria willei and Phormidium purpurescence* were observed only in monsoon season.

2.4 Discussion

The annual net primary production by phytoplankton from the oceans of the world amounts to 15-18 x 10^9 t of carbon (Koblentz-Mishke *et al.*, 1970). The study of seasonal and spatial variation of planktonic cyanobacteria, forms a fundamental aspect in their ecology. Odate *et al.*, (1989) studied the distribution of cyanobacteria and other picophytoplankton in the western North Pacific Ocean. They found that cyanobacteria were the most abundant forms in the surface of subtropical water.

The remarkable adaptability of cyanobacteria to various habitats is, well known (Hof and Fremy, 1933; Desikachary, 1959; Van Baalen, 1962; Fogg *et al.*, 1973; Carr and Whitton, 1982). There are reports proving that cyanobacteria could grow in salinities ranging from 0-99% (Prabaharan, 1988). Despite the fact that this group of photosynthetic prokaryotes is structurally simple, it is physiologically diverse and well adapted to environmentally extreme conditions, especially nutrient deprivation and excesses (Paerl, 1999). The cyanobacteria have developed an array of biochemical and ecological mechanisms to access the limiting nutrients; the foremost is the ability of numerous genera to fix atmospheric nitrogen. They have also exploited anthropogenic alterations of coastal environments causing eutrophication.

The present investigation shows that Cochin estuary is endowed with morphologically diverse group of cyanobacterial forms. Most of the species had shown wide salinity tolerance, as they were found distributed in almost all the stations and in all the seasons. Therefore, It was difficult to strictly segregate most of the species into exactly saline and freshwater species as

can be done with other algal forms. In all, 75 species of cyanobacteria from 24 genera were obtained from the study sites. The family Oscillatoriaceae was represented by maximum number of species (40 species) and in all the sampling stations, nonheterocystous filamentous forms dominated, whereas, only two heterocystous forms could be observed throughout the study period. On an analysis of seasonal variation it was noted that maximum number of cyanobacteria occurred during pre-monsoon season. Pre-monsoon period characterized by high temperature and light intensity, supported maximum growth of cyanobacteria. All the stations exhibited almost a similar pattern of cyanobacterial distribution. About 44 to 54 species were observed except in station 9, i.e., the seashore region that was significantly different from other sites. Only 14 species were obtained from here, which shows that low nutrients and sandy substratum are not much favourable for cyanobacterial growth.

Similar reports on cyanobacterial distribution are available from different parts of India. Laloraya and Mitra (1973) studied the cyanobacteria in the paddy fields of India and identified 122 forms belonging to different families. Prasad and Khanna (1986) explored the cyanophycean flora of Sikkim and 24 species belonging to the family *Oscillatoriaceae* were recorded of which 14 species belonged to *Oscillatoria*, two to *Phormidium*, six to *Lyngbya* and one each to the genus *Spirulina* and *Microcoleus*. Anand *et al.*, (1986) made an attempt to study the blue green algae occurring in estuaries, backwaters and salt marshes of South India. A total number of 25 taxa were recorded of which four were unicellular or colonial forms, 19 were nonheterocystous filamentous forms and two were heterocystous filamentous forms. In the present study also, the distribution pattern showed that nonheterocystous filamentous forms dominated in all the sampling sites. In general, saline habitats harboured more of nonheterocystous filamentous forms than unicellular or heterocystous filamentous forms. Similar observations were made by Iyengar and Desikachary, 1944 , and Anand and Venkatesan, 1985.

Thajuddin and Subramanian (1992) studied the cyanobacterial flora of the east coast of India and found that the distribution of cyanobacteria depends upon the habitat. They have reported 130 species, of which 26 were categorized as constant species, 37 as seasonal species and the rest were rare species. Heterocystous forms were observed to be less in number in comparison to nonheterocystous forms. They observed that the shore in the Bay of Bengal region was essentially sandy and, therefore, there were only 11 species of cyanobacteria. The same observation could be made in the present investigation also. In station 9 (Kannamaly), the coastal area, cyanobacterial distribution was very poor and this might be attributed to the occurrence of sandy shore, rough waves, and absence of nutrient-rich muddy substratum at this site. When compared to the unicellular and non heterocystous filamentous forms, the heterocystous forms were very poor in numbers in the east coast of India and this might be due to the high levels of combined nitrogen in the sea (Thajuddin and Subramanian, 1990). Similar observation could be made in the present study also.

The study of cyanobacteria of Cochin estuary has been quite rewarding not only in giving some idea of the diversity and occurrence of cyanobacteria in the area but also in providing an opportunity to understand their distribution, abundance and versatility. A profound cyanobacterial culture collection has also been established in the laboratory.

A wide variety of morphological, physiological and biotic survival and opportunistic competitive strategies can be found in this phylum. As such, cyanobacteria have been able to take advantage of natural (geological, climatic) and man-induced (eutrophication) environmental change in the world's oceans. Growth, numerical dominance and bloom dynamics are controlled by the complex interplay of nutritional, physical and biotic factors characterizing the world's estuarine, coastal and open ocean habitats. Cyanobacteria are particularly adept in exploiting physically and chemically

"extreme" but "stable" environments, such as highly-stratified, excessively-illuminated, nutrient-deprived or nutrient-enriched surface waters, tropical and polar hypersaline ponds, sulfidic benthic environments and unique biotic associations.

As human influences and perturbations such as climate change, nutrient enrichment, stoichiometric imbalances and xenobiotic pollutant discharge continue to encroach the coastal zone and beyond, there are reasons to believe that cyanobacterial dominance will increase in stressed waters. In part, the opportunistic "behaviour" typifying cyanobacteria can be attributed to a long evolutionary history that has endowed this group with remarkable survival and adaptational qualities. As human environmental influence on the world's ocean increases, so will the biogeochemical and trophic roles of cyanobacteria. In many ways, cyanobacteria are the proverbial 'canary in the mine' indicator of environmental change, except instead of dying, this indicator may well benefit from such a change.

CHAPTER 3

Seasonal and spatial distribution of cyanobacteria in Cochin estuary: Quantitative analysis

3.1 Introduction

Cyanobacteria are significant as well as dominant contributors to estuarine, coastal and open ocean primary production and fixed nitrogen inputs (Paerl, 1999). They are well recognized as major component of biomass in all aquatic ecosystems. Morphologically and functionally diverse cyanobacterial taxa are found in planktonic and benthic habitats. The high diversity and broad geographic distribution of cyanobacteria are attributed to a long evolutionary history, dating back to the Precambrian, about 2 billion years ago, when the earth underwent the transition from anoxic to oxic atmosphere conditions (Cloud, 1976; Knoll, 1979; Schopf and Walter, 1982; Schopf, 1992). This geochemical transition was initiated by cyanobacteria themselves, being the first oxygenic phototrophs to inhabit the planet (Schopf and Walter, 1982).

The routine biological monitoring of various habitats for cyanobacteria has generally been treated as separate problem in environmental studies. Changes in the concentration of cells with location and time are monitored. Usually, estimations of population densities have been done directly on preserved water samples. The two basic methods used were settling techniques (Lund, 1959), or the membrane filter procedure (APHA, 1998). However, viability was not assessed with either of these two methods. The most probable number (MPN) technique described by McCurdy and Hodgson (1973) allows the enumeration of viable cyanobacteria in natural populations based on standard microbiological techniques. Cyanobacteria are typically identified and enumerated under microscope using a calibrated counting chamber and generally reported as cells per millilitre (Lasett *et al.*, 1997).

One of the requirements for proficient enumeration of cyanobacteria is the development and implementation of reliable sampling techniques (Lawton *et al.*, 1999). The samples that have been collected should be preserved immediately at the sampling site by the addition of acidified Lugol's solution

(Vollenweider, 1969; Lawton *et al.*, 1999). Water quality in an unpreserved sample will be altered within hours, and grazing zooplankton can reduce cyanobacterial numbers (Hotzel and Croome, 1998). These changes may consequently reduce the accuracy of the count. The iodine in the solution not only preserves the cyanobacterial cells in the sample, but also increases their specific weight. This, in turn, facilitates the sedimentation process that is commonly used in the concentration of cyanobacteria in water samples. Iodine can also stain these organisms (Shaw and Smith, 2000). This, of course, presupposes familiarity with freshwater cyanobacteria, because loss of structural detail may occur (Lund *et al.*, 1958). The break-up of filaments and colonies during processing is often unavoidable (Hotzel and Croome, 1998).

Only very high concentration of natural cyanobacteria could be enumerated directly. Therefore, the samples usually need to be concentrated before enumeration and then the identification is performed (SCOR Working Group, 1974). The three most common methods employed for sample concentration are sedimentation, centrifugation and membrane filtration (SCOR Working Group, 1974; Hotzel, 1998; APHA, 1998). Sedimentation is the benchmark method of phytoplankton concentration, which is nonselective and nondestructive.

The Sedgwick - Rafter (S-R) chamber with a standard compound microscope can be used for cyanobacterial enumeration and identification. The S-R chamber consists of a 20mm X 50mm microscope slide, with a grid floor consisting of 1000 fields and a raised well, capable of holding 1 ml sample (Hotzel, 1998). The number of fields examined depends upon the cyanobacterial density of the sample and upon the level of accuracy required (Shaw and Smith, 2000). A minimum of 30 fields must be counted to ensure that 90-95 % of the cyanobacterial species present within the S-R chamber are detected (McAlice 1971; Hotzel, 1998).

The cells within each trichome should be counted (or estimated), wherever possible, in case of filamentous cyanobacteria. Some species form large colonies so that only an estimate of cells per colony could be made, unless the colonies are dispersed and individual cells are counted. Traditional techniques for the identification of cyanobacteria have relied primarily on morphological characteristics observed under the microscope. The morphology of cyanobacteria may change depending on environmental conditions and the phase of growth of the organism (Lu *et al.*, 1997; Murayama-kayano *et al.*, 1998). This morphological variation may lead to difficulty and errors in cyanobacterial identification (Murayama-kayano *et al.*, 1998). Current developments in cyanobacterial enumeration and identification are molecular techniques and flow cytometry (Shaw and Smith, 2000).

This chapter deals with identification and quantification of cyanobacteria present in Cochin estuary. The addition of the preservative, iodine, and further processing of sample may distort the actual structure of cyanobacterial cells. Hence, it was very difficult to identify them at species level. However, an attempt has been made to identify and enumerate the cyanobacteria at species level and the community structure of cyanobacteria was studied.

Sanders (1968) established a fundamental relation between species diversity and nature of the environment. Since then, ecologists started assessing species diversity as a powerful tool in community studies. Four different terms of diversity indices with different ecological importance were used. They were richness, evenness, diversity and dominance indices. The richness (Margalef's index) is used to estimate the total number of species observed in a given area. It is a straightforward count of the number of species. More the number of species in the sample from a particular site, the greater will be the richness. The term evenness expresses how evenly the individuals are distributed among the different species. High evenness occurs, when the species present are virtually in equal abundance, which is

conventionally equated with high diversity. The less numerically equal the species are, the less diverse the sample is or, conversely, the greater dominance in the fauna (Sanders, 1968). Dominance is the relative occurrence of one or few species with others. The dominant one will have high relative occurrence. The term diversity is used for the number of species per number of individuals. The highest species diversity is possible when only one individual represents every species and the lowest diversity possible is when community consists of only one species (Soetaert and Heip, 1990). Diversity measurements of cyanobacteria are often more informative than their counts alone because they take into account two factors, richness and evenness.

Hierarchial clustering and Multidimensional scaling (MDS) start explicitly from a triangular matrix of similarity coefficients computed between every pair of samples. In former case, representation of the communities for each sample is by a dendrogram. The coefficient is usually some simple algebraic measure of how close the abundance levels are for each site and defined such that 100% represents total similarity and 0%, complete dissimilarity. The latter method attempts to place the samples on a "map", usually in two dimensions, in such a way that the rank order of the distances between samples on the map exactly agrees with the rank order of the matching (dis) similarities.

3.2 Materials and Methods

3.2.1 Sampling

Details of sampling sites and the methods used for sampling are presented in section 2.2 of chapter 2. Surface and bottom water samples were collected using Niskin water sampler. From station 9 and 10, only surface water was collected.

3.2.2 Preservation of samples

Samples were preserved at the sampling site by the addition of acidified Lugol's solution (Shaw and Smith, 2000). Acidified Lugol's solution was made by dissolving 20g potassium iodide and 10g iodine crystals in 200 ml distilled water containing 20 ml glacial acetic acid (Vollenweider, 1969; Lasett *et al.*, 1997).

3.2.3 Sample concentration technique

Samples were concentrated by sedimentation using a measuring cylinder (Shaw and Smith, 2000). The cylinder was filled with the preserved sample without forming a vortex and kept vibration-free at 20^0C for 3 days. After sedimentation, 90% of top volume was carefully siphoned off without disturbing the sedimented cyanobacteria. The remainder of the sample was taken for identification and enumeration.

3.2.4 Enumeration

The enumeration of cyanobacterial cells was carried out using Sedgewick-Rafter chamber and identified at 400X magnification by using a phase contrast microscope. In case of filamentous bacteria, the cells within each trichome were counted. The final result, expressed as cells per millilitre was calculated as;

$$\text{Cells per ml} = \frac{\text{Number of cells counted x 1000}}{\text{Sample concentration factor x No. of fields examined}}$$

3.2.5 Identification

Identification of cyanobacteria at species level was done as explained in section 2.2.4.

3.2.6 Data analysis

The data set was treated with various univariate and multivariate analyses of PRIMER-5 (Plymouth Routines in Multivariate Ecological Research) for community structure study. Diversity indices such as Margalef's index (species richness-d), Pielou's index (species evenness-J'), Shannon index (species diversity-H') and Simpson's index (species dominance-λ') were estimated. Multivariate analysis was performed on taxa by station data matrix, following the approach of Clarke (1993) and Clarke and Warwick (1994). Q-mode (site similarity) analysis using the Bray-Curtis similarity measure was applied to species-abundance data to group the samples with similar community composition or ecological conditions, as described by Clarke and Warwick (1994). The stations were plotted on a 2-dimensional map by non-metric multidimensional scaling (MDS) ordination based on the similarity matrix.

3.3 Results

3.3.1 Distribution pattern

3.3.1.1 Seasonal and spatial variation

The study showed that pre-monsoon season was characterized by high level of organisms, whereas, cell count was very low in monsoon season. Monthly variation in total cell count is given in Table 3.1a and 3.1b (Appendix 1) and seasonal variation is shown in Fig 3.1 & 3.2. Table 3.2 (Appendix 1) shows the abundance of each genus of cyanobacteria in various seasons for both surface and bottom samples. Total cell count was high at station 10 (Puduvaippu) and ranged between 1163 – 2080 cells/ml, whereas, it was very less in station 9, where the range was 81 - 315 cells/ml only. Cyanobacterial count in the samples from industrially polluted areas such as Eloor (stn.1), Vaduthala (stn.2), Varapuzha (stn.3) and Aroor (stn.8) was low (295 - 495 cells/ml) when compared to other backwater stations. Higher saline areas

such as Barmouth (station 5), Mattanchery (station 6) and Thevara (station 7) demonstrated higher cyanobacterial count (525 – 825 cells/ml).

Fig. 3.1 Seasonal variation in cyanobacterial count at various stations – Surface water

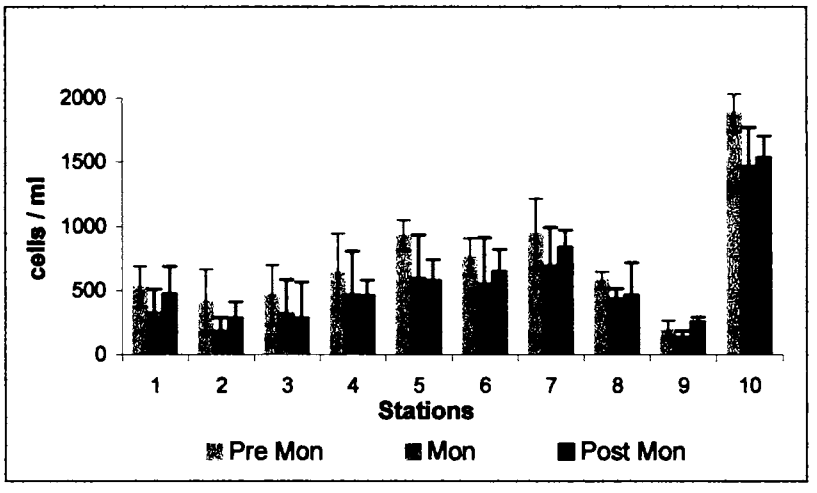
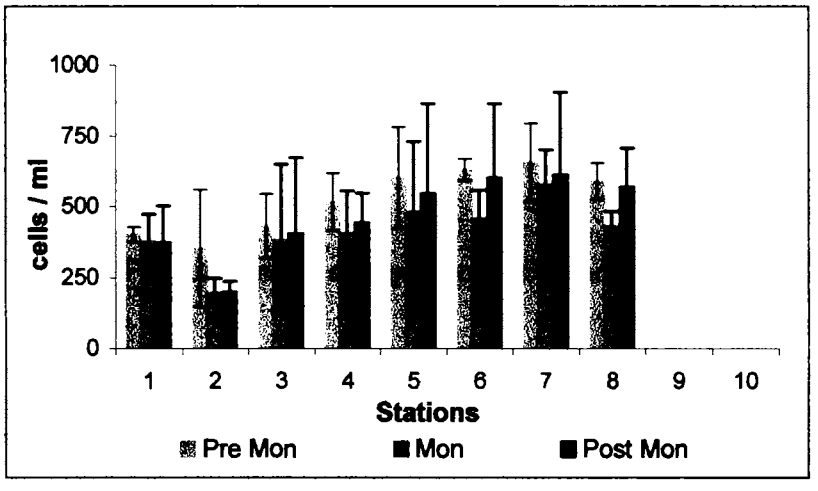


Fig. 3.2 Seasonal variation in cyanobacterial count at various stations – Bottom water



3.3.1.2 Vertical variation

Cell count

The average cell count in the surface and bottom samples at station 1 is 445 and 384 cells/ml respectively, whereas, that in other stations are as follows (values for surface and bottom samples are given in brackets); station-2 (296/251), station-3 (359/407), station-4 (525/456), station-5 (703/544),

station-6 (655/564), station-7 (825/616), station-8 (495/531). In general, cyanobacteria were more in surface water. However, stations 3 (Vaduthala), 7 (Thevara) and 8 (Aroor) which are comparatively shallow regions with an average depth of 1.4 to 2.1 m, bottom water was having slightly elevated levels of cell count.

Generic Composition

Generic composition of cyanobacteria in surface and bottom water is shown in Fig 3.3 and Fig 3.4.

Fig 3.3 Generic composition – Surface water

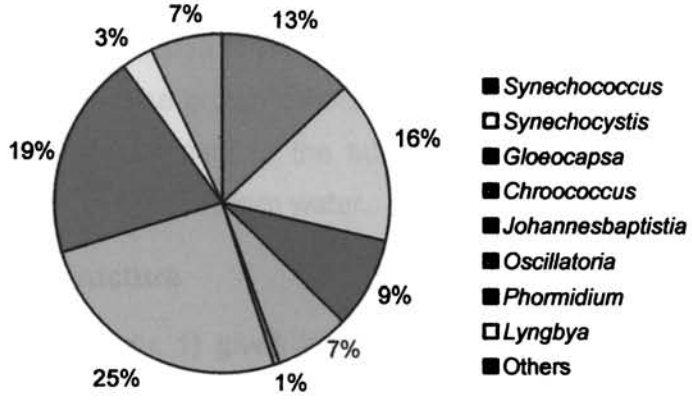
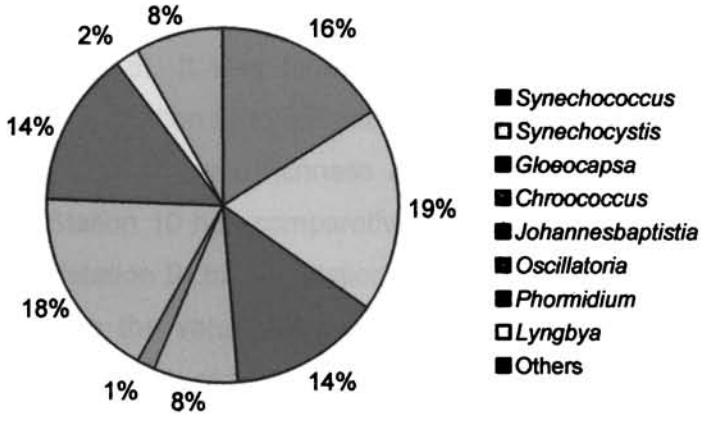


Fig 3.4 Generic composition – Bottom water



Totally, 24 genera were recorded from the study sites. The most abundant genus in the surface water was *Oscillatoria* whereas, in bottom water, the predominant form was *Synechocystis.* Other major forms include *Phormidium, Synechococcus, Gloeocapsa, Johannesbaptistia, Chroococcus* and *Lyngbya.* In surface samples, 25% was from *Oscillatoria* and 19% from *Phormidium* followed by *Synechocystis* (16%), *Synechococcus* (13%), *Gloeocapsa* (9%), *Chroococcus* (7%), *Lyngbya* (3%) and *Johannesbaptistia* (1%), while 7% was represented by "others" which include *Aphanocapsa, Aphanothece, Coelosphaerium, Dactylococcopsis, Eucapsis, Gloeothece, Microcystis, Chlorogloeoa, Dermocarpa, Myxosarcina, Spirulina, Arthrospira, Anabaena* and *Pseudanabaena.* In bottom samples, 19% was from genus *Synechocystis* followed by *Oscillatoria* (18%), *Synechococcus* (16%), *Phormidium* (14%), *Gloeocapsa* (14%), *Chroococcus* (8%), *Lyngbya* (2%) and *Johannesbaptistia* (1%). The group "others" represented 8%. The filamentous cyanobacteria were predominant in the surface water, whereas, unicellular forms were the major forms in bottom water.

3.3.2 Community structure

Table 3.3 (Appendix 1) gives the value of richness index, evenness index, diversity index and dominance index for all the stations in various seasons.

3.3.2.1 Species richness

Margalef's richness index (d) was estimated for each station during various seasons (Fig.3.5). It was found that in pre-monsoon season, the values varied from 1.1 (station 9) to 5.7 (station 5) with mean value of 4.5. All stations except station 9 showed richness ≥ 4.0 and stations 3,5,6,7 and 8 had values ≥ 5.0. Station 10 had comparatively less index (4.0). In monsoon, the range was 1.6 (station 9) to 5.5 (station 8) with an average of 4.4 and in post-monsoon season the values ranged between 1.0 (station 9) and 5.3 (station 3) with a mean value of 3.9. The result obtained in these seasons was in the same pattern as that of pre-monsoon season.

3.3.2.2 Species evenness

Pielou's evenness index (J') for samples from all the ten stations in various seasons was determined (Fig. 3.6). It was found that in pre-monsoon season, the J' values varied from 0.9 (station 6) to 0.96 (station 9) with an average of 0.91. In monsoon, the index varied from 0.91 (station 10) to 0.961 (station 4) with a mean value of 0.94 and in post-monsoon season, the values ranged between 0.91 (station 7) and 0.97 (station 3) with an average of 0.937.

3.3.2.3 Species diversity

Shannon index or diversity index (H) in pre-monsoon season, varied from 2.68 (station 9) to 4.91 (station 5) with an average of 4.52 (Fig.3.7). All the stations except station 9 showed H value greater than 4.5. In monsoon, the index varied from 3.1 (station 9) to 4.9 (station 7) with an average of 4.5. In post-monsoon season, the values ranged between 2.6 (station 9) and 4.9 (station 3) with an average of 4.3 and all the stations except station 9 showed H' value ≥ 4.0. Station 10 has shown comparatively less diversity index (3.9).

3.3.2.4 Species dominance

Simpson's index or species dominance (λ') was determined for various seasons (Fig 3.8). In pre-monsoon season, the values varied from 0.04 (station 6) to 0.16 (station 9). The mean index value was 0.06. Station 9 recorded very high λ value whereas, all other stations showed less than 0.06. In monsoon, it varied from 0.04 (station 1) to 0.13 (station 9) with a mean value of 0.06 (station 10). All other stations except station 9 showed very low λ' value, less than 0.06. In post-monsoon season, the values ranged between 0.04 (station 3) and 0.17 (station 9) with an average of 0.07.

3.3.2.5 Similarity index and cluster analysis

The results of the comparison of the community at the species level using cluster analysis is based upon the Bray – Curtis index of similarity and group dendrogram which are given in Fig 3.9 and Table 3.4 (Appendix 1).

Community structure for various seasons

Fig. 3.5 Richness index

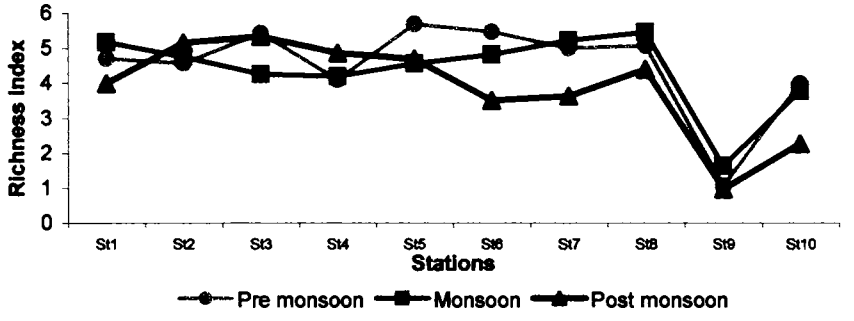


Fig. 3.6 Evenness index

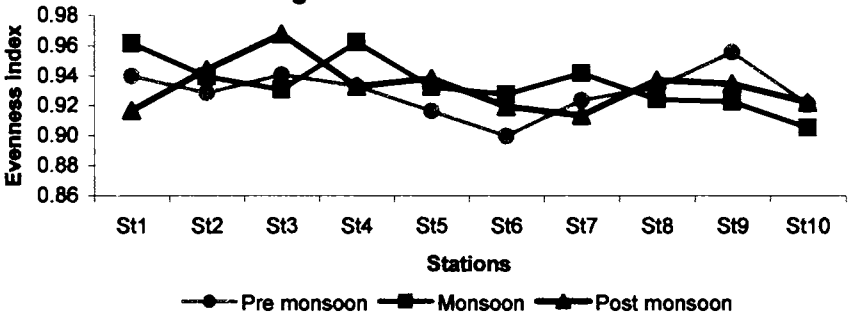


Fig 3.7 Diversity index

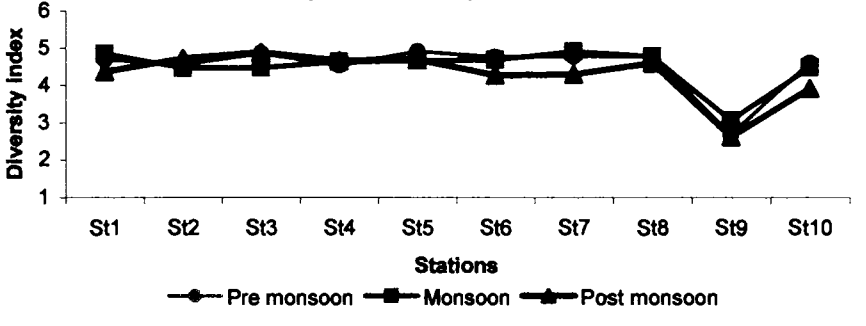


Fig 3.8 Dominance index

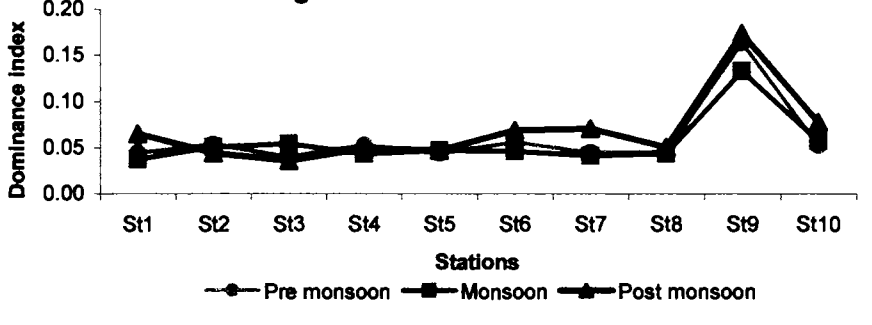
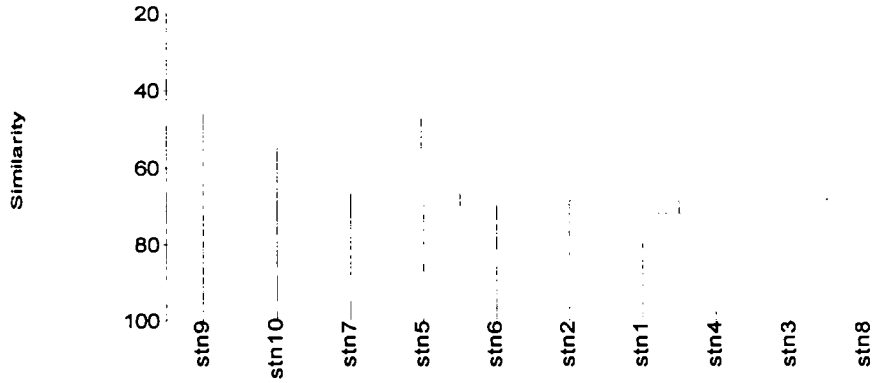
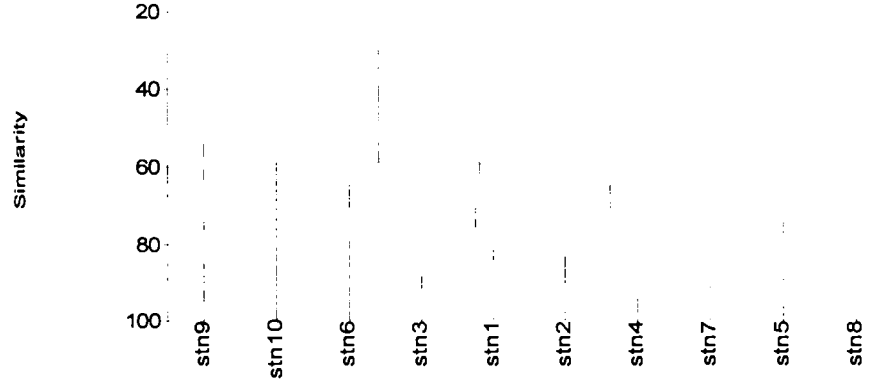


Fig 3.9 Dendrogram for hierarchial clustering with respect to cyanobacteria

Premonsoon season



Monsoon season



Postmonsoon season

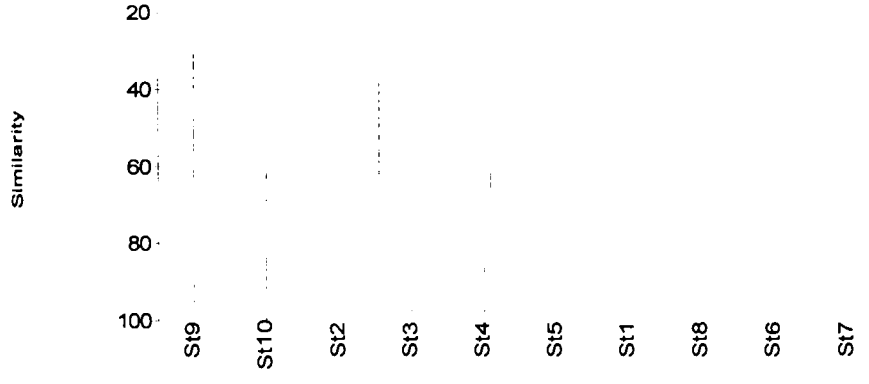
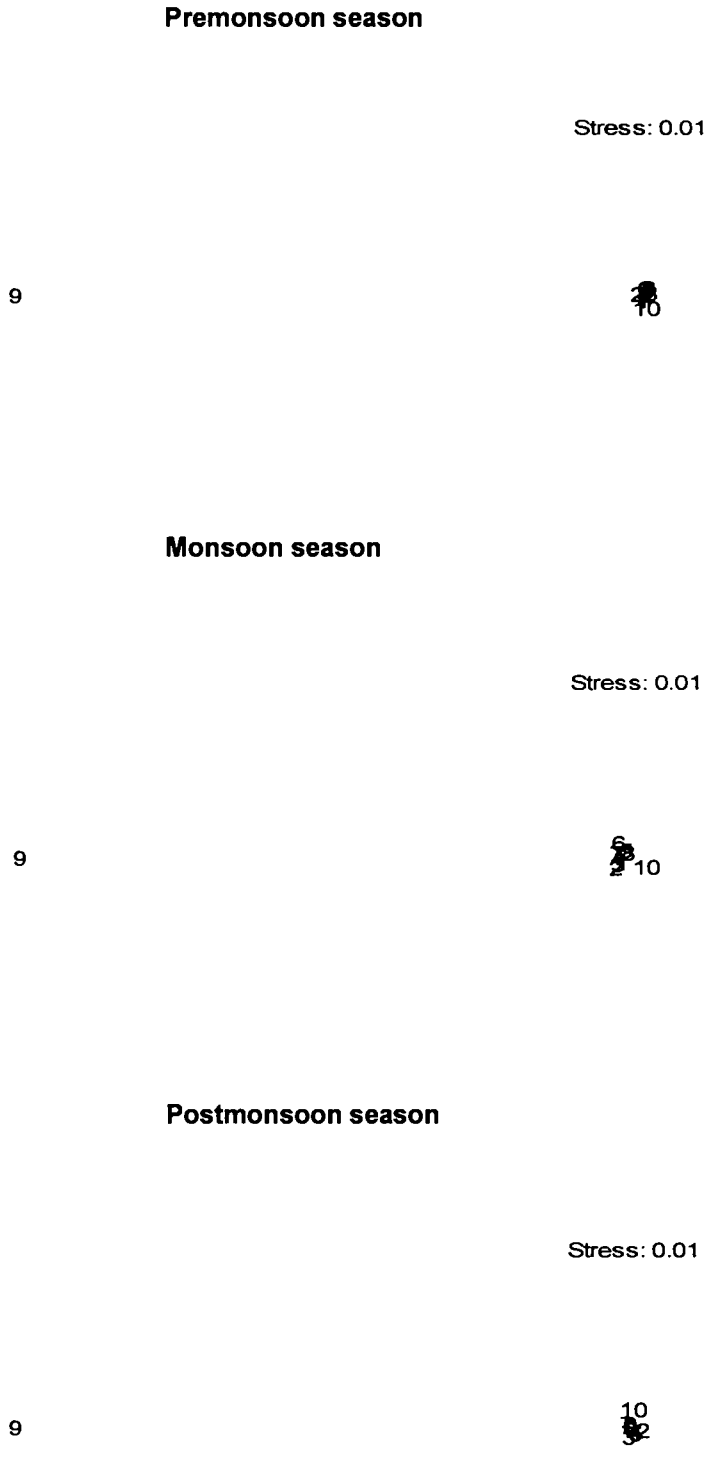


Fig 3.10 MDS ordination of the community in various seasons



In pre-monsoon season, similarity index ranged between 27.2 (between station 3 & 9) and 71.71 (between station 1 & 4). Station 9 exhibited very less similarity (< 37%) with all the other estuarine sites. Similar results were observed in monsoon and post-monsoon season also. The maximum similarity in monsoon season was between stations 4 & 7 (76.61%) whereas, in post-monsoon season, it was between station 6 & 7 (83.42 %).

From the dendrogram (Fig 3.9) it is clear that all the stations, except station 9 and 10, could be grouped together. Station 10 was slightly dissimilar to the major group. But station 9 was distinctly apart from all other stations. The result was same for all the seasons.

3.3.2.6 Multidimensional scaling (MDS)

In the present study, the ordinations depicted from similarity matrix revealed the same patterns as seen in the cluster analysis (Fig 3.10). Station 1,2.3,4,5,6,7,8 and 10 were similar as they were closely placed in the plot even though station 10 was slightly distant from others. Station 9 was significantly isolated from all other stations

3.4 Discussion

3.4.1 Distribution pattern

3.4.1.1 Spatial variation

The spatial distribution of cyanobacteria showed that mangrove environment (station 10) supported abundant quantity of cyanobacteria where the total cell count ranged between 1163 and 2080 cells/ml with an average count of 1630 cells/ml. The probable reasons that could be attributed to this are the organic rich muddy substratum, relatively stagnant shallow water conditions, sheltered nature (hence reduced water movement), high phosphate content and optimum saline conditions (15–30ppt) (Ramachandran, 1982). All these factors ensure a good growth of these forms

here. Mangrove station chosen for the study was characterized by high content of phosphate due to domestic sewage disposal and application of fertilizer in the nearby cultivation farms, which would also contribute to the high cell number. Previous workers also have reported the dominance of cyanobacteria in other mangrove regions (Ramachandran, 1982; Thajuddin and Subramanian, 1992). The members of the family Oscillatoriaceae were the dominant flora in these habitats (Thajuddin and Subramanian, 1992). Wood (1965) attributed the abundance of cyanobacteria in mangrove areas to the presence of anaerobic mud. He also showed rapid growth of marine cyanobacteria in media with low redox potentials. The decomposition of organic matter in the mangrove environment promotes bacterial production of hydrogen sulfide, resulting in a reducing environment. Stewart and Pearson (1970) showed that trace amount of sulfide and the associated reducing conditions were favourable for growth and nitrogen fixing activity of cyanobacteria.

Total cell count was very low in the coastal area, ranged between 81 cells/ml in June'02 and 315 cells/ml in May'03 with an average count of 196 cells/ml. This may be because of sandy shore, rough waves, and scarcity of nutrients in the water. In a similar study, Thajuddin and Subramanian (1992) reported the distribution of cyanobacterial flora in different shores in the southern east coast of India such as the shores of the Bay of Bengal region, those of the Palk Strait region, Gulf of Mannar region, Mandapam, Kurusadai Island and Cape Comorin. This study also revealed that in the case of sandy shores, the cyanobacterial population was very poor due to the rough tides, absence of substratum, low nutrient content of water etc.

As per the present investigation, average cyanobacterial counts in other backwater stations ranged between 296 cells/ml (station-2) to 825 cells/ml (station-7). Backwater and mangrove ecosystems showed high population of cyanobacteria when compared to seashore station, probably because, these habitats remain undisturbed for relatively long periods,

particularly these systems are protected from severe wave action. The saline areas such as stations 5, 6 and 7 were having elevated cell count, which shows that cell count was more in saline waters when compared to fresh water regions.

3.4.1.2 Seasonal variation

The average cyanobacterial concentration for pre-monsoon, monsoon, and post-monsoon seasons in the estuary were 732, 523 and 583 cells/ml respectively. Cell count was maximum during pre-monsoon season. The reduction of cell count during monsoon season might be due to the dilution of water body with land runoff.

3.4.1.3 Vertical variation

In general, cyanobacteria were more in surface water, where there is enough sunlight for photosynthesis. Light is the source of energy, which enables photosynthesis. The quantity and quality of the ambient light has a great influence on the growth, distribution and productivity of cyanobacteria. Slightly elevated levels of cyanobacteria, observed in the bottom of shallow regions such as stations 3, 7 & 8 of average depth of 1.4m to 2.1m might be due to the sufficient light availability at the bottom. The dominance of filamentous cyanobacteria in the surface waters could be due to the phototactic movement exhibited by them since the filamentous forms have a tendency to accumulate at the surface level (Desikachary, 1959; Walsby, 1972). Out of 24 genera obtained, *Oscillatoria, Synechocystis, Phormidium, Synechococcus* and *Gloeocapsa* were the dominant flora in the estuary.

3.4.2 Community structure

When community structure based on cyanobacteria was taken in to consideration using PRIMER 5 analysis, not much seasonal variation in diversity index could be observed. But, spatial variation was prominent at station 9, which is located at the seashore site and was significantly different

from all the other sampling sites. Eventhough the total cell count was very high in station 10 (mangrove station), the diversity indices were almost similar to that of other sites except station 9. This is because of the fact that index value is not only based on the total number of cells obtained, but also the number of species observed in that site. The number of species present in station 10 was less, but the number of individuals in each species was of high value. That is why the richness/Margalef's index, which explains the total number of species, was comparatively low at this station. High species richness and species diversity of cyanobacteria were observed at station 5, whereas, low species richness and diversity were noticed at station 9. Vallet and Dauvin (1998) attributed the high species richness and species diversity to the more stable physical condition. In both cluster analysis and multidimensional scaling maps, all the stations except station 9 were clustered together. So it was confirmed that station 9, which is the seashore area, was significantly dissimilar to other backwater sites with respect to cyanobacterial community.

When direct counting methods were used, resuspended sediment generally obscured the few cyanobacterium present, as the estuarine system studied was shallow and wind-stirred. Therefore, the order of magnitude of the real number of species present is uncertain. Moreover, the morphological variation due to the variant environmental conditions and distortion of cell shapes by adding preservatives lead to difficulty and errors in cyanobacterial identification (Murayama-kayano *et al.*, 1998). Hence it is suggested to employ modified or advanced techniques for the quantitative analysis of cyanobacteria.

CHAPTER 4

Hydrography of the study area

4.1 Introduction

In natural phytoplankton populations, cyanobacteria are well expected to make a significant contribution to photosynthesis, i.e. biological productivity (Morris *et al.*, 1981), which, in turn, is dependent on complex physico-chemical and biological processes (Gopinathan *et al.*, 2001). Investigations on phytoplankton, the prime synthesizers of all organic matter, have assumed great significance the world over as their occurrence and abundance fluctuate in relation to definite environmental factors and as they serve as an important and convenient basis for assessing the stock of other organisms.

Considerable information is available on the physico-chemical and biological characteristics of the Arabian Sea along the west coast of India (Ramamirtham and Jayaraman, 1963; Patil and Ramamirtham, 1963; Sharma, 1966; Radhakrishna, 1969; Qasim *et al.*, 1972b; Qasim, 1982; Bhattathiri and Devassy, 1979; Pant, 1992; Pillai, 1983). Krey and Babernad (1976) and Sumitra and Krishnakumari (1989) have given a general picture of the distribution of chlorophylls along the west coast. Balachandran *et al.* (1989) studied the distribution and profile of chlorophyll-a in the inshore waters of Cochin and Laccadive Sea during the monsoon season.

Several physico-chemical and biological investigations have been carried out in various estuaries of India. Roy (1949, 1955) made a preliminary study on certain aspects of the physico-chemical characteristics of the Hooghly estuary, West Bengal. Physico-chemical and biological parameters of the Chilka Lake, Orissa, were investigated by Devasundaram and Roy (1954), Ramanandham *et al.* (1964), Banerjee and Choudhury (1966), Dehadrai and Bhargava (1972), Parulekar and Dwivedi (1972), Sigbal (1973), Mohanty (1975) and Varma *et al.* (1975). Seasonal cycle of nutrients in Goa estuary was studied by De Sousa *et al.* (1981). De Sousa and Gupta (1986) conducted studies on the various hydrographic and biological parameters of Zuari estuary, Goa and concluded that area at the freshwater end showed relatively higher oxygen concentration than the areas nearer to the sea.

Purushothaman and Venugopalan (1972), Vijayalakshmi and Venugopalan (1973), Krishnamurthy and Sundararaj (1973) and Mohan (2000) investigated different aspects of the Vellar estuarine ecology. Joseph (1982) observed that in Vellar estuary temporal variation of biota is largely dependent on the salinity and to a lesser extent on the ambient water temperature.

Sastry and Chandramohan (1990) studied the physico-chemical features of Vasishta-Godavari estuary and Gouda and Panigrahy (1992) investigated the seasonal distribution and behaviour of silicate in the Rushikulya estuary. Daniels and Boyd (1993) studied nitrogen, phosphorus and silicate replenishment in backwater and other adjoining water bodies. Physico-chemical features of Bahuda estuary were investigated by Mishra *et al.* (1993). Rao and Sharma (1995) described the temperature and salinity structure of Gosthani estuary. Prabhadevi *et al.* (1996) related the water quality with the benthic fauna of the Kayamkulam backwaters. Das *et al.* (1997) studied sediment variation and some physico-chemical parameters in the Mahanadi estuary. Padma and Periakali (1999) and Padmavathi and Satyanarayana (1999) made an investigation on physico-chemical factors in Pulicat and Godavari estuary respectively.

A detailed study of different aspects of hydrography of Cochin estuary was carried out by Ramamirtham and Jayaraman (1963), Cheriyan (1967), Qasim and Gopinathan (1969), Sankaranarayanan and Qasim (1969), Wellershaus (1971), Josanto (1971), Balakrishnan and Shynamma(1976) and Nair *et al.* (1993). Variation in salinity and temperature in Cochin estuary was investigated by Balakrishnan (1957), George (1958), George and Kartha(1963), Haridas *et al.* (1973), Joseph and Kurup (1990) and George *et al.* (1996). Lakshmanan *et al.* (1982) studied the hydrological conditions of Cochin backwaters, which are greatly influenced by seawater intrusion and influx of river water. Sediment characteristics and its distribution in Cochin estuary was described by Gopalan *et al.* (1983) and Saraladevi (1989), whereas, nutrient distribution was investigated by Murthy and Veerayya

(1972), Manikoth and Salih (1974), Lakshmanan *et al.* (1987) and Anirudhan and Nambisan (1990). Distribution and seasonal variation of phytoplankton was studied by Gopinathan (1972), Joseph and Pillai (1975) and Kumaran and Rao (1975). Arun (2002) compared the hydrography of the stations on either side of Thannermukkom bund in the estuary. Primary production and fishery potential of the Panangad region in the Cochin estuarine system was investigated by Renjith *et al.* (2004).

Studies on phytoplankton conducted on the Indian coasts have usually been limited to their systematics and/or seasonal occurrence (Menon, 1945; Subramanian 1946; Chacko, 1950; Prasad, 1954; Prasad and Ramachandran, 1963; Desikachary and Prema, 1987; Desikachary *et al.*, 1987). In a few studies, phytoplankton productivity has been correlated with the prevailing hydrological conditions (Ramamurthy, 1953; Rajagopal, 1981; Varshney *et al.*, 1983; Rao and Valsaraj 1984; Verlencar, 1984; Segar and Hariharan, 1989 and Valsaraj and Rao, 1994). Gopinathan *et al.* (2001) studied the distribution of chlorophyll 'a' and 'b' in the eastern Arabian Sea (west coast of India) in relation to nutrients during post-monsoon. Studies on the interrelationships among phytoplankton and the general factors governing their distribution are scarce with almost no report on specific factors controlling phytoplankton species succession or distribution in the Indian waters.

As nutrient load increases, species that can utilize increased nutrients efficiently take advantage and multiply very rapidly at the expense of the less efficient species, which eventually are reduced numerically (Valsaraj and Rao, 1999). The ability of cyanobacteria to grow in seawater was presumably related to a preference for alkaline conditions (Fogg, 1973). Petrov (1974) observed that the most significant factors, which affect the vertical and horizontal distribution of benthic forms, were euphotic depth, movement of water, temperature, chemical composition and grazing. Sanders (1979)

observed that salinity differences play a major role in deciding the algal assemblages.

Consistent monitoring of the environment is essential to determine the quality of water. For the last decade, there have been no detailed investigations on the physico-chemical characteristics of a continuous stretch of Cochin estuary and hence detailed studies are called for to fill up the gap in our knowledge on these aspects. Moreover, no detailed study showing the cyanobacterial distribution and abundance in Cochin estuary, with respect to physico-chemical parameters, has been carried out so far. Hence, an attempt has been made to comprehend the environmental factors that control cyanobacterial abundance and species succession in Cochin estuary.

4.2 Materials and Methods

Surface and bottom water samples were collected from ten stations of Cochin estuary for two years, beginning from April 2001, and analyzed for temperature, salinity, pH, dissolved oxygen, euphotic depth, NO_3-N, NO_2-N, PO_4-P, SiO_3-Si and phytoplankton productivity in terms of chlorophyll-a. Details of parameters analysed and the methodologies followed in the analyses are given below (Table 4.1).

Statistical analysis

To study the relationship between parameters, Matrix of Correlation was constructed for each station. Significant positive correlation implies co-existence of the two parameters and significant negative correlation implies lack of co-existence of the two parameters.

In order to determine significant difference, if any, in physico-chemical parameters between seasons and between stations, the results were analysed using Two-factor ANOVA by Duncan's multiple comparison of the means using SPSS (Statistical Package for Social Sciences) 10.0 for windows. Significant differences were indicated at $p< 0.05$.

Table 4.1 Methodology used for estimating physico-chemical parameters

Physico-chemical parameters	Methodology followed
Temperature	Standard mercury thermometer
Salinity	Salinometer
pH	pH meter
Dissolved Oxygen	Winkler method, Strickland and Parsons (1972)
Euphotic depth	Secchi disc
Productivity (Chlorophyll -a)	APHA 1998
Nitrate	Grasshoff *et al.* (1983)
Nitrite	Grasshoff *et al.* (1983)
Phosphate	Grasshoff *et al.* (1983)
Silicate	Grasshoff *et al.* (1983)

4.3 Results

4.3.1 Temperature

Seasonal variation in temperature for both surface and bottom samples is shown in Figs 4.1a, 4.1b and Table 4.2a and monthly variation is shown in Tables 4.3a and 4.3b of Appendix 2. Temperature showed highly significant variation between seasons, whereas, between sites, the variation was not significant. No major difference was observed between the surface and bottom temperature. The temperature found in various study areas was in the range of 24^0C (Station 10 in December) to 34^0C (Station 7 and 8 in April'03 and station 3 and 10 in March'04). Pre-monsoon showed maximum temperature at all the stations and monsoon showed minimum, except at station 10, where the minimum temperature was observed during the post-monsoon.

Temperature showed significant negative correlation ($p<0.05$) with nutrients and positive correlation with chlorophyll-a, dissolved oxygen, salinity and pH at some stations (Table 4.13, Appendix 2).

Fig 4.1a Seasonal variation in temperature at various stations – Surface water

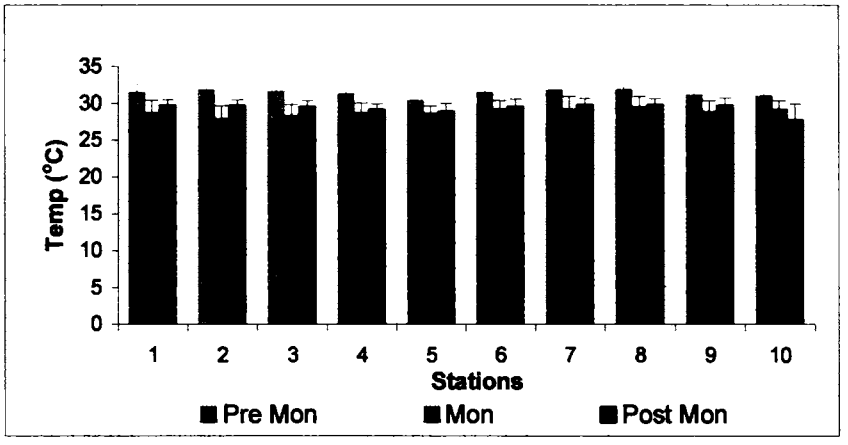


Fig 4.1b Seasonal variation in temperature at various stations – Bottom water

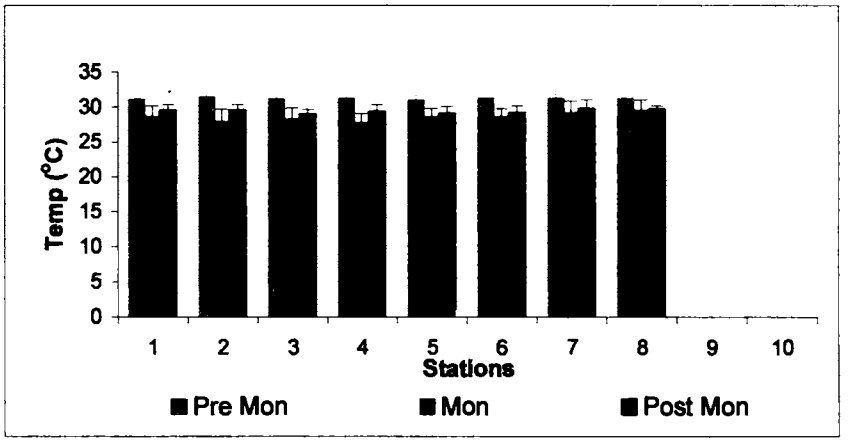


Table 4.2a Temperature
Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	31.42±1.11^{C}	31.75± 0.88^{C}	31.58± 1.43^{C}	31.25± 1.33^{C}	30.33± 1.03^{C}	31.42± 1.50^{C}	31.75± 1.54^{C}	31.83± 1.47^{C}	31.08± 0.92^{C}	31.0± 2.10^{C}
Mon	28.67±1.75^{A}	27.83± 1.83^{A}	28.25± 1.60^{A}	28.67± 1.37^{A}	28.58± 1.02^{A}	29.17± 1.17^{A}	29.17± 1.72^{A}	29.50± 1.38^{A}	28.83± 1.47^{A}	29.17± 1.17^{A}
Post-mon	29.67±0.82^{B}	29.67± 0.82^{B}	29.50± 0.84^{B}	29.08± 0.80^{B}	28.83± 1.17^{B}	29.50± 1.05^{B}	29.75± 0.88^{B}	29.75± 0.88^{B}	29.67± 1.03^{B}	27.67± 2.25^{B}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	31.0± 1.10^{C}	31.42±1.02^{C}	31.08±1.28^{C}	31.17± 1.17^{C}	30.92± 0.80^{C}	31.17± 0.75^{C}	31.17± 0.68^{C}	31.17± 1.17^{C}
Mon	28.5±1.64^{A}	27.83±1.83^{A}	28.17±1.69^{A}	27.67± 1.37^{A}	28.5± 1.22^{A}	28.5 ±1.22^{A}	29.17± 1.72^{A}	29.5± 1.52^{A}
Post Mon	29.5±0.84^{B}	29.5± 0.84^{B}	29.0 ±0.63^{B}	29.33± 1.03^{B}	29.08 ±1.02^{B}	29.17± 0.98^{B}	29.75± 1.25^{B}	29.67± 0.52^{B}

Values with same superscripts do not vary significantly ($p<0.05$)

Two-Factor ANOVA and further comparisons by Duncan's analysis (Table 4. 2a) revealed that significant difference existed between different seasons, but not between stations. All the stations showed a similar pattern of seasonal changes.

4.3.2 Salinity

Seasonal variation in salinity for both surface and bottom samples is shown in Figs 4.2a, 4.2b and Table 4.2b and monthly variation is shown in Tables 4.4a and 4.4b of Appendix 2. The salinity of various study areas was in the range of 0 to 37 ppt. Salinity was very low, almost reaching to freshwater condition, at stations 1,2 and 3. All other stations were characterized by high salinity. Station 9, which was a coastal area, showed maximum salinity of 37 ppt (Dec'02 and July'03). Seasonal variation was observed at all the stations and monsoon showed the lowest average value. In pre-monsoon and post-monsoon season the average values of salinity were almost similar. However, higher salinity was observed in post-monsoon season except at stations 7, 8 and 9. Salinity was always high in bottom water than in surface water.

Salinity showed significant correlation ($p<0.05$) with other parameters at some stations. There was positive correlation with pH and productivity, whereas, negative correlation existed with salinity and nutrients (Table 4.13, Appendix 2).

Two-way analysis of variance and subsequent comparisons by Duncan's analysis (Table 4.2b) revealed that monsoon showed significantly low salinity, whereas, in pre-monsoon and post-monsoon almost same average values were obtained. Salinity significantly varied from station to station except between stations1 and 2, and stations 5 and 6. Station 7 did not show a significant difference in salinity from stations 4 and 8 eventhough they differed significantly from each other. There was a significant increase in salinity in the order, stations (1,2) < 3 < (4,7) < (7,8) < 10 < (5,6) < 9.

Fig 4.2a Seasonal variation in salinity at various stations – Surface water

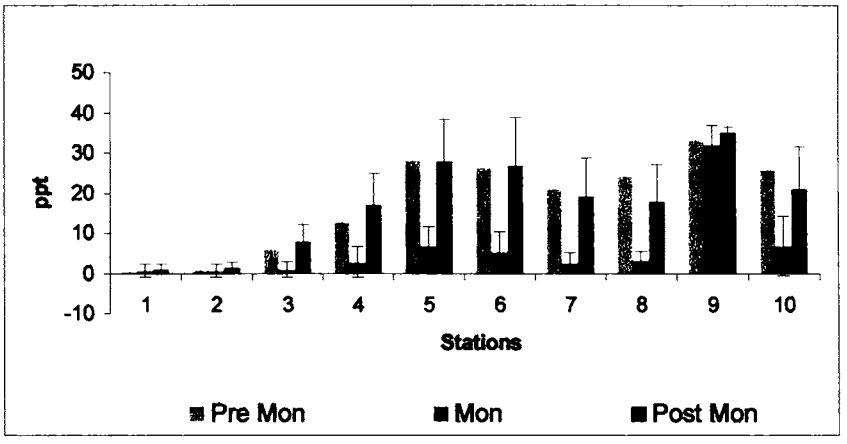


Fig 4.2b Seasonal variation in salinity at various stations –Bottom water

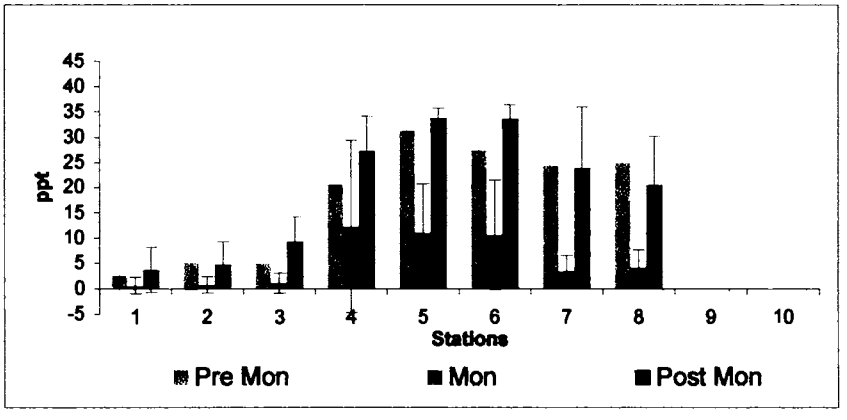


Table 4.2b Salinity

Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	0.33 ± 0.52^{Be}	0.83 ± 1.60^{Be}	6.0 ± 7.59^{Bb}	12.67 ± 7.61^{Bc}	28.0 ± 4.05^{Be}	26.17 ± 3.66^{Be}	21.0 ± 5.51^{Bcd}	24.17 ± 2.40^{Bd}	33.33 ± 2.66^{Bf}	25.83 ± 8.89^{Bde}
Mon	0.67 ± 1.63^{Aa}	0.67 ± 1.63^{Aa}	1.0 ± 2.00^{Ab}	2.83 ± 3.82^{Ac}	6.83 ± 4.83^{Ae}	5.33 ± 4.93^{Ae}	2.67 ± 2.58^{Acd}	3.17 ± 2.32^{Ad}	32.17 ± 4.71^{Af}	6.83 ± 7.41^{Ade}
Post-mon	1.0 ± 1.26^{Ba}	1.40 ± 1.34^{Ba}	7.83 ± 4.45^{Bb}	17.0 ± 7.85^{Bc}	27.83 ± 10.57^{Be}	26.83 ± 12.07^{Be}	19.2 ± 9.62^{Bcd}	17.83 ± 9.37^{Bd}	35.17 ± 1.47^{Bf}	21.0 ± 10.66^{Bde}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	2.67 ± 3.08^{Ba}	5.17 ± 5.34^{Ba}	5.00 ± 4.86^{Ba}	20.67 ± 9.61^{Bbc}	31.33 ± 3.14^{Bd}	27.50 ± 3.73^{Bcd}	24.50 ± 4.04^{Bb}	25.00 ± 2.37^{Bb}
Mon	0.67 ± 1.63^{Aa}	0.83 ± 1.60^{Aa}	1.17 ± 1.94^{Aa}	12.33 ± 17.00^{Abc}	11.17 ± 9.64^{Ad}	10.67 ± 10.80^{Acd}	3.50 ± 3.08^{Ab}	4.17 ± 3.49^{Ab}
Post Mon	3.67 ± 4.41^{Ba}	4.83 ± 4.49^{Ba}	9.33 ± 4.93^{Ba}	27.17 ± 7.03^{Bbc}	33.83 ± 1.83^{Bd}	33.67 ± 2.73^{Bcd}	23.83 ± 12.06^{Bb}	20.50 ± 9.71^{Bb}

Values with same superscripts do not vary significantly ($p<0.05$)

4.3.3 pH

Seasonal variation in pH for both surface and bottom samples is shown in Figs 4.3a, 4.3b and Table 4.2c and monthly variation is shown in Tables 4.5a and 4.5b of Appendix 2. The average pH values obtained from various study areas were in the range of 6.5 and 8.5.The freshwater areas (station 1,2 and 3) showed slightly acidic pH, whereas, saline environments (station 4,5,6,7, and 8) always recorded slightly alkaline pH. The seashore and mangrove area also showed alkaline pH. Distinct seasonal stratification was not evident in the case of pH. However, it has been observed that pre-monsoon season was characterized by maximum average pH with slight variation. Vertical variation of pH was not conspicuous. The maximum value of surface pH recorded during the period of study was 9.1 at station 7 in April'02 and the minimum was 6.22 at station 2 in Jan'04. While, for bottom, pH 8.9 was recorded as the maximum at station 7 and 8 in April'02 and 6.3 as the minimum in Sep'03 at station 2.

pH showed significant negative correlation ($p<0.05$) with nitrite, nitrate and silicate, whereas, positive correlation was observed with temperature, dissolved oxygen, salinity and phosphate at some stations (Table 4.13, Appendix 2).

Two-Factor ANOVA and further analysis (Table 4.2c) revealed that there was a significant increase in pH between monsoon and pre-monsoon as well as monsoon and post-monsoon season. Station 1,2 and 3 showed significantly lower ($p> 0.05$) pH compared to all the remaining stations. There was no significant difference between stations 5,6,7,8 and10. Station 9 gave significantly higher value compared to other stations.

Fig 4.3a Seasonal variation in pH at various stations –Surface water

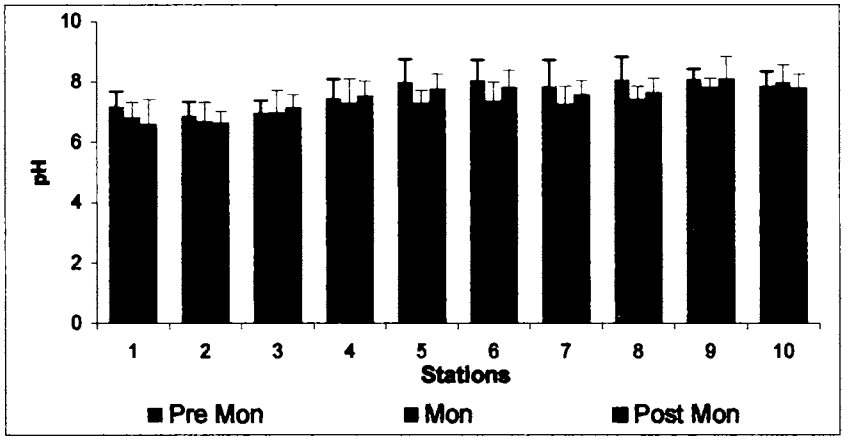


Fig 4.3b Seasonal variation in pH at various stations –Bottom water

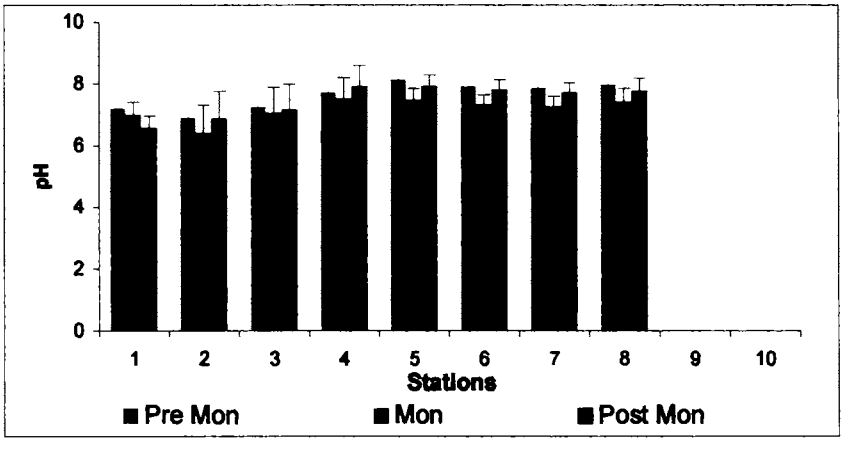


Table 4.2.c. pH
Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	7.20±0.48^{B}	6.90± 0.44^{B}	7.00±0.38^{B}	7.45±0.63^{B}	8.01±0.74^{B}	8.06±0.66^{B}	7.86±0.87^{B}	8.07±0.75^{B}	8.10±0.33^{B}	7.89±0.45^{B}
Mon	6.84±0.48^{A}	6.72±0.60^{A}	6.99±0.71^{A}	7.33±0.77^{A}	7.32±0.37^{A}	7.38±0.60^{A}	7.29±0.55^{A}	7.44±0.41^{A}	7.84±0.29^{A}	8.01±0.54^{A}
Post-mon	6.61±0.80^{AB}	6.66±0.36^{AB}	7.17±0.41^{AB}	7.54±0.49^{AB}	7.76±0.51^{AB}	7.83±0.56^{B}	7.58±0.46^{AB}	7.64±0.49^{AB}	8.09±0.74^{AB}	7.80±0.46^{AB}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	7.21±0.60$^{B\,ab}$	6.91± 0.38$^{B\,a}$	7.27 ±0.46$^{B\,b}$	7.73± 0.72$^{B\,c}$	8.15± 0.59$^{B\,c}$	7.92 ±0.69$^{B\,c}$	7.87± 0.69$^{B\,c}$	7.98 ±0.78$^{B\,c}$
Mon	7.02 ±0.38$^{A\,ab}$	6.43 ±0.88$^{A\,a}$	7.07 ±0.82$^{A\,b}$	7.55 ±0.66$^{A\,c}$	7.50 ±0.36$^{A\,c}$	7.34 ±0.31$^{A\,c}$	7.28 ±0.33$^{A\,c}$	7.44 ±0.40$^{A\,c}$
Post Mon	6.56 ± 0.36B^{ab}	6.67± 0.11$^{B\,a}$	7.17 ±0.42$^{B\,b}$	7.94± 0.40$^{B\,c}$	7.93± 0.24$^{B\,c}$	7.82± 0.37$^{B\,c}$	7.71± 0.57$^{B\,c}$	7.78± 0.55$^{B\,c}$

Values with same superscripts do not vary significantly ($p<0.05$)

4.3.4 Dissolved oxygen

Seasonal variation in dissolved oxygen for both surface and bottom samples is shown in Figs 4.4a, 4.4b and Table 4.2d and monthly variation is shown in Tables 4.6a and 4.6b of Appendix 2. Dissolved oxygen always showed a lower value (average -2.5ml/L) at station-1 (Eloor), which is an industrial belt. The maximum value was recorded in April'02 (6.714 ml/L) at station 7. There was not much variation in average dissolved oxygen at other stations, which ranged between 3.5 and 4.2 ml/L. It was found that the values varied from season to season. Maximum dissolved oxygen was observed during monsoon at stations 2,4, 6 and 7 while at stations 1, 5, 8 and 9 pre-monsoon season showed maximum value. It was high in bottom water than in surface water in relatively polluted stations like Eloor, Varapuzha and Vaduthala. But, in the case of other stations, dissolved oxygen values were either almost similar in surface and bottom water or higher in surface water.

Dissolved oxygen content showed significant negative correlation ($p<0.05$) with temperature, salinity, nitrite and chlorophyll-a, whereas, positive correlation existed with pH, nitrate and silicate at some stations (Table 4.13, Appendix 2).

Two-way ANOVA and subsequent analysis (Table 4.2d) showed that there was no significant difference in dissolved oxygen between seasons. Station 1 gave significantly least ($p< 0.05$) dissolved oxygen content compared to all the other stations.

4.3.5 Euphotic Depth

Seasonal variation in euphotic depth is shown in Figs 4.5 and Table 4.2e and monthly variation is shown in Tables 4.7 of Appendix 2. The depth of light penetration at different stations of Cochin estuary ranged between 0.2m at station 7 in August'02 to 1.85m at station 1 in April'02. Light penetration

Fig 4.4a Seasonal variation in dissolved oxygen at various stations –Surface water

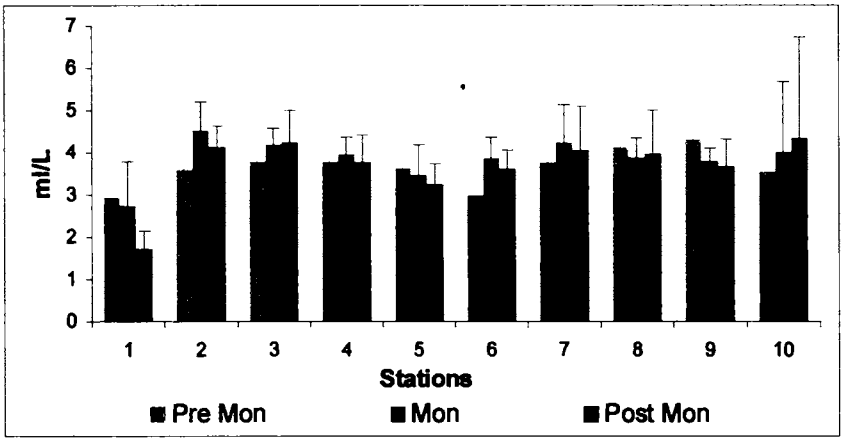


Fig 4.4b Seasonal variation in dissolved oxygen at various stations –bottom water

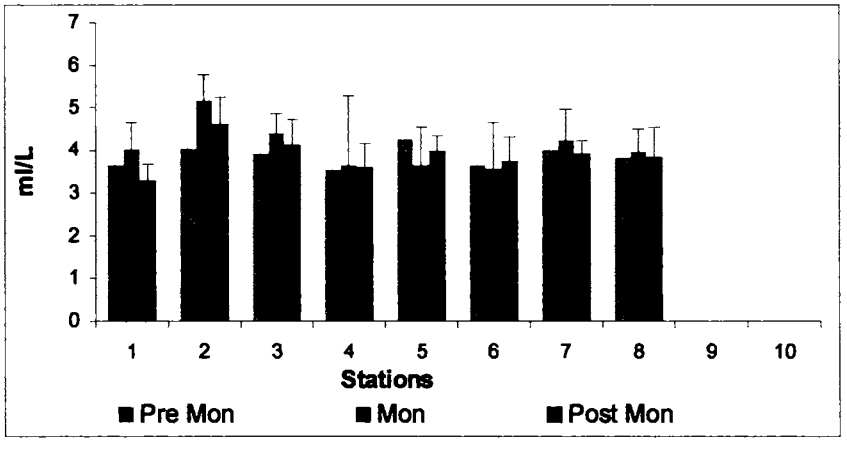


Table 4.2.d Dissolved oxygen

Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	2.93±0.69^{a}	3.6±0.48^{b}	3.79±0.70^{b}	3.79±0.51^{b}	3.63±0.74^{b}	2.98±0.48^{b}	3.77±1.65^{b}	4.13±1.42^{b}	4.32±1.16^{b}	3.54±1.86^{b}
Mon	2.74±1.04^{a}	4.53±0.67^{b}	4.20±0.38^{b}	3.97±0.40^{b}	3.48±0.71^{b}	3.87±0.48^{b}	4.25±0.89^{b}	3.89±0.46^{b}	3.81±0.29^{b}	4.01±1.66^{b}
Post-mon	1.71±0.44^{a}	4.13±0.51^{b}	4.24±0.76^{b}	3.78±0.64^{b}	3.25±0.49^{b}	3.61±0.44^{b}	4.06±1.05^{b}	3.97±1.03^{b}	3.68±0.64^{b}	4.34±2.39^{b}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	3.66±0.59^{ab}	4.04±0.42^{c}	3.94±0.32^{b}	3.55±0.39^{a}	4.27±0.52^{ab}	3.67±0.25^{ab}	4.02±0.90^{ab}	3.83±0.88^{ab}
Mon	4.02±0.63^{ab}	5.17±0.60^{c}	4.41±0.45^{b}	3.66±1.61^{a}	3.66±0.88^{ab}	3.58±1.07^{ab}	4.24±0.73^{ab}	3.98±0.52^{ab}
Post Mon	3.29±0.38^{ab}	4.62±0.63^{c}	4.12±0.60^{b}	3.6±0.55^{a}	3.99±0.35^{ab}	3.75±0.56^{ab}	3.93±0.30^{ab}	3.65±0.68^{ab}

Values with same superscripts do not vary significantly ($p<0.05$)

was maximum in pre-monsoon and minimum in monsoon season at all the stations.

Euphotic depth showed significant negative correlation ($p<0.05$) with nutrients and dissolved oxygen, and positive correlation with temperature, salinity and productivity at some stations (Table 4.13, Appendix 2).

Two-factor ANOVA and further analysis by Duncan's multiple comparison of the means (Table 4.2e) revealed that monsoon season showed significantly least euphotic depth. No significant difference between stations could be observed.

4.3.6 Chlorophyll-a

Seasonal variation in chlorophyll-a is shown in Figs 4.6 and Table 4.2f and monthly variation is shown in Tables 4.8 of Appendix 2. The quantity of photosynthetic pigment, chlorophyll-a in the estuary was determined. It ranged between 0.2 μg/L at station 2 in August'02 and 192 μg/L at station 10 in June '02. Station 10 was strikingly different from other stations as it showed very high chlorophyll content. Seasonal variation was not uniform at all the stations.

Chlorophyll-a showed significant negative correlation ($p<0.05$) with dissolved oxygen, nitrate and phosphate whereas, it showed positive correlation with temperature and salinity at some stations (Table 4.13, Appendix 2).

Two-way ANOVA and subsequent analysis (Table 4.2f) showed that there was no significant difference between seasons. Station 10 gave significantly higher ($p< 0.05$) productivity than the remaining stations.

Fig 4.5 Seasonal variation in euphotic depth at various stations

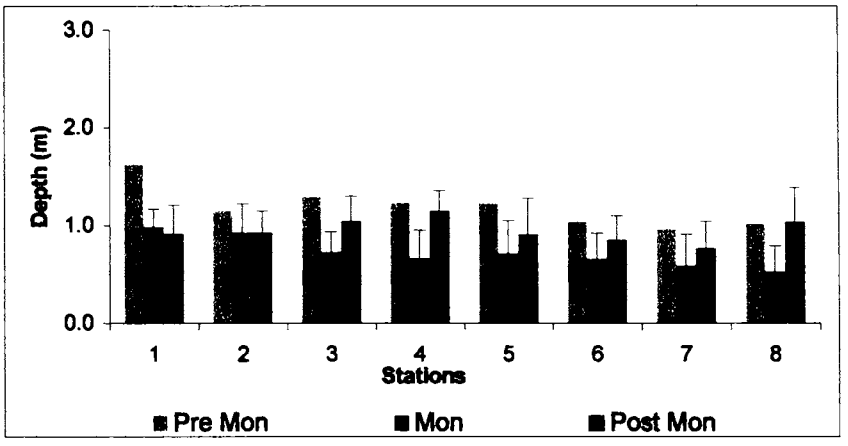


Table 4.2.e Euphotic depth

Stn	1	2	3	4	5	6	7	8
Pre Mon	1.62±^{B}0.96	1.16±0.92^{B}	1.30±1.13^{B}	1.24±1.00^{B}	1.23±0.85^{B}	1.04±0.87^{B}	0.96±1.19^{B}	1.02±0.99^{B}
Mon	0.99±0.16^{A}	0.93±0.29^{A}	0.73±0.21^{A}	0.67±0.26^{A}	0.71±0.34^{A}	0.66±0.26^{A}	0.6±0.31^{A}	0.53±0.26^{A}
Post Mon	0.91±0.30^{AB}	0.93±0.22^{AB}	1.04±0.26^{AB}	1.15±0.21^{AB}	0.91±0.37^{AB}	0.85±0.25^{AB}	0.77±0.28^{AB}	1.04±0.35^{AB}

Fig 4.6 Seasonal variation in chlorophyll-a at various stations

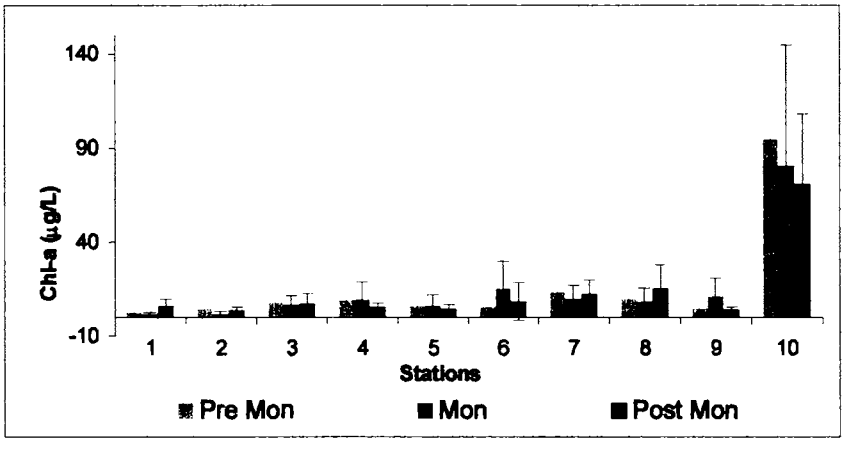


Table 4.2.f. chlorophyll a

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	2.67±1.83^{a}	4.41±4.75^{a}	7.86±6.00^{a}	9.43±5.97^{a}	5.95±3.48^{a}	5.31±3.37^{a}	13.39±8.55^{a}	9.88±4.47^{a}	4.81±3.05^{a}	94.73±56.80^{b}
Mon	1.81±0.83^{a}	1.76±1.30^{a}	7.11±4.39^{a}	9.66±9.28^{a}	6.26±5.55^{a}	15.27±14.41^{a}	9.94±7.09^{a}	8.61±6.90^{a}	11.21±9.64^{a}	80.98±63.57^{b}
Post-mon	6.17±3.50^{a}	3.85±1.47^{a}	7.47±5.43^{a}	5.34±2.26^{a}	4.42±2.15^{a}	8.36±10.17^{a}	12.52±7.27^{a}	15.46±12.32^{a}	4.14±1.33^{a}	70.9±37.02^{b}

Values with same superscripts do not vary significantly ($p<0.05$)

4.3.7 Nitrite

Seasonal variation in nitrite for both surface and bottom samples is shown in Figs 4.7a, 4.7b and Table 4.2g and monthly variation is shown in Tables 4.9a and 4.9b of Appendix 2. Nitrite originates in water by the reduction of nitrates and by the conversion of ammonia into nitrites. Nitrite concentration was maximum at station 7 in July'03 (2.08 μmol/L) and minimum at station 6 in Nov'03 (0.19 μmol/L). The nitrite content of the bottom samples varied between a minimum of 0.17 μmol/L at station 6 and 7 in Nov'03 and a maximum of 2.504 μmol/L at station 5 in June '02. The seasonal variation was erratic at all the stations. Slight increase in the value was noticed in bottom samples when compared to surface samples. However, the pattern of seasonal and spatial distribution was similar in both samples.

Concentration of nitrite showed significant negative correlation ($p<0.05$) with temperature, salinity and dissolved oxygen and positive correlation with chlorophyll-a and nutrients such as nitrate, phosphate and silicate at some stations (Table 4.13, Appendix 2).

Two-way ANOVA and subsequent analysis (Table 4.2g) showed that there was no significant difference in nitrite concentration between stations. Pre-monsoon season showed significantly higher value ($p< 0.05$) than other seasons.

4.3.8 Nitrate

Seasonal variation in nitrate for both surface and bottom samples is shown in Figs 4.8a, 4.8b and Table 4.2h and monthly variation is shown in Tables 4.10a and 4.10b of Appendix 2. Nitrate concentration varied from below detectable level to 79.5 μmol/L for surface samples. Maximum nitrate value was obtained from station 1 in all the seasons while saline regions such

Fig 4.7a Seasonal variation in Nitrite at various stations – surface water

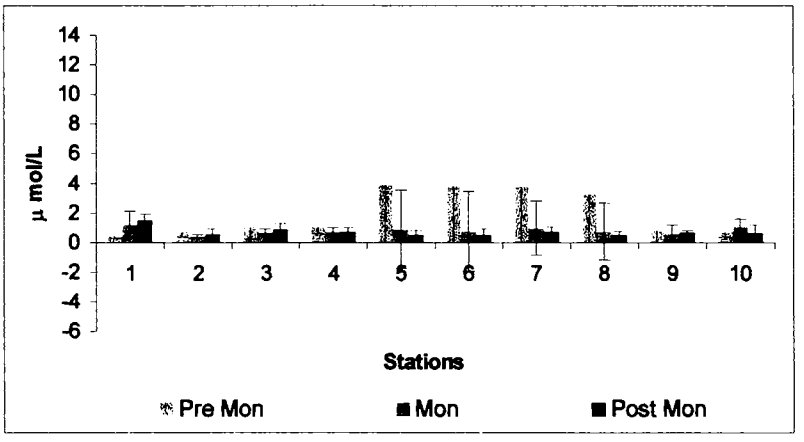


Fig 4.7b Seasonal variation in Nitrite at various stations – bottom water

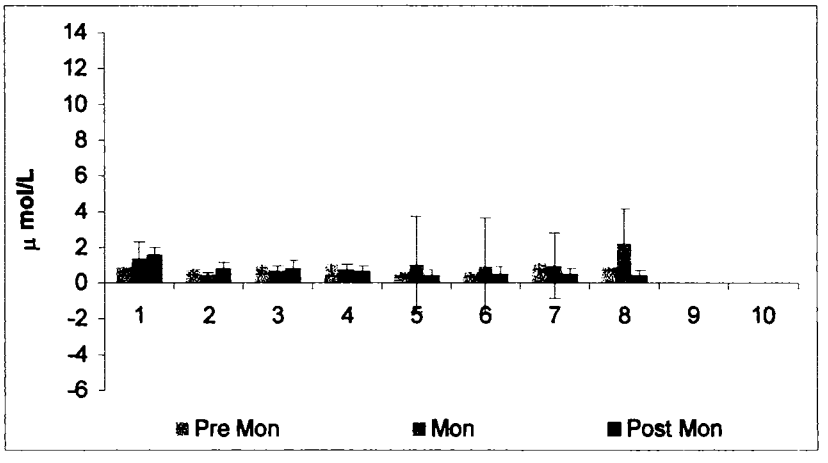


Table 4.2. g Nitrite

Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	0.46±0.19^{B}	0.71±0.34^{B}	1.03±0.79^{B}	1.05±0.45^{B}	0.76±7.82^{B}	0.56±7.87^{B}	1.85±5.25^{B}	1.08±5.74^{B}	0.85±0.85^{B}	0.73±0.36^{B}
Mon	1.2±0.90^{A}	0.41±0.10^{A}	0.69±0.22^{A}	0.73±0.29^{A}	0.89±2.66^{A}	0.75±2.71^{A}	0.96±1.83^{A}	0.72±1.93^{A}	0.61±0.55^{A}	1.08±0.48^{A}
Post-mon	1.49±0.41^{A}	0.57±0.34^{A}	0.89±0.40^{A}	0.72±0.29^{A}	0.54±0.28^{A}	0.58±0.39^{A}	0.73±0.31^{A}	0.52±0.25^{A}	0.67±0.12^{A}	0.66±0.52^{A}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	0.92±0.51^{A}	0.81±0.35^{A}	1.05±0.53^{A}	1.13±0.72^{A}	0.64±0.32^{A}	0.64±0.31^{A}	1.15±0.38^{A}	0.91±0.19^{A}
Mon	1.38±1.00^{A}	0.47±0.08^{A}	0.72±0.30^{A}	0.76±0.31^{A}	1.05±0.85^{A}	0.94±0.66^{A}	0.97±1.11^{A}	2.23±3.77^{A}
Post Mon	1.58±0.55^{A}	0.80±0.34^{A}	0.84±0.40^{A}	0.67±0.32^{A}	0.45±0.30^{A}	0.52±0.46^{A}	0.51±0.40^{A}	0.45±0.24^{A}

Values with same superscripts do not vary significantly ($p<0.05$)

Fig 4.8a Seasonal variation in Nitrate at various stations – surface water

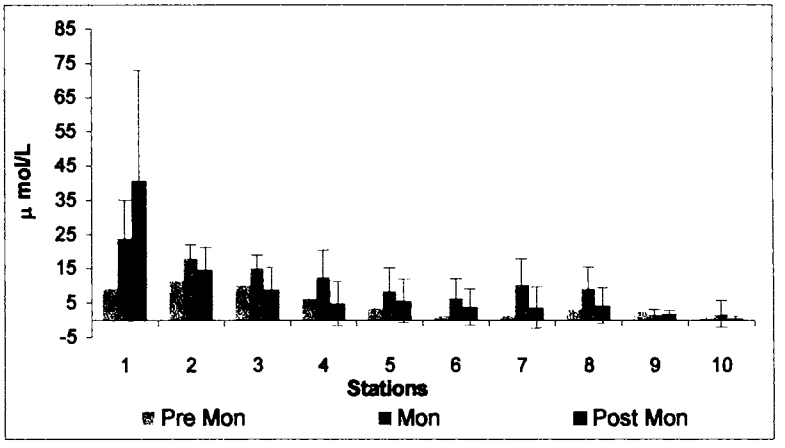


Fig 4.8b Seasonal variation in Nitrate at various stations – bottom water

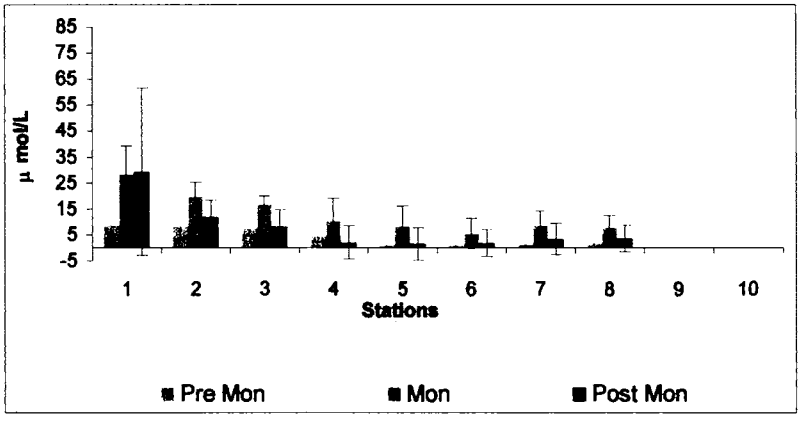


Table 4.2 h Nitrate

Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	9.35± 5.98^{Ae}	11.49±4.51^{Ad}	10.13±2.84^{Acd}	6.2±2.13^{Abc}	3.45±4.99^{Aab}	1.08±1.67^{Aab}	1.28±2.74^{Aab}	3.12±3.28^{Aab}	2.65±4.76^{Aab}	0.65±0.84^{Aa}
Mon	23.9±11.13^{Be}	18.1±3.84^{Bd}	15.3±3.60^{Bcd}	12.56±7.7^{Bbc}	8.6±6.63^{Bab}	6.44±5.57^{Bab}	10.3±7.55^{Bab}	9.29±6.23^{Bab}	1.7±1.48^{Bab}	1.93±3.88^{Ba}
Post-mon	40.6±32.18^{Be}	14.7±6.55^{Bd}	8.94±6.48^{Bcd}	4.79±6.42^{Bbc}	5.57±6.29^{Bab}	3.83±5.27^{Bab}	3.63±6.00^{Bab}	4.22±5.16^{Bab}	1.89±0.96^{Bab}	0.53±0.66^{Ba}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	8.64±4.15^{Ad}	8.17±4.86^{Ac}	7.6±3.90^{Abc}	4.43±3.30^{Aab}	1.01±1.99^{Aa}	0.98±1.63^{Aa}	1.22±2.88^{Aa}	1.81±2.11^{Aa}
Mon	28.4±10.92^{Bd}	19.9±5.50^{Bc}	16.7±3.31^{Bbc}	10.6±8.41^{Bab}	8.2±7.91^{Ba}	5.52±5.74^{Ba}	8.67±5.66^{Ba}	7.7±4.73^{Ba}
Post Mon	29.3±^{d}34.32	11.98±10.33^{Ac}	8.32±6.93^{Abc}	2.02±2.26^{Aab}	1.56±1.32^{Aa}	1.83±2.53^{Aa}	3.26±5.39^{Aa}	3.55±5.42^{Aa}

Values with same superscripts do not vary significantly ($p<0.05$)

as stations 5,6,7,9 and 10 demonstrated below detectable level at least once during the period of study. In the case of bottom water samples, the distribution of nitrate was similar to that of surface water. Season-wise studies have shown that monsoon showed maximum value except in station 1 where highest nitrate content was observed in post-monsoon. Minimum value was recorded in pre-monsoon season at all the stations throughout the period of study. A general trend that has been observed in all the seasons was a gradual decrease in the value of nitrate content from station 1, which is at the northern part of the estuary, towards the south. Station 10 showed lowest seasonal average for nitrate.

Nitrate level showed significant negative correlation ($p<0.05$) with temperature, salinity, pH and chlorophyll-a, whereas, positive correlation existed with dissolved oxygen and other nutrients at some stations (Table 4.13, Appendix 2).

Two-factor ANOVA and further comparisons by Duncan's multiple analyses of the means (Table 4.2h) revealed that pre-monsoon season showed significantly least ($p< 0.05$) nitrate content. Station 10 gave significantly lower ($p< 0.05$) value than the remaining stations whereas, station 1 gave significantly higher value compared to other stations.

4.3.9 Phosphate

Seasonal variation in phosphate for both surface and bottom samples is shown in Figs 4.9a, 4.9b and Table 4.2i and monthly variation is shown in Tables 4.11a and 4.11b of Appendix 2. Inorganic phosphate content in surface waters varied from the minimum of 0.06 μmol/L at station 5 in January and March'04 to a maximum of 37.1 μmol/L at station 10 in July'02 while in bottom samples the variation was from 0.053 μmol/L at station 2 in July'02 to 16.31 μmol/L at station 1 in June'02. The highest concentration of phosphate

Fig 4.9a Seasonal variation in phosphate at various stations – surface water

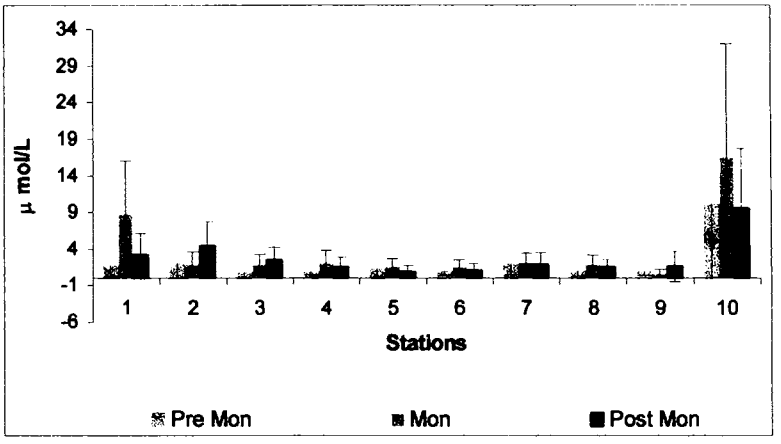


Fig 4.9b Seasonal variation in phosphate at various stations – bottom water

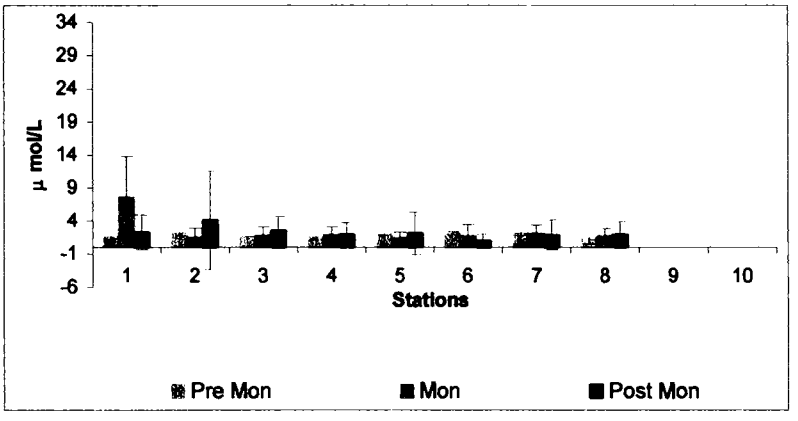


Table 4. 2. i Phosphate
Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	1.58±1.29^{b}	1.95±2.88^{ab}	0.67±0.69^{ab}	0.87±0.35^{ab}	1.16±0.96^{a}	0.98±0.51^{a}	2.35±1.42^{ab}	0.99±0.55^{ab}	0.98±0.50^{a}	10.69±11.14^{c}
Mon	8.73±7.28^{b}	1.83±1.72^{ab}	1.86±1.38^{ab}	2.10±1.69^{ab}	1.56±1.08^{a}	1.53±0.96^{a}	2.19±1.23^{ab}	1.83±1.29^{ab}	0.61±0.54^{a}	16.54±15.50^{c}
Post-mon	3.167±2.99^{b}	4.40±3.25^{ab}	2.47±1.78^{ab}	1.48±1.36^{ab}	0.85±0.90^{a}	1.05±0.95^{a}	1.87±1.64^{ab}	1.49±1.10^{ab}	1.59±2.07^{a}	9.52±8.15^{c}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	1.52±1.16^{A}	2.18±2.96^{A}	1.65±2.33^{A}	1.55±1.48^{A}	1.97±2.59^{A}	2.40±3.43^{A}	2.10±1.66^{A}	1.37±0.91^{A}
Mon	7.58±6.20^{A}	1.60±1.38^{A}	1.82±1.28^{A}	1.93±1.22^{A}	1.50±0.81^{A}	1.78±1.68^{A}	2.10±1.27^{A}	1.70±1.10^{A}
Post Mon	2.29±2.66^{A}	4.10±7.50^{A}	2.60±2.04^{A}	1.92±1.81^{A}	2.12±3.18^{A}	0.98±1.06^{A}	1.88±2.23^{A}	1.91±1.93^{A}

Values with same superscripts do not vary significantly ($p<0.05$)

was obtained from mangrove region (Station-10) during April'02 to August'02, at which the range was between 14.5 and 37.1μmol/L. However, when other backwater stations were taken into consideration, for all the seasons, the highest values of phosphate were recorded at station 1 which is at the northern part of the estuary and the value gradually decreased towards south. Seasonal variation in phosphate concentration at different stations did not show any distinct pattern. At some stations, monsoon showed maximum value while in others, pre-monsoon was characterised by high values. The pattern of seasonal and spatial distribution was similar in both surface and bottom samples.

Phosphate showed significant negative correlation ($p<0.05$) with temperature, salinity and chlorophyll-a, whereas, positive correlation existed with pH and other nutrients at some stations (Table 4.13, Appendix 2).

Two-factor ANOVA and further comparisons by Duncan's multiple analyses of the means (Table 4.2i) revealed that there was no significant difference ($p< 0.05$) between seasons. Station 5,6 and 9 didn't vary significantly with respect to phosphate content but showed significantly least ($p< 0.05$) value from the rest. Station 10 gave significantly higher value ($p< 0.05$) compared to other stations.

4.3.10 Silicate

Seasonal variation in silicate for both surface and bottom samples is shown in Figs 4.10a, 4.10b and Table 4.2j and monthly variation is shown in Tables 4.12a and 4.12b of Appendix 2. Silicate content in surface water varied from 0.775 μmol/L at station 9 in Sep'02 to 110 μmol/L at station 3 in May'02, whereas, in bottom samples, the range was between 0.9 μmol/L at station 2 in Dec'02 to 117 μmol/L at station 8 in Aug'02. Station 9 showed least seasonal average. The values for bottom samples were slightly higher than that for

Fig 4.10a Seasonal variation in silicate at various stations – surface water

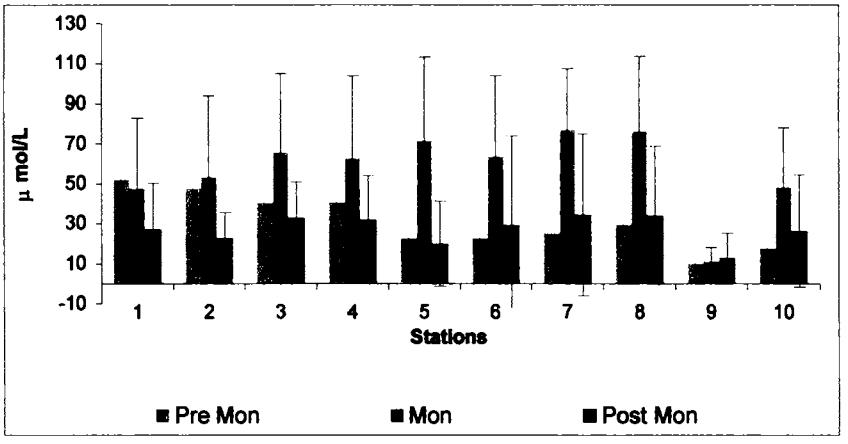


Fig 4.10b Seasonal variation in silicate at various stations – bottom water

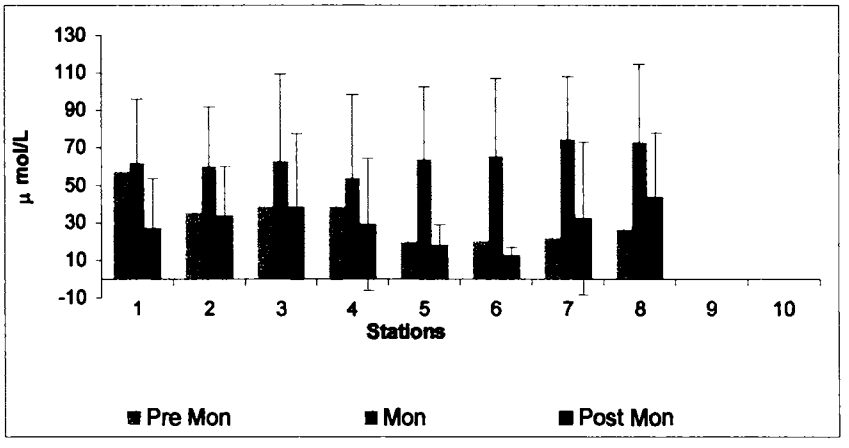


Table 4. 2 j Silicate

Surface water

Stn	1	2	3	4	5	6	7	8	9	10
Pre mon	52.04± 35.89^{Bb}	47.59± 37.67^{Bb}	40.42± 35.20^{Bb}	40.80 ±37.31^{Bb}	22.65± 13.56^{Bb}	22.59 ± 12.94^{Bb}	24.87± 12.89^{Bb}	29.44 ±13.32^{Bbb}	10.11± 9.11^{Bb}	17.85± 17.57^{Bb}
Mon	47.67± 35.04^{Ab}	53.40± 40.49$^{A\,b}$	65.63± 9.23$^{A\,b}$	62.86± 40.97$^{A\,b}$	71.45± 41.51$^{A\,b}$	63.74± 40.09$^{A\,b}$	76.99± 30.30^{Ab}	76.22± 37.26$^{A\,b}$	11.08± 6.94$^{A\,a}$	48.18± 29.68A^{ab}
Post-mon	27.15± 23.19^{Bb}	22.99± 12.51$^{B\,b}$	32.76± 18.30$^{B\,b}$	32.05± 21.86$^{B\,b}$	19.91± 21.25$^{B\,b}$	29.25± 44.72$^{B\,b}$	34.34± 40.49^{Bb}	34.20± 4.45$^{B\,b}$	12.86± 12.31^{Ba}	26.22± 27.93^{Bab}

Bottom water

Stn	1	2	3	4	5	6	7	8
Pre Mon	57.41±28.31^{A}	35.34 ±39.16^{A}	39.06± 28.99^{A}	38.91± 39.48^{A}	19.88± 10.82^{A}	20.11± 13.26^{A}	21.84± 13.16^{A}	26.52± 14.25^{A}
Mon	61.99 ±33.97^{B}	60.09 ±31.71^{B}	63.16 ±46.23^{B}	54.20 ±44.33^{B}	63.95 ±38.39^{B}	65.50 ±41.37^{B}	74.67 ±33.14^{B}	73.06 ±41.51^{B}
Post Mon	26.93 ±26.64^{A}	33.91± 26.12^{A}	38.61 ±38.98^{A}	29.18 ±35.43^{A}	17.87 ±11.24^{A}	12.62 ±4.01^{A}	32.34 ±40.79^{A}	43.70 ±34.06^{A}

Values with same superscripts do not vary significantly ($p<0.05$)

surface water. Monsoon season was characterized by high silicate content at all the stations.

Silicate showed significant correlation ($p<0.05$) with other parameters at some stations. There was positive correlation with dissolved oxygen, pH and other nutrients, whereas, negative correlation existed with temperature and salinity (Table 4.13, Appendix 2).

Two-way ANOVA and subsequent analysis (Table 4.2j) revealed that both seasonal and spatial variation exist in the case of silicate content. Monsoon season showed significantly high value from the other seasons and station 9 showed significantly least ($p< 0.05$) value from all the rest.

4.4 Discussion

It is well known that physico-chemical parameters such as temperature, salinity, pH, dissolved oxygen, abundance of chlorophyll and nutrients in the water column are of paramount importance for determining the biological productivity and potential resources. Light penetration decides the depth of the euphotic zone, while the nutrients, especially the nitrates and phosphates, indicate the fertility of the water to promote productivity, and availability of photosynthetic pigments reveal the production at the primary level. These factors also play an important role in the distribution of cyanobacteria in any ecosystem.

Temperature

Temperature enhances the rate of metabolism and growth of cyanobacteria upto a certain level. High temperature inhibits photosynthesis by causing damage to enzymes and cell structure. The Cochin estuary is subject to constant changes in physico-chemical parameters. The mixing of inflowing freshwater and tidally-influenced seawater plays an important role in the hydrography of the estuary. Processes like exchange of heat with

atmosphere and other localized phenomena are also likely to influence the distribution of temperature. Talikhedkar *et al.*, (1976), Beninger and Lucas (1984) and Modassir (1990) observed that in estuaries comparatively high temperature prevails during pre-monsoon and low temperature during monsoon periods. Similar results regarding the variation of temperature have been obtained in the present study. Low temperature during the monsoon could be due to strong land sea breeze, rainfall and cloudy sky (Ramamirtham and Jayaraman, 1963; Gopinathan, 1972). The upwelling of subsurface cold, nutrient-rich waters, which occurs during the monsoon season, is also an important factor. (Santhanam and Perumal, 2003). The high temperature during pre-monsoon can be attributed to high solar radiation.

Salinity

Salinity is an important hydrographic parameter affecting growth, distribution, species succession and productivity in the aquatic environment, especially in estuary that is more susceptible to variations as compared to the offshore waters. Salinity is a function of evaporation, precipitation, land-runoff, melting of ice and upwelling. High salinity value during pre-monsoon season was due to the high solar radiation and upwelling (Thangaraj, 1984) and the low value during monsoon season is due to the higher effect of land-runoff and freshwater influx. Similar result was reported by Qasim and Gopinathan (1969); Talikhedkar *et al.* (1976); Nair *et al.* (1978); Joseph and Madhyastha (1984) and Rao (1988). Salinity was always more in bottom water than in surface water, which may be attributed to the incursion of cold dense saline water from Arabian Sea into deep layers of Cochin backwaters (Ramamirtham and Jayaraman, 1963).

pH

In the present study, pH showed considerable variation from freshwater to saline stations. It was found that at station 1,2 and 3, the pH obtained was acidic, which may be attributed to the regular discharge of riverine water. Ramamurthy's (1963) finding that the decrease in pH is possibly influenced by

the floodwater from river, lends much support to the present observation. Tinu and Prezant (1992) found that river water remained acidic throughout the year. The slightly basic pH recorded at remaining stations could be attributed to the intrusion of saline water from the sea. Similar observations were made by Bose (1956) in Hooghly estuary, Prabhadevi *et al.* (1996) in Kayamkulam estuary, Padma and Periakali (1999) in Pulicat Lake. pH was high during pre-monsoon season because of the uptake of carbondioxide by phytoplankton and was low during monsoon due to the influence of freshwater, reduction in salinity and decomposition of organic matter. Santhanam and Perumal (2003) made similar observations in the south east coast of India.

Dissolved oxygen

Seasonal variation in dissolved oxygen was obvious in the present study. The dissolved oxygen level of the estuary is mainly dependent on the phytoplankton photosynthesis. A significant negative correlation existed between salinity and dissolved oxygen in station 1 and 6. Similar observations were made by Haridas *et al.* (1973) and Kumaran and Rao (1975) in Cochin estuary; Nair and Sivankutty (1971) in Kayamkulam estuary; Dehadrai and Bhargava (1972) in Mandovi and Zuari estuary, Vijayalakshmi and Venugopalan (1973) in Vellar estuary.

Euphotic depth

Light is the source of energy which enables photosynthesis. The quantity and quality of the ambient light has a great influence on the growth, distribution and productivity of cyanobacteria. The penetration of light into the water body depends largely on the turbidity. In monsoon, it always showed low level as the rainfall makes the water column turbid.

Chlorophyll

It is well known that photosynthetic pigments are the index of primary production of an area and they play a significant role in the ecological characteristics of an ecosystem. The biological productivity of the coastal and oceanic regions is dependent on the distribution and abundance of

photosynthetic pigments in the euphotic zone (Gopinathan *et al.*, 2001). In the present study, a tremendous increase could be found in the chlorophyll content at station 10 at which the water column was with a very conspicuous layer of blue greens. This could be due to the relatively higher tidal influence observed at this region, which might bring in more nutrients for the growth and multiplication of phytoplankton from the open estuarine system.

Nutrients

The concentration of nutrients in the estuary indicated large inputs from industrial units and agricultural runoffs. Discharge of urban waste which is considered to be the main contributor of nitrogen and phosphorous, greatly influences the growth of phytoplankton population and other dependent communities in estuaries. Nutrient distribution is principally determined by the mixing processes between freshwater of high nutrient content and seawater of low nutrient content, leading to a gradual decrease in nutrient concentration towards sea in the estuarine region. Thus the nutrient content of the estuary is determined by the concentration in its riverine and coastal water sources and the properties of each in the mixture. These nutrients are also introduced into the estuarine waters through land-runoff or as a result of microbial decomposition of the dead organisms and their waste products. The microbial decomposition often occurs at the bottom and the resultant nutrients are brought up to the surface layer by upwelling. Highly significant positive correlation observed between nitrite and nitrate at some stations clearly substantiates the conversion of nitrate (NO_3N) from nitrite (NO_2-N). Significantly high value of nitrite and phosphate observed in station 1 is due to discharge of effluents from the industrial units located close to the station. Relatively higher values of phosphate recorded at station 10 (Puduvaippu) due to domestic discharge indicated the higher fertility status of this region. Silicate is an important nutrient salt required by diatoms and silicoflagellates, which constitute 77% of the total planktonic algae in the coastal waters of the south west coast of India. The availability of silicate in Cochin estuary was

determined in order to find out whether it has any obvious influence on the growth and distribution of cyanobacteria.

Higher concentrations of nutrient salts during the monsoon season in the coastal waters of the south west coast of India was due to the upwelling of cold-nutrient-rich waters, land drainage and input of fertilizers from the catchment areas associated with heavy rains during this season. As the run-off slowed down afterwards, the nutrients also decreased and became very low during pre-monsoon. Furthermore, the utilisation of dissolved nutrients by phytoplankton decreases their concentration during these seasons (Krishnamurthy, 1961 and Santhakumari, 1971). The increased water salinity in the estuarine system promotes the growth and multiplication of primary and secondary producers resulting in the reduction in nitrite and nitrate values due to their utilisation by primary producers (Venkatesan *et al.*, 2001). Similar trend for seasonal change in nutrients has been reported by Balakrishnan and Shynamma (1976) and Joseph (1974) in Cochin estuary. Nitrite concentration was always less than the nitrate concentration at all the stations and for all periods, as observed by Sankaranarayan and Qasim (1969) and Pillai *et al.* (1975).

The present study shows that the hydrographical conditions of the Cochin estuary are greatly influenced by the seasons, seawater intrusion and influx of river water.

CHAPTER 5

Effect of physico-chemical factors on distribution of cyanobacteria in Cochin estuary

5.1 Introduction

Distribution of microorganisms in any environment depends upon various physical and chemical factors such as temperature, salinity, pH, light, nutrients etc. Cyanobacteria are important primary producers in all aquatic ecosystems. This necessitates the study of ecobiological properties of cyanobacteria with respect to different hydrobiological parameters. The discovery of the prokaryotic nature of cyanobacteria coupled with the knowledge that a number of these organisms are unique in not only having a trophic independence for carbon but also nitrogen, evoked considerable interest in these organisms among scientists around the world.

Most of the earlier work on ecobiology had been at the total planktonic level, primarily at the productivity and other conventional ecobiological aspects (Srinivasan, 1946; Dutt *et al.*, 1954; Subramanian and Sarma, 1960; Ryther *et al.*, 1966; Hulbert, 1967; Jitts, 1969; Devassy and Gopinathan, 1970; Mefferet and Krambeck, 1977; Tilman *et al.*, 1982; Smith and Attinson, 1984; Shim *et al.*, 1985; Shim and Lee, 1987; Subramaniyam and Bhavanarayana, 1989). Studies concentrating essentially on cyanobacteria include the works reported by Borongerrsma-Sanders, 1957; Prabhu *et al.*, 1965; Ramamurthy, 1968; Qasim, 1970; Kristiansan, 1972; Jayaraman, 1972; Fogg, 1973 and 1982; Sakthivel and Haridas, 1974; Dunston and Hofford, 1977; Devassy *et al.*, 1978 and 1979; Nair *et al.*, 1980; Krempin and Sullivan, 1981; Marshall, 1981; Ramachandran, 1982; El-Hag and Fogg, 1986; Baker, 1987; Devassy, 1987; Thajuddin and Subramanian, 1990 and 1991.

The remarkable physiological, morphological and ecological adaptability of cyanobacteria is well known. (Hof and Fremy, 1933; Desikachary, 1959; Van Baalen, 1962; Fogg *et al.*, 1973; Carr and Whitton, 1982; Gallon, 1992; Paerl and Millie, 1996). Physiologically, marine cyanobacteria are well adapted to nutrient deprivation, a condition characterizing much of the world's oceans (Dugdale, 1967; Ryther and

Dunstan, 1971; Carpenter and Capone, 1983). In particular, cyanobacteria have developed an array of biochemical and ecological mechanisms and strategies to access those essential nutrients most often limiting growth, including nitrogen (N), phosphorus (P) and iron (Fe). Foremost is the ability of numerous genera to fix atmospheric nitrogen (N_2), a capability that has opened up vast segments of the oligotrophic open ocean and coastal waters to cyanobacterial exploitation (Fogg, 1982; Carpenter and Capone, 1983; Capone *et al.*, 1997). Numerous taxa are encapsulated in mucilaginous sheaths and slimes that exhibit anti-desiccation, strong irradiance-absorbing ("sunscreen") and selective gas diffusion characteristics (Fogg *et al.*, 1973; Garcia-Pichel and Casten-holz, 1998). These protective mechanisms ensure survival during extremely long unfavourable growth periods.

Cyanobacteria are also well adapted to environmental excesses. A contemporary example of cyanobacterial opportunism is the development and proliferation of harmful blooms in nutrient-enriched estuarine and coastal ecosystems (Horstmann, 1975; Fogg, 1982; Paerl, 1988; Larsson *et al.*, 1990; Kahru *et al.*, 1994; Sellner, 1997). The tolerance to saline situations generally shown by these algae and the resistance, which many species show towards osmotic shock, variation in temperatures and reducing conditions of the environment, were particularly suitable for their existence in a variety of intertidal habitats (Pillai, 1954; Stewart, 1977; Megue, 1977). Desikachary (1959) attributed the capability of cyanobacteria to thrive in halotolerant conditions to the peculiar characteristics of their protoplasm, especially plasmolysis and deplasmolysis, which are quite distinct from what is seen in other plant cells.

Fogg (1973) suggested that the ability of cyanobacteria to grow in seawater was presumably related to a preference for alkaline conditions. Petrov (1974) reported that the most significant factors, which affect the vertical and horizontal distribution of benthic forms were transparency, movement of water, temperature, chemical composition and grazing. Dunston

and Hofford (1977) presumed that the factors such as water column stability, temperature and nutrient levels favour bloom formation. Ramachandran (1982) reported that the difference in the composition of algal mats observed in the mangrove area was probably due to the effect of salinity. Sanders (1979) observed that the salinity differences played a major role in deciding the algal assemblages. Subramanian and Thajuddin (1995) reported that the maximum diversity of the cyanobacterial flora in the Gulf of Mannar region correlated well with the higher salinity, pH and nutrient content of the water.

Little (1973) revealed that the cyanobacterial community is more abundant on soft, porous rocks such as sand stones. Thajuddin and Subramanian (1992) noted that in the case of sandy shores, the cyanobacterial population was very poor due to the rough tides, absence of substratum, low nutrient content of water etc. The stagnated seawater ponds and puddles showed rich populations of cyanobacteria growing in the form of thick mats, because these habitats remain undisturbed for relatively long periods. Ramachandran (1982) observed that mangrove habitats supported abundant growth of benthic cyanobacteria with richness and diversity of species.

Most of the cyanobacteria possess broad tolerance to different environments and survive through most of the seasons. Thajuddin and Subramanian (1992) observed that 75 of the species recorded from the southern east coast of India have originally been reported from freshwater sources by earlier workers (Biswas, 1949; Smith, 1950; Prescott, 1951; Desikachary, 1959; Tilden, 1968; Hum and Wicks, 1980; Anand, 1989). It was difficult to strictly segregate most of the cyanobacteria into marine and freshwater species as can be done with other algal forms (Subramanian and Thajuddin, 1995).

Many of the earlier workers have concentrated their attention only on a limited number of factors in a particular season or area and have indicated the

importance of those factors on the level of phytoplankton. Jayaraman and Seshappa (1957) positively correlated phosphate level with phytoplankton content. Confield (1983), Confield *et al.* (1985) and Sakamoto *et al.* (1989) found both nitrogen and phosphorus influencing phytoplankton abundance. Braarud (1951), Pearse and Gunter (1957), Provasoli (1958), Smayda (1958) and Munda (1978) emphasized the role of salinity in phytoplankton maintenance. Shim and Lee (1987) found water temperature as the most significant factor for the distribution of phytoplanktons.

In order to examine the environmental barriers to cyanobacterial growth and proliferation in Cochin estuary, systematic monitoring of their distribution, abundance and physico-chemical parameters such as temperature, salinity, pH, dissolved oxygen, euphotic depth, photosynthetic pigments and nutrients of the study sites were made. The present chapter evaluates how these parameters influence the growth and proliferation of cyanobacteria. An attempt has also been made to delineate the subtle relationship between cyanobacteria and the environmental conditions within this ecosystem.

5.2 Results and Discussion

Correlation between hydrography and Cyanobacterial cell count

In order to study how the physicochemical parameters affect cyanobacterial abundance in Cochin estuary, correlation between hydrography and cyanobacterial cell count for both the surface and bottom samples were determined by statistical analysis. Table 5.1 shows the correlation between hydrography and cyanobacterial cell count for both the surface and bottom samples.

Table 5.1 Correlation between Hydrography and Cyanobacteria

Values in bold digits are significant at p < 0.05%

Stn No.	Temp	Salinity	D.O.	pH	Chl-a	Nitrite	Nitrate	Phosphate	Silicate	Euph. depth
Surface water										
1	0.238	-0.315	0.049	0.255	0.272	-0.092	0.131	-0.365	-0.027	0.060
2	**0.470**	-0.200	-0.084	0.193	0.039	0.169	-0.212	0.039	-0.128	-0.012
3	**0.540**	-0.043	**-0.525**	-0.327	0.049	-0.227	-0.290	**-0.650**	-0.258	0.319
4	**0.546**	0.087	-0.360	-0.364	0.407	-0.291	**-0.528**	**-0.578**	**-0.573**	0.226
5	**0.573**	0.339	-0.157	0.039	-0.044	-0.213	-0.376	-0.140	-0.452	0.277
6	0.438	0.201	**-0.550**	-0.241	0.175	**-0.468**	-0.269	-0.189	-0.392	0.346
7	**0.590**	0.427	-0.041	0.222	-0.120	-0.017	**-0.471**	**-0.475**	-0.459	0.402
8	0.391	**-0.061**	-0.019	-0.113	**-0.015**	0.191	**0.038**	-0.385	0.155	-0.098
9	-0.020	0.474	0.014	0.125	-0.517	0.382	0.492	0.156	0.222	NIL
10	0.312	0.419	-0.116	0.064	0.340	-0.071	-0.245	-0.151	-0.284	NIL
Bottom water										
1	-0.253	0.077	0.242	0.287	0.360	-0.074	-0.261	0.178	0.065	0.1110
2	0.432	0.036	-0.376	0.120	0.169	-0.169	-0.190	-0.121	-0.272	-0.2549
3	0.459	0.254	-0.241	-0.281	0.110	-0.113	-0.283	-0.445	-0.302	0.3939
4	0.236	0.125	-0.199	-0.204	-0.023	-0.041	-0.216	-0.230	-0.379	0.0874
5	0.376	0.312	-0.416	0.028	-0.067	-0.288	-0.287	-0.229	-0.329	0.1999
6	**0.484**	**0.483**	-0.230	0.031	-0.158	-0.120	-0.272	-0.118	**-0.476**	**0.5145**
7	0.228	0.009	0.078	-0.022	-0.289	0.200	-0.026	0.320	0.108	0.0895
8	0.208	**0.503**	0.054	0.192	0.338	-0.379	**-0.479**	-0.194	-0.442	0.4220

5.2.1 Temperature

It was found that out of ten parameters studied, temperature was the prime factor, which showed significant correlation with cyanobacteria. Positive correlation existed in all the stations except at station 9 and at the bottom of station 1. The relation was very significant in most of the stations, which implies that both the factors coexisted. From the details of the hydrography data, given in Fig 4.1a and 4.1b in chapter 4, it was evident that temperature was low in monsoon and then increased to its peak in pre-monsoon season. Likewise, a progressive increase in abundance of cyanobacteria from monsoon to pre-monsoon has been observed in Fig. 3.1 and 3.2. Therefore, it

appears that temperature enhances growth and multiplication of cyanobacteria within the temperature range of the study. The scatter diagrams (Fig 5.1a and 5.1b) also support the same observation. As demonstrated by the scatter diagrams, cell count was high at temperatures between 26 - 32^{0}C.

Fig 5.1a Scatter diagram:Temperature versus cell count for surface water

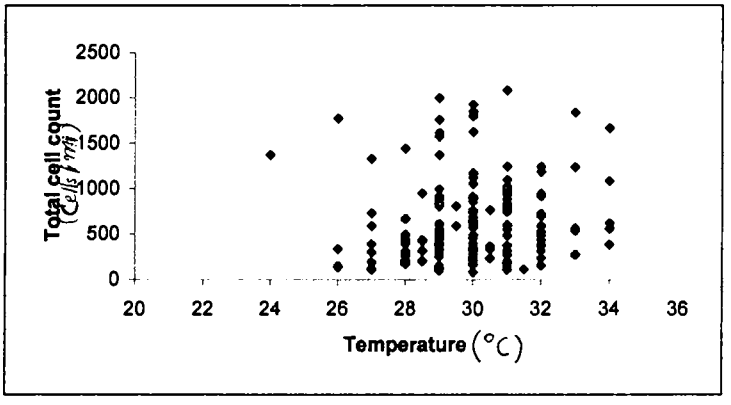
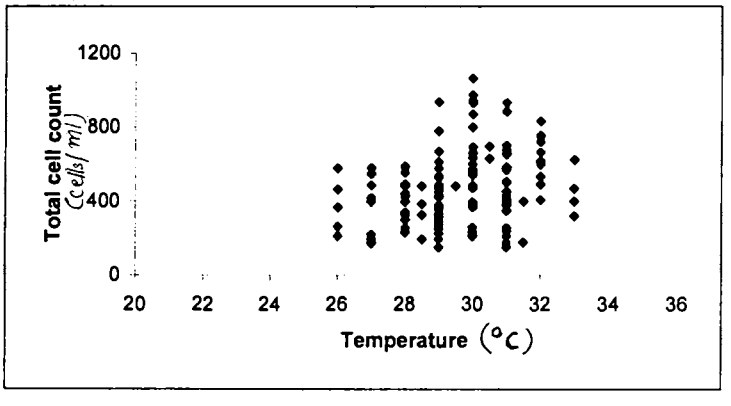


Fig 5.1b Temperature versus cell count for bottom water



5.2.2 Salinity

Salinity was found to exhibit positive correlation with cyanobacterial numbers with a few exceptions. Positive correlation between cell count and salinity at the bottom samples of station 6 and 8 was very much significant. Salinity increased progressively from station 1 to 5 and then decreased

slightly up to station 8. A similar trend was observed in the case of total cell count indicating that salinity favours cyanobacterial growth. However, station 9, the highest saline area, showed very few cells and very less diversity which might be due to the effect of sandy shore and rough waves. Station 10 showed optimum saline conditions, 15 – 30 ppt, where abundant quantity of cyanobacteria was recorded. The scatter diagrams of salinity verses cell count for both surface and bottom water reveal that cyanobacteria possess broad tolerance to salinity ranging from 0 to 37 ppt (Fig 5.2a and 5.2b). Therefore, it was difficult to strictly segregate most of the species into saline and freshwater forms.

Fig 5.2a Salinity versus cell count for surface water

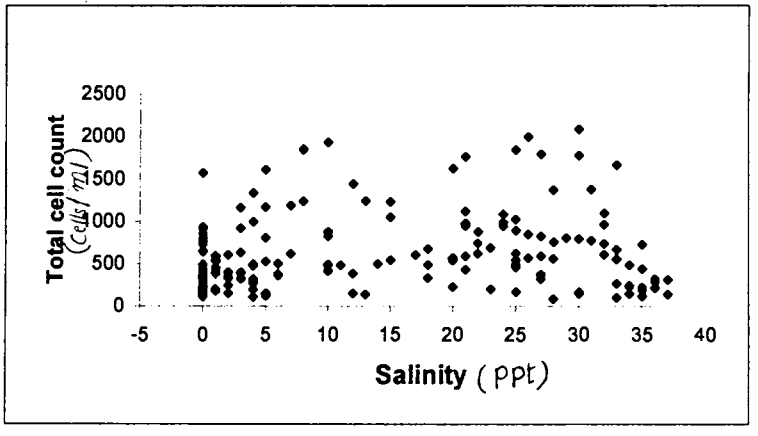
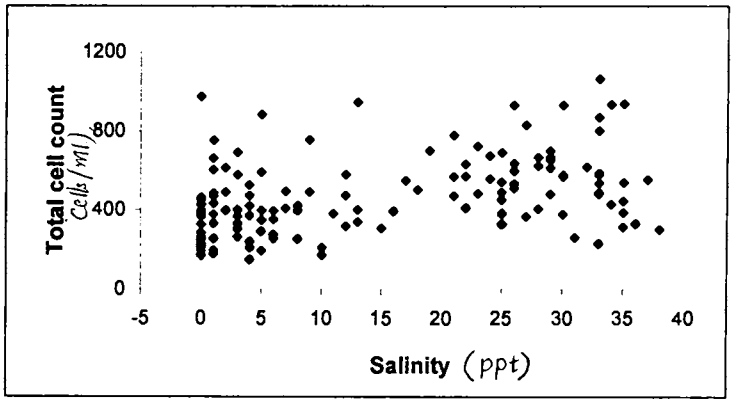


Fig 5.2b Salinity versus cell count for bottom water



5.2.3 Dissolved Oxygen

Correlation between dissolved oxygen and cell count was always negative, except at station 1,9 and the bottom samples of station 7 and 8. Cell count was high when dissolved oxygen was less than 4 ml/L. Similar observations of low oxygen concentration with high phytoplanktonic population levels were made in North Sea (Rosenberg, 1985, Howarth *et al.*, 1988) and in Gulf of Mannar region of the Indian coast specifically in Mandapam – Rameswaram – Kurusadai island region (Thajuddin, 1991). From the scatter diagrams (Fig. 5.3a and 5.3b), it was clear that cyanobacteria were more concentrated at the sites where the dissolved oxygen content ranged between 3 to 5 ml/L.

Fig 5.3a Dissolved oxygen versus cell count for surface water

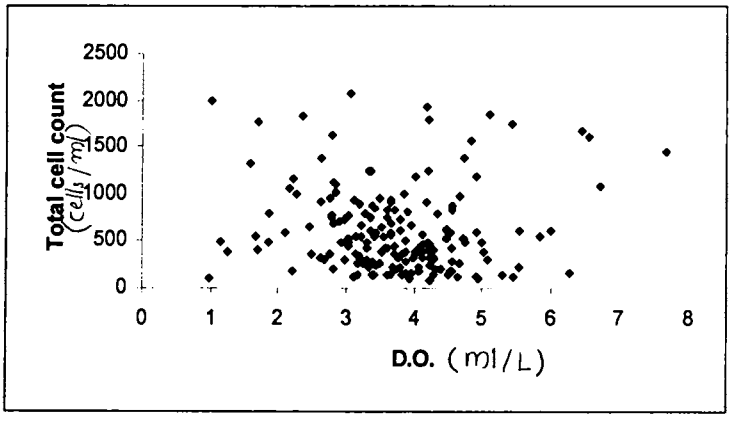
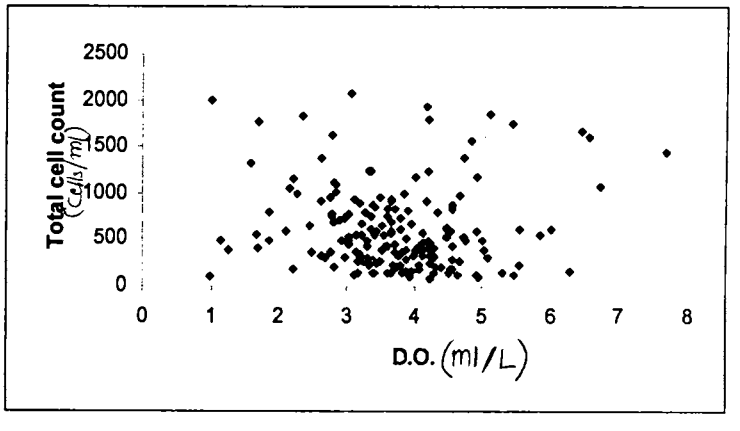


Fig 5.3b Dissolved oxygen versus cell count for bottom water



5.2.4 pH

No significant correlation could be observed between cyanobacterial number and pH of the sampling stations. Their abundance was more or less same at all the pH values within the range of the study. The pH variation observed in the sampling stations was not very predominant. Fig 5.4a and 5.4b show that cyanobacteria were more abundant at sites where pH values ranged between 6.5 and 8.5.

Fig 5.4a pH versus cell count for surface water

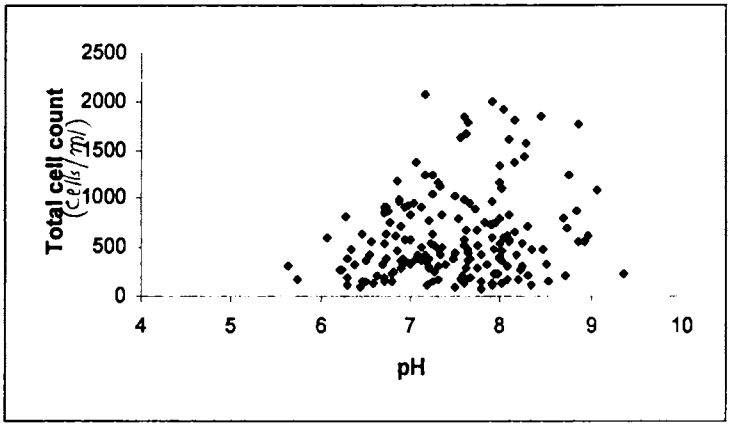
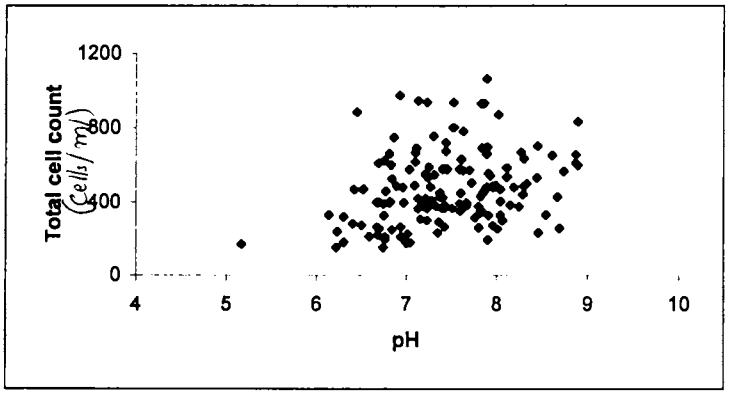


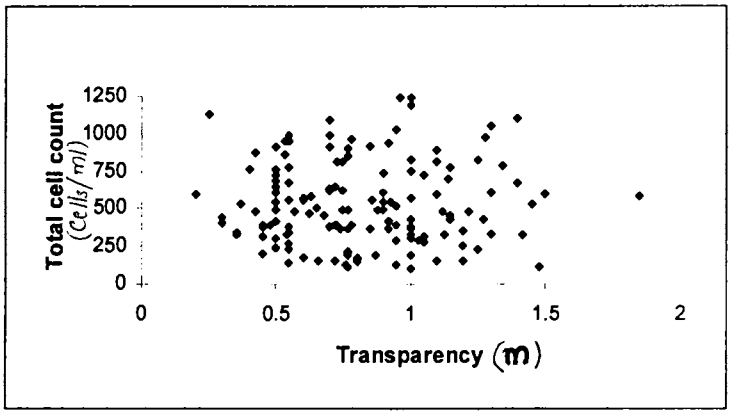
Fig 5.4b pH versus cell count for bottom water



5.2.5 Euphotic depth

In general, total cell count was positively correlated with light penetration which may be the reason for large population of cyanobacteria observed in surface water than in bottom. Slightly elevated levels of cyanobacteria, observed in the bottom of shallow regions such as station 3, 7 and 8 could be attributed to the sufficient light availability at the bottom. Fig. 5.5 shows that cyanobacteria are distributed throughout the Cochin estuary where the euphotic depth ranged between 0.5m to 1.5m.

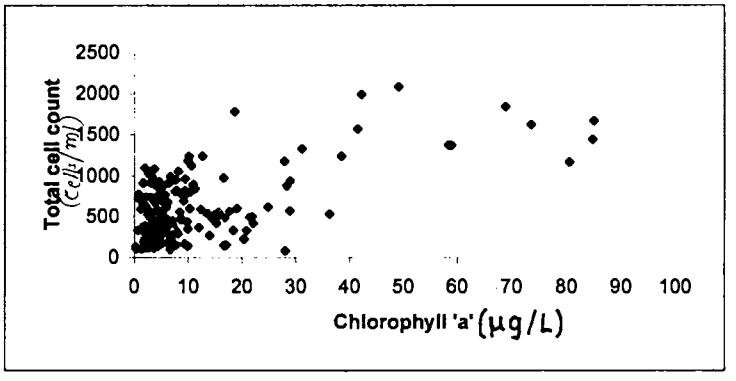
Fig 5.5 Euphotic depth versus cell count



5.2.6 Chlorophyll-a

The distribution of chlorophyll-a along Cochin estuary did not show a direct correlation with cyanobacterial abundance. Cell count was more or less same except at station 10 where it was strikingly high. Only at station 10, a direct correlation between cyanobacterial count and chlorophyll-a could be observed which shows that the main contributor of chlorophyll-a at this station was cyanobacteria, whereas, in other stations, other algae might be the major contributors of chlorophyll-a. The scatter diagram (Fig 5.6) also shows the same pattern. As is clear from the scatter diagram, cyanobacteria were more abundant in areas having chlorophyll-a content 1 to 10 μg/L.

Fig 5.6 Chlorophyll 'a' versus cell count



5.2.7 Nutrients

In the present investigation, concentration of nutrients such as nitrate, nitrite, phosphate and silicate from various sites of Cochin estuary showed negative correlation with cyanobacterial abundance. Greater availability of nutrients coincided with low to moderate values of cell count. The availability of nutrients was largely due to upwelling and river run off and also drainage from the land. Very often, these nutrients available in the water column are not fully utilized by the phytoplankton and high concentration was detected in water samples and this substantiates the negative correlation between cell count and nutrient distribution observed at the study site. Figs. 5.7 to 5.10 show the same pattern. Cell count was less in the areas where concentration of nutrients such as nitrate,. nitrite, phosphate and silicate were high. Cyanobacteria were more abundant when nitrate concentration in the water was less than 5 µmol/L, nitrite less than 0.8 µmol/L and phosphate and silicate less than 3 µmol/L and 25 µmol/L respectively.

Fig 5.7a Nitrate versus cell count for surface water

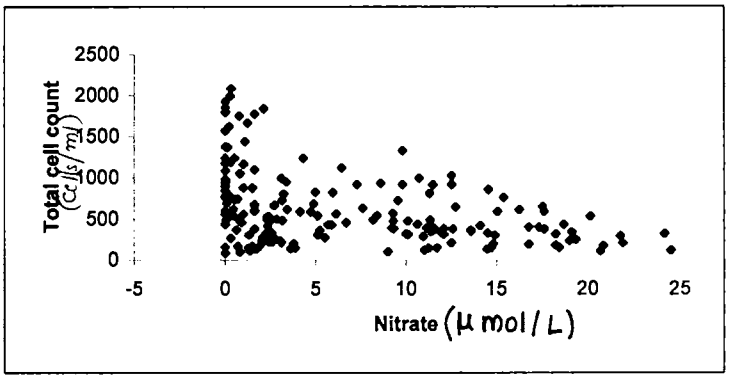


Fig 5.7b Nitrate versus cell count for bottom water

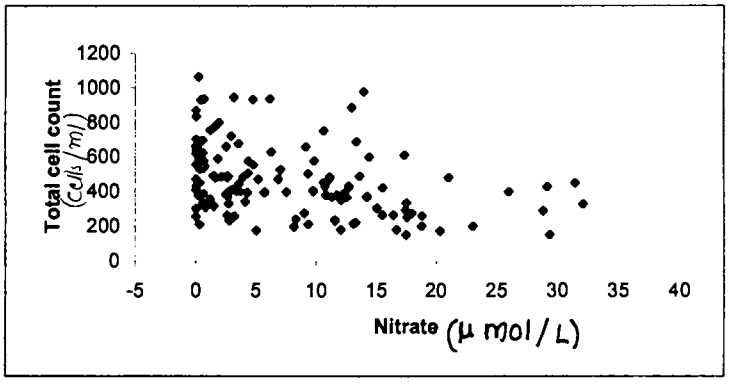


Fig 5.8a Nitrite versus cell count for surface water

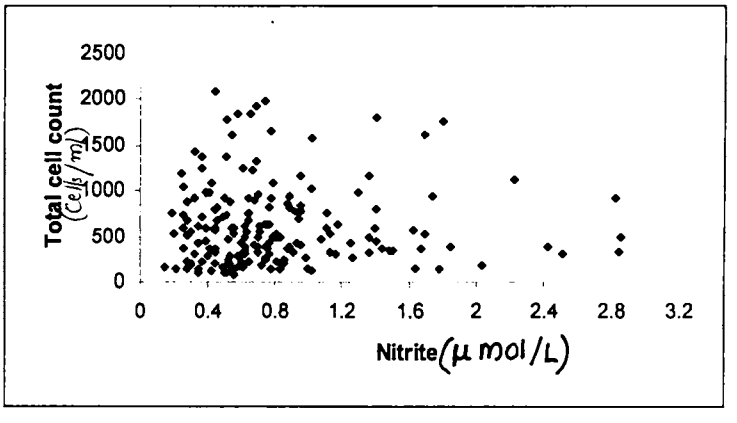


Fig 5.8b Nitrite versus cell count for bottom water

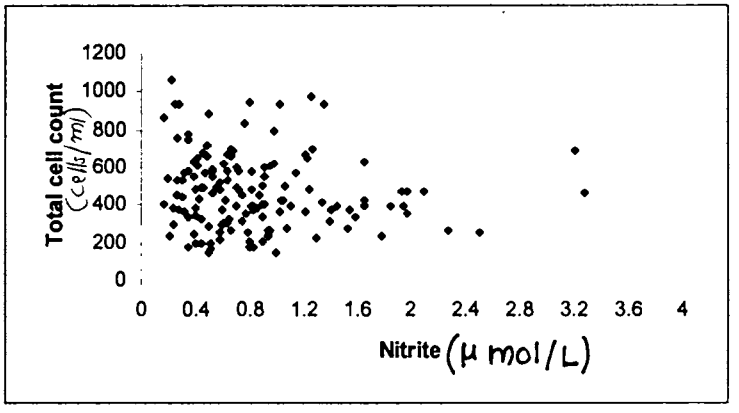


Fig 5.9a Phosphate versus cell count for surface water

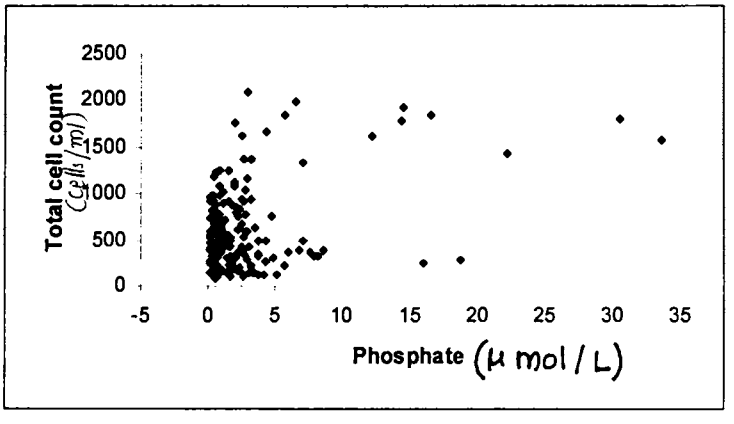


Fig 5.9b Phosphate versus cell count for bottom water

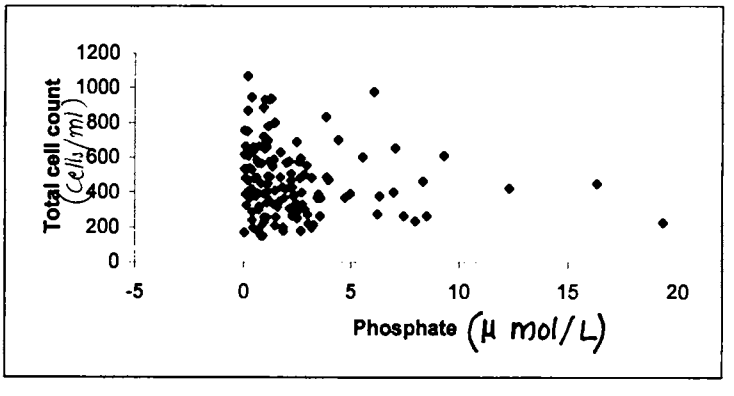


Fig 5.10a Silicate versus cell count for surface water

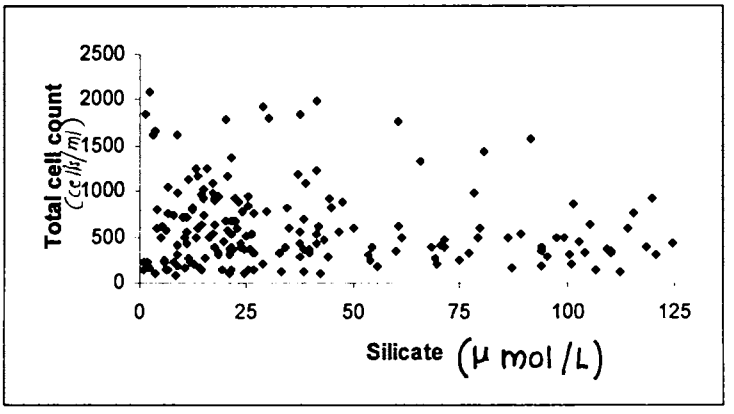
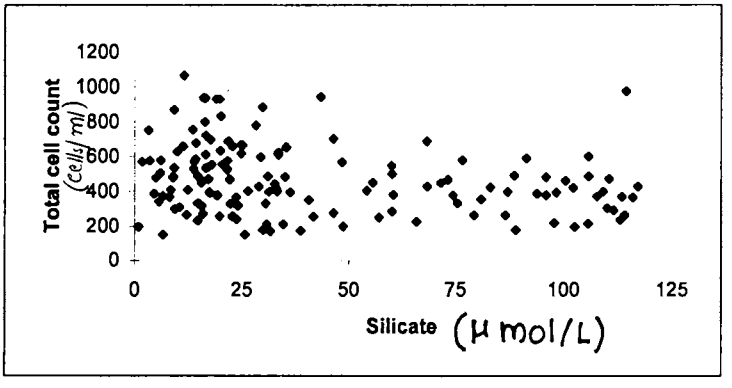


Fig 5.10b Silicate versus cell count for bottom water



Compared to the unicellular and non-heterocystous filamentous forms, the heterocystous forms were very poor in number and this may be due to the high levels of combined nitrogen in the estuary (up to 79.5 μmol/L NO_3^- and 2.08 μmol/LNO_2^-).

It was a difficult task to strictly evaluate the factors responsible for the variation of cyanobacteria, because of their erratic nature. Although hydrobiological parameters do exert some influence, they have, as yet, not shown statistically significant effect on the abundance of cyanobacteria. Changes in population structure were affected not only by the dynamics of processes, internal to environments, but also by the addition and superposition of changes in the surrounding environments of the estuary.

CHAPTER 6

Biochemical evaluation of selected strains of cyanobacteria

6.1 Introduction

Cyanobacteria are distributed in the whole aquatic ecosystem serving as the primary food source for all growth stages of bivalves, and for the larvae of some crustaceans and fishes (Brown and Farmer, 1994). The biochemical composition of the algae plays an important role in their nutritional value and is crucial to the growth and development of these animals. Besides, cyanobacteria have the potential to produce a wide range of fine chemicals including polyunsaturated fatty acids, carotenoids, biloproteins, antibiotics, vitamins, polysaccharides, bioflocculants, biosurfactants, growth promoters etc. and thus can be utilised on a commercial scale.

The cyanobacterium *Spirulina* has been commercially exploited in several countries (Benemann, 1988; Venkataraman and Becker, 1985). It has been used as food for many centuries in Central America. For the past two decades *S.platensis* has been a focus of interest among researchers in various fields because of its commercial importance as a source of proteins, vitamins, essential aminoacids and fatty acids. (Ciferri and Tiboni, 1985; Vonshak and Richmond, 1988; Vonshak, 1990; Tanticharoen *et al.*, 1994) and more recently, for its potential in therapeutic effects (Amha Belay *et al.*, 1993). Basic studies on this species include studies on the growth kinetics by Ogawa *et al.* (1971) and Iehana (1983); growth and growth yield by Aiba and Ogawa (1977) and Ogawa and Aiba (1978).

Although work on the screening and selection of efficient *Spirulina* strains for their nutritive value as well as production of particular end products has been done, much attention has not been paid to screening and selection of other cyanobacteria. Cyanobacteria such as *Phormidium, Chroococcus* and *Nostoc* have regularly been used as food in Mexico, Mongolia, China, Fiji and Thailand (Cannell, 1989). However, the commercial significance of cyanobacteria was realized only very recently and so it is in its infancy. Now-a-days, scientists are motivated to search for more potential species available

in nature for exploiting them in a variety of ways to meet our needs. It needs an extensive screening, which is expected to result in the discovery of better cyanobacterial strains of industrial interest.

The merits of an organism for commercial exploitation are maximum yield and utility of cellular constituents (Borowitzka and Borowitzka, 1988). Higher growth rate and nutrient profile of cyanobacteria make them a potentially valuable source of nutrients (Cannell, 1989). Growth of a living organism is defined as an increase in mass or size accompanied by synthesis of macromolecules, leading to the production of a newly organized structure. In unicellular cyanobacteria, the increase in number of cells is a measure of growth. In filamentous species, growth may include differentiation to produce cells for particular function. Any cell of such a filament is potentially capable of binary fission, and hence, growth in length of the chain is mainly the sum of growth of the individual cells. Therefore, it is very complicated to measure the growth of cyanobacteria in terms of increase in cell numbers by microscopic observation.

Microbiologists use a variety of techniques to quantify microbial growth other than determining direct cell count. These include the measurement of macromolecules in the cells (Healy and Henzdel, 1976), the cell quota of specific elements (Rhee and Gotham, 1980), or the kinetic parameters for nutrient uptake (Zevenboom *et al.* 1982). Photosynthetic rate was higher in cultures grown at faster rates. Although relative incorporation into protein was constant, the absolute rate of protein incorporation increased at higher growth rates because the photosynthetic rate had increased. Chlorophyll-a is another component of biomass, which can be estimated as a measure of growth (Kobayasi, 1961).

The various phases of growth in a microbial culture constitute a typical growth curve. The growth cycle in the system passes through four phases such as lag, log, stationary and death phase. Lag phase is the initial period in

which there appears to be no growth. Although cells are not dividing during the lag phase they are metabolically active, repairing cellular damage and synthesizing enzymes. The lag phase is followed by a period of rapid balanced growth, the logarithmic or the exponential growth phase commonly called the log phase. Next is stationary phase, during which no new growth is apparent and finally there is decline in the viable population until all microbial cells die, i.e. the decline or death phase. Very often, one or more of these phases may not be recognizable. The presence and duration of various phases of growth are indicative of the eco-physiological response of the organism to the physicochemical parameters to which the culture is exposed.

Venkataraman and Mahadevaswamy (1992) pointed out that good culture management with suitable strain is one of the basic needs to get promising yields with quality material on commercial scale. Therefore, cultivation techniques are to be improved with the main objective of obtaining higher algal biomass that exhibits specific qualities (Lobban and Harrison, 1994). Mass cultivation of cyanobacteria is essentially a complex process involving a large number of variables and for the successful growth of cyanobacteria, the environment must be conditioned to meet as many of the essential requirements of that organism as possible. The limitations imposed in the cultivation process can be due to the physical (photon, nutrients, temperature and pH), physiological (organism-environmental-interrelationship) and economic constraints (Radha Prasanna *et al.*, 1998). The environmental factors may be either physiological such as salinity and pH or chemical which provide all the raw materials used for structural and protoplasmic synthesis of cyanobacterial cells (Becker, 1994). Physical and chemical factors such as temperature, salinity and light (Lobban and Harrison, 1994), aeration (Chen and Johns, 1991) or nutrient concentration (Bjornsater and Wheeler, 1990) influence the biochemical composition, physiological status and ultrastructure of the cyanobacteria. Culture medium has been found to play a significant role in the growth kinetics of algae, since it has to stimulate the natural conditions as closely as possible. Cyanobacteria are known to exhibit a wide adaptability

to pH and salinity, but for mass cultivation of cyanobacteria, it is essential to determine their optimal conditions.

The measurement of photosynthetic pigments and other products can be used to monitor their physiological state (Li *et al.*, 1980; Smith and Morris, 1980; Morris *et al.*, 1981; Konopka, 1982), but by itself, it will not provide conclusive information on the nutritional status of the population (Konopka, 1983). Evaluation of their physiological as well as biochemical characteristics lead to the selection of more prospective strains.

The aim of the present investigation was to screen and select the most promising cyanobacteria. They were investigated both physiologically and biochemically. Determination of the most favourable pH, salinity and nutritional requirements for low cost production of selected cyanobacterial strains has been done. Preliminary screening was done based on their biochemical components such as total sugars, proteins and lipids, maintaining optimal growth conditions. Further characterisation of the selected species was carried out by studying the growth kinetics, pigment composition and productivity.

6.2 Materials and Methods

6.2.1 Optimisation of Culture conditions

6.2.1.1 Salinity and pH

The effect of salinity and pH on growth of cyanobacteria was studied to find out the optimum salinity and pH for maximum growth. Three filamentous species identified as *Phormidium tenue, Phormidium angustissimum, Oscillatoria salina* and one unicellular species, *Gloeocapsa livida*, were chosen for the study. Experiments were carried out in Allen and Nelson medium. Effect of salinity was examined at 0,10, 20, 30 and 40 ppt. and that of pH was done at pH 6,7,8 and 9.

6.2.1.2 Medium

The influence of various culture media such as Allen and Nelson medium, BG11 medium, Sea Water Enrichment medium and Nitrogen free medium on growth rate of the above four species of cyanobacteria was studied.

The compositions of these media are given below: -

6.2.1.2a Allen and Nelson

Composition of Allen and Nelson medium is given in section 2.2.2

6.2.1.2b BG11 (Stanier *et al.*, 1971)

Table 6.1 Composition of BG 11 medium

	Ingredients	**Quantity /L**
Solution - A	$NaNO_3$	1.5 g
	Na_2CO_3	20 mg
	$K_2HPO_4.\ 3H_2O$	40 mg
	$MgSO_4.\ 7H_2O$	95 mg
	$CaCl_2.\ 2H_2O$	36 mg
	Citric acid	6 mg
	Ferric ammonium citrate	6 mg
	EDTA	1 mg
Solution - B	Boric.acid	2.86 g
	$MnCl_2.\ 4\ H_2O$	1.81 g
	$ZnSO_4.\ 7H_2O$	222 mg
	Sodium molybdate	390 mg
	Copper sulphate	79 mg
	Calcium nitrate	49.4 mg

Solution A and B were prepared separately and autoclaved. 1ml of solution B was added to 1000 ml of solution 'A' prepared in seawater.

6.2.1.2c Sea Water Enrichment Medium (SWEM) (Subramanian *et al.*, 1999)

Table 6.2 Composition of Seawater enrichment medium

	Ingredients	Quantity
Solution – A	$NaNO_3$	0.5 g
	Na_2HPO_4. $2H_2O$	100mg
	Sea Water	100ml
Solution - B	KNO_3	2.02g
	K_2HPO_4	0.35g
	$FeCl_3$	9.7mg
	$MnCl_2$	0.75mg
	EDTA	100mg
	Thiamine HCl	0.1mg
	Sea water	75ml
	Distilled water	25ml

Solution A and solution B were prepared separately and autoclaved. 2 ml solution A and 1ml solution B were added to 1000ml sterilised seawater.

6.2.1.2d Nitrogen free medium

The source of combined nitrogen (KNO_3) in the Allen and Nelson medium was omitted and replaced by corresponding chlorides to prepare the nitrogen free medium.

The observation on growth using chlorophyll-a as the biomass component was made upto 30 days at time intervals of 3 days as per the method of Strickland and Parsons (1972). Experiments were carried out in triplicate.

10ml of the culture filtrate was filtered through GF/C filter paper under moderate vacuum, the filter paper was then transferred into a clean stoppered test tube and 10ml of 90% acetone was added. The test tube was refrigerated

for about 24 hrs, in order to facilitate the complete extraction of chlorophyll. The chlorophyll-acetone solution was centrifuged for about 10 minutes at 5000 rpm and the absorbance of the clear solution was measured at 665, 645 and 630 nm wavelengths using U – 2001 spectrophotometer taking 90% acetone solution as the blank. The absorbance of the sample was also obtained at 750nm, which was substracted from the values at 665, 645 and 630 nm, thus minimizing the error in chlorophyll measure. Chlorophyll-a was estimated in μg/ml using the equation given below.

$Ca = 11.85\ E_{665} - 1.54\ E_{645} - 0.08\ E630$ ---------- Eqn -1

where E is the absorbance of chlorophyll samples at the respective wavelength.

Chlorophyll -a (μg/ml) = (Ca x v) / (V x 1)

where:

v = volume of acetone (ml)

V = volume of water sample filtered (L)

1 = path length of the cuvette (cm)

6.2.2 Biochemical composition, Growth kinetics, Pigment composition and productivity

Biochemical characterization of various cyanobacteria species were done under optimal conditions.

6.2.2.1 Biochemical composition

The biochemical composition (total sugars, proteins and lipids) of forty pure cultures of cyanobacteria were determined. Out of these, 12 were *Oscillatoria sp.*, 10 *Phormidium sp.*, 5 *Lyngbya sp.*, 4 *Gloeocapsa sp.*, 2 *Synechococcus sp.*, and one species each from the genera of *Synechocystis, Gloeothece, Chroococcus, Microcystis, Chlorogloea, Tolypothrix*, and *Pseudanabaena.*

Total sugar content was determined by Anthrone method (Roe, 1955) after hydrolysing dried samples in 2.5 N HCl.

Protein was analysed by Lowry's method (Lowry *et al.*, 1951). Extraction was done using 1N NaOH.

Lipid content was extracted in chloroform methanol mixture and assay was done using sulphophosphovanillin method (Frings and Dunn, 1970).

6.2.2.2 Growth kinetics

In order to study growth characteristics, twenty strains of cyanobacteria were selected based on their biochemical composition and yield, of which ten were *Oscillatoria sp.*, five *Phormidium sp.*, three *Lyngbya sp.* and two *Gloeocapsa sp.* Growth as chlorophyll content was measured as per the methodology given in the section 6.2.1 and was expressed in terms of μg chlorophyll/ml.

6.2.2.3 Pigment composition

The composition of pigments such as chlorophyll-a, carotenoids, and phycobiliproteins of 20 strains of cyanobacteria were analysed. Concentration of phycobiliproteins such as C-phycocyanin, allophycocyanin and C-phycoerythrin were determined separately. Cells were harvested in the exponential and early stationary growth phases and these parameters were analysed.

6.2.2.3a Chlorophyll-a

Chlorophyll-a was measured as per the methodology given in the section 6.2.1 and was expressed as mg chlorophyll/100mg wet weight of the sample.

6.2.2.3b Carotenoids

Carotenoids present in the samples were extracted using 90% acetone. After complete extraction, samples were centrifuged for about 10 minutes at 5000 rpm, and the absorbance of the clear solution was measured

at 480 and 510 nm wavelengths using U – 2001 spectrophotometer, taking 90% acetone solution as the blank (Strickland and Parsons, 1972). The absorbance of the sample was also obtained at 750nm, which was substracted from the values at 480 and 510 nm nm, thus minimizing the error. The amount of carotenoids was determined using the equation:

Total carotenoids (μg/ml) = 7.6 x (E480 – 1.49 E510)

6.2.2.3c Phycobiliproteins

Known volumes of cyanobacterial suspensions were centrifuged and the pellets were suspended in 3 ml of phosphate buffer. The contents were repeatedly frozen and thawed and centrifuged in order to facilitate complete extraction. The supernatants were pooled and the absorbance was measured at 565, 620 and 650 nm against phosphate buffer blank (Seigelman and Kycia, 1978). Calculations were done using the following equations,

C-Phycocyanin (PC) mg/ml = A620 – (0.7 x A 650) / 7.38

Allophycocyanon (APC) = A650 – (0.208 x A 620) / 5.09

C-Phycoerythrin (PE) = A562 – 2.41 (PC) – 0.849 (APC) / 9.62

6.2.2.4 Productivity

Productivity of cyanobacterial cultures was estimated by light and dark bottle oxygen technique (Gaarder and Gran, 1927). The difference in oxygen concentration between the light (LB) and dark bottles (DB) was converted into its carbon equivalents using a PQ (photosynthetic quotient) of 1.25 for obtaining gross production values.

Calculation

Gross production (mg C/l/hr) = Oxygen content of LB – DB / PQ x T

where 'T' is the time of incubation

6.2.3 Data analysis

One-way ANOVA was done by Duncan's multiple comparison of the means using SPSS 10.0 for window in order to analyze significant difference, if any, between the various culture conditions chosen.

6.3 Results

6.3.1 Optimisation of Culture conditions

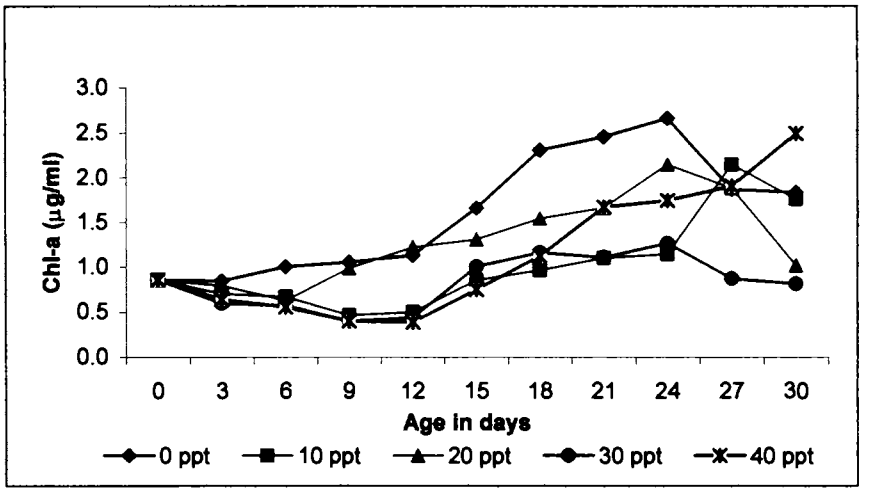
6.3.1.1 Salinity tolerance

Salinity tolerance of different species of cyanobacteria viz. *Phormidium tenue, P. angustissimum, Oscillatoria salina, Gloeocapsa livida* was studied by growing the cultures in Allen and Nelson medium having different salinity levels (0-40ppt) and measuring growth as chlorophyll-a content upto 30 days at three day time interval.

Phormidium tenue

Chlorophyll content of *P.tenue* grown at zero, 20 and 30 ppt salinity began to decline markedly after 24th day of the experiment [Fig. 6.1 and Table 6.3 (Appendix 3)]. At 10 ppt, decline of growth was observed beyond 27th day. However, at 40 ppt, cells kept growing until the end of the experiment. When the species was grown at salinity 0 ppt the lag phase was very short or absent whereas, at 10, 30 and 40 ppt lag phase extended for 12 days and for 20 ppt it was upto 6 days. The specific growth rate or 'k' value obtained at various time intervals obviously showed the same trend. Negative values for 'k' indicated that final values were less than the initial values and therefore, a clear decline in cell biomass. The highest specific growth rate was obtained at 0 ppt, on the 18th day of the experiment. The maximum chlorophyll content obtained was 2.66 μg/ml at 0 ppt. However, growth was found to occur at all levels of salinity indicating that the species can tolerate wide fluctuations in salinity. ANOVA result substantiated the same finding that there was no significant difference ($p < 0.05$) between growth at various salinities.

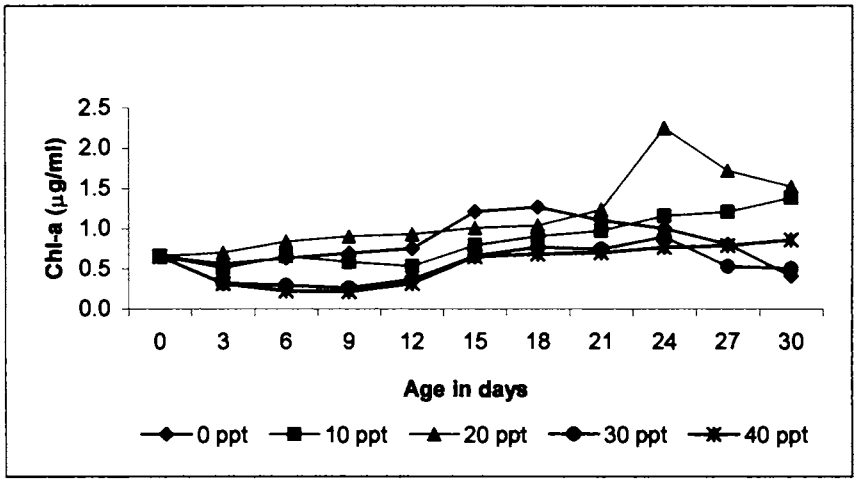
Fig. 6.1. Salinity tolerance of *Phormidium tenue*



Phormidium angustissimum

Phormidium angustissimum exhibited growth at all salinities initially [Fig. 6.2 and Table 6.4 (Appendix 3)]. The onset of death phase was on 21st day at zero ppt , 24th day for both 20 and 30ppt . At salinity 10 and 40ppt, cells kept growing until the end of the experiment.

Fig. 6.2. Salinity tolerance of *Phormidium angustissimum*



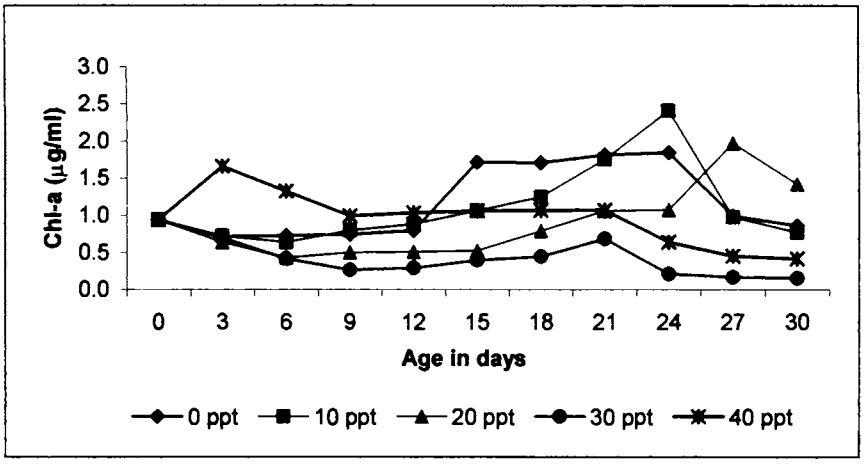
Log phase started from 3,12,0, 9 and 9th day at 0,10, 20, 30, 40 ppt salinity respectively. Very short lag phase upto 3 days could be observed at zero ppt and it was absent at 20ppt. The highest growth rate was obtained at

20ppt (0.066 day^{-1}). The maximum chlorophyll content obtained was 2.25 µg/ml at 20 ppt. It was found that at 20 ppt, yield as well as growth rate increased to double fold when compared to other salinities. One-way ANOVA showed that 20ppt gave significantly higher ($p \leq 0.05$) growth than all other salinities except zero ppt. Very low growth was observed in 30 and 40ppt.

Oscillatoria salina

From Fig. 6.3 and Table 6.5 (Appendix 3) it is clear that for *O.salina* at zero ppt, the lag phase was upto 3 days followed by log phase upto 24th day and proceeded to death phase. The maximum chlorophyll concentration obtained at the end of the log phase was 1.84µg/ml. At 10 and 20 ppt lag phase was upto 6th day whereas, at 30 and 40 ppt there was no growth. At 10ppt, the species exhibited a log phase from 6th day to 24th day and reached the maximum chlorophyll concentration of 2.41µg/ml whereas, the log phase was upto 27th day at 20ppt.

Fig. 6.3. Salinity tolerance of *Oscillatoria salina*

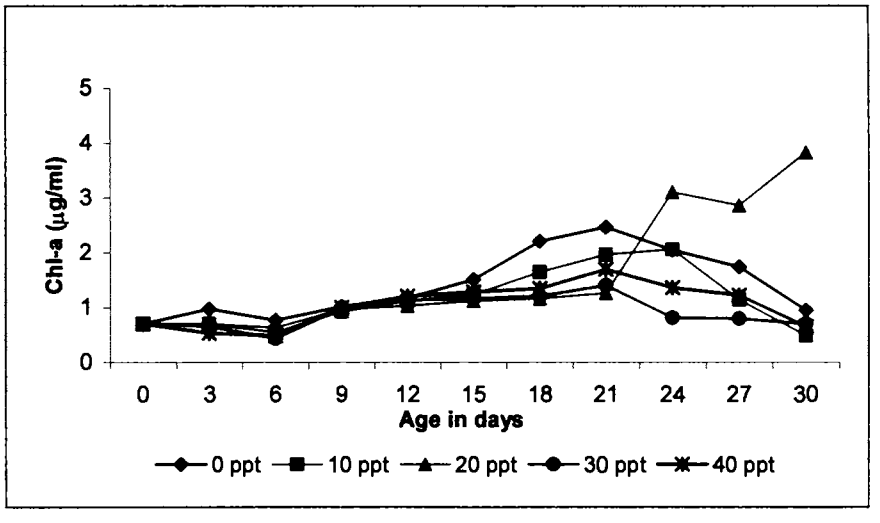


The maximum specific growth rates obtained at 0, 10 and 20 ppt were 0.052, 0.061 and 0.038day^{-1} respectively. The species could not survive at 30 and 40ppt. Maximum yield was obtained at 10 ppt. Duncan analysis revealed that the species could show significant ($p < 0.05$) growth at the salinities 0 to 20 ppt.

Gloeocapsa livida

Almost similar pattern of salinity tolerance was exhibited by *G. livida* [Fig. 6.4 and Table 6.6 (Appendix 3)]. At zero ppt, the onset of log phase was on 3rd day and it sustained upto 24th day and at 10ppt, it was from 6th day to 24th day and at 20 ppt from 6th to 27th day. There was no growth at 30 and 40 ppt. Maximum chlorophyll content obtained was 2.46 µg/ml at 0 ppt whereas, it was 2.06 at 10 ppt, and 3.82 at 20 ppt. The maximum specific growth rate was obtained at 20 ppt (0.1 day^{-1}) followed by zero (0.084 day^{-1}) and then by 10 ppt (0.06 day^{-1}). From these results, it was inferred that *Gloeocapsa livida* is tolerant to salinity range of zero to 20 ppt. The optimum salinity was found to be 20 ppt at which the species showed maximum growth rate and maximum yield. One-way ANOVA revealed that there was no significant difference ($p < 0.05$) between growth at salinities 0-20 ppt and species could show good growth throughout the experiment period.

Fig. 6.4. Salinity tolerance of *Gloeocapsa livida*



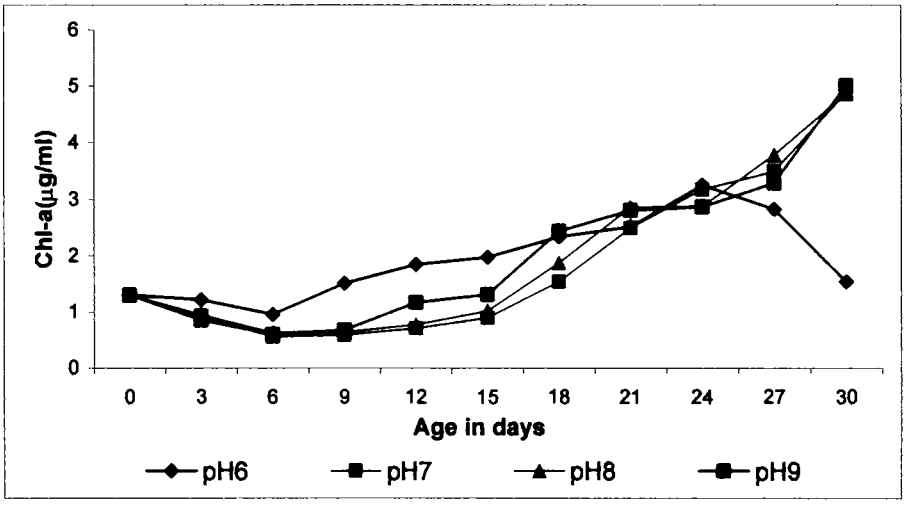
6.3.1.2 pH

The effect of pH on growth of *Phormidium tenue*, *P. angustissimum*, *Oscillatoria salina*, and *Gloeocapsa livida* were studied for 30 days by inoculating the cultures in Allen and Nelson medium having varying pH (6-9)

Phormidium tenue

Fig. 6.5 and Table 6.7 (Appendix 3) explain pH tolerance of *Phormidium tenue*. It was clear that at pH 6 the lag phase was upto 6 days followed by log phase upto 24th day and then proceeded to death phase. The maximum chlorophyll concentration obtained was 3.25µg/ml on 24th day. Lag phase at pH 7, 8 and 9 were also upto 6 days but log phase extended till the end of the experiment. The highest concentration of chlorophyll at these pH were almost same, i.e., 4.88, 4.86 and 5.01 µg/ml respectively. Maximum specific growth rate obtained at pH 6 and 7 was 0.081 day^{-1} while at pH 8 and 9, it was 0.119 and 0.124 day^{-1} respectively. ANOVA result showed that the growth at various pH did not show any significant difference ($p< 0.05$).

Fig 6.5 pH tolerance of *Phormidium tenue*

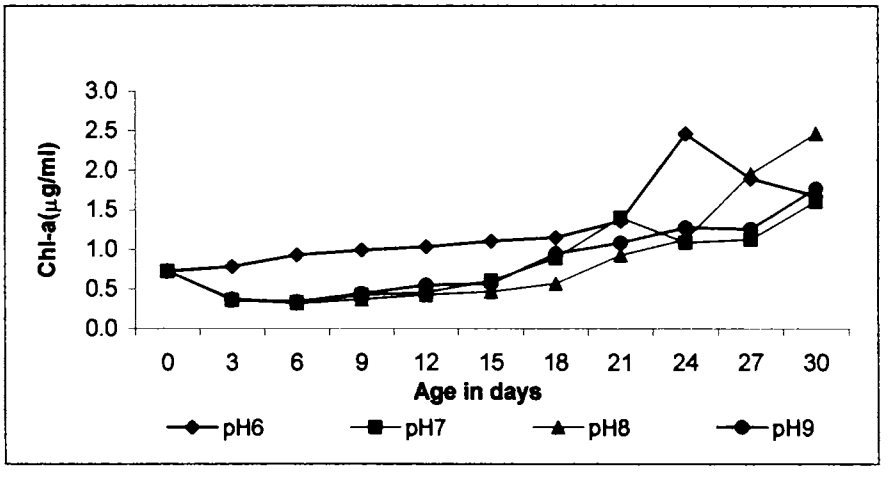


Phormidium angustissimum

Phormidium angustissimum exhibited growth at all pH [Fig. 6.6 and Table 6.8 (Appendix 3)]. At pH 6, the onset of death phase was on 24th day whereas, at pH 7, 8 and 9, cells kept growing until the end of the experiment. There was no lag phase observed at pH 6 whereas, at all other pH lag phase extended upto 6th day. The maximum chlorophyll content obtained was 2.46, 1.61, 2.46 and 1.76 µg/ml at pH 6,7, 8 and 9 respectively. The maximum

specific growth rate at pH 6,7,8, and 9 were 0.072, 0.032, 0.058 and 0.035day^{-1} respectively. Highest growth rate was obtained at pH 6. From this experiment it was inferred that eventhough the species showed broad pH tolerance, pH 6 was the optimum one. pH 8 was also giving the same growth, but slower. No significant difference ($p < 0.05$) could be observed between the growth at various pH conditions when Duncan analysis was done.

Fig 6.6 pH tolerance of *Phormidium angustissimum*

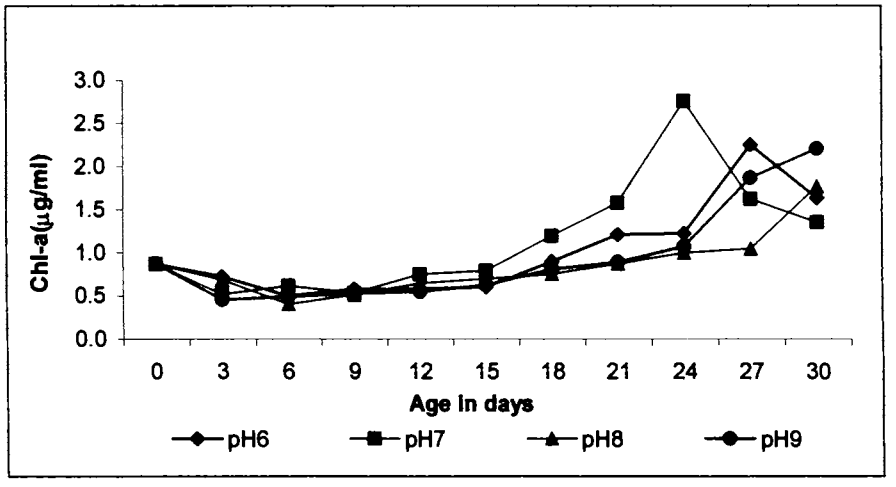


Oscillatoria salina

From Fig. 6.7 and Table 6.9 (Appendix 3)], it is clear that chlorophyll content of *Oscillatoria salina* grown at pH 6 began to decline markedly after 27th day and that at pH 7, it declined after 24th day of the experiment. At pH 8 and 9, cells kept growing until the end of the experiment. The onset of log phase was on the 6th day at pH 6 and 8 and for cultures grown at pH 7 and 9, it was on the 3rd day. The maximum specific growth rate of the species at pH 6 was 0.051 per day, at pH 7, it was 0.079, at pH 8, 0.03, and at pH 9, the value was 0.044 per day. Therefore, the highest specific growth rate was obtained at pH 7. The maximum chlorophyll contents obtained were 2.25, 2.75, 1.77 and 2.2 µg/ml at pH 6, 7, 8 and 9 respectively. From these results, it was concluded that the optimum pH for *O.salina* was pH 7. However, the

species showed broad pH tolerance. ANOVA revealed the same fact that no significant difference ($p < 0.05$) in growth could be seen at various pH levels.

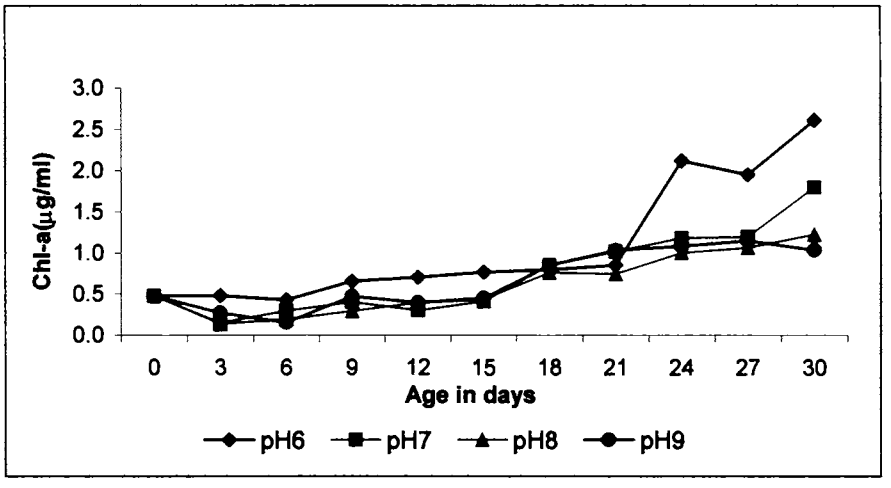
Fig 6.7 pH tolerance of *Oscillatoria salina*



Gloeocapsa livida

Fig. 6.8 and Table 6.10 (Appendix 3)] reveal that at pH 6, 7 and 8 *Gloeocapsa livida* cells were growing constantly until the end of the experiment. At pH 9, a slight decline in growth was observed on the last day. Lag phase was absent at pH 6, whereas, it was upto 3 days at pH 7 as well as pH 8 and upto 6 days at pH 9. The maximum chlorophyll content obtained was 2.61 µg/ml at pH 6, whereas, it was 1.8, 1.22 and 1.15 at pH 7, 8 and 9 respectively. Maximum specific growth rate was 0.071 day^{-1} at pH 6, 0.044 day^{-1} at pH 7, 0.025 day^{-1} at pH 8 and 0.026 day^{-1} at pH 9.Therefore, for *Gloeocapsa livida*, the optimum pH was found to be 6 at which the species showed maximum growth rate. Maximum yield was also obtained at the same pH. However, single factor ANOVA revealed that there was no significant difference ($p \leq 0.05$) in growth between various pH levels.

Fig 6.8 pH tolerance of *Gloeocapsa livida*



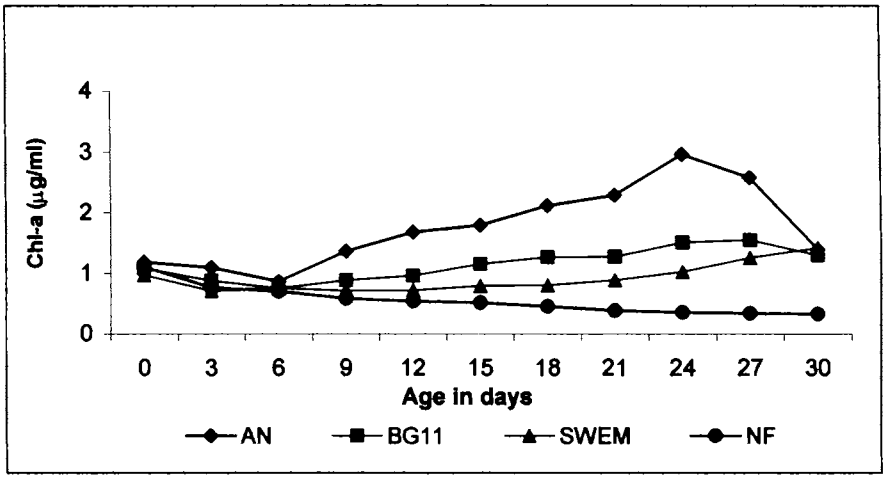
6.3.1.3 Medium

The ability of cyanobacteria to grow in different media, Allen and Nelson medium, BG11 medium, Sea Water Enrichment Medium (SWEM) and Nitrogen-free medium were tested.

Phormidium tenue

Fig. 6.9 and Table 6.11 (Appendix 3) reveal that in Allen and Nelson (AN) medium, *Phormidium tenue* showed a log phase between 6th to 24th days and in BG11, it was between 6 -27 days. The log phase extended upto the end of the experiment from 9th day in SWEM whereas, in Nitrogen-free medium, the species showed no growth. In Allen and Nelson medium, maximum specific growth rate (0.074 day^{-1}) and highest chlorophyll content (2.96 µg/ml) were obtained on 24th day. In BG11, these factors were 0.018 day^{-1} and 1.548 µg/ml and in SWEM 0.015 day^{-1} and 1.418 µg/ml respectively. But in NF medium, k value was negative indicating that the species could not grow in nitrogen free medium, as it was unable to fix nitrogen. From these results, it was found that AN medium was the best medium for the growth of this species. One-way ANOVA also revealed the same.

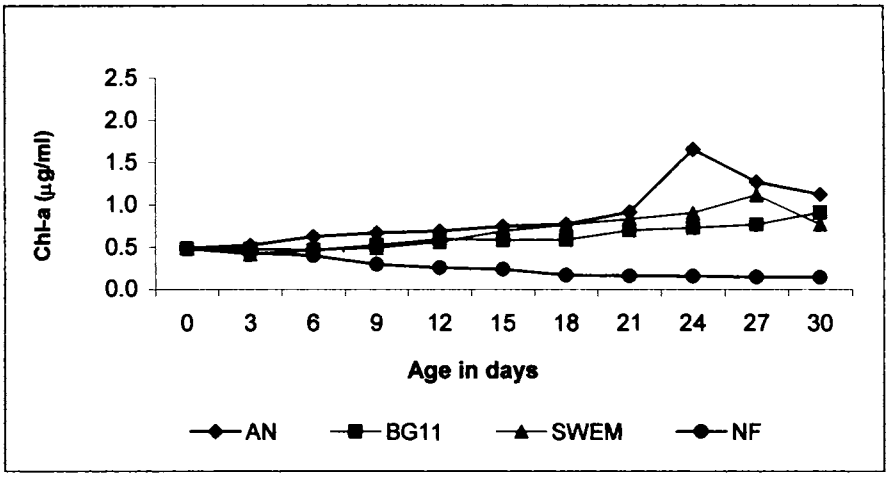
Fig 6.9 Growth characteristics of *Phormidium tenue* in different media



Phormidium angustissimum

Growth characteristics of *Phormidium angustissimum* in various media are shown in Fig. 6.10 and Table 6.12 (Appendix 3). In AN medium, growth curve was in such a way that lag phase was completely absent and log phase started directly from zero day and extended upto 24th day which was followed by a death phase. K_{max} was 0.049day^{-1} and chlorophyll content reached the peak value of 1.656 µg/ml. In BG11 medium, lag phase was found upto 6th day followed by log phase upto the end of the experiment. Maximum specific growth rate of 0.014 day^{-1} and chlorophyll content of 0.909 µg/ml were obtained on the 30th day of the experiment. In SWEM, lag phase was upto the 3rd day followed by log phase upto 27th day followed by the death phase. K_{max} of 0.024 day^{-1} and chlorophyll content of 1.117 µg/ml were obtained in this medium. In nitrogen-free medium, no growth was obtained. From these results, it was inferred that AN medium was the optimum one for the growth of *P. angustissimum* and it was also found that the species was unable to fix nitrogen as it could not grow in NF medium. Duncan analysis demonstrated that AN medium showed significantly higher ($p \leq 0.05$) growth than BG 11. The species could give comparatively good growth in SWEM also.

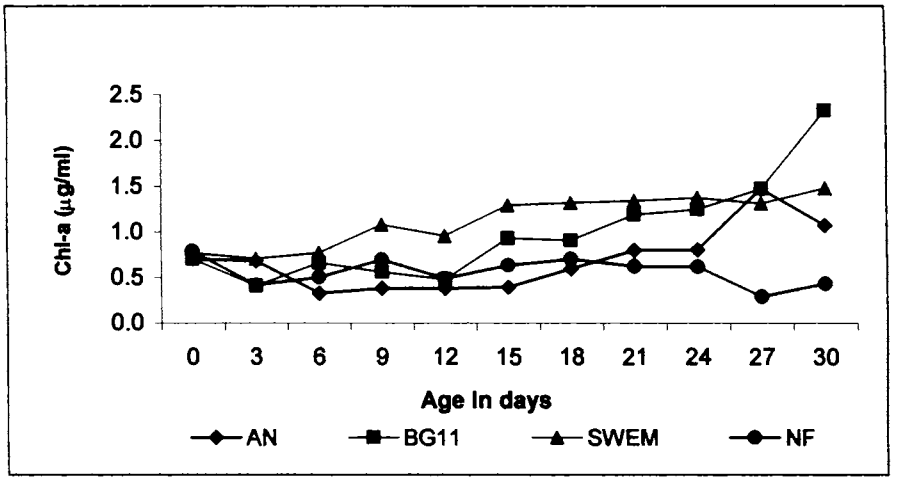
Fig 6.10 Growth characteristics of *Phormidium angustissimum* in different media



Oscillatoria salina

Fig. 6.11 and Table 6.13 (Appendix 3) reveal that in AN medium, *Oscillatoria salina* showed exponential phase from 6th day to 27th day and then an immediate death phase whereas in BG 11 medium, cells were growing constantly until the end of the experiment and in SWEM the species attained maximum growth without much delay and the stationary phase was prolonged afterwards. In this case also, in NF medium, no growth was observed. The maximum chlorophyll content obtained was 1.473 µg/ml in AN medium, whereas, it was 2.327 and 1.48 in BG11 and SWEM respectively. Maximum specific growth rate obtained in these media were 0.028, 0.054 and 0.035 day^{-1} respectively. Therefore, for *Oscillatoria salina* the best medium for growth was found to be BG11 at which the species showed maximum growth rate and yield. However, the species could show considerable growth in both AN medium and SWEM and it was also found that the species did not have the capacity to fix nitrogen as it could not grow in NF medium. One-way ANOVA revealed that the organism showed significantly good ($p \leq 0.05$) growth in SWEM and least growth in NF medium.

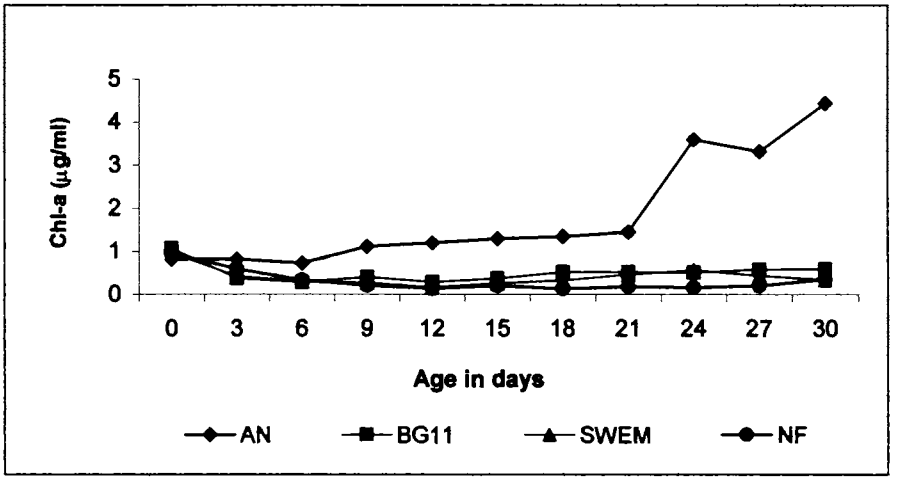
Fig 6.11 Growth characteristics of *Oscillatoria salina* in different media



Gloeocapsa livida

Gloeocapsa livida also exhibited significant growth in AN medium [Fig. 6.12 and Table 6.14 (Appendix 3)]. The onset of log phase was on 6th day and was extended upto the end of the experiment. K_{max} was 0.121day^{-1} and chlorophyll content reached the peak value of 4.438 µg/ml. In BG11 medium, SWEM, and NF medium k value was found always negative indicating that there was no growth in these media. From this, it was concluded that AN medium was the only suitable medium for the favourable growth of this unicellular species. Single-factor ANOVA substantiated the same finding.

Fig 6.12 Growth characteristics of *Gloeocapsa livida* in different media



6.3.2 Biochemical composition, Growth kinetics, Pigment composition and Productivity

In order to select the most promising strains, biochemical studies of the isolated cyanobacterial species were carried out in the laboratory keeping them in Allen and Nelson medium at the optimum salinity (20 ppt) and pH (7). Cultures were grown at 28^0C with an illumination of 2000 lux for 12 hours duration and were harvested in the exponential /early stationary growth phase (24^{th} day of incubation).

6.3.2.1 Biochemical composition

The biochemical composition of forty strains of cyanobacteria has been determined. The results are expressed as percentage of dry weight and are given in Figs 6.13 to 6.15 and Table 6.15 .

6.3.2.1a Total sugars

Sugar content of various species was found to be in the range of 1.1 – 13% (Fig 6.13) and *Oscillatoria pseudogeminata* (C81) yielded the highest amount of sugars (13.02%). *O.accuta* (C49), *O. jasorvensis* (C23), *O. willei* (C30), *O. fremyi* (C32), *O.foreauii* (C72), *O.limnetica* (C77), *Phormidium dimorphum* (C9), *P. corium* (C13), *Lyngbya semiplena* (C34), *Gloeocapsa gelatinosa* (C28), *G. compacta* (C52), *G. quaternata* (C94) , and *Tolypothrix tenuis* (C113) were also composed of comparatively higher amount of sugars (more than 8%).

6.3.2.1b Proteins

Protein content was found to be in the range of 1.84 – 39% (Fig 6.14); maximum was encountered in *Phormidium dimorphum* (C9 - 38.88%). Other species with high protein content (more than 20%) were *Oscillatoria salina* (C21), *O.foreauii* (C72), *O. pseudogeminata* (C81), *O.laete-virens minimum* (C85), *O.accuminata* (C112), *Phormidium abronema* (C11), *P. corium* (C13), *Lyngbya martensiana* (C1), *L. semiplena* (C34) and *Gloeothece rupestris* (C19).

Fig 6.13 Total sugars of selected cyanobacterial species

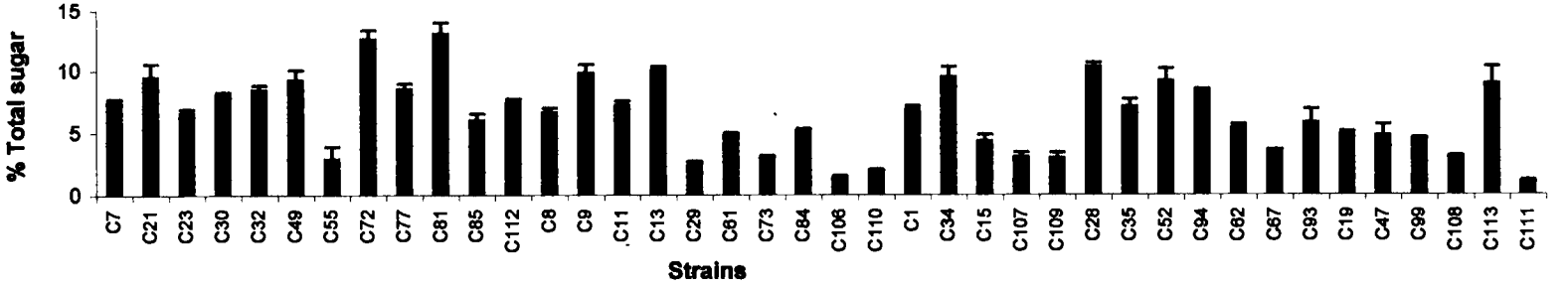


Fig 6.14 Protein content of selected species

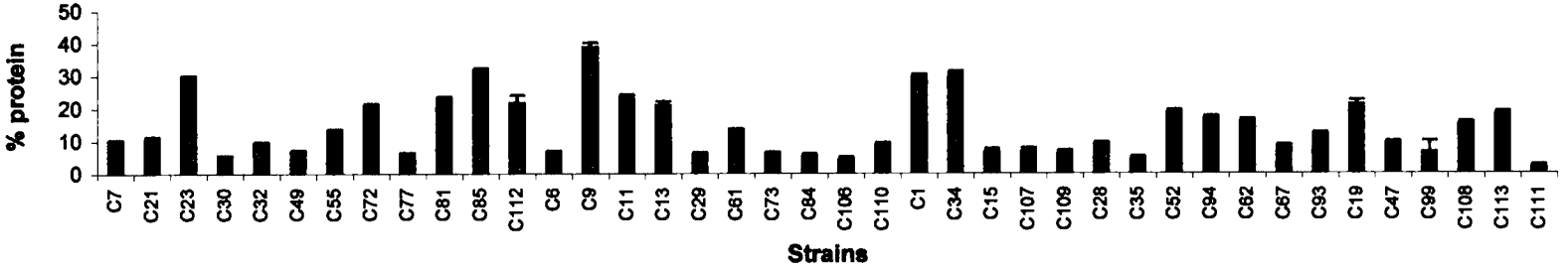


Fig 6.15 Lipid content of selected species

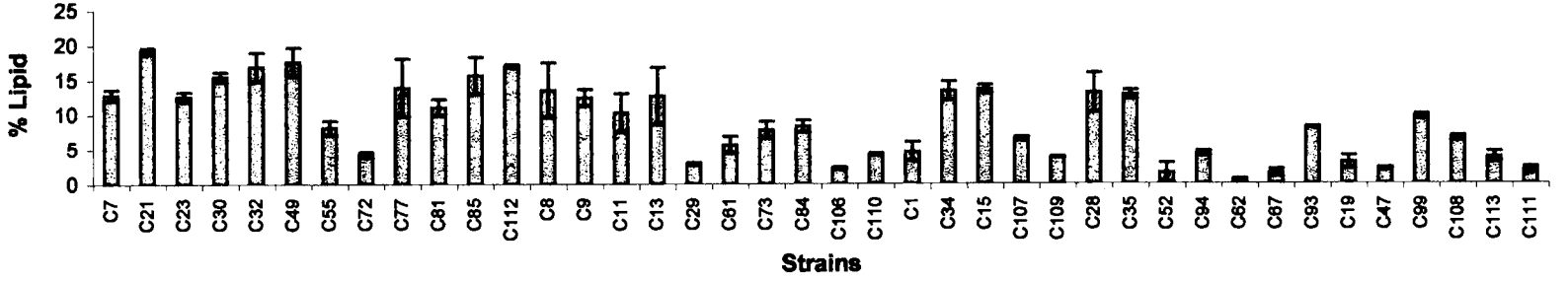


Table 6.15 - Biochemical composition of forty different species of cyanoabcteria

Expressed as % of dry weight of sample

S.No	Strain No.	Name of the species	Total sugars	Proteins	Lipids
1	C 7	***Oscillatoria cortiana***	7.51 ± 0.22	10.26 ± 0.22	12.90 ± 0.79
2	C 21	***Oscillatoria salina***	9.50 ± 1.07	10.53 ± 0.89	19.25 ± 0.42
3	C 23	***Oscillatoria jasorvensis***	6.58 ± 0.31	30.02 ± 0.15	12.62 ± 0.67
4	C 30	***Oscillatoria willei***	8.04 ± 0.22	5.61 ± 0.05	15.51 ± 0.69
5	C 32	***Oscillatoria fremyi***	8.45 ± 0.37	9.58 ± 0.21	16.95 ± 2.07
6	C 49	***Oscillatoria ac uta***	9.24 ± 0.80	6.82 ± 0.42	17.66 ± 2.07
7	C 55	*Oscillatoria minnesotensis*	2.90 ± 0.98	13.41 ± 0.23	8.16 ± 1.02
8	C 72	***Oscillatoria foreauii***	12.58 ± 0.73	21.17 ± 0.34	4.25 ± 0.40
9	C 77	***Oscillatoria limnetica***	8.52 ± 0.41	6.42 ± 0.11	13.95 ± 4.15
10	C 81	***Oscillatoria pseudogeminata***	13.02 ± 0.92	23.31 ± 0.38	11.10 ± 1.17
11	C 85	***Oscillatoria laete-virens minimus***	6.02 ± 0.49	31.93 ± 0.45	15.69 ± 2.64
12	C112	*Oscillatoria ac uminata*	7.41 ± 0.30	21.82 ± 2.32	17.07 ± 0.14
13	C 8	***Phormidium tenue***	6.65 ± 0.31	6.92 ± 0.14	13.59 ± 3.93
14	C 9	***Phormidium dimorphum***	9.80 ± 0.68	38.88 ± 1.30	12.50 ± 1.19
15	C 11	***Phormidium abronema***	7.22 ± 0.34	23.51 ± 0.85	10.34 ± 2.76
16	C 13	***Phormidium corium***	10.01 ± 0.30	21.07 ± 1.10	12.74 ± 4.10
17	C 29	*Phormidium mucicola*	2.68 ± 0.03	5.82 ± 0.69	2.90 ± 0.21
18	C 61	*Phormidium purpurescens*	4.85 ± 0.12	13.60 ± 0.14	5.64 ± 1.21
19	C 73	*Phormidium foveolarum*	3.05 ± 0.10	6.47 ± 0.12	7.82 ± 1.19
20	C 84	***Phormidium angustissima***	5.15 ± 0.15	5.36 ± 0.75	8.35 ± 0.86
21	C106	*Phormidium molle*	1.36 ± 0.19	4.39 ± 0.76	2.25 ± 0.13
22	C110	*Phormidium bohneri*	1.99 ± 0.06	8.71 ± 0.77	4.26 ± 0.16
23	C 1	***Lyngbya martensiana***	6.83 ± 0.30	29.68 ± 0.70	4.67 ± 1.39
24	C 34	***Lyngbya semiplena***	9.45 ± 0.80	30.71 ± 0.70	13.45 ± 1.34
25	C 15	***Lyngbya aerugineo***	4.32 ± 0.49	6.64 ± 1.05	13.71 ± 0.61
26	C107	*Lyngbya cryptovaginata*	3.06 ± 0.35	6.82 ± 1.02	6.49 ± 0.29
27	C109	*Lyngbya putealis*	2.96 ± 0.42	6.21 ± 0.90	3.85 ± 0.08
28	C 28	***Gloeocapsa gelatinosa***	10.26 ± 0.32	8.89 ± 0.70	13.17 ± 2.86
29	C 35	***Gloeocapsa livida***	7.08 ± 0.57	4.40 ± 0.85	12.90 ± 0.63
30	C 52	*Gloeocapsa compacta*	9.11 ± 0.98	18.65 ± 0.83	1.60 ± 1.40
31	C 94	*Gloeocapsa quaternata*	8.40 ± 0.04	16.74 ± 0.93	4.37 ± 0.37
32	C 62	*Synechocystis aquatilis*	5.36 ± 0.29	15.90 ± 0.74	0.59 ± 0.10
33	C 67	*Synechococcus cedrorum*	3.61 ± 0.00	8.13 ± 0.71	1.56 ± 0.52
34	C 93	*Synechococcus elongatus*	5.77 ± 1.10	11.86 ± 0.70	8.15 ± 0.12
35	C 19	*Gloeothece rupestris*	4.82 ± 0.25	21.16 ± 1.32	3.10 ± 0.94
36	C 47	*Chroococus tenax*	4.76 ± 0.87	9.12 ± 0.75	2.14 ± 0.17
37	C 99	*Microcystis orissica*	4.55 ± 0.02	6.47 ± 3.50	9.68 ± 0.34
38	C108	*Chlorogloea fritschii*	3.05 ± 0.08	15.14 ± 0.80	6.54 ± 0.34
39	C113	*Tolypothrix tenuis*	8.90 ± 1.35	18.09 ± 0.95	3.84 ± 0.78
40	C111	*Pseudanabaena schmidlei. Robusta*	1.08 ± 0.09	1.84 ± 0.76	1.90 ± 0.50

Species selected for further study are given in bold letters

6.3.2.1c Lipids

Lipid content varied between 0.6-19.25% (Fig 6.15) and the maximum being in *Oscillatoria accuta* (C49), for which 19.25% of the total biomass was lipid. *Oscillatoria cortiana* (C7), *Oscillatoria salina* (C21), *Oscillatoria jasorvensis* (C23), *Oscillatoria willei* (C30), *Oscillatoria fremyi* (C32), *Oscillatoria limnetica* (C77), *Oscillatoria pseudogeminata* (C81), *Oscillatoria laete-virens minimum* (C85), *Oscillatoria accuminata* (C112), *Phormidium tenue* (C8), *Phormidium dimorphum* (C9), *Phormidium abronema* (C11), *Phormidium corium* (C13), *Lyngbya semiplena* (C34), *Lyngbya aerugineo* (C15), *Gloeocapsa gelatinosa* (C28), *and Gloeocapsa livida* (C35) were also composed of comparatively higher amount (more than 11%) of lipid.

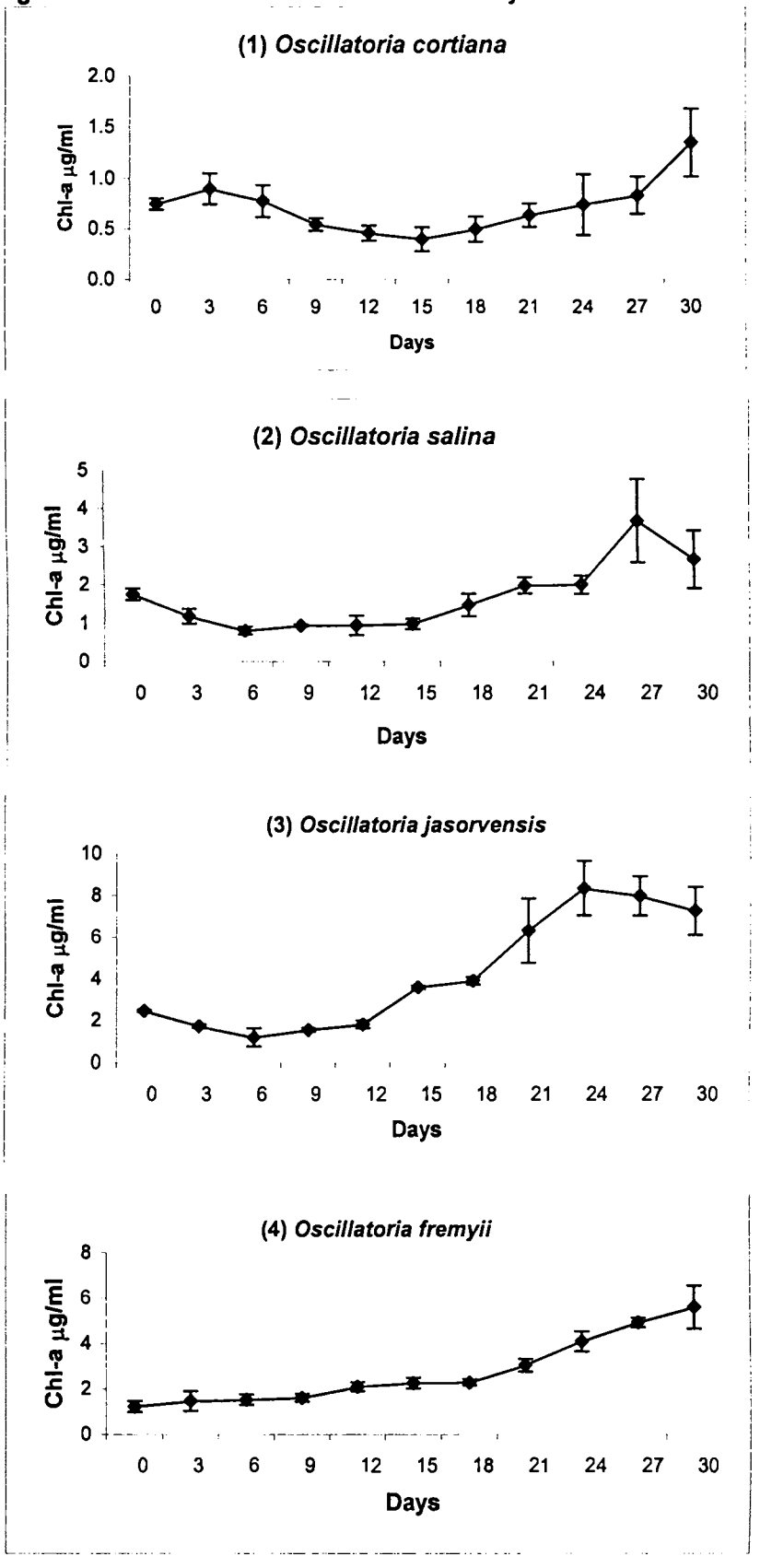
Species with high composition of all the three components *viz.* total sugars, proteins and lipids were *Oscillatoria pseudogeminata* (C81), *Phormidium dimorphum* (C9), *Phormidium corium* (C13) *and Lyngbya semiplena* (C34).

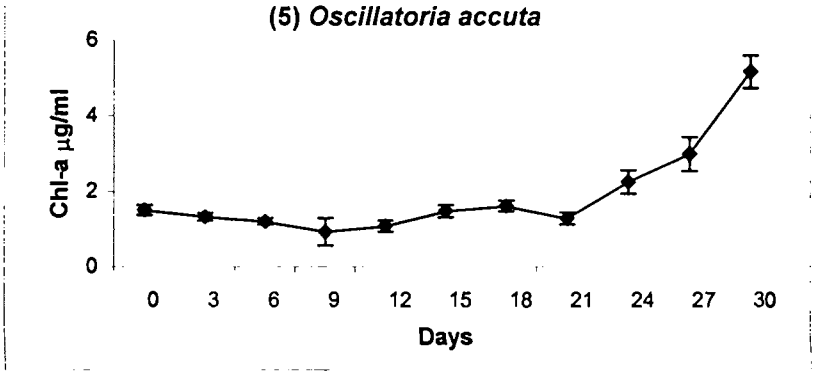
6.3.2.2 Growth profile

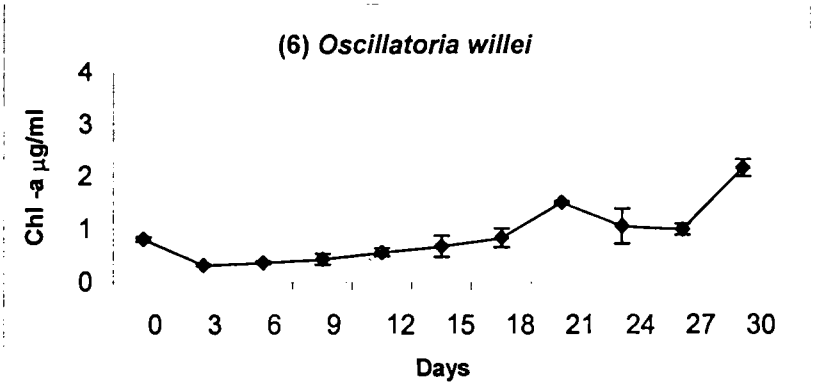
Twenty species of cyanobacteria with high composition of carbohydrates, proteins and lipids were selected for the growth characterization. The cultures were kept under optimum conditions. Growth was measured in terms of chlorophyll-a content. Figs 6.16 (1-20) and Tables 6.16 (1-20) (Appendix 3) depict growth characteristics of various species of cyanobacteria. From these results, the following inferences were made·

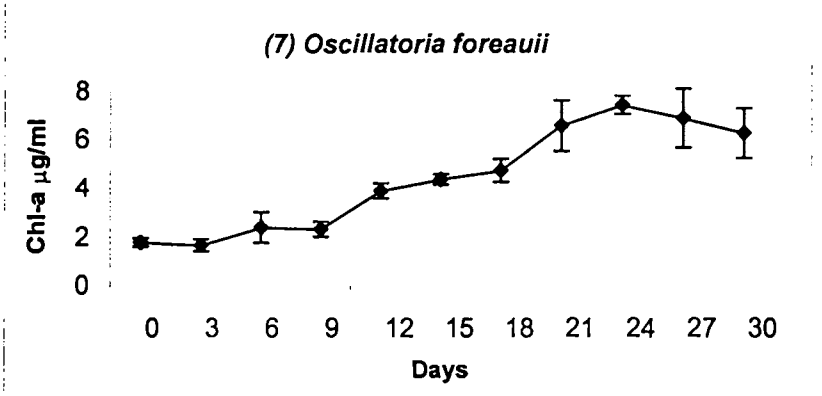
Out of ten *Oscillatoria sp.* studied, *O. laete-virens minimum* (C85), *O.jasorvensis* (C23) and *O. foreaui* (C72) showed maximum specific growth rate and the values were 0.301, 0.244 and 0.235day^{-1} respectively. The cultures were continuously growing upto 24th day and reached their zenith and then a diminution was observed.

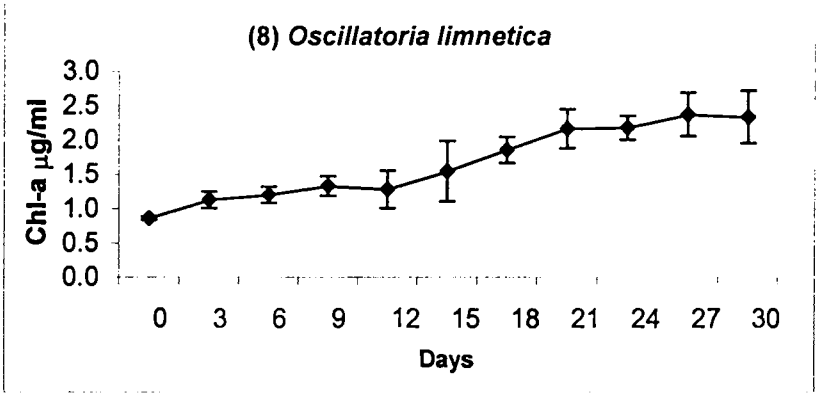
Fig 6.16 Growth characteristics of selected cyanobacterial strains



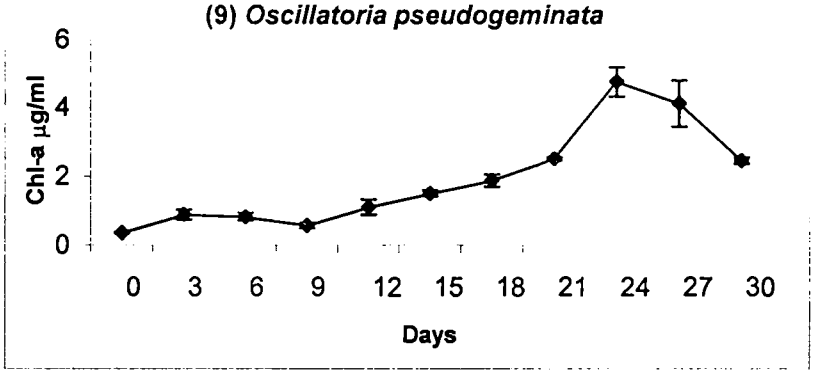

(5) Oscillatoria accuta
Chl-a µg/ml
0
2
4
6
0 3 6 9 12 15 18 21 24 27 30
Days


(6) Oscillatoria willei
Chl -a µg/ml
0
1
2
3
4
0 3 6 9 12 15 18 21 24 27 30
Days

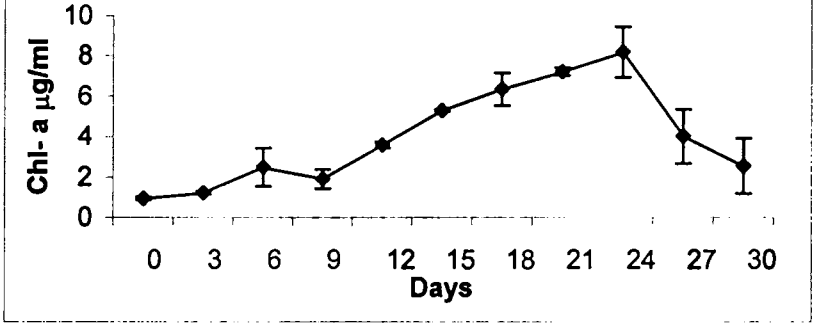

(7) Oscillatoria foreauii
Chl-a µg/ml
0
2
4
6
8
0 3 6 9 12 15 18 21 24 27 30
Days


(8) Oscillatoria limnetica
Chl-a µg/ml
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0 3 6 9 12 15 18 21 24 27 30
Days

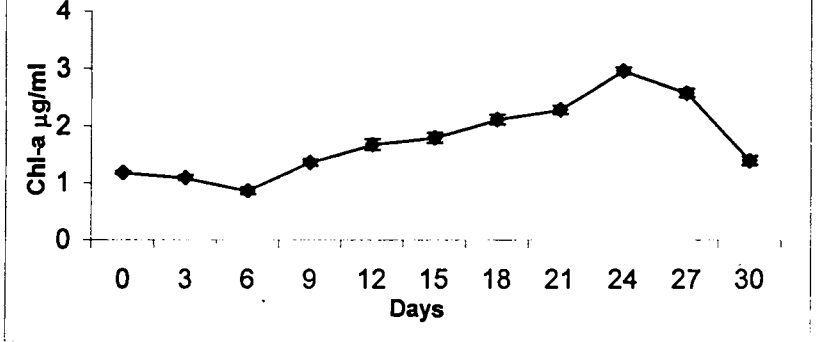
(9) *Oscillatoria pseudogeminata*



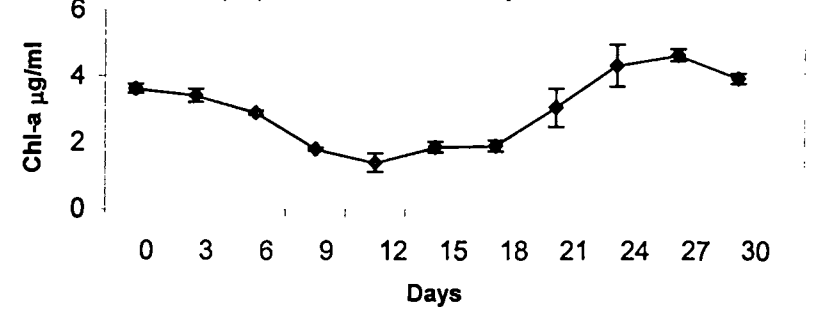
(10) *Oscillatoria leaetevirens minimum*

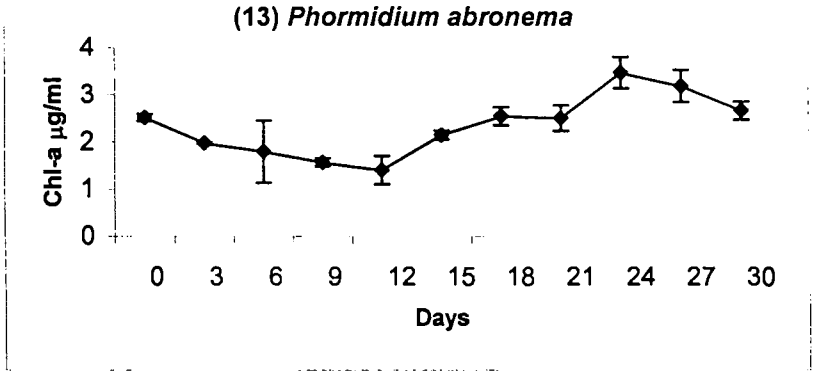


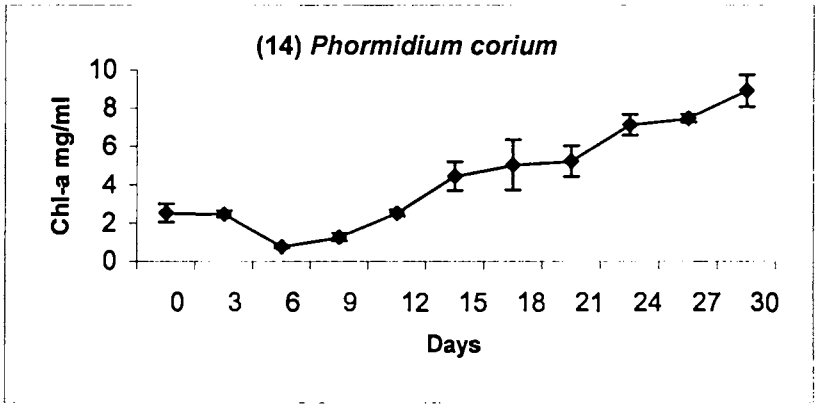
(11) *Phormidium tenue*

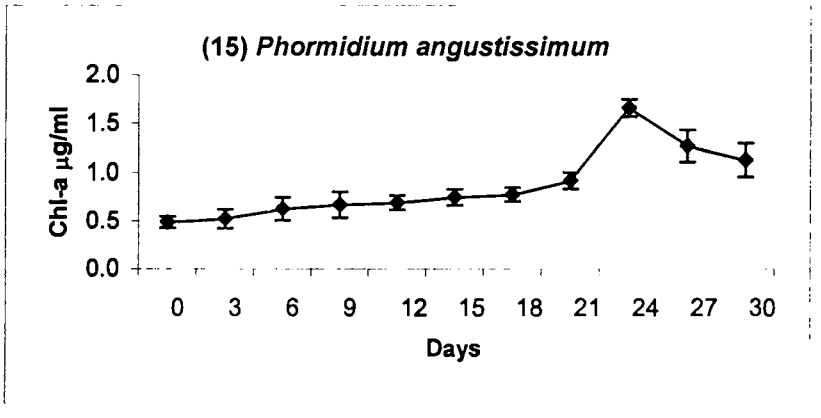


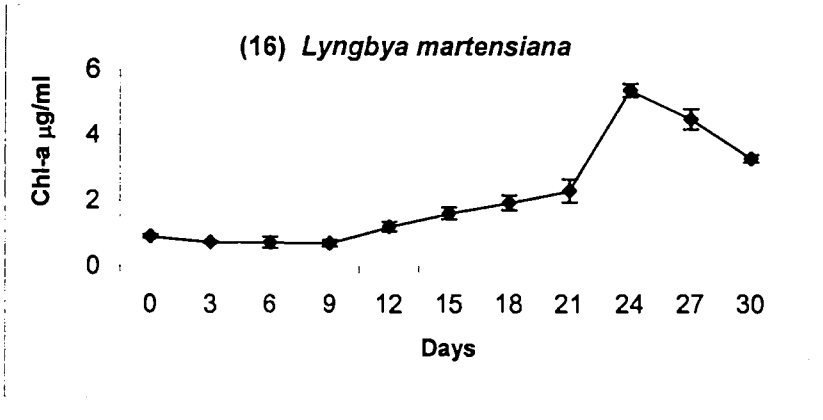
(12) *Phormidium dimorphum*

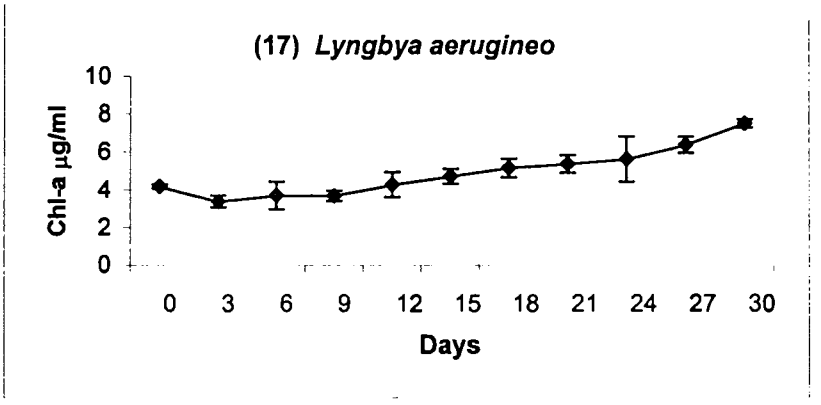


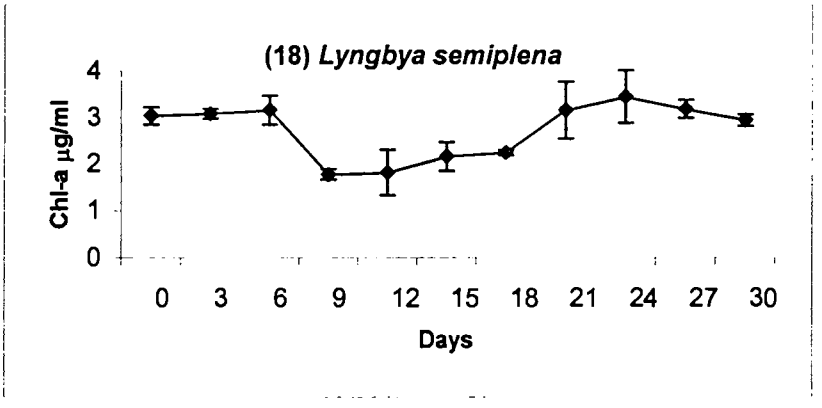

(13) Phormidium abronema
Chl-a µg/ml
0
1
2
3
4
0 3 6 9 12 15 18 21 24 27 30
Days

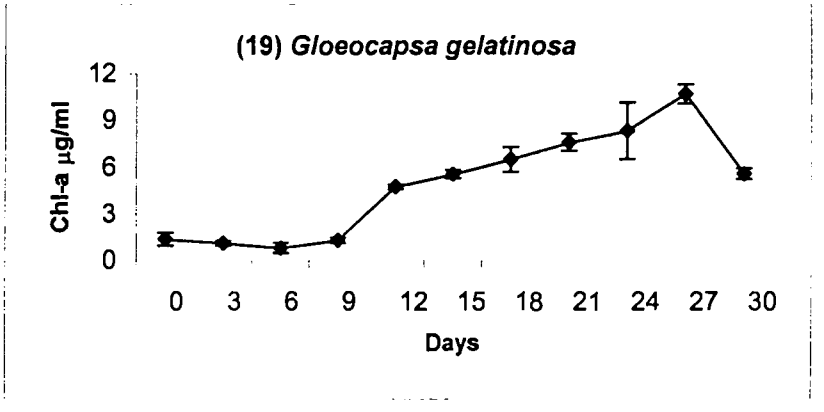

(14) Phormidium corium
Chl-a mg/ml
0
2
4
6
8
10
0 3 6 9 12 15 18 21 24 27 30
Days

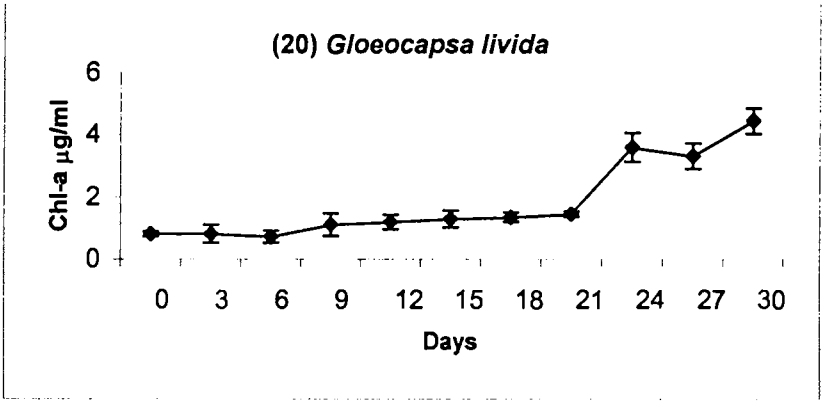

(15) Phormidium angustissimum
Chl-a µg/ml
0.0
0.5
1.0
1.5
2.0
0 3 6 9 12 15 18 21 24 27 30
Days


(16) Lyngbya martensiana
Chl-a µg/ml
0
2
4
6
0 3 6 9 12 15 18 21 24 27 30
Days


(17) Lyngbya aerugineo
Chl-a µg/ml
10
8
6
4
2
0
0 3 6 9 12 15 18 21 24 27 30
Days


(18) Lyngbya semiplena
Chl-a µg/ml
4
3
2
1
0
0 3 6 9 12 15 18 21 24 27 30
Days


(19) Gloeocapsa gelatinosa
Chl-a µg/ml
12
9
6
3
0
0 3 6 9 12 15 18 21 24 27 30
Days


(20) Gloeocapsa livida
Chl-a µg/ml
6
4
2
0
0 3 6 9 12 15 18 21 24 27 30
Days

Phormidium corium (C13) was the outstanding species in the genus *Phormidium* as it showed high specific growth rate, 0.213day^{-1}. *P. corium* showed continuous growth from the 6th day to the end of the experiment. Of the species selected from *Lyngbya*, *L. martensiana* (C1) showed excellent growth with a Kmax of 0.186 day^{-1}. But the species reached the log phase very late, i.e., beyond 9th day. In case of *Gloeocapsa, G. gelatinosa* (C28) was grown with the maximum specific growth rate of 0.345 day-1. *Gloeocapsa gelatinosa* and *Oscillatoria laete-virens minimum* (C85) showed maximum growth rate among the tested cyanobacteria.

6.3.2.3 Pigment composition

Percentage composition of pigments such as chlorophyll-a, carotenoids and phycobiliproteins of 20 species of selected cyanobacteria were analyzed. Figs 6.17 to 6.21 and Table 6.18 (Appendix 3) show the pigment composition in various species.

Chlorophyll- a

Out of 20 species studied, *Oscillatoria jasorvensis* (C23), *O. laetevirens minimum* (C85), *Phormidium corium* (C13) *and Gloeocapsa gelatinosa* (C28) were composed of more than 3% chlorophyll (Fig 6.17).

Carotenoids

Carotenoids were more in *O. fremyii* (C32), *Phormidium corium* (C13), *Phormidium angustissima* (C84), *Lyngbya aerugineo* (C15) *and Gloeocapsa gelatinosa* (C28) in which more than 0.6% could be obtained (Fig 6.18).

Phycobiliproteins

Phycocyanin was maximum in *Lyngbya semiplena* (C34) and in *Phormidium corium* (C13), it was 10 % of the weight, whereas, *Gloeocapsa gelatinosa* (C28), 7 % and *Oscillatoria foreaui* (C72), 5.5% and others were with less than 2.5% phycocyanin (Fig 6.19). *Lyngbya semiplena* (C34), *Phormidium corium* (C13), *Gloeocapsa gelatinosa* (C28) *Oscillatoria willei*

Fig 6.17 Chlorophyll- a content of selected cyanobacterial species

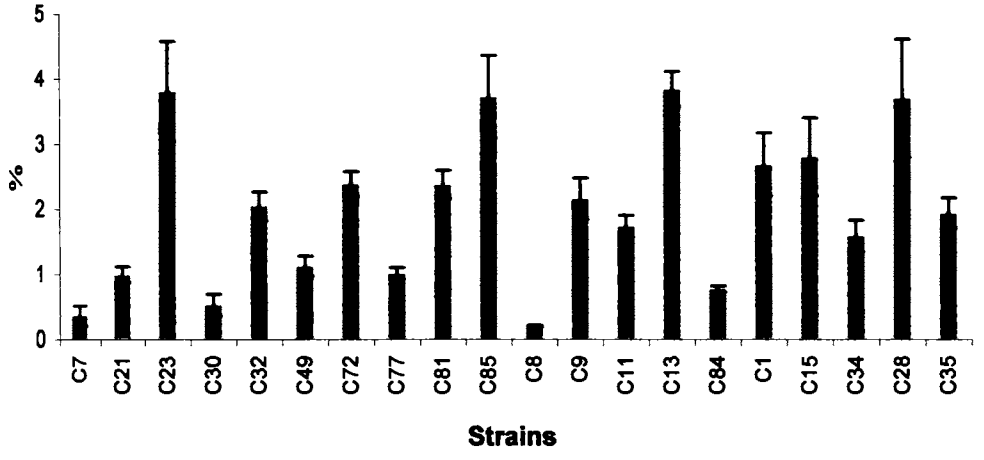


Fig 6.18 Carotenoid content of selected cyanobacterial species

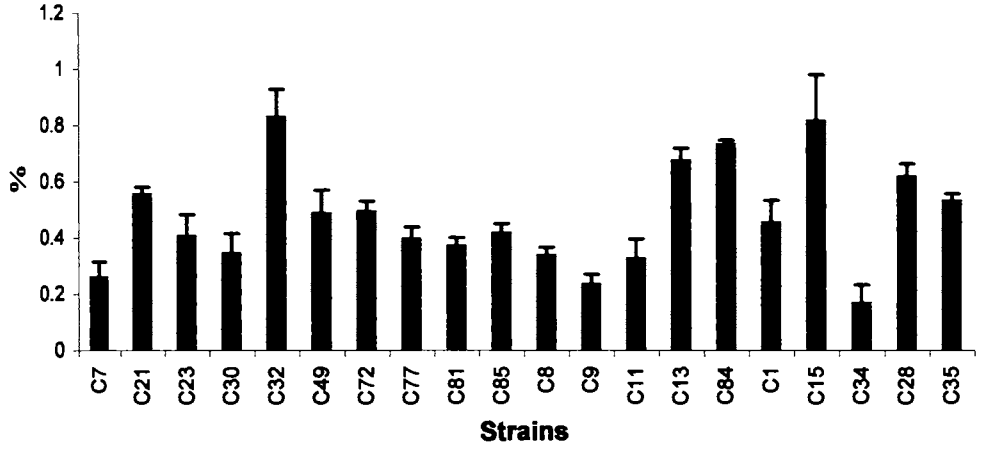


Fig 6.19 C- Phycocyanin content of selected strains

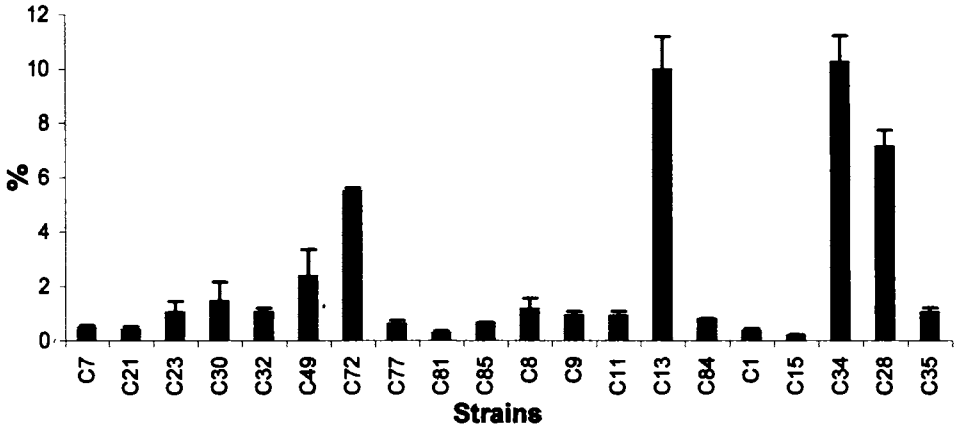


Fig 6.20 Allophycocyanin content of selected strains

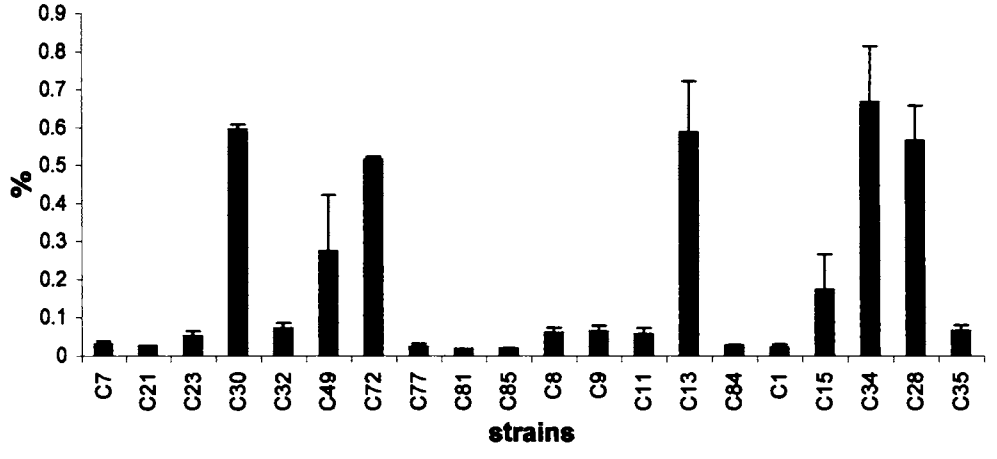
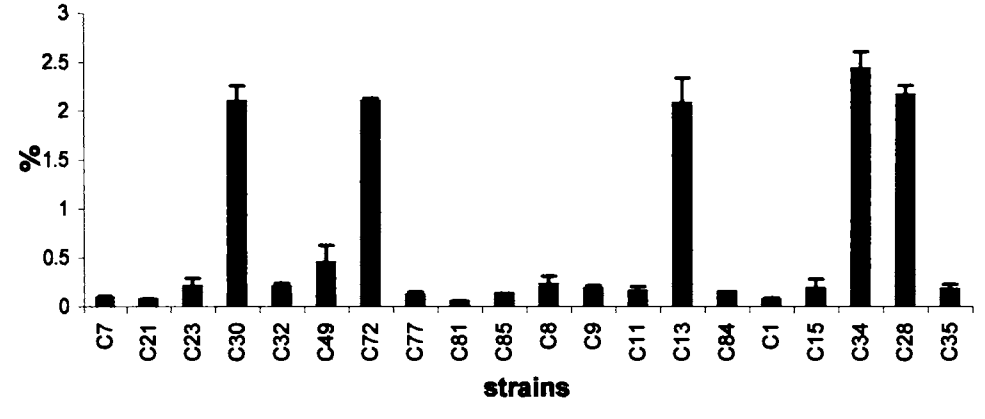


Fig 6.21 C- Phycoerythrin content of selected strains



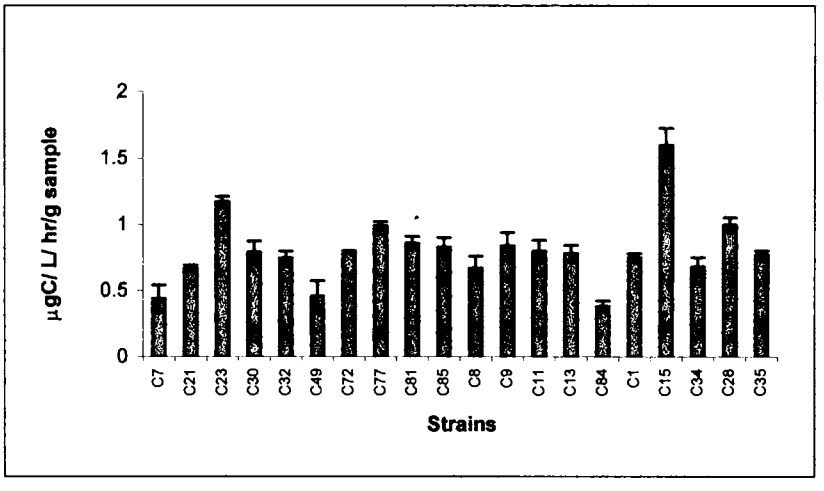
(C30) and *Oscillatoria foreaui* (C72) were composed of more than 0.5% allophycocyanin (Fig 6.20). Phycoerythrin was also present in high level in these strains where more than 2% of the weight was phycoerythrin (Fig 6.21)

From the analysis, it was found that the prominent pigments present in cyanobacteria are chlorophyll and phycobiliproteins whereas, carotenoids were comparatively less. The proportion of phycobiliproteins to chlorophyll was more than one in case of species like *O.cortiana* (C7), *O.willei* (C30), *O.accuta* (C49), *O.foreaui* (C72), *P.tenue* (C8), *P.corium* (C13), *P.angustissima* (C84), *L. semiplena* (C34) and *G. gelatinosa* (C28) as phycobiliproteins were more in these species.

6.3.2.4 Productivity

Production of organic substances by 20 different species of cyanobacteria grown in Allen and Nelson medium with salinity 20 ppt and temperature 28^0C were studied. The cells were harvested on 24^{th} day. Known weight of the samples were taken for the analysis and productivity was estimated in terms of µg C / L/ hr/ gram wet weight of sample. The results are presented in Fig 6.22 and Table 6.19 (Appendix 3). Gross production was high in *Lyngbya aerugineo* (C15) and *Oscillatoria jasorvensis* (C23).

Fig 6.22 Productivity of selected species of cyanobacteria



6. 4 Discussion

6.4.1 Optimisation of culture conditions

All the four species of cyanobacteria studied in the present investigation showed almost identical requirements of salinity and pH. Salinity upto 20 ppt was found to be optimum for all species. Growth was found to occur at all levels of salinity indicating that the species can tolerate wide fluctuations of salinity. This is in agreement with earlier studies reported on cyanobacteria (Kaushik and Sharma, 1997, Subramanian and Thajuddin, 1995 and Newby, 2002). However, higher salinity was found to be unsuitable for survival as it affects the protein content. Kaushik and Sharma (1997) studied the effect of salinity stress on the halotolerant forms such as *Nostoc linckia, Westiellopsis prolifica and Tolypothrix ceylonica* and found that there was a 3-5% reduction in total protein content when these forms were grown in 100mM NaCl. It was found that the growth of cyanobacteria in the presence of NaCl depends on the availability of nutrients and the rate and mode of carbon fixation (Atre, 1998).

Cyanobacteria showed a wide range of adaptability and flexibility in their response to pH. The pH of the medium plays an important role in culturing as it determines the solubility of CO_2 and minerals in the medium, which in turn, directly or indirectly influence the metabolism of the algae (Markl, 1977). Cyanobacteria vary, not only in their preference for different pH but also in their qualitative response which is dependent upon the composition and buffering capacity of the medium, amount of CO_2 dissolved, temperature and metabolic activity of the cell. Venkataraman (1972), Subramanian and Shanmugasundaram (1987) and Radha Prasanna *et al.* (1998) studied the pH tolerance of cyanobacteria. The organisms have been reported to grow well in a range of pH 7.5 - 10 (Gerolff *et al.*, 1950; Kratz and Myers, 1955; Okuda and Yamaguchi, 1956; Prasad *et al.*, 1978; Roger and Reynaud, 1979). However, there are reports of cyanobacteria growing at pH as low as 3.5

(Aiyer, 1965). In the present study, the strains exhibited broad tolerance to pH.

Growth kinetics of four cyanobacterial strains in various media proved that Allen and Nelson medium was significantly superior to other media in terms of both chlorophyll and growth and was very simple to prepare. All the media showed good growth except nitrogen-free medium in which no growth was observed. Unicellular species *Gloeocapsa livida* could grow well only in Allen and Nelson medium.

6.4.2 Biochemical composition, Growth kinetics, Pigment composition and Productivity

Cyanobacteria constitute a large proportion of the world's organic matter. They offer the most efficient means of fixing solar energy in the form of biomass. The cyanobacterium *Spirulina* has already been commercially exploited because of its merits *viz.* maximum yield and utility of cellular constituents (Borowitzka and Borowitzka, 1988). Therefore, it would be appropriate if the growth characteristics and biochemical composition of the presently investigated species could be compared with those of *Spirulina*. *Spirulina* strains from CFTRI, India were found to have protein content within the range of 40 – 55 % and total lipid content between 2 – 7 % (Tasneem Fatma *et al.*, 1999). Phycocyanin was reported in them between 10 and 19 % of the weight. In the present study, out of forty strains, *Phormidium dimorphum* (C9) contained total proteins of 39% of the dry weight, which was comparable to *Spirulina*, the reference species. Eighteen species were reported with more than 11% lipid content, which was very much greater than that of *Spirulina*. In the present study, phycocyanin was estimated as 10% of the weight in *Lyngbya semiplena* and *P. corium* (C13). Highest amount of carbohydrate (13.02%) was present in *Oscillatoria pseudogeminata* (C81) whereas, carbohydrate content of dried Spirulina (CFTRI) could be improved only upto 10% as per Venkataraman, 1989. Vaidya and Mehta, 1989 reported that the dried matter of blue green algae like *Anabaena* and *Nostoc* contained

total carbohydrates varying from 14.6% to 20.2%. When nitrogen fixing Cyanobacteria were grown under diazotrophic conditions, protein, carbohydrate, and lipid comprised 37-52%, 16-38% and 8-13% of the dry weight respectively (Vargas *et al.*, 1998). Kebede and Ahlgren (1996) found maximum chlorophyll content of 2.4% of dry weight in *S.platensis* and also reported that the ratio of carotenoids to chlorophyll-a was within the range of 0.27 to 0.64 in them. In the present investigation, of the twenty strains studied, four strains contained more than 3% of chlorophyll. Seven species were reported having the ratio of phycobiliprotein to chlorophyll greater than one. In *Phormidium tenue,* the ratio of carotenoids to chlorophyll was greater than one.

Since the culture conditions are known to change the biochemical composition of the algae (Ciferri, 1983), the constituents can be improved upon further by manipulating culture conditions. The overall results showed that, of the strains undertaken for the detailed biochemical evaluation, *Oscillatoria pseudogeminata* (C81), *Phormidium dimorphum* (C9), *Phormidium corium* (C13), *Gloeocapsa gelatinosa* (C28) *Oscillatoria foreauii* (C72) and *Lyngbya semiplena* (C34) are suitable for mass cultivation as a source of food/ feed as they showed not only good growth and productivity but also high levels of valuable biochemical constituents and pigments, very much similar to that of *Spirulina.*

The techniques of genetic manipulation are likely to be applied to cyanobacterial biotechnology in the near future. Genetic manipulation may lead to increased production of valuable primary and secondary metabolites, faster growth rates, tolerance of extreme growth conditions and so on (Lewin, 1983). Considerable variation in growth and cellular constituents justify the importance of strain selection over the complex and time-consuming genetic engineering process involved in strain improvement. Cyanobacteria represent an ecologically sensible form of technology, and it is hoped that, with enduring research and development, their enormous potential would be realized by all.

CHAPTER 7

Antioxidant activity of cyanobacteria against ethanol-induced peroxidative damage in *Oreochromis mossambicus* (Peters)

7.1 Introduction

Recently, there has been a tremendous increase in the interest in the value-added products of cyanobacteria. In the past, as drug research was focus'ed mostly on organisms such as actinomycetes, fungi and higher plants, scientists could only isolate known compounds or their close analogues. Cyanobacteria are sources of new compounds, not found in higher plants and traditional drug sources. In addition, they are recognized as a primary source of some exciting molecules found in marine invertebrates (Shimizu, 1996). At present, emphasis is on large-scale production of cyanobacterial metabolites of therapeutic value by culturing them under controlled conditions. In the present study, an attempt has been made to investigate the therapeutic effect of cyanobacteria with reference to its antioxidant property.

In biological systems, a number of powerful oxidising agents can cause damage to cells. Electron stealing molecules known as free radicals as well as highly reactive forms of oxygen such as singlet oxygen are produced in the body during various normal metabolic processes. Within the cells, free radicals can damage DNA, proteins and lipid membranes leading to ageing and a number of pathological conditions including carcinogenesis (Moody and Hassan, 1982), infant retinopathy (Phelps, 1987), atherogenesis (Steinberg *et al.*, 1989), age related muscular degeneration (Gerster, 1991) and other degenerative diseases including Alzheimer's disease, Parkinson's disease and Hodgkin's disease (Jenner, 1991).

Lipid peroxidation has been known to cause many pathological effects. Lipid peroxides are produced by oxidation of all the common unsaturated fatty acids found in naturally occurring lipids. Hydroperoxides of fatty acids are known to be one of the active oxygen species and are produced by enzyme catalysed oxidation, autooxidation and light-induced oxidation. The most important reaction in lipid peroxidation is the autooxidation of unsaturated fattyacids. Autooxidation is known to proceed by a radical chain reaction

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(Frankel, 1979). A lipid radical (L.) is formed from lipid (LH) in the initiation step. The lipid radical then reacts with oxygen to form a lipid peroxyl radical (LOO.), which reacts with an additional lipid molecule to give a lipid hydroperoxide (LOOH) in the propagation step. Lipid peroxyl radicals may combine to give nonradical compounds in the termination step.

Initiation:

$$LH \longrightarrow L.$$

Propagation:

$$L. + O_2 \longrightarrow LOO.$$

$$LOO. + LH \longrightarrow LOOH + L.$$

Termination:

$$LOO. + LOO. \longrightarrow \text{nonradical products}$$

Chemical compounds and reactions capable of generating reactive toxic oxygen species/free radicals are referred to as prooxidants. On the other hand, compounds and reactions disposing off these species, scavenging them, suppressing their formation or opposing their actions are called antioxidants. In a normal cell, there is an appropriate prooxidant : antioxidant balance. However, this balance can be shifted towards the prooxidant when production of oxygen species is increased or when levels of antioxidants are diminished. This state is called oxidative stress and can result in serious cell damage if the stress is massive or prolonged.

The antioxidant defence mechanism

Antioxidant defence mechanism is operated to detoxify or scavenge highly reactive oxygen species (ROS). It comprises of different types of functional components classified as first line, second line and third line defences.

Cells convene substantial resources to protect themselves from the *potentially damaging effects of reactive oxygen species. Several vitamins and micronutrients, which are active at quenching these free radical species or*

required for their enzymic detoxification, as well as enzymes, such as superoxide dismutases (SODs), glutathione peroxidases (GPXs) and catalases (CATs), constitute a first line of defence against ROS, and are generally refered to as primary antioxidants.

Superoxide dismutase mainly acts by quenching superoxide (O_2^-), active oxygen radical, produced in different aerobic metabolism. Catalase is a tetrameric enzyme, present in most of the cells, and acts by catalysing the decomposition of H_2O_2 to water and oxygen. Glutathione peroxidase (GPX) is a selenium containing enzyme which catalyses the reduction of H_2O_2 and lipid hydroperoxide (LO_2H), generated during lipid peroxidation, to water using reduced glutathione as substrate. In cytosolic and mitochondrial compartments the oxidized glutathione (GSSG) is reduced at the expense of NADPH by the ubiquitous flavin containing enzyme glutathione reductase. Glutathione-S-transferases (GSTs) are a group of detoxifying enzymes that catalyse the conjugation of reduced glutathione with a variety of compounds bearing suitable electrophilic centers in them (Boyland and Chasseaus, 1969).

The antioxidants belonging to second line of defence include glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E (mainly α-tocopherol), carotenoids, flavanoid and ubiquinol. Glutathione(GSH) is the most abundant non-protein thiol, synthesised in the liver and acts as a substrate for glutathione peroxidase enzyme. This also serves as a scavenger of different free radicals. Similarly β-carotene (Pro-vitamin A), vitamin C and vitamin E are some important scavenging antioxidant vitamins, which cannot be synthesised by most mammals including human beings, and therefore, are required from diet.

Third line antioxidants are a complex group of enzymes for repair of damaged DNA, damaged protein, oxidised lipids and peroxides and also to stop chain propagation of peroxyl lipid radical. E.g. Lipase, proteases, DNA repair enzymes, transferase, methionine sulphoxide reductase etc.

The detection of oxidative stress has relied largely on the quantification of compounds such as conjugated dienes, hydroperoxides as well as malondialdehyde (MDA), which are formed by the degradation of initial products of free radical attack.

Biological antioxidants are compounds that protect biological systems against the harmful effects of the free radicals (Palozza and Kinsky, 1992). Cyanobacteria, like all photosynthesising plants, are exposed to a combination of light and high oxygen concentrations, which lead to the formation of free radicals and other strong oxidizing agents (Dykens *et al.*, 1992). The elements of photosynthetic apparatus are especially vulnerable to photodynamic damage, because polyunsaturated fatty acids are important structural components of the thylakoid membrane (Sukenik *et al.*, 1993). The absence of such damage in cyanobacteria, in spite of the proximity of the photosynthetically produced oxygen and suitable targets within the photosynthetic apparatus, suggests that these cells have protective antioxidant compounds and mechanisms. The cyanobacterial cells possess an antioxidant defence system which causes removal of peroxides, free radicals such as superoxide anions ($O_2^{\cdot-}$) generated during photosynthesis and other metabolic process. (Karni *et al.*, 1984). Normally this system provides the conditions required for nitrogen fixation and other metabolic events by removing peroxides (Karni *et al.*, 1984).

Vitamin E and carotenoids are very important biological antioxidants that act in both preventive and radical scavenging roles. Blue green algae contain a significant amount of carotenoids, namely, β-carotene, lycopene and lutein. By their quenching action on reactive oxygen species, antioxidants carry intrinsic anti-inflammatory properties. Blue green algae also have specific anti-inflammatory properties because of their high phycocyanin content which can be up to 15% of dryweight. C-phycocyanin is a free radical scavenger (Bhat and Madyasta, 2000) and has significant hepatoprotective effect. Phycocyanin was shown to inhibit inflammation in mouse ears and

prevent acetic acid induced colitis in rats. This was attributed to the reduced formation of leukotriene B4, an inflammatory metabolite of arachidonic acid (Romay *et al.*, 1999).

In a study on rats, the cyanobacterium, *Aphanizomenon flos-aquae* was shown to decrease the plasma level of arachidonic acid. It contains significant amounts of the omega-3 alpha-linolenic acid, which had been shown to inhibit the formation of inflammatory prostaglandins and arachidonate metabolites (Kushak *et al.*, 2000).

Another carotenoid pigment, astaxanthin plays a role in many essential metabolic functions such as protection against oxidation and UV light, vision, immune response, pigmentation, reproduction and development. Ability of astaxanthin to quench singlet oxygen and scavenge free radicals has been demonstrated by a number of *in vitro* studies, which suggest that it can be ten times more powerful than other carotenoids and hundred times than vitamin E (Miki, 1991). The ubiquitous microalgae, *Haematococcus pluvialis* is able to accumulate the highest levels of astaxanthin in nature (Inborr, 1998). *Haematococcus pluvialis* can accumulate as much as 30g of astaxanthin kg^{-1} of dry biomass, a level that is 1000-3000 fold higher than that found in Salmon fillets.

The occurrence of phenolic compounds in *Spirulina* is well known and these groups of compounds possess antioxidant activity in biological systems (Ho, 1992 and Miranda *et al.*, 1998). Components with antioxidant activities can be found in other cyanobacteria also, and are poorly studied. There are still a large number of cyanobacteria, which need to be screened for this attribute.

The objective of the present study was to screen the cyanobacteria, isolated from Cochin estuary, for antioxidant activity by evaluating their influence on lipid peroxidation *in vitro*. The selected strains were further

analysed for their bioactivity against peroxidative damage in an animal model, a teleost fish, Tilapia (*Oreochromis mossambicus*).

The common freshwater teleost *O.mossambicus* is extensively cultured and its ability to withstand difficult environmental conditions including resistance to disease infections is well known (Huet, 1975; Nielson, 1975; Bardach, 1985). *O.mossambicus* is of particular interest because of its high food conversion rates and high growth rates.

In the present investigation, lipid peroxidation was induced in *O.mossambicus* by treating them with ethanol, a prooxidant to living beings. Alcoholism is now considered as one of the most serious addictive diseases prevalent in our society. Chronic alcohol intake produces a variety of physiological and physical changes in humans. The liver is one of the important organs, severely damaged as a result of chronic alcohol intake. There appears to be increasing evidence that alcohol toxicity may be associated with increased oxidative stress and free radical associated injury (Cederbaum, 1989). Generation of oxygen metabolites such as superoxide (O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) is believed to be important in the pathogenesis of alcoholic tissue injury (Thurman and Handler, 1989).

In acute ethanol intoxication, liver microsomal metabolism of ethanol was accompanied by hydroxyl radical ($^{\cdot}OH$) generation by cytochrome p 450 system. Hydroxyl radicals are responsible for the conversion of ethanol to acetaldehyde. The alcoholic liver injury appears to be generated by the effects of ethanol metabolism and the toxic effects of acetaldehyde, which may be mediated, by acetaldehyde altered proteins (Ishak *et al.*, 1991). Chronic alcohol intake is known to produce hypercholesterolemia, hyperlipidemia and hypertriglyceridemia (Baraona *et al.*, 1979; Loeper *et al.*, 1983). In chronic lipid accumulation the liver cells become fibrotic and leads to impaired liver function. Ethanol increases triglycerides and cholesterol levels thus inducing imbalance in lipid metabolism in liver, heart, kidney and other organs and this

could explain the reason for the increase in lipid peroxidation in these organs. Recently free radical induced lipid peroxidation has gained much importance because of its involvement in several pathologies (Salin and McCord, 1975; Rowley and Halliwell, 1983). Protection of cell membrane from lipid peroxidation has become a necessity to prevent, cure or delay the aforesaid diseases.

Effect of ethanol toxicity on various animals such as Siamese fighting fish (*Betta splendens*), rainbow trout and mice was studied by Galizio *et al.*, 1985; Rustay *et al.*, 2001.

The role of dietary antioxidants and their potential benefits in health and disease have attracted great attention (Kehrer and Smith, 1994). The use of synthetic antioxidants has decreased due to their suspected activity as promoters of carcinogenesis (Namiki, 1990). There is a current worldwide interest in finding new and safe antioxidants from natural sources to prevent oxidative deterioration of food and to minimise oxidative damage to living cells (Pratt, 1992). Therefore, screening and selection of cyanobacteria with high antioxidant property with a view to producing formulated feed offers tremendous scope.

Against this background, the present investigation was undertaken with the following objectives:

i. Screening and selection of cyanobacteria based on their antioxidant activity

ii. Maintenance of the test organism Tilapia (*Oreochromis mossambicus*)

iii. Determination of LC_{50} of ethanol in Tilapia

iv. Preparation of fish feed by incorporating live cyanobacteria

v. Evaluation of the effect of cyanobacteria against ethanol-induced lipid peroxidation in *Oreochromis mossambicus*.

7.2 Materials and Methods

7.2.1 Screening and selection of cyanobacteria based on their antioxidant activity *in vitro*

Twelve strains of cyanobacteria isolated from Cochin estuary, which showed better growth, productivity and elevated levels of valuable biochemical constituents and pigments were taken for determining their antioxidant activity *in vitro*. They were *Oscillatoria cortiana*(C7), *Oscillatoria fremyi*(C32), *Oscillatoria acuta*(C49), *Oscillatoria foreauii*(C72), *Oscillatoria limnetica*(C77), *Oscillatoria pseudogeminata*(C81), *Phormidium dimorphum*(C9), *Phormidium corium*(C13), *Phormidium angustissima*(C84), *Lyngbya semiplena*(C34), *Lyngbya aerugineo*(C15) and *Gloeocapsa gelatinosa*(C28).

7.2.1.1 Extraction of antioxidant substances

The cells were harvested at their exponential phase and extracted by continuous maceration with acetone (solvent: mycelia = 100:1, v/w) for 30 min in a separatory funnel. The solvent layer was separated by passing it through Whatmann no.1 filter paper and evaporated to dryness in vacuum (Yen and Chang, 2003).

7.2.1.2 Antioxidant activity

The antioxidant activity of the crude acetone extracts in inhibiting linoleic acid peroxidation was assayed using the thiocyanate method (Mitsuda *et al.*, 1966). 0.5ml methanol solution of the extract was mixed with linoleic acid emulsion (2.5 ml, 0.02M, pH 7.0) and phosphate buffer (2ml, 0.2M, pH7.0). The linoleic acid emulsion was prepared by mixing 0.28 g of Tween-20 as emulsifier and 50 ml phosphate buffer, and then the mixture was homogenised. The reaction mixture was incubated at 37 ^{0}C to accelerate oxidation. The levels of oxidation were determined by measuring the absorbance at 500 nm after reaction with ferrous chloride and ammonium

thiocyanate. The antioxidant activity was expressed as percentage of inhibition of peroxidation (IP%):

IP% = 1- (absorbance of sample at 500 nm)/(absorbance of control at 500 nm) x 100.

All tests were performed in triplicate and results averaged.

7.2.1.3 Statistical analysis

One-way ANOVA was done followed by Duncan's multiple range test in order to determine the significant differences between means. P values ≤0.05 were considered statistically significant.

7.2.2 Maintenance of test organisms

Oreochromis mossambicus with an average weight of 15±3 g and an average length of 11±3 cm were collected from nearby ponds in and around Cochin and from the culture ponds of Rice Research Institute, Vyttila. Collected fishes were immediately transported to the laboratory, in plastic carriers with the same pond water and were acclimated in dechlorinated waters in large tanks of 1000 L capacity. The water quality parameters were checked and controlled at the optimum level. Dissolved oxygen content was kept at 7.6mgl^{-1}; pH 7.5; temperature 26 ^{0}C and salinity at 0 ppt. The fishes were fed *ad libitum* with commercial feed from Higashimaru Pvt. Ltd. and were maintained in tanks for more than a week prior to the experiment. For experimentation the laboratory acclimated fishes were sorted into batches of eight each and kept in fiber tanks of 30 L capacity. Water exchange was done daily and the fishes were maintained with adequate aeration.

7.2.3 Determination of LC_{50} of ethanol in *O.mossambicus*

Bioassay test was carried out by the method described by Doudoroff *et al.*, 1953. For LC_{50} determination, the acclimated fishes of 8 numbers each were exposed to various concentrations (10,11,12,13,14,15,16,17,18,19 ml/L) of ethanol with dechlorinated tap water separately along with controls in

duplicates. Fishes were checked for mortality at every 24 hrs interval. The mortality of the fish in each concentration was recorded and percentage mortality was calculated on 96 hr exposure period and LC_{50} level was determined as per the method of Finney (1971) by doing probit analysis.

7.2.4 Preparation of fish feed by incorporating live cyanobacteria

Experimental feed was prepared from the commercially available feed from Higashimaru Pvt. Ltd. The feed pellets were well-powdered, mixed with adequate amount of water, autoclaved and then mixed thoroughly with 15% of live cyanobacteria, having high antioxidant property. Newly formulated diet was prepared in pellet form with the help of a laboratory pellet press and was allowed to air dry. Egg white,a natural binder was coated over the pellets in order to strongly bind all the components of the feed together.

Three experimental feeds namely, F28, F34 and F72 were prepared by mixing cyanobacterial strains, *Gloeocapsa gelatinosa* (C28), *Lyngbya semiplena* (C34) and *Oscillatoria foreaui* (C72) respectively. Control feed was also made without incorporating cyanobacteria (Plate 9).

7.2.5 Effect of cyanobacteria in lipid peroxidation *in vivo*

Comparison of antioxidant status of the alcohol exposed fish fed with experimental diet and those given control diet was done by determining the level of antioxidants and antioxidant enzymes in the tissues. In order to assess long term sublethal toxicity of ethanol to the fish $1/10^{th}$ of the LC_{50} value was selected for treatment. A set of fishes supplied with experimental diets, but not exposed to ethanol was also tested for their antioxidant status.

7.2.5.1 Experimental design

The test organism, *O.mossambicus* were divided into 8 separate groups of which 4 groups were exposed to sub-lethal concentration of ethanol by mixing with fresh water and the other 4 sets kept without exposing to

F 28

F 34

F 72

F C

Experimental Feeds

PLATE-9

ethanol. Each group consisted of six fishes and the whole experiment was designed as follows:

Group I : Control feed (fishes fed with control diet)

Group II : Control feed + Ethanol (Ethanol treated fishes fed with control diet)

Group III : F28 (Fishes fed with *Gloeocapsa gelatinosa* (C 28) incorporated diet)

Group IV : F28+Ethanol (Ethanol treated fishes fed with *Gloeocapsa gelatinosa* (C28) incorporated diet

Group V : F34 (Fishes fed with *Lyngbya semiplena* (C 34) incorporated diet)

Group VI : F34 + Ethanol (Ethanol treated fishes fed with *Lyngbya semiplena* (C34) incorporated diet)

Group VII: F72 (Fishes fed with *Oscillatoria foreaui* (C 72) incorporated diet)

Group VIII: F72 + Ethanol (Ethanol treated fishes fed with *Oscillatoria foreaui* (C72) incorporated diet)

The experimental animals were dosed for 21 days. Water exchange and ethanol dosage was done daily, so as to avoid any possible degradation or evaporation. They were fed on the same diet twice daily. The experimental set up for bioassay is shown in Plate 10.

7.2.5.2 Preparation of tissue homogenate for biochemical analysis

The fishes were killed by pithing (by damaging the brain and severing the spinal cord between the head and the trunk region using a sharp needle) after the experimental period (21 days) and the tissues *viz.* liver, gill, heart, muscle and kidney were removed from its body, wiped thoroughly using blotting paper to remove blood and other body fluids. Then they were washed, weighed and homogenised in ice-cold 0.1M Tris-HCl buffer of pH 7.4, using a

Experimental set up for Bioassay

PLATE-10

glass tissue homogeniser. The homogenate was centrifuged and supernatant was used for assessing lipid peroxidation.

7.2.5.3 Assessment of lipid peroxidation and antioxidant status of the fish

Lipid peroxidation was assessed in terms of thiobarbituric acid reactive substances (TBARS), hydroperoxides and conjugated dienes (CD). Antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-s-transferase and the non-enzymic antioxidant substance, glutathione in various tissues were determined. The concentrations of enzymes and glutathione were estimated and expressed per milligram of protein in the corresponding tissues and therefore protein content of the tissues was also determined. The following methods are used for the biochemical analysis.

7.2.5.3 a Estimation of superoxide dismutase (SOD)

Superoxide dismutase in different tissues was determined using the method of Kakkar *et al.*, (1984).

Reagents

0.33 M sucrose, n-butanol, 0.052µM sodium pyrophosphate buffer (pH 8.3), 90% ammonium sulphate, 0.0025M Tris HCl buffer (pH 7.4), 186 µM phenazine methosulphate (PMS), 300 µM Nitro blue tetrazolium (NBT), 780 µM NADH and glacial acetic acid.

Procedure

The tissue homogenate was subjected to differential centrifugation under cold conditions to obtain the cytosol fraction. Before estimating the activity, an initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulphate and this fraction was then dialysed against 0.0025 M Tris-HCl buffer (pH 7.4). The supernatant was used as the enzyme source. Assay mixture contained 1.2 ml of sodium pyrophosphate

buffer, 0.1 ml of PMS, 0.3 ml of NBT, 1.3 ml of distilled water and 0.1 ml of the enzyme source. The tubes were kept at 30^0C for 1 minute and then 0.2ml NADH was added and incubated at 30^0C for 90 sec and the reaction was stopped by the addition of 1 ml glacial acetic acid. Reaction mixture was shaken vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. The upper butanol layer was removed. Absorbance of the chromogen in butanol was measured at 560nm against n-butanol blank. A reagent blank without the enzyme source served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in one minute under the assay conditions and specific activity is expressed as units/mg protein.

7.2.5.3 b Estimation of Catalase (CAT)

Catalase level in different tissues was determined using the method of Machly and Chance (1955).

Reagents

0.01 M phosphate buffer (pH 7.0), 30 mM H_2O_2.

Procedure

The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction mixture contained 0.01 M phosphate buffer, 30 mM hydrogen peroxide and the enzyme extract prepared by homogenising the tissue in phosphate buffer and centrifuging at 5000 rpm. Specific activity was expressed as International Units(IU) / mg protein. 1 IU = change in absorbance/ min / extinction coefficient (0.021)

7.2.5.3 c Estimation of total reduced Glutathione (GSH)

Glutathione was measured by its reaction with DTNB to give a compound that absorbs at 412 nm as per Ellman's method (Ellman,1959) .

Reagents

a. Metaphosphoric acid: 1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30g NaCl in 100ml water
b. 0.4M Na_2HPO_4
c. DTNB reagent: 40mg DTNB in 100 ml of 1% trisodium citrate
d. Standard glutathione: 20mg reduced glutathione was dissolved in 100ml water

Procedure

1ml tissue homogenate was mixed with 4ml of metaphosphoric acid. The precipitate was removed by centrifugation. To 2ml of the supernatant, 2ml disodium hydrogen phosphate and 1ml of DTNB reagent were added. The absorbance was read within 2min at 412nm against a reagent blank (without tissue homogenate). A set of standards was also treated in the above manner. The amount of glutathione was expressed as µg/mg protein.

7.2.5.3 d Estimation of Glutathione peroxidase (GPX)

Glutathione peroxidase in different tissues was estimated by the method of Rotruck (1973).

Reagents

0.4 M Tris buffer (pH 7.0) 10mM sodium azide solution, 10% Trichloroacteic acid (TCA), 0.4 mM Ethylene diamine tetra acetic acid (EDTA), 0.2 mM Hydrogen peroxide (H_2O_2), 2 mM glutathione solution (GSH).

Procedure

To 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1 ml sodium azide and 0.5 ml tissue homogenate were added and mixed well. To this mixture 0.2 ml of GSH followed by 0.1 ml H_2O_2 solution were added. The contents were mixed and incubated at 37 °C for 10 minutes along with a control containing all reagents except tissue homogenate. After 10 minutes the reaction was arrested by the addition of 0.5 ml of 10% TCA. Tubes were centrifuged and the supernatant

was assayed for GSH as per the method given in the section 7.5.2.3c. The values were expressed as µg of GSH / min / mg protein.

7.2.5.3 e Estimation of Glutathione-S-Transferase (GST)

Glutathione-S-transferase in different tissue was determined using the method of Beutler (1986).

Reagents

0.5 M phosphate buffer (pH 6.5), 30mM of 1-chloro-2, 4- dinitro benzene (CDNB) in 95% ethanol, 30 mM glutathione (GSH).

Procedure

The reaction mixture containing 1ml of phosphate buffer, 0.1ml CDNB, and 1.8ml distilled water was taken in the control tube and 1ml phosphate buffer, 0.1ml CDNB, 0.1ml tissue extract and 1.7ml distilled water were taken in the sample test tubes. Then the tubes were incubated at 37 ^{0} C for 5 minutes. After the incubation, 0.1ml of reduced glutathione was added to all the tubes. Increase in absorbance was noted at 340 nm for 5 minutes in a quartz cuvette of 1 cm path length in a UV-visible spectrophotometer. Values were expressed in µmoles of CDNB complexed / min/ mg protein. The extinction coefficient between CDNB-GSH conjugate and CDNB is 9.6 mM^{-1} cm^{-1}.

7.2.5.3 f Estimation of Glutathione reductase (GR)

Glutathione reductase was estimated by the method of Bergmeyer *et al.* (1974).

Reagents

0.067 M phosphate buffer (pH 6.6), 0.06% NADPH, 15mM EDTA, 1.15% glutathione (GSSG)

Procedure

The decrease in absorbance of the solution containing glutathione (GSSG) (oxidized), NADPH, EDTA and phosphate buffer was noted for 3-5 minutes at 340nm using a UV-visible spectrophotometer. The controls were run with distilled water instead of GSSG. Enzyme activity was expressed as units / mg protein. One unit is defined as the change in absorbance /minute

7.2.5.3 g Estimation of Hydroperoxides

Hydroperoxides were estimated by the method of Mair and Hall (1977).

Reagents

Potassium iodide (0.80 g/ml), 0.5% cadmium acetate

Procedure

1 ml of the tissue homogenate was mixed thoroughly with 5 ml of chloroform: methanol (2:1) followed by centrifugation at 1000g for 5 minutes to separate the phases. 3ml of the lower chloroform layer was recovered using a syringe and placed in a test tube and dried in a 45 ^{0}C water bath under a stream of Nitrogen. 1 ml of acetic acid: chloroform (3:2) mixture followed by 0.05 ml of KI was quickly added and the test tubes were stoppered and mixed. The tubes were placed in dark at room temperature for exactly 5 minutes followed by the addition of 3 ml of cadmium acetate. The solution was mixed and centrifuged at 1000 g for 10 minutes. The absorbance of the upper phase was read at 353 nm against a blank containing the complete assay mixture except the tissue homogenate. Molar extinction coefficient of Hydroperoxides is 1.73 x 10^{4} M^{-1} cm^{-1}. The values were expressed as millimoles / 100 g wet wt. of tissue.

7.2.5.3 h Estimation of Conjugated Dienes (CD)

The concentration of conjugated dienes was estimated according to the method of Retnagal and Ghoshal (1966).

Procedure

Membrane lipids were extracted and evaporated to dryness as described for the iodometric assay for hydroperoxides. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance at 233nm was determined against a cyclohexane blank. Molar extinction coefficient of conjugated dienes is 2.52 x 10^4 M^{-1} cm^{-1}. The values were expressed as millimoles / 100 g wet wt. tissue.

7.2.5.3 i Estimation of Malondialdehyde (MDA)

Malondialdehyde was estimated by the method of Nihaeus and Samuelson (1958).

Reagents

TCA-TBA-HCl reagents: 15% (w/v) Trichloroacetic acid. 0.375 % (w/v) Thiobarbituric acid (TBA) in 0.25 N HCl, 0.1 M Tris-HCl buffer (pH 7.5).

Procedure

The tissue homogenate of different tissues were prepared in Tris HCl buffer and was combined with thiobarbituric acid and mixed thoroughly and heated for 15 minutes in a boiling water bath. It was then cooled and centrifuged for 10 minutes at 600 g. The absorbance of the sample was read spectrophotometrically at 535 nm against a reagent blank that did not contain tissue extract. The extinction coefficient for malondialdehyde is $1.56x10^5$ $M^{-1}cm^{-1}$. The values were expressed as millimoles / 100 g wet wt. tissue.

7.2.5.3 j Estimation of protein

Protein was estimated by the method of Lowry *et al.* (1951), using Bovine Serum Albumin (BSA) as standard protein.

Reagents

a. Alkaline copper reagent

i. Reagent A: 2% Na_2CO_3 in 0.1N NaOH

ii. Reagent B: 0.5% $CuSO_4.5H_2O$ in 2% aqueous solution of sodium potassium tartarate

The alkaline copper reagent was made by mixing 50 ml of reagent A and 1 ml of reagent B.

b. Folin's reagent

Folin Ciocalteau reagent is commercially available which is diluted with distilled water in the ratio 1:2.

Procedure

Graded volumes (0.02 - 0.1mg /ml) of protein solutions were pipetted out into a series of test tubes and made up to 1ml using distilled water. 0.25 ml of the test solution was pipetted out into a series of test tubes and made up to 1 ml using distilled water. 5 ml alkaline copper reagent was pipetted out in to all the tubes. It is mixed well and allowed to stand at room temperature for 10 minutes, 0.5 ml Folin's reagent was added to all the tubes, mixed well and incubated at room temperature for 30minutes. The absorbance was read at 660nm.

7.2.5.4 Statistical analysis

Statistical evaluation was done using two-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the SPSS statistical software package version 10.0. The level of significance was set at $p< 0.05$.

7.3 Results

7.3.1 Screening and selection of cyanobacteria based on their antioxidant activity *in vitro*

Antioxidant activities of 12 strains of cyanobacteria in the lipid peroxidation system were determined by the thiocyanate method and the results are shown in Fig 7.1 and Table 7.1. *Gloeocapsa gelatinosa* (C28) exhibited 64% inhibition of linoleic acid peroxidation (IP%) and *Oscillatoria*

Fig 7.1 Antioxidant activity of twelve strains of cyanobacteria in the lipid peroxidation system

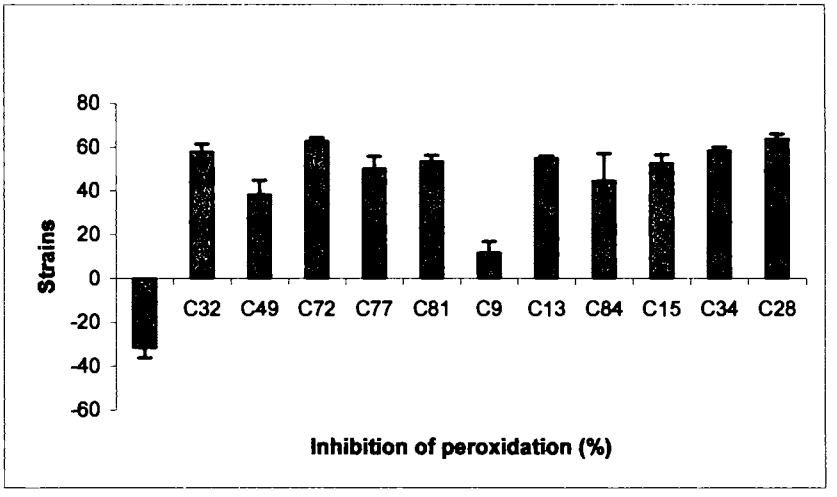

Table 7.1 Antioxidant activity of cyanobacteria in the lipid peroxidation system

Strains	Cyanobacteria	% IP
C7	*Oscillatoria cortiana*	-31.77 ± 4.4782 [a]
C32	*Oscillatoria fremyii*	57.84 ± 3.5515 [ef]
C49	*Oscillatoria acuta*	38.31 ± 6.4347 [c]
C72	***Oscillatoria foreaui***	**62.75** ± 1.5187 [f]
C77	*Oscillatoria limnetica*	50.2 ± 5.532 [de]
C81	*Oscillatoria pseudogeminata*	53.47 ± 2.6792 [ef]
C9	*Phormidium dimorphum*	11.76 ± 4.9712 [b]
C13	*Phormidium corium*	54.9 ± 1.033 [ef]
C84	*Phormidium angustissima*	44.55 ± 12.429 [cd]
C15	*Lyngbya aerugineo*	52.53 ± 3.9085 [e]
C34	***Lyngbya semiplena***	**58.36** ± 1.5304 [ef]
C28	***Gloeocapsa gelatinosa***	**63.69** ± 2.2301 [f]

Values with the same superscripts do not vary significantly ($p<0.05$)
Species given in bold letters were selected for bioassay studies

foreaui (C72) exhibited the IP% of 63%. *O. fremyii*(C32), *O. limnetica* (C77), *O. pseudogeminata* (C81), *Phormidium corium* (C13) and *Lyngbya aerugineo*(C15) showed more than 50% inhibition of peroxidation whereas *O.cortiana* (C7) displayed negative IP% as the absorbance of sample at 500nm was higher than that of control indicating that it could not inhibit but augment lipid peroxidation. One-way ANOVA followed by Duncan's analysis revealed that C28 and C72 displayed significantly high activity in the linoleic acid peroxidation system followed by C13, C32, C34 and C81 which did not vary each other significantly and C7 exhibited significantly least value of IP%. Based on these results, three strains were selected for further study with respect to their antioxidant property and are *Gloeocapsa gelatinosa* (C28), *Lyngbya semiplena* (C34) and *Oscillatoria foreaui* (C72). They have been demonstrated to possess significant antioxidant activity, thereby suggesting potential use as a value added ingredient for stabilising food matrices against lipid peroxidation reactions.

7.3.2 Determination of LC_{50} of ethanol in Tilapia

Lethal toxicity studies give information about the relative lethality of a toxicant. LC_{50} test was designed to determine the highest concentration of ethanol that was sufficient to kill 50% of *O.mossambicus*. In the present investigation, the effect of various concentrations of ethanol on *O.mossambicus,* as a function of different exposure periods indicated that the mortality of fish was dose and duration dependent. On exposing 8 fishes to different concentrations of ethanol (10 – 20 ml/L), it was found that at 15ml/L dose, 50% of the fishes were dead within 96 hrs. (Table 7.2). As per probit analysis, LC_{50} was recorded at 14.61ml/L. The 95% confidence limit ranged between 13.95 and 15.27 ml/L (Appendix 4).

The fish appeared lethargic after exposure to lethal concentration of ethanol. The exposed fish showed erratic movements. The other signs of toxicity such as loss of equilibrium and gradual onset of inactivity were also

observed. Discharge of large quantity of mucous substance through the mouth was also noticed which caused the water turbid.

Table 7.2 Mortality of *O.mossambicus* at different concentrations of ethanol after 96 hr. exposure period

Sl. No.	Ethanol concentration (ml/L)	No. of fishes exposed	% Mortality	LC_{50} value
1	Control	8	0.0	
2	10	8	0.0	
3	11	8	0.0	
4	12	8	12.5	
5	13	8	25.0	
6	14	8	37.5	
7	15	8	50.0	**15 ml/L**
8	16	8	75.0	
9	17	8	87.5	
10	18	8	100.0	
11	19	8	100.0	
12	20	8	100.0	

7.3.3 Effect of cyanobacteria in lipid peroxidation *in vivo*

The activities of the antioxidant enzymes (SOD, CAT, GPx, GST and GR) the level of antioxidant (glutathione) and the concentration of the lipid peroxidation products (hydroperoxides, CD and MDA) in the liver, gill, kidney, heart and muscle tissues of *O.mossambicus* subjected to sublethal concentration of ethanol (1.5ml/L) and fed with cyanobacterial feed for a time period of 21 days are presented in Tables 7. 3 to 7.11 and Figures 7.2 to 7.10. Data are expressed as mean ± S.D. of six fishes in each group.

7.3.3a Superoxide dismutase (SOD) activity of *O.mossambicus*

Two-Factor ANOVA and further comparisons by Duncan's multiple range analysis revealed that SOD activity was significantly increased ($P<0.05$)

Fig 7.2 Activity of SOD in various tissues of *O.mossambicus* treated with ethanol and fed with experimental feed

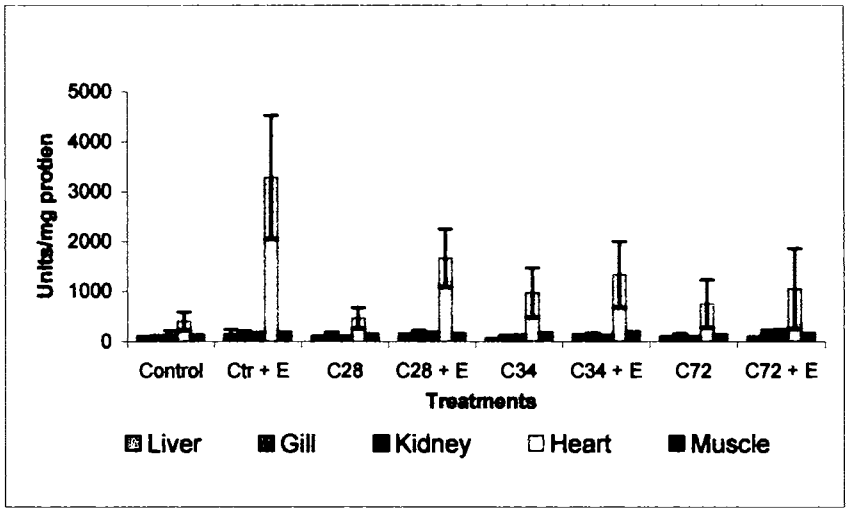

Table 7.3 Effect of cyanobacterial feed on ethanol-induced SOD activity in *O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	90.88 ± 18.68 $^{A\,a}$	95.28 ± 20.11 $^{A\,a}$	136.03 ± 81.11 $^{A\,a}$	407.68 ± 181.20 $^{B\,a}$	112.45 ± 18.89 $^{A\,a}$
Ctr + E	152.22 ± 92.30 $^{A\,e}$	176.14 ± 39.69 $^{A\,e}$	147.75 ± 39.56 $^{A\,e}$	3286.30 ± 1239.67 $^{B\,e}$	153.19 ± 41.07 $^{A\,e}$
C28	91.25 ± 23.77 $^{A\,a}$	156.47 ± 37.87 $^{A\,a}$	81.83 ± 32.05 $^{A\,a}$	469.02 ± 209.33 $^{B\,a}$	110.67 ± 43.50 $^{A\,a}$
C28 + E	107.74 ± 47.77 $^{A\,d}$	177.26 ± 46.64 $^{A\,d}$	147.17 ± 44.94 $^{A\,d}$	1667.62 ± 579.78 $^{B\,d}$	134.79 ± 24.59 $^{A\,d}$
C34	59.38 ± 5.64 $^{A\,abc}$	113.08 ± 11.84 $^{A\,abc}$	107.52 ± 28.32 $^{A\,abc}$	976.57 ± 494.45 $^{B\,abc}$	140.77 ± 38.32 $^{A\,abc}$
C34 + E	105.71 ± 40.31 $^{A\,cd}$	146.15 ± 28.76 $^{A\,cd}$	112.63 ± 20.96 $^{A\,cd}$	1336.28 ± 660.19 $^{B\,cd}$	161.62 ± 38.04 $^{A\,cd}$
C72	73.61 ± 25.80 $^{A\,ab}$	141.12 ± 15.11 $^{A\,ab}$	84.62 ± 19.69 $^{A\,ab}$	755.72 ± 478.51 $^{B\,ab}$	116.88 ± 23.88 $^{A\,ab}$
C72 + E	76.78 ± 23.23 $^{A\,bcd}$	177 ± 59.93 $^{A\,bcd}$	169.06 ± 80.82 $^{A\,bcd}$	1057.32 ± 801.76 $^{B\,bcd}$	145.80 ± 24.49 $^{A\,bcd}$

Values are expressed as units / mg protein
One unit is defined as the amount of enzyme, which gives 50% inhibition of the formazan formation/minute

Values with the same superscripts do not vary significantly ($p<0.05$)

in group II (control feed + ethanol treated) when compared to all other groups. On comparing all the ethanol treated groups, there was a significant increase in SOD activity in the order, group VIII (F72 + Ethanol) < group VI (F34 +Ethanol) < group IV (F28 + Ethanol) which in turn showed significantly lower ($P<0.05$) activity than group II (control feed + ethanol) and therefore it was understood that F72 could reduce the enhanced SOD activity due to ethanol exposure, more effectively. There was no significant difference between the groups, which were not exposed to ethanol. i.e., the SOD activities showed by F28, F34 and F72 fed groups were similar to that of ethanol unexposed control group. There was no significant difference between the SOD activities of various tissues with the exception of heart, which showed significantly high ($P<0.05$) activity (Table 7.3 and Fig 7.2).

7.3.3b Catalase (CAT) activity of *O.mossambicus*

Two-way analysis of variance and subsequent comparison by Duncan's multiple analysis revealed that there was no significant difference ($P<0.05$) between various treatments with the exception of group II (control feed + ethanol) which showed significantly high activity. It was clear that even though ethanol exposure increased catalase activity in control groups, the experimental feed could suppress its level bringing it to that of normal fish. The cyanobacteria-treated groups without ethanol exposure showed similar activity to that of control. Significant difference was noted between tissues with the exception of gill and heart. There was a significant decrease in catalase activity in the order liver > kidney > gill and heart > muscle (Table 7.4 and Fig 7.3).

7.3.3c Level of Glutathione (GSH)

Two-factor ANOVA and subsequent analysis showed that glutathione level significantly increased ($P<0.05$) in group II (control feed + ethanol) when compared to all other groups. F28 and F34 fed fishes among the ethanol treated groups (group VI and VI) showed significantly lower glutathione than F72 + ethanol treated ones (group VIII) which in turn showed lower value

Fig 7.3 Activity of Catalase in various tissues of *O.mossambicus* treated with ethanol and fed with experimental feed

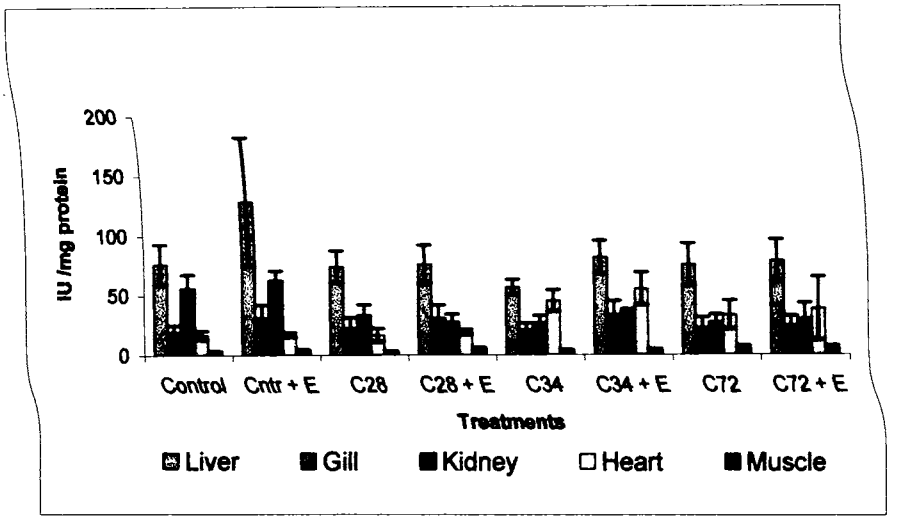

Table 7.4 Effect of cyanobacterial feed on ethanol-induced catalase activity in *O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	76.15 ± 16.68^{Da}	19.54 ± 5.49^{Ba}	55.88 ± 11.7^{Ca}	16.81 ± 3.6^{Ba}	2.07 ± 1.42^{Aa}
Ctr + E	128.55 ± 53.84^{Db}	31.24 ± 10.9^{Bb}	62.77 ± 8.21^{Cb}	17.25 ± 1.81^{Bb}	3.52 ± 1.27^{Ab}
C28	74.34 ± 13.41^{Da}	22.73 ± 8.68^{Ba}	33.17 ± 9.19^{Ca}	16.91 ± 5.45^{Ba}	2.85 ± 0.7^{Aa}
C28 + E	76.30 ± 15.87^{Da}	30.95 ± 11.2^{Ba}	27.75 ± 6.5^{Ca}	19.50 ± 2^{Ba}	5.18 ± 0.96^{Aa}
C34	56.98 ± 6.39^{Da}	21.10 ± 5.33^{Ba}	26.63 ± 6.44^{Ca}	45.64 ± 8.96^{Ba}	3.21 ± 1.03^{Aa}
C34 + E	81.99 ± 13.8^{Db}	33.93 ± 11.4^{Bb}	36.40 ± 1.91^{Cb}	55.54 ± 13.76^{Bb}	3.69 ± 1.09^{Ab}
C72	75.70 ± 17.77^{Da}	22.96 ± 8.54^{Ba}	26.99 ± 6.88^{Ca}	33.42 ± 12.06^{Ba}	5.59 ± 1.81^{Aa}
C72 + E	78.91 ± 17.7^{Da}	24.66 ± 7.79^{Ba}	29.69 ± 13.87^{Ca}	38.68 ± 26.72^{Ba}	5.64 ± 1.74^{Aa}

One IU = Change in absorbance at 230 nm / min, Extinction coefficient = 0.021

Values with the same superscripts do not vary significantly ($p<0.05$)

Fig 7.4 Level of Glutathione in various tissues of *O.mossambicus* treated with ethanol and fed with experimental feed

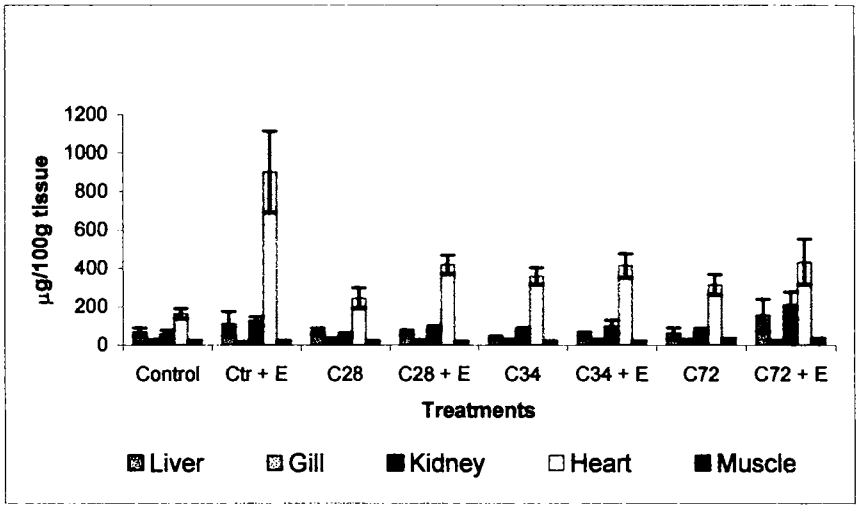

Table 7.5 Effect of cyanobacterial feed on ethanol-induced Glutathione level in *O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	66.57 ± 21.57 ^{Ba}	24.28 ± 3.24 ^{Aa}	53.89 ± 21.6 ^{Ba}	163.09 ± 26.02 ^{Ca}	23.97 ± 2.54 ^{Aa}
Ctr + E	109.19 ± 65.33 ^{Be}	17.42 ± 0.73 ^{Ae}	124.11 ± 23.59 ^{Be}	901.51 ± 210.3 ^{Ce}	24.02 ± 2.66 ^{Ae}
C28	70.71 ± 17.14 ^{Bab}	33.07 ± 2.99 ^{Aab}	50.57 ± 12.21 ^{Bab}	244.84 ± 53.89 ^{Cab}	23.37 ± 0.92 ^{Aab}
C28 + E	63.66 ± 13.37 ^{Bc}	25.71 ± 2.08 ^{Ac}	86.91 ± 11.95 ^{Bc}	420.13 ± 49.57 ^{Cc}	20.31 ± 1.3 ^{Ac}
C34	37.22 ± 8.04 ^{Bbc}	26.52 ± 3.49 ^{Abc}	75.39 ± 13.28 ^{Bbc}	359.78 ± 44.27 ^{Cbc}	22.99 ± 0.69 ^{Abc}
C34 + E	51.67 ± 14.95 ^{Bc}	25.48 ± 4.48 ^{Ac}	96.71 ± 32.19 ^{Bc}	415.39 ± 62.2 ^{Cc}	21.64 ± 2.58 ^{Ac}
C72	60.79 ± 28.26 ^{Bbc}	26.68 ± 1.96 ^{Abc}	73.50 ± 13.18 ^{Bbc}	316.03 ± 53.38 ^{Cbc}	38.11 ± 0.52 ^{Abc}
C72 + E	155.20 ± 84.55 ^{Bd}	22.93 ± 1.91 ^{Ad}	207.51 ± 70.38 ^{Bd}	434.55 ± 119.1 ^{Cd}	35.80 ± 1.73 ^{Ad}

Values are expressed as µg / 100 g tissue

Values with the same superscripts do not vary significantly ($p<0.05$)

than control + ethanol groups (group II). Glutathione content in group III (F28) was almost same as that of control whereas in group V and VII (F34 and F72) its level was higher than that in the control. There was no significant difference between gill and muscle and between liver and kidney whereas heart showed significantly high glutathione content (Table 7.5 and Fig 7.4).

7.3.3d Glutathione peroxidase (GPx)

Ethanol exposed control group (Group-II) showed significantly high activity of glutathione peroxidase when compared to all other groups. The experimental feeds could repress increased peroxidase activity due to ethanol exposure when all the ethanol treated groups were considered. There was significant difference between peroxidase activity in various tissues of which muscle showed least activity whereas, heart showed the maximum (Table 7.6 and Fig 7.5).

7.3.3e Glutathione -s- transferase (GST)

GST showed an increasing trend in ethanol treated groups when compared to the corresponding normal ones. Two factor ANOVA and subsequent analysis showed that GST activity was significantly increased ($P<0.05$) in group II (control feed+ethanol) when compared to all other groups. Significant reduction in the enzyme activity was exhibited when experimental feeds were supplied, of which F72 was more effective. When tissues were considered, muscle and kidney showed least GST activity whereas, heart showed the highest value. There was no significant difference between muscle, kidney and gill and between liver and gill (Table 7.7 and Fig 7.6).

7.3.4f Glutathione reductase (GR)

Ethanol treated groups always showed significantly higher glutathione reductase activity than the corresponding normal groups without ethanol exposure. Group II (control feed + ethanol) showed highest activity, which meant that cyanobacterial feed could suppress the production of the enzyme in the tissues caused by ethanol exposure. Two way analysis of variance and

Fig 7.5 Activity of Glutathione peroxidase in various tissues of O.mossambicus treated with ethanol and fed with experimental feed

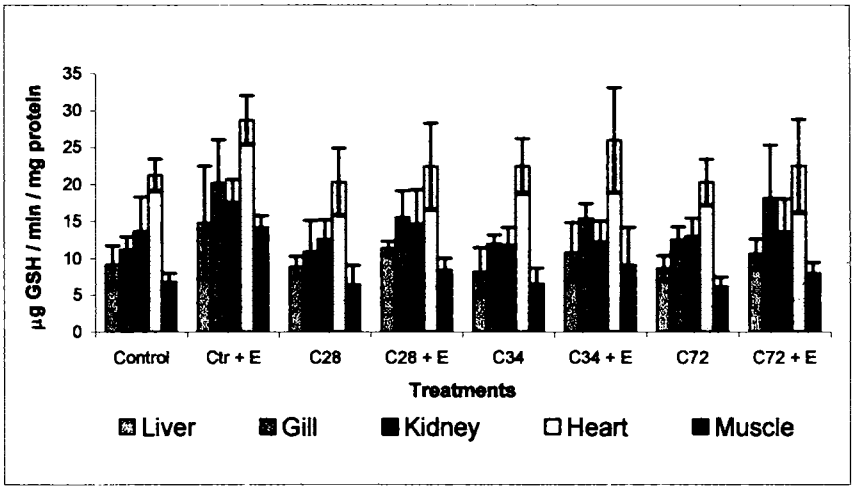

Table 7.6 Effect of cyanobacterial feed on ethanol-induced GPx activity in *O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	9.16 ± 2.52 ^{Ba}	11.20 ± 1.75 ^{Da}	13.64 ± 4.64 ^{Ca}	21.22 ± 2.17 ^{Ea}	6.84 ± 1.1 ^{Aa}
Ctr + E	14.83 ± 7.62 ^{Bc}	20.20 ± 5.84 ^{Dc}	17.54 ± 3.09 ^{Cc}	28.71 ± 3.32 ^{Ec}	14.21 ± 1.59 ^{Ac}
C28	8.86 ± 1.42 ^{Ba}	10.90 ± 4.24 ^{Da}	12.58 ± 2.62 ^{Ca}	20.35 ± 4.51 ^{Ea}	6.49 ± 2.56 ^{Aa}
C28 + E	11.40 ± 0.9 ^{Bb}	15.56 ± 3.53 ^{Db}	14.64 ± 4.58 ^{Cb}	22.42 ± 5.85 ^{Eb}	8.44 ± 1.56 ^{Ab}
C34	8.20 ± 3.23 ^{Ba}	11.96 ± 1.22 ^{Da}	11.78 ± 2.38 ^{Ca}	22.46 ± 3.7 ^{Ea}	6.60 ± 2.05 ^{Aa}
C34 + E	10.80 ± 4.01 ^{Bb}	15.41 ± 1.98 ^{Db}	12.23 ± 2.8 ^{Cb}	25.96 ± 7.11 ^{Eb}	9.16 ± 5.02 ^{Ab}
C72	8.68 ± 1.67 ^{Ba}	12.53 ± 1.71 ^{Da}	9.96 ± 2.48 ^{Ca}	20.28 ± 3.09 ^{Ea}	6.19 ± 1.27 ^{Aa}
C72 + E	10.64 ± 1.96 ^{Bb}	18.14 ± 7.16 ^{Db}	13.57 ± 4.45 ^{Cb}	22.49 ± 6.3 ^{Eb}	8.02 ± 1.43 ^{Ab}

Values are expressed as µg of GSH / min / mg protein

Values with the same superscripts do not vary significantly ($p<0.05$)

Fig 7.6 Activity of glutathione-s- transferase in various tissues of *O. mossambicus* treated with ethanol and fed with experimental feed

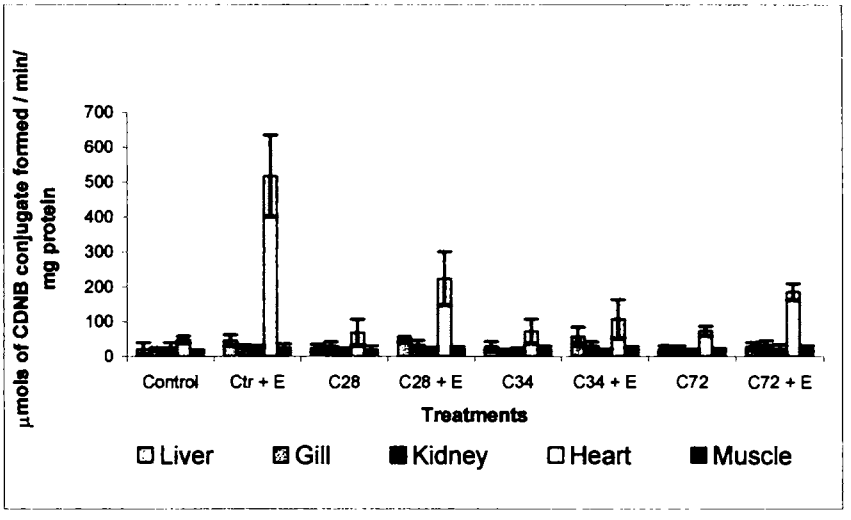

Table 7.7 Effect of cyanobacterial feed on ethanol-induced GST activity in *O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	21.01 ± 18.07 $^{B\,a}$	21.39 ± 3.13 $^{AB\,a}$	24.72 ± 15.55 $^{A\,a}$	47.61 ± 10.41 $^{C\,a}$	14.64 ± 3.81 $^{A\,a}$
Ctr + E	46.46 ± 15.16 $^{B\,e}$	30.91 ± 2.73 $^{AB\,e}$	28.15 ± 3.57 $^{A\,e}$	517.74 ± 116.5 $^{C\,e}$	25.35 ± 10.74 $^{A\,e}$
C28	23.87 ± 10.68 $^{B\,ab}$	29.99 ± 12.09 $^{AB\,ab}$	17.73 ± 6.94 $^{A\,ab}$	68.71 ± 37.51 $^{C\,ab}$	19.88 ± 10.74 $^{A\,ab}$
C28 + E	48.00 ± 8.01 $^{B\,d}$	32.40 ± 14 $^{AB\,d}$	23.64 ± 3.1 $^{A\,d}$	223.73 ± 77.09 $^{C\,d}$	22.79 ± 5.49 $^{A\,d}$
C34	28.72 ± 13.62 $^{B\,ab}$	17.39 ± 3.18 $^{AB\,ab}$	18.58 ± 6.48 $^{A\,ab}$	71.68 ± 35.18 $^{C\,ab}$	20.62 ± 9.33 $^{A\,ab}$
C34 + E	57.70 ± 26 $^{B\,c}$	30.39 ± 11.28 $^{AB\,c}$	20.34 ± 1.83 $^{A\,c}$	106.65 ± 55.83 $^{C\,c}$	24.01 ± 5.15 $^{A\,c}$
C72	21.02 ± 9.75 $^{B\,ab}$	23.89 ± 6.18 $^{AB\,ab}$	16.20 ± 4.8 $^{A\,ab}$	72.79 ± 13.74 $^{C\,ab}$	19.67 ± 4.18 $^{A\,ab}$
C72 + E	29.10 ± 10.92 $^{B\,bc}$	33.68 ± 11.8 $^{AB\,bc}$	21.23 ± 13.15 $^{A\,bc}$	185.68 ± 23.48 $^{C\,bc}$	23.89 ± 8.01 $^{A\,bc}$

Values are expressed in µ moles of CDNB complexed / min / mg protein

Values with the same superscripts do not vary significantly ($p<0.05$)

Fig 7.7 Activity of Glutathione reductase in various tissues of *O.mossambicus* treated with ethanol and fed with experimental feed

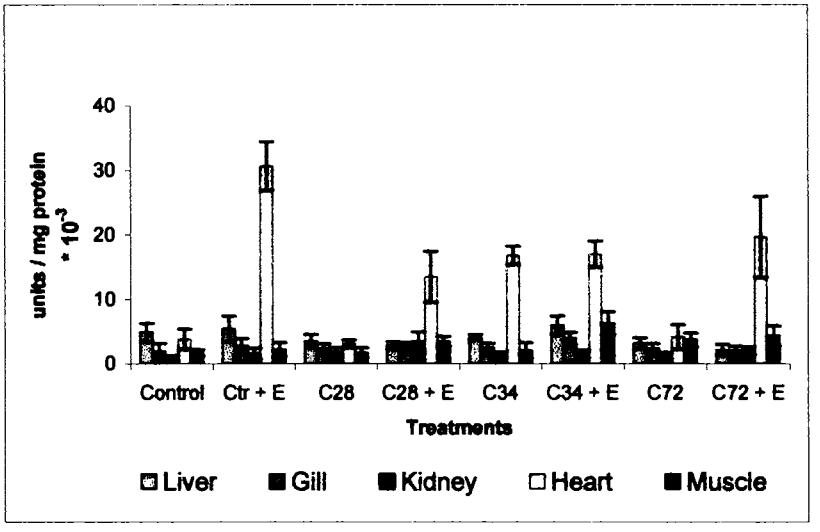

Table 7.8 Effect of cyanobacterial feed on ethanol-induced GR activity *in O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	4.896 ± 1.41^{Ca}	1.911 ± 1.242^{Ba}	0.906 ± 0.39^{Aa}	3.838 ± 1.565^{Da}	1.752 ± 0.482^{Ba}
Ctr + E	5.450 ± 1.97^{Cd}	2.821 ± 1.11^{Bd}	1.580 ± 0.83^{Ad}	30.665 ± 3.764^{Dd}	2.240 ± 1.047^{Bd}
C28	3.530 ± 1.02^{Ca}	2.340 ± 0.781^{Ba}	1.807 ± 0.72^{Aa}	3.100 ± 0.603^{Da}	1.760 ± 0.747^{Ba}
C28 + E	2.883 ± 0.6^{Cb}	2.829 ± 0.5^{Bb}	3.434 ± 1.55^{Ab}	13.522 ± 3.952^{Db}	3.454 ± 0.797^{Bb}
C34	4.182 ± 0.41^{Cb}	2.514 ± 0.695^{Bb}	1.437 ± 0.4^{Ab}	16.853 ± 1.403^{Db}	2.069 ± 1.228^{Bb}
C34 + E	6.015 ± 1.42^{Cc}	3.967 ± 0.935^{Bc}	1.550 ± 0.61^{Ac}	17.019 ± 2.022^{Dc}	6.310 ± 1.784^{Bc}
C72	3.192 ± 0.87^{Ca}	2.386 ± 0.767^{Ba}	1.302 ± 0.49^{Aa}	4.183 ± 1.901^{Da}	3.728 ± 1.081^{Ba}
C72 + E	2.159 ± 0.86^{Cb}	2.023 ± 0.682^{Bb}	2.026 ± 0.63^{Ab}	19.665 ± 6.258^{Db}	4.359 ± 1.532^{Bb}

Values are expressed as units / mg protein

Unit is defined as the change in absorbance at 340 nm / min/ mg protein

Values with the same superscripts do not vary significantly ($p<0.05$)

subsequent comparison by Duncan's multiple analysis revealed that both F28 and F72 were effective in this regard when compared to F34. There was a significant difference between tissues with the exception of gill and muscle and it was maximum in heart followed by liver, gill, muscle and kidney (Table 7.8 and Fig 7.7).

7.3.3g Conjugated dienes (CD)

The levels of conjugated dienes were significantly higher in the tissues of alcohol treated animals as compared with those of the normal control groups. However, administration of cyanobacteria incorporated feed to alcohol treated animals could reduce CD in tissues when compared to the groups fed by control feed and dosed with ethanol (group II). Two-way analysis of variance and subsequent comparison by Duncan's multiple analysis revealed that there was no significant difference ($P<0.05$) between normal groups that were supplied by control feed and experimental diets. However, significant production of CD was noted in ethanol treated groups when compared to other groups. The highest CD level was observed in ethanol treated control groups and F28, F34 and F72 could significantly reduce CD formation due to ethanol treatment. From the results, it was evident that F34 was more effective when compared to other experimental feeds. Significant difference was noted between different tissues with the exception of liver and heart, which showed maximum CD level and of muscle and gill, which showed least level (Table 7.9 and Fig 7.8).

7.3.3h Hydroperoxides

Two-factor ANOVA and subsequent analysis showed that group II (ethanol treated control groups) showed significantly higher production of hydroperoxides when compared to control fishes. This was the case for all other pairs supplemented with experimental diets. The experimental feeds could inhibit the formation of hydroperoxide. Of these, F34 was found to be most effective followed by F28 and F72. There was very clear difference between peroxidase activity in various tissues of which muscle and gill

Fig 7.8 Level of Conjugated dienes (CD) in various tissues of *O.mossambicus* treated with ethanol and fed with experimental feed

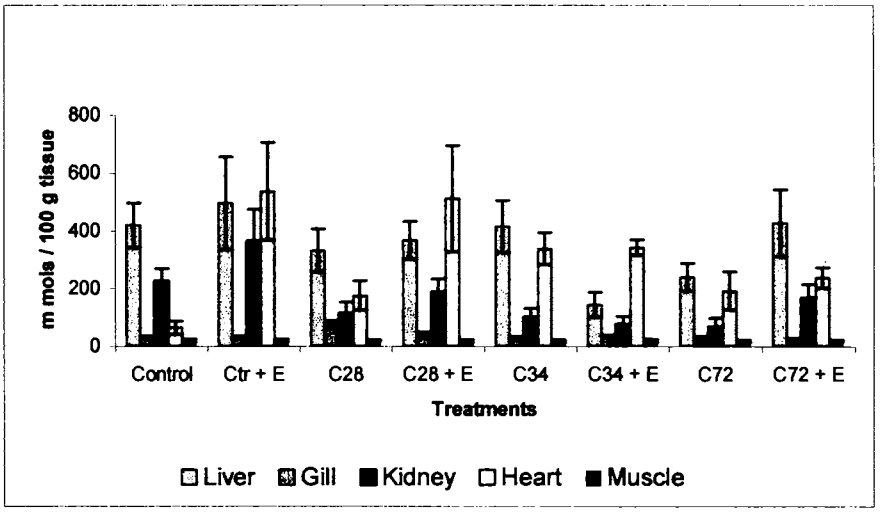

Table 7.9 Effect of cyanobacterial feed on ethanol induced CD level in *O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	419.69 ± 78.13 ^{Ca}	31.75 ± 4.34 ^{Aa}	226.69 ± 43.32 ^{Ba}	64.67 ± 23.7 ^{Ca}	18.54 ± 3.43 ^{Aa}
Ctr + E	496.23 ± 159.7 ^{Cc}	31.32 ± 4.49 ^{Ac}	364.58 ± 109 ^{Bc}	536.73 ± 168.6 ^{Cc}	20.69 ± 3.08 ^{Ac}
C28	332.18 ± 75.52 ^{Ca}	79.20 ± 9.84 ^{Aa}	114.77 ± 37.99 ^{Ba}	175.78 ± 51.03 ^{Ca}	19.49 ± 1.5 ^{Aa}
C28 + E	368.16 ± 64.28 ^{Cb}	42.97 ± 7.17 ^{Ab}	189.13 ± 45.78 ^{Bb}	511.98 ± 184.5 ^{Cb}	17.67 ± 2.26 ^{Ab}
C34	416.32 ± 89.66 ^{Ca}	29.69 ± 6.46 ^{Aa}	102.05 ± 28.92 ^{Ba}	339.35 ± 55.61 ^{Ca}	16.31 ± 4.01 ^{Aa}
C34 + E	144.65 ± 43 ^{Ca}	36.06 ± 4.35 ^{Aa}	77.00 ± 26.48 ^{Ba}	343.19 ± 25.59 ^{Ca}	18.60 ± 3.45 ^{Aa}
C72	240.43 ± 48.26 ^{Ca}	27.44 ± 7.81 ^{Aa}	68.42 ± 29.65 ^{Ba}	193.13 ± 66.42 ^{Ca}	17.31 ± 4.48 ^{Aa}
C72 + E	427.31 ± 115.4 ^{Cb}	23.27 ± 5.89 ^{Ab}	168.72 ± 45.53 ^{Bb}	237.98 ± 34.68 ^{Cb}	18.50 ± 1.78 ^{Ab}

Values are expressed as m mols / 100 g tissue

Values with the same superscripts do not vary significantly ($p<0.05$)

Fig 7.9 Level of Hydroperoxide in various tissues of *O.mossambicus* treated with ethanol and fed with experimental feed

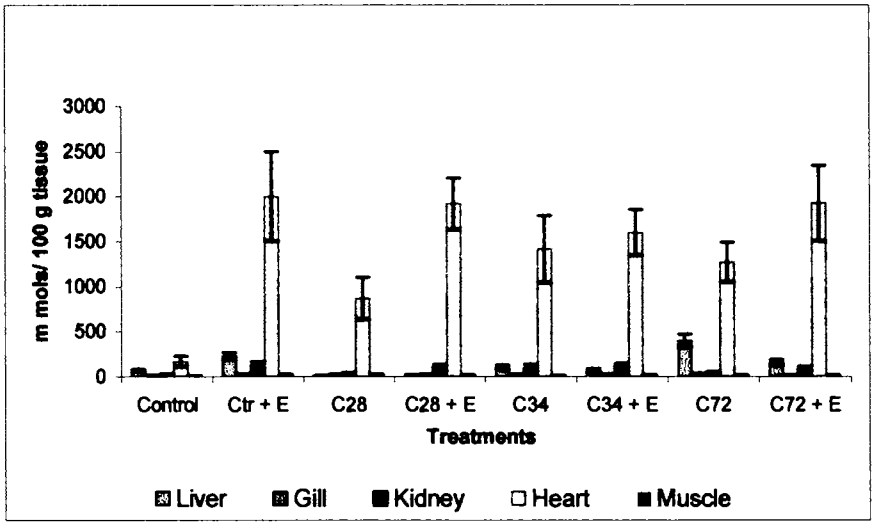

Table 7.10 Effect of cyanobacterial feed on ethanol induced Hydroperoxide level in *O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	70.55 ± 10.5 $^{B a}$	14.70 ± 3.33 $^{A a}$	25.38 ± 7.73 $^{B a}$	168.6 ± 58.1 $^{C a}$	10.29 ± 3.33 $^{A a}$
Ctr + E	228.68 ± 36.1 $^{B f}$	29.79 ± 6.21 $^{A f}$	128.37 ± 35.83 $^{B f}$	2003.7 ± 497 $^{C f}$	16.04 ± 7.95 $^{A f}$
C28	11.08 ± 4.83 $^{B b}$	21.41 ± 5.28 $^{A b}$	39.31 ± 7.81 $^{B b}$	869.3 ± 233.8 $^{C b}$	23.42 ± 5.98 $^{A b}$
C28 + E	15.16 ± 3.05 $^{B def}$	23.49 ± 4.27 $^{A def}$	104.96 ± 26.54 $^{B def}$	1918.6 ± 283.5 $^{C def}$	14.22 ± 4.82 $^{A def}$
C34	104.55 ± 22.1 $^{B c}$	21.88 ± 2.58 $^{A c}$	94.11 ± 37.73 $^{B c}$	1418.4 ± 371.3 $^{C c}$	11.65 ± 3.94 $^{A c}$
C34 + E	66.22 ± 20.7 $^{B cde}$	29.61 ± 6.12 $^{A cde}$	126.68 ± 22.01 $^{B cde}$	1601.0 ± 254.4 $^{C cde}$	15.90 ± 1.97 $^{A cde}$
C72	394.74 ± 71 $^{B cd}$	28.55 ± 9.41 $^{A cd}$	48.43 ± 8.09 $^{B cd}$	1271.6 ± 223.3 $^{C cd}$	15.33 ± 1.9 $^{A cd}$
C72 + E	160.27 ± 30.5 $^{B ef}$	15.26 ± 5.2 $^{A ef}$	89.98 ± 21.84 $^{B ef}$	1926.1 ± 416.4 $^{C ef}$	15.59 ± 5.59 $^{A ef}$

Values are expressed as m mols / 100 g tissue

Values with the same superscripts do not vary significantly ($p<0.05$)

Fig 7.10 Lipid peroxidation (level of MDA) in various tissues of *O.mossambicus* treated with ethanol and fed with experimental feed

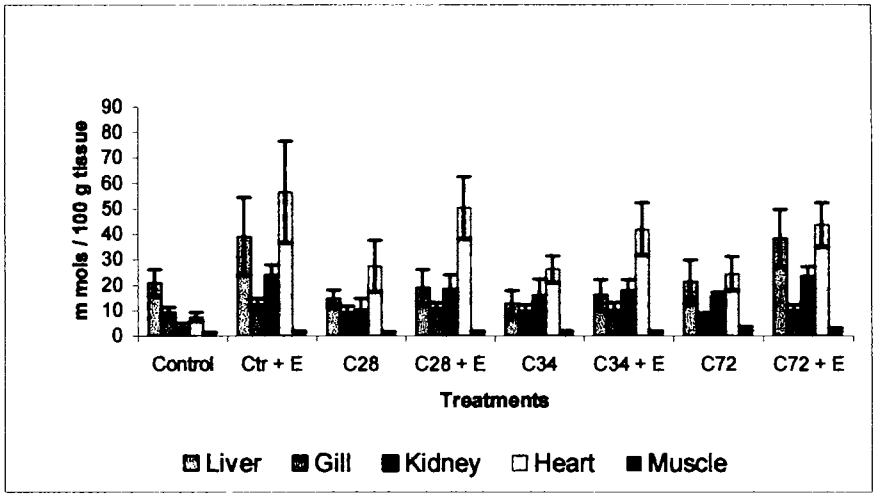

Table 7.11 Effect of cyanobacterial feed on ethanol induced lipid peroxidation (level of MDA) *in O.mossambicus*

Treatment	Liver	Gill	Kidney	Heart	Muscle
Control	20.775 ± 5.339 ^{Da}	9.126 ± 2.03 ^{Ba}	3.32 ± 1.654 ^{Ca}	7.41 ± 1.83 ^{Ea}	1.282 ± 0.166 ^{Aa}
Ctr + E	39.163 ± 15.22 ^{Dd}	12.227 ± 2.4 ^{Bd}	24.00 ± 4.026 ^{Cd}	56.62 ± 20 ^{Ed}	1.751 ± 0.227 ^{Ad}
C28	14.586 ± 3.463 ^{Db}	9.244 ± 2.35 ^{Bb}	10.28 ± 4.418 ^{Cb}	27.43 ± 10.13 ^{Eb}	1.352 ± 0.213 ^{Ab}
C28 + E	19.015 ± 7.105 ^{Dc}	10.976 ± 2.07 ^{Bc}	18.43 ± 5.52 ^{Cc}	50.41 ± 12.18 ^{Ec}	1.518 ± 0.448 ^{Ac}
C34	12.697 ± 5.166 ^{Db}	9.666 ± 2.52 ^{Bb}	15.80 ± 6.372 ^{Cb}	26.21 ± 5.34 ^{Eb}	1.769 ± 0.194 ^{Ab}
C34 + E	16.140 ± 5.872 ^{Dc}	10.173 ± 2.78 ^{Bc}	17.83 ± 4.301 ^{Cc}	42.01 ± 10.22 ^{Ec}	1.554 ± 0.422 ^{Ac}
C72	21.258 ± 8.603 ^{Db}	7.778 ± 1.23 ^{Bb}	15.58 ± 1.586 ^{Cb}	24.41 ± 6.75 ^{Eb}	2.810 ± 0.734 ^{Ab}
C72 + E	38.289 ± 11.4 ^{Dd}	9.915 ± 2.15 ^{Bd}	23.45 ± 3.776 ^{Cd}	43.71 ± 8.75 ^{Ed}	2.508 ± 0.587 ^{Ad}

Values are expressed as m mols / 100 g tissue

Values with the same superscripts do not vary significantly ($p<0.05$)

showed least activity whereas, heart showed the maximum. No significant difference was found between liver and kidney (Table 7.10 and Fig 7.9).

7.3.3i Malondialdehyde (MDA)

Ethanol treated groups always produced significantly higher levels of malondialdehyde than the corresponding normal groups, without ethanol exposure. Group II (control feed + ethanol) animals showed significantly high production of MDA whereas cyanobacterial feed could suppress its formation in the tissues. However group VIII (F72 + ethanol) did not show a significant reduction in malondialdehyde as other cyanobacteria incorporated feeds did. There was a significant reduction in the levels of MDA in tissues in the order muscle >gill > kidney > liver >heart (Table 7.11 and Fig 7.10).

7.4 Discussion

Free radicals play a major role in the progression of a wide range of pathological disturbances and it can be scavenged by the addition or supplementation of antioxidants to food or to the biological system (Venkateswarlu, *et al.*, 2003). Cyanobacteria are source of a wide variety of compounds with a potential of antioxidant activity. There are reports that β-carotene from algae could prevent cancer because of their antioxidant property (Schwartz and Shklar, 1987 and Fedkovic *et al.*, 1993). Schwartz and Shklar (1987) showed that the algal extract was more effective on hamster cancer regression than β-carotene alone and concluded that there was a possible synergistic effect of the extract, as components other than β-carotene also have a decisive action in the oxidation inhibition. Tutour (1990) proposed that some compounds such as vitamin C, phenols, amines and phospholipids from algae possess antioxidant activity. The levels of antioxidant compounds such as phenolic acids, tocopherols and carotenoids were determined by Miranda *et al.* (1998) from *Spirulina*.

In the present study, on screening different species of cyanobacteria for evaluation of antioxidant activity some potent strains could be obtained. The selected cyanobacteria, *Gloeocapsa gelatinosa* (C28), *Lyngbya semiplena* (C34) and *Oscillatoria foreaui* (C72) were shown to inhibit lipid peroxidation by 64, 63 and 58% respectively.

Further study was done to evaluate the effect of the selected strains on controlling tissue lipid peroxidation and the antioxidant status in experimental toxicity *in vivo*. As reported by Kappus (1985) and Di Guilo *et al.* (1989) antioxidant defence consists of enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transferase (GST). One of the important features of these enzymes is their inducibility under conditions of oxidative stress.

Exposure to sublethal concentration of ethanol i.e.1.5ml/L to *O.mossambicus* for 21 days resulted in a significantly elevated levels of tissue malondialdehyde, conjugated dienes and hyroperoxides and elevated activities of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione related enzymes such as glutathione peroxidase (GPx), glutathione S- transferase (GST) and glutathione reductase (Gred) as compared with the control fish. Supplementation of antioxidant cyanobacterial feeds to the alcohol treated fishes significantly lowered the enzymic and non-enzymic antioxidants as compared with the alcohol treated fishes supplemented with control feed.

Antioxidant enzymes help in the detoxification of reactive oxygen species formed from ethanol by decreasing the peroxide levels or by maintaining a steady supply of metabolic intermediate like glutathione (GSH) and NADPH (Kappus, 1985). Therefore, in the present study, the increased activities of SOD, CAT, GPx and Gred could serve as protective responses to eliminate reactive free radicals formed from ethanol and thereby protect the cell from further injury (Karakoc *et al.*, 1997; Sachin *et al.*, 1997). The

increased activity of GST- a multifunctional protein suggests that it increases the conjugation of epoxides of polycyclic aromatic hydrocarbons with the tripeptide glutathione, the resulting conjugates being water soluble and thus more easily excretable, thereby minimising the cellular damages. (Camus *et al.*, 2002). In addition, the increased GST activity serves to reduce the possibility of electrophilic compounds covalently binding to important cellular macromolecules such as DNA (Cheung *et al.*, 2001).

GSH as an oxyradical scavenger, is important in antioxidant defence. GSH is a co-substrate for hydrogen peroxide on hydroperoxide decomposition to their corresponding alcohols, through GPx activity (Seis, 1993). In the present study a significant increase in GSH was observed in the tissues of *O.mossambicus* exposed to 1.5 ml/L of ethanol.

Thus it is conceivable that higher activities of antioxidant enzymes and glutathione in the experimental animals treated with sublethal concentration of ethanol may represent a protective trial to inhibit lipid peroxidation and is considered as the first line of defence against tissue damage.

Lipid peroxidation, as measured by the concentration of malondialdehyde, significantly increased in tissues of fishes exposed to ethanol when compared to the control group, which might be due to the enhanced generation of reactive oxygen species (Francesco *et al.*, 1998). In our study, significantly elevated levels of MDA, CD and hydroperoxides were observed which demonstrated enhanced lipid peroxidation due to ethanol. These results are in line with the observations of previous researchers (Khan *et al*, 1997 and Balasubramanian *et al*, 2003) who reported a significant increase in lipid peroxidation in the tissues of mice that received ethanol.

In the present study, a comparison between ethanol treated groups and normal fishes supplemented with Cyanobacterial feed (F28, F34 and F72) was also done. There was enhanced lipid peroxidation in stressed groups as

MDA, CD and hydroperoxides were significantly higher when compared to the normal ones. The enzymic and nonenzymic antioxidants also stimulated in fishes exposed to ethanol. This may be a general adaptive defence response of the animals (Karakoc *et al.*, 1997; Sachin *et al.*, 1997). However, malondialdehyde, conjugated dienes and hydroperoxides were significantly reduced in groups treated with both experimental diets and ethanol when compared to the group treated with both control feed and ethanol. This revealed that the incorporation of cyanobacteria in the diet significantly reduced lipid peroxidation of ethanol treated groups compared to the ethanol exposed groups supplemented with control feed. Experimental diets could effectively bring down the requirement of defensive antioxidant enzymes in various tissues indicating that cyanobacteria could act as an antioxidant by scavenging free radicals produced during ethanol exposure and thus it could inhibit lipid peroxidation.

Antioxidants show protective effect by scavenging highly reactive free radicals and/or blocking peroxidation chain reactions,eventually inhibiting lipid peroxidation. The present study, has proved that the selected cyanobacterial strains could act as a very good antioxidant in ethanol induced Tilapia.The presence of natural compounds with antioxidant properties such as flavanoids, phenolic acids, vitamin E, vitamin C, phycocyanin, β-carotene and other carotenoid molecules in cyanobacteria give them the specific property (Miki, 1991; Ho, 1992; Miranda *et al.*, 1998; Bhat and Madyasta, 2000).

With regard to the antioxidant status of various tissues of the animal, results indicated that maximum level of SOD, glutathione, GST and Gred were observed in the heart tissue, whereas, GPX was high in both heart and gill. Catalase was maximum in liver followed by kidney. The concentration of CD was very high in both heart and liver followed by kidney. The concentrations of hydroperoxides and MDA was high in heart followed by liver and kidney. Muscle showed least activity for all the compounds studied.

From the foregoing results, it can be perceived that the selected cyanobacteria, *Gloeocapsa gelatinosa* (C28), *Lyngbya semiplena* (C34) and *Oscillatoria foreaui* (C72) could protect *O.mossambicus* from lipid peroxidation and from subsequent tissue damage due to their antioxidant property. In addition, the chemical composition of these species indicated that they have high nutritional value due to the presence of high contents of carbohydrates, proteins, lipids and pigments. Therefore it will be profitable if these species could be cultured commercially for use as natural food source or feed additives in aquaculture and also as source of valuable chemicals such as antioxidant compounds.

Even though the antioxidant potential of these cyanobacteria was proved both *in vitro* and *in vivo* the actual mechanism of action is not clear. Further research is essential to determine the actual constituents of these species, which make them a good source of antioxidants.

CHAPTER 8

Summary

Cyanobacteria play a key role in the productivity of aquatic ecosystems and constitute one of the basis of food chain. The chemical composition of cyanobacteria indicates that they have high nutritional value due to the presence of a wide range of essential nutrients, such as vitamins, minerals, carbohydrates and proteins. Moreover, they contain poly-unsaturated fattyacids, provitamins, β-carotene, phycobiliproteins and phenolic compounds, which are known to exhibit antioxidant properties.

The main objectives of the present investigation were to evaluate the qualitative and quantitative distribution of natural cyanobacterial population and their ecobiological properties along the Cochin estuary and their application in aquaculture systems as a nutritional supplement due to their nutrient-rich biochemical composition and antioxidant potential. This thesis presents a detailed account of the distribution of cyanobacteria in Cochin estuary, an assessment of physico-chemical parameters and the nutrients of the study site, an evaluation of the effect of physico-chemical parameters on cyanobacterial distribution and abundance, isolation, identification and culturing of cyanobacteria, the biochemical composition and productivity of cyanobacteria, and an evaluation of the potential of the selected cyanobacteria as antioxidants against ethanol induced lipid peroxidation.

To determine the distribution and abundance of cyanobacteria in Cochin estuary, ten stations were selected for the study and sampled for two years. Out of the ten stations, three were freshwater regions, five saline regions, one seashore area and one was mangrove area. Temporal and spatial variations in cyanobacterial population and physico-chemical parameters such as temperature, salinity, pH, dissolved oxygen, euphotic depth, chlorophyll-a and nutrients along the Cochin estuary were determined. Further, the subtle relationship between cyanobacteria and the environmental conditions were delineated to examine the environmental barriers to cyanobacterial growth and proliferation in the ecosystem.

The pH, salinity and nutritional requirements were optimised for low-cost production of the selected cyanobacterial strains. Preliminary screening was done based on their biochemical components such as total sugars, proteins and lipids, maintaining optimal growth conditions. Further, the selected species were characterised by studying the growth kinetics, pigment composition and productivity.

The cyanobacteria, which showed better growth, productivity and elevated levels of valuable biochemical constituents and pigments, were selected for the determination of their antioxidant activity *in vitro*. These strains were further analysed for their bioactivity against peroxidative damage in an animal model, a teleost fish, Tilapia (*Oreochromis mossambicus*).

The important findings of the study are:

- Cyanobacteria are widely distributed in Cochin estuary.
- A total number of 75 species of cyanobacteria from 24 genera across 7 families and 4 orders of the class Cyanophyceae were recorded from Cochin estuary and its nearshore waters. Thirty one of these were unicellular colonial forms, 43 nonheterocystous filamentous forms and two were heterocystous filamentous forms. This is the first report on the diversity of cyanobacteria from Cochin estuary.
- Species diversity was maximum at station 6, Mattanchery (a saline area) where 54 species belonging to 20 genera were obtained.
- Total cell count was very high in mangrove station whereas, it was very less in the seashore area.
- Pre-monsoon season was characterised by high density of organisms, whereas, cell count was very low during monsoon season.
- The distribution pattern showed that non-hetrocystous filamentous forms dominated in the surface waters and unicellular forms in the bottom water. The predominant species observed were *Chroococcus turgidus, Chroococcus tenax, Synechococcus elongatus,*

Synechocystis salina, Oscillatoria foreaui, Oscillatoria fremyii, Oscillatoria pseudogeminata, Oscillatoria subtillissima, Oscillatoria willei, Phormidium purpurescens, and Phormidium tenue.

- Most of the species showed broad tolerance to different environments and could survive through all the seasons.
- It was difficult to strictly segregate the species into saline and freshwater forms.
- *Gloeothece rhodochlamys* was the only freshwater species.
- *The species* from saline environment included *Aphanocapsa littoralis, Chroococcus coharens, Eucapsis minuta, Gloeocapsa dermochroa, Dermocarpa olivaceae, Oscillatoria laete-virens, Oscillatoria limnetica, Oscillatoria schultzii, Oscillatoria tenuis, Phormidium abronema, Phormidium jadinianum, Phormidium mucicola, Lyngbya cryptovaginata, Lyngbya putealis* and *Tolypothrix tenuis.*
- Atmospheric temperature, salinity and euphotic depth had a positive correlation with the cyanobacterial density, whereas, nutrients had a negative impact when they were in excess.
- The cyanobacterial species studied showed wide tolerance to salinity ranging from 0 to 20 ppt.
- The tested strains, *Phormidium tenue, P. angustissimum, Oscillatoria salina* and *Gloeocapsa livida* showed good growth at varying pH (6 to 9).
- Allen and Nelson medium was the best medium for the growth of these cyanobacteria, even though they could give good growth in other media as well.
- Forty strains of cyanobacteria were screened for their biochemical composition. *Oscillatoria pseudogeminata* yeilded the highest amount of total sugars (13.02%). Highest protein content was obtained from *Phormidium dimorphum* (38.88%). Lipid was maximum in *Oscillatoria acuta* in which 19.25% of the total biomass was lipid.

- Species with high composition of all the three components like carbohydrates, proteins and lipids were *Oscillatoria pseudogeminata, Phormidium dimorphum, Phormidium corium* and *Lyngbya semiplena.*
- It was found that *G. gelatinosa* and *Oscillatoria sp.* exhibited very high growth rate when compared to other species tested.
- The prominent pigments present in cyanobacteria were chlorophyll (0.2 – 3.8%) and phycobiliproteins (0.3 – 4.1%). Phycobiliproteins were found in very high concentration (12.6 % of its weight) in *P. corium.* The concentration of carotenoids ranged between 0.24 and 0.83%.
- Productivity was maximum in *Lyngbya aerugineo* (C15) and *Oscillatoria jasorvensis* (C23) which was estimated in terms of μg C /L/ hr/ gram fresh weight of the sample.
- The cyanobacteria strains such as *Oscillatoria pseudogeminata* (C81), *Phormidium dimorphum* (C9), *Phormidium corium* (C13), *Gloeocapsa gelatinosa* (C28) *and Lyngbya semiplena* (C34) were found to be suitable for application as food or feed as they showed good growth and productivity as well as elevated levels of valuable biochemical constituents and pigments.
- *Gloeocapsa gelatinosa* (C28) exhibited 64% inhibition of linoleic acid peroxidation (IP%) and *Oscillatoria foreaui* (C72) exhibited the IP% of 63%. *O. fremyii* (C32), *O. limnetica* (C77), *O. pseudogeminata* (C81), *Phormidium corium* (C13) and *Lyngbya aerugineo*(C15) showed more than 50% inhibition of peroxidation whereas *O.cortiana* (C7) displayed negative IP%.
- LC_{50} of ethanol to *Oreochromis mossambicus* was found to be 15ml/L dose at which 50% of the fishes were dead within 96 hrs.
- Experimental diets containing cyanobacteria could effectively bring down the lipid peroxidation and requirement of defensive antioxidant enzymes in various tissues indicating that cyanobacteria could act as an antioxidant by scavenging free radicals produced during exposure to ethanol.

- The selected cyanobacteria, *Gloeocapsa gelatinosa* (C28), *Lyngbya semiplena* (C34) and *Oscillatoria foreaui* (C72) could protect *O.mossambicus* from lipid peroxidation and from subsequent tissue damage due to their antioxidant property.

The present study provides an insight into the distribution, abundance, diversity and ecology of cyanobacteria of Cochin estuary. From the results, it is evident that the ecological conditions of Cochin estuary support a rich cyanobacterial wealth. The chemical composition of these species indicated that they have high nutritional value and of all the species, *Gloeocapsa gelatinosa* (C28), *Lyngbya semiplena* (C34) and *Oscillatoria foreaui* (C72) deserve special attention due to their antioxidant potential. These species could be cultured for commercial application as a source of natural food, feed additives and antioxidant compounds in aquaculture. However, for selecting the right strains for application in aquaculture and as animal feed, further studies on mass culturing of these cyanobacterial strains, mechanism of their antioxidant activity, their amino acid composition, fatty acid profile, animal toxicity etc. are inevitable.

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