ALGAE IN INDUSTRIAL EFFLUENT TREATMENT

Thesis submitted in partial fulfilment of the requirements for the Degree of

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By

VALSAMMA JOSEPH

SCHOOL OF ENVIRONMENTAL STUDIES
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 016

APRIL, 1998

Certificate

This is to certify that the results presented in this thesis entitled **ALGAE IN INDUSTRIAL EFFLUENT TREATMENT** are based on the original research carried out by Ms. Valsamma Joseph, under my guidance in the School of Environmental Studies, Cochin University of Science and Technology in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title or recognition.

Kochi - 682 016
April, 1998

Dr. Ammini Joseph
Supervising Guide
Senior Lecturer
School of Environmental Studies
Cochin University of Science
& Technology, Kochi - 682 016

DECLARATION

I, Ms. Valsamma Joseph, hereby declare that the results presented in this Ph.D. thesis entitled **Algae in Industrial Effluent Treatment** are based on original research work carried out by me under the guidance of Dr. Ammini Joseph, Senior Lecturer, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682016 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship , fellowship, or any other similar title or recognition.

Kochi - 682 016
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Valsamma Joseph

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Valsamma Joseph

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INTRODUCTION

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INTRODUCTION

Throughout history social and economic development, and the stability of culture and civilization were dependent on the availability of water. The population is projected to reach 7.9 to 9.1 billion by 2015, which has to depend on the same water resources, which were being used by 1 billion population about two centuries ago. Moreover due to the impact of the human activity, environmental disturbances on the water cycle are on the increase.

The fight against water pollution has become a major issue in terms of health, environment, and economy. Although volume wise the domestic sewage constitutes about 75% of the total effluent generated, it is the industrial effluent which contain high concentration of pollutants, either toxic or non-toxic that are of greater concern (Bhavanisankar, 1994). The main approaches to pollution control in industries are:

- efficient process control or modification to minimise the strength of the wastes
- waste minimisation by proper handling of raw materials and finished products
- minimising resource utilisation

The internal control measures will only reduce the pollutant generation, but will not eliminate it's generation. Therefore, the external measures, *i.e.*, the treatment of the effluent is an inevitable part in pollution control. The various processes in effluent treatment are pretreatment, primary treatment, secondary treatment, and tertiary treatment (Sax,1974).

Pretreatment usually includes preliminary processes to remove large aggregates of floating and suspended solids matter, grit, and much of oil and grease content, and the equalisation and storage of the effluent from different waste streams. The primary treatment consists of both physical and chemical methods including flotation, sedimentation, neutralisation, chemical addition and coagulation. Primary treatment removes settleable solids, suspended solids and biochemial oxygen demand. Following primary treatment the wastewater is processed in secondary treatment phases which include activated sludge process, trickling filtration, contact stabilisation, rotating discs, fluidised beds, and lagoons of various types. Biological processes are employed in secondary treatment where organic wastes are metabolised by living organisms.

Waste stabilisation ponds are low-cost, low technology, but highly efficient method of wastewater treatment (Mara and Pearson,1988). Stabilisation ponds have been employed for the treatment of wastewater for over 3000 years. The first recorded construction of a pond system in United States was at San Antonio, Texas in 1901. Today, large number of pond systems are used throughout the world (Reed *et al.*,1995). There are three main types of ponds: anaerobic, facultative and aerobic ponds. Anaerobic ponds are several meters deep, are free of oxygen, and have high BOD rates. Facultative ponds have aerobic conditions on the surface and anaerobic conditions in the bottom layers. Aerobic ponds, also called high-rate ponds are shallow, completely oxygenated, and are best for algal growth (Venkataraman *et al.*,1994a). Pond biology is described in terms of mutualistic relationship between algae and bacteria where algae produce oxygen required by aerobic heterotrophic bacteria to oxidise the organic matter which releases inorganic nutrients to sustain algal growth.

The effluent after secondary treatment may contain suspended and colloidal solids, organic materials which are resistant to or have escaped from biological treatment and which are by-products of bacterial metabolism, nutrients primarily phosphorus and nitrogen, dissolved solids such as chlorides, and other mineral salts, bacteria and viruses (Pavoni and Perrich, 1977). The secondary effluent loaded with inorganic nitrogen and phosphorus causes eutrophication, and long term problems arise because of refractory organics, and heavy metals that are discharged (de la Noue *et al.*,1992).

Advanced wastewater treatment is required when the removal of substances beyond the limits normally achieved by conventional primary and secondary processes are necessary. The common advanced treatment techniques include biological nitrification- denitrification, filtration, reverse osmosis, carbon absorption, chemical addition, and ion exchange (Pelczar *et al.*,1993). The relative cost of the treatment doubles for each additional step following primary treatment. A complete tertiary treatment process for the removal of ammonium, phosphate, and nitrate will be about four times more expensive than primary treatment. Quarternary treatment intended for the removal of refractory organics and toxicants, and quinary treatment leading to the removal of heavy metals , organic compounds and soluble minerals will be about eight and sixteen times costlier than that of the primary treatment, respectively (Oswald,1988). In addition, the physico-chemical methods cause secondary pollution problems.

The presence of toxic substances in wastewaters has always been a matter of concern in conventional biological treatment. Growing awareness of the biodegradation pathways of xenobiotics, type of organisms that can be useful, and their selective conditions are the most promising areas of investigation to successfully remediate many complex wastes. Microalgae

are envisaged to provide a tertiary biotreatment system for the treatment of urban, industrial, and agricultural effluents (de la Noue *et al.*, 1992). The benefits of algae in treatment systems are oxygenation and mineralisation in addition to their role as producers in the trophic system (Elnabaraway and Welter, 1984). The microalgae have the ability to use inorganic nitrogen and phosphorus for their growth (Oswald,1988). They have also the capacity to remove heavy metals (Rai *et al.*,1981; Becker,1994) as well as some of the toxic organic compounds (Redalje *et al.*, 1989). Algae can specifically accumulate and thereby remove toxic compounds from industrial waters (Cannell, 1990).

The two major issues in the treatment of toxic wastes are the assessment of environmentally safe concentrations, and biodegradability. The algae can be used in the assessment and abatement of pollution. The effects of pollutants on the biological community can be considered as an early warning system for potential pollutants (Walsh,1980). They also respond to intermittent pollution missed by chemical surveillance programme, and new or suspected pollutants in the environment. The bioaccumulation of chemicals and their concentrations in certain organisms reflect the environmental pollution over time (Mason, 1990). The algae being the primary producers can indicate the trophic status of effluent treatment systems and receiving streams. Algal growth is either inhibited or stimulated depending on the toxicity of the effluents. Algae appear to act directly in the degradation of organic chemicals or mediate photolysis. The mitigation of contaminant effects on other organisms by algae is effected by sorption or rendering toxic chemicals unavailable by degradation to a harmless form (Boyle, 1984).

The present research is based on two broader aspects of pollution assessment ,and treatability of petroleum and petrochemical effluents by algae. The objectives of the investigation were to:

- study the algal ecology and trophic status of an oil refinery effluent holding pond
- isolate and identify pure cultures of algae
- study the role of algae in petroleum and petrochemical effluent treatment
- develop strains of algae tolerant to toxic effluents
- study the biotreatment potential of the tolerant algal strains developed

The thesis comprises of **six chapters**. The **first chapter** gives the significance and objectives of the present study. The **second chapter** describes the methodology, and results of studies on the algal ecology, and trophic status of the effluent holding pond of Cochin Refineries Ltd., Ambalamugal, Kochi. The **third chapter** deals with the isolation, and development of pure cultures of algae, the algal bioassay of the refinery effluent, and the analyses of Chitrapuzha river water. The analysis, and assessment of the algal growth potential of the petrochemical effluent of Hindustan Organic Chemicals Ltd., Ambalamugal, Kochi, are summarised in the **fourth chapter** The **fifth chapter** deals with the algal growth potential in phenol and phenolic effluent, and subsequent absorption of phenol and total dissolved solids. The summary and conclusion of the present study are given in the **sixth chapter**.

CHAPTER 2

ALGAL ECOLOGY OF AN OIL REFINERY EFFLUENT HOLDING POND

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CHAPTER 2

ALGAL ECOLOGY OF AN OIL REFINERY EFFLUENT HOLDING POND

2.1. INTRODUCTION

The refining of petroleum is conducted world-wide, the industry being more concentrated in the areas of crude extraction and industrial centres. World production of petroleum in 1994 was about 66.7 million barrels/day, and petroleum remained the most heavily used source of energy (Famighetti, 1996). The enormous demand of petroleum products is being tackled by enhanced exploration and production of crude oil and augmented refining capacity. According to Sridhar (1997) the crude output during 1992-1997 was 154 million tonnes. The indigenous production of crude oil is currently 34 million tonnes with a target of 44 million tonnes by 2006-2007. The refining capacity required to meet the demand of petroleum products will be over 120 million tonnes by the turn of the century.

The process of refining in a simple petroleum industry involves catalytic reforming and treating processes, in addition to crude oil distillation. The more complex refining process includes catalytic cracking, polymerization, alkylation, asphalt oxidation as well as other selected unit operations. The processes in a very complex refinery are high vacuum filtration, solvent extraction, deasphalting, dewaxing, and treating processes in addition to the basic unit processes (Vernick *et al.*,1984).

The petroleum industry uses an average of 18 gpm water per gallon of crude processed, 80-90% of which is used for indirect cooling purposes (Wall, 1980). The refinery waste water consists of cooling water and process wastewater together with sanitary and storm water. The pollution potential of a refinery effluent can be measured in terms of oil and grease, ammonia, phenol compounds, sulphides, cyanides, biochemical oxygen demand, chemical oxygen demand, total organic carbon, and total solids. The major processes employed in the refinery effluent treatment are API gravity oil separation, chemical addition and coagulation, biological treatments such as activated sludge treatment, trickling filters, oxidation ponds, and advanced tertiary treatment processes. Despite the treatment , large scale extraction, refining, and use of oil results in the contamination of seas, rivers and lakes by oil and petrochemicals (Nemerow, 1978; Green and Trett, 1989; Botkin and Keller, 1995).

Oxidation ponds are usually incorporated in the refinery effluent treatment either as a major treatment process, or as polishing process after other treatments. Pond systems are basically classified into anaerobic, facultative, and aerobic according to the physico-chemical environment in the pond (Mara and Pearson, 1988). The pond type is selected based on the nature of the effluent generated. The performance of oxidation ponds is dependent on the biotic community present.

The application of ecological principles in the study of oxidation ponds was recognized only recently, because of the preference for engineering approaches. The organisms are sensitive indicators of water quality and their performance can give as much information as the chemical data itself about the state of depuration of the effluent (Wong, 1995). In this context the phytoplankton of wastewater lagoons are quite significant (Soler *et al.*, 1991).

The species diversity of the algae in waste stabilisation ponds were correlated negatively to the organic loading by Palmer (1969). He described Chlorophyta and Euglenophyta, and to a lesser extent Chrysophyta, and Cyanophyta to be typical of these ponds. The seasonal succession of algal flora in waste stabilisation ponds treating sewage has been studied by Govindan (1990). Among 115 taxa listed, 67 species were of Chlorophyta, and 24 of Cyanophyta followed by Bacillariophyta, and Euglenophyta. The biological features of waste stabilisation ponds treating specific industrial effluents have also been investigated (Copeland and Dorris, 1962; Thripathi and Pandey,1990). Provided sufficient solar radiation is available, non-toxic waste waters of various origin and nature, can support good algal growth. However, the community composition depends upon the adaptability of the species to organically rich and polluted waters.

The waste stabilisation ponds are examples of artificial fresh water environments. The study of physico-chemical and biological parametes in the ponds can be used to assess the trophic status as in natural systems. The community function index like productivity/ respiration (P/R) ratio can be used to monitor the degree of stabilisation of organic matter in polluted water bodies (Odum, 1971).

This **chapter** contains the data on the physico-chemical and biological variables of a polishing pond, which is the final effluent holding pond in the Effluent Treatment Plant of Cochin Refineries Ltd.

2.2. MATERIALS AND METHODS

The Sampling Site

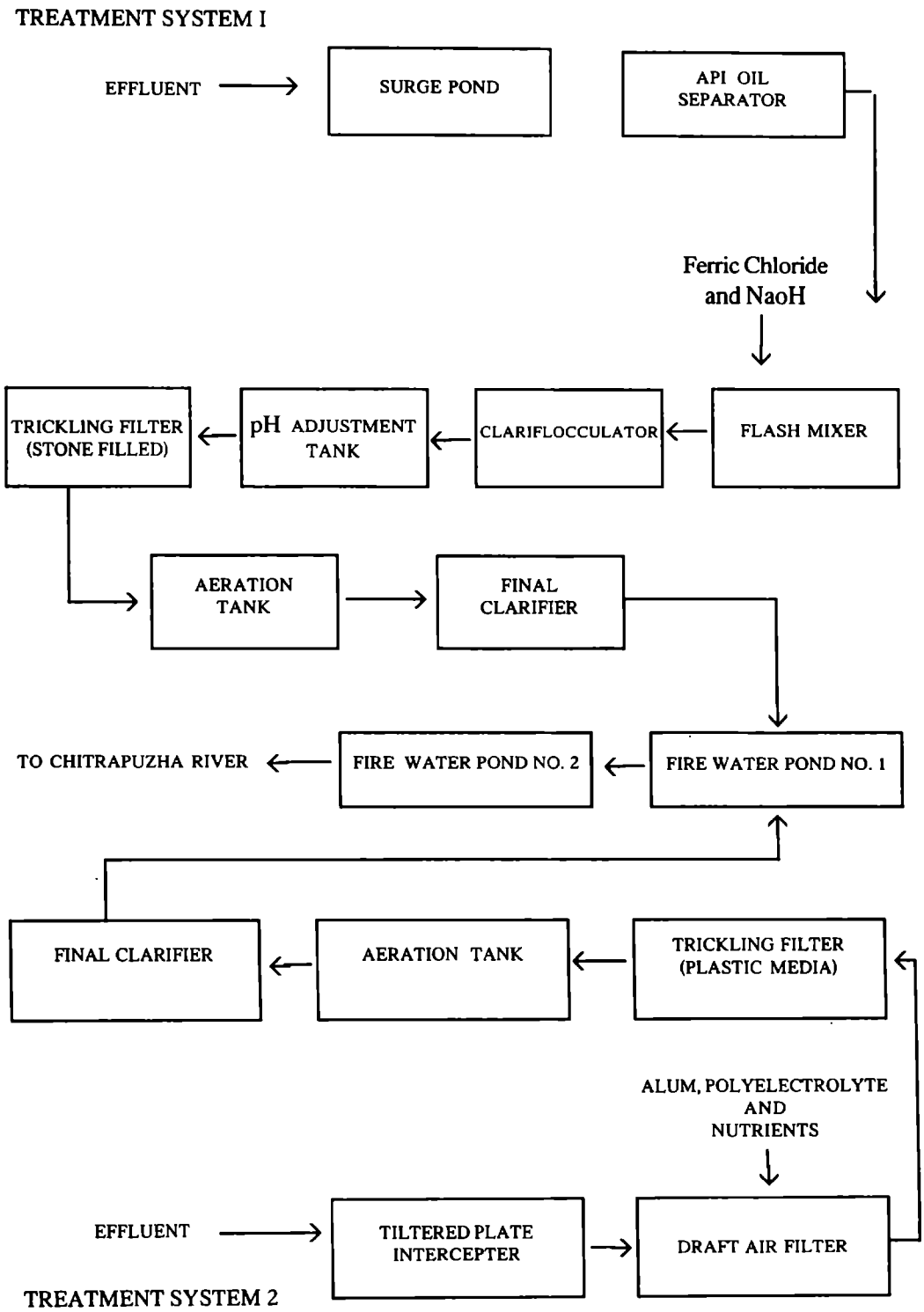
The Cochin Refineries Ltd. (CRL), located at Ambalamugal about 14 km away from

Ernakulam, was selected for the study. The refinery had an original design capacity of 2.5 million tonnes per annum, which was increased to 3.3 million tonnes per annum by 1973, and to 4.5 million tonnes per annum by 1985. The production of petrochemicals, benzene and toluene was started in 1989. The refining capacity was further expanded to 7.5 million tonnes per annum in 1994. The secondary processing facilities were enhanced from 1 million tonnes to 1.4 million tonnes per annum, maximising the yield of distillates like LPG, petrol and diesel. The refining capacity will be enhanced to 10.5 million tonnes per annum in future. Since November 1997, the refinery started processing indigenous offshore Bombay high crude along with various types of imported crudes.

The crude oil is refined by fractional distillation to separate various hydrocarbons, by the application of heat and pressure to alter the molecular structure of some of the distillates produced, and by chemical and mechanical treatment of various fractions to remove impurities. The major process units in the refinery are crude distillation unit, vacuum distillation unit, naphtha splitter unit, naphtha and kerosene hydrodesulfurisation unit, catalytic reformation unit, aromatic recovery unit, raffinate purification unit, sulfur recovery unit, fluidness catalytic cracking unit, light ends and feed preparation unit. The major products include LPG, petrol, diesel, benzene, toluene, bitumen, and furnace oil.

The effluent emanating from the process units of the refinery contains sulphides, hydrocarbons, phenols, and other organic matter. There are two systems of wastewater treatment in CRL (Block diagram 1). The effluent sources of one stream are waste merox, spent caustic recirculation, pump discharge, sour water stripper, fluid catalytic cracking unit, pump cooling water, floor washing, bitumen unit, and steam drum blow down. The outlet flow rate is 100 m^3/h. The effluent flows to a surge pond with an oil skim device provided

Block Diagram 1. Effluent Treatment Plant of Cochin Refineries Ltd.



for the equalisation and cooling of flows. In the next phase the effluent is passed to API gravity oil separator and then to chemical treatment section. Ferric chloride and spent caustic are added in the flash mixer for the removal of sulphides, cyanides, and emulsified oil. The effluent then passes to a mechanical clariflocculator where the precipitated solids settle and the clear overflowing liquid passes on to the next stage.

The clariflocculator overflow passes through a pH adjustment tank before biological treatment. The sanitary wastes emanating from various parts of the plant are mixed with the process wastes at this point. The combined effluent gravitates into a trickling filter filled with stones where the effluent is stabilised by the biological film. The effluent then passes to an aeration tank where nutrients such as urea and diammonium phosphate are added to ensure healthy microbial growth. The effluent passes to the fire water ponds No.1 and No.2 for polishing of the effluent before discharge.

The effluent from the crude distillation units 1 and 2 is treated separately in the other treatment system. The effluent passes to a tiltered plate interceptor for oil separation and then to a draft air filter where alum, polyelectrolyte and nutrients are added for the removal of emulsified oil. The effluent then gravitates through a trickling filter filled with plsatic media. The next stage is an aeration tank from where the effluent passes to the fire water ponds after final clarification.

The effluent is discharged to Chitrapuzha river from the fire water pond No.2. The fire water ponds are natural ponds with a depth of about 2 m. The capacity of the fire water pond No.2 is 60,000 m^3 The effluent is retained for 4 days before discharge. The water is also used for fire-fighting purposes. The total water consumption in the refinery is about 19,200 m^3/day. The discharge rate of the effluent is 325 m^3/h.

Sampling Procedure

Water samples were collected from the fire water pond No.2 of CRL every fortnight during the period from February 1994 to February 1995. The sampling device was Van Dorn sampler of one litre capacity. Surface and bottom water samples were collected from different locations in the pond. Temperature was read at the spot using thermometer. A small quantum of the water sample was immediately fixed using manganous sulphate and alkali-iodide-azide reagent solutions for the determination of oxygen. The water samples for the rest of the chemical and biological analyses were transferred to polyethylene bottles and brought to the laboratory immediately, and analysed without delay. The transparency of the pond was measured each time using a secchi disc by immersing the secchi disc in the water column and determining the depth at which the disc disappeared from sight and then reappeared. The water samples for the measurement of primary productivity, chlorophyll, and phytoplankton were collected from the euphotic zone.

Analytical Methods

The water samples were analysed for pH, dissolved oxygen, biochemical oxygen demand, primary productivity, chlorophyll, phosphate, nitrate, ammonia and phytoplankton. The pH was measured using Elico pH meter. The dissolved oxygen was determined by azide modified Winkler method (APHA, 1992).

The biochemical oxygen demand(BOD) was estimated as the difference in the dissolved oxygen content of the initial and final samples following incubation at 20^0C in a BOD incubator for five days as detailed in APHA (1992).

The primary productivity was estimated by Oxygen method (APHA, 1992). The water samples from the surface and bottom layers of the euphotic zone were transferred to sets of

three bottles and stoppered air tight. The sample for the determination of initial dissolved oxygen was immediately fixed with manganous sulphate and alkali-iodide-azide reagent. A second set of samples were incubated for one hour under artificial light. The third set was kept in darkness throughout the incubation period. The period of incubation was so selected that no gas bubbles formed in the light exposed bottles. Thegross primary productivity was calculated from the difference inthe dissolved oxygen content between the light exposed and dark incubated bottles, using the following equation:

$$\text{mg C fixed /m}^3 = \text{mg } O_2 \text{ released/L x } 12/32 \text{ x K x } 1000$$

where 12/32 = the factor to convert oxygen to carbon

K = photosynthetic quotient, here taken as 1.2

The difference in the oxygen content between the initial and dark incubated samples was taken as a measure of community respiration.

The chlorophyll content was estimated by spectrophotometric method. Water samples of 50 mL were filtered through Sartorius membrane filters (pore size 0.45 μm). Prior to filtration ,a few drops of magnesium carbonate suspension was added to the filter paper. The filter paper after draining under suction,was placed in darkened screw-cap tubes to which 4 mL of dimethyl sulfoxide were added. These samples were incubated at 65°C for 10 minutes (Burnison, 1980). The samples, on attaining room temperature were centrifuged at 3,000 rpm. The clear extract was made upto 10 mL with 90% acetone. The clarified extract was transferred to a cuvette and the optical density was read in a Hitachi UV-VIS spectrophotometer at 750 nm, 664 nm and 647 nm.

The samples for the estimation of phosphate, nitrate and ammonia were filtered through Whatman GF/C filter paper (0.45 μm), and the filtrate was used for analysis. The phosphate

was estimated by ascorbic acid method (APHA, 1992). Determinatin of ammonia was done by phenol-hypochlorite method (Solo'rzano, 1969). The hydrazine sulfate reduction method was used for the estimation of nitrate (APHA, 1992).

The effluent samples for phytoplankton analyses were fixed in Lugol's iodine and the cell count was determined in a haemocytometer. The identification of algae was done following monographs on algae (Desikachary, 1959; Prescott,1954; Philipose, 1967).

Data Analyses

The monthly averages of the physico-chemical data were integrated to obtain the water column mean values. One -way ANOVA (analysis of variance) was used to compare the monthly variation of the variates studied. The primary productivity of the surface and bottom samples were integrated mathematically and the results expressed in terms of g $C/m^2/day$ The ecology of phytoplankton production in the pond was interpreted utilising correlation analysis of physico-chemical varaites against productivity and chlorophyll content.

The data on composition of phytoplankton community was analysed to define the dominant forms. The species which formed more than 75% of the population were grouped in the category 'dominant'. The species that formed 40-75% of the population were ranked 'common', and those less than 40% in the category 'present'.

The values for similarity index (S) were calculated for different months based on the equation

$$S = \frac{2C}{a+b} x100$$

where, C = the number of species common to both months

a = the number of species present during one month

b = the number of species present during the other month (Sorensen, 1948).

2.3. RESULTS

Physical Variates

The mean depth of the sampling pond was 2 m and the euphotic zone was quite above the pond bottom in the range 0.82 m-1.59 m There was significant difference in light penetration into the pond at various periods of the year (Table 2.1). The depth of light penetration was inversely related to algal biomass. The highest amount of chlorophyll *a* and the lowest depth of penetration of light were recorded in February, 1994 (Fig.2.1, Fig 2.2).

The temperature of the pond was 30 -33°C (Table 2.1, Fig 2.3). The highly significant seasonal variation did not correlate with chlorophyll *a* and productivity (Table.2.2).

Table 2.1. Variables studied, ranges, means, F ratio and probability of the monthly data pertaining to the fire water pond No.2 of CRL

Variables	Minimum	Maximum	Mean	*F* ratio	Probability
pH	7.13	7.94	7.47	0.859	0.364
Temperature (°C)	30	33	31.75	549.16	1.400E-13**
Euphotic depth (m)	0.82	1.59	1.19	25.93	4.218E-05**
Dissolved oxygen (mg/L)	1.22	6.26	4.39	3.48	0.0755
BOD(mg/L)	7.11	51.85	29.03	22.78	9.154E-05**
Phosphate (mg/L)	0.08	1.63	0.57	31.78	7.143E-05**
Ammonia (mg/L)	2.23	28.68	12.45	5.36	0.0304*
Nitrate (mg/L)	0.074	0.65	0.24	36.05	4.829E-06**
Gross primary productivity ($g\ C/m^2/day$)	1.76	13.00	7.14	0.23	0.6403
Community respiration ($g\ C/m^2/day$)	1.07	7.91	3.56	5.95	0.0233*
Chlorophyll *a* (mg/m^3)	55.66	452.04	220.57	51.63	3.345E-07**
Chlorophyll *b* (mg/m^3)	8.40	151.32	60.59	20.99	1.460E-04**

* significant at 5% level
** significant at 1% level

Table 2.2. Correlation coefficient between physico-chemical variables, gross primary productivity and chlorophyll *a* in CRL pond

Variables	Gross primary productivity	Chlorophyll *a*
pH	-0.14011	0.41031
Temperature	-0.30200	0.42944
Euphotic depth	-0.26650	-0.54678
Dissolved oxygen	0.63854	0.23787
BOD	0.60938	-0.24975
Phosphate	-0.21414	0.54011
Ammonia	0.42679	-0.57185
Nitrate	-0.17445	0.00381

Critical value (2-tail, 0.05)=+/-0.57600

Chemical Variates

The annual mean pH of the effluent in the fire water pond was 7.47 with neither significant monthly variation, nor any correlation with chlorophyll *a* and primary productivity (Table 2.1, Table 2.2 and Fig.2.4). The concentration of dissolved oxygen was low especially in the bottom (Fig.2.5). The overall integrated value of dissolved oxygen was in the range of 1.22 mg/L to 6.26 mg/L. The monthly variation as revealed by ANOVA was not significant (Table 2.1). However, the BOD of the effluent fluctuated significantly over the year; the minimum value recorded was 7.11 mg/L and maximum 51.85 mg/L (Table 2.1, Fig 2.6).

There was quite significant monthly variation in the nutrient concentrations (Table2.1). The premonsoon phosphate concentrations were higher than the rest of the year (Fig.2.7). Nitrogen was present more as ammonia than as nitrate (Fig.2.8 and Fig.2.9). The monthly mean concentration of ammonia was 12.45 mg/L with the values ranging from 2.23 mg/L to 28.68 mg/L. The highest levels of ammonia were recorded in the postmonsoon months.

Algal Variates

The depth integrated values of algal biomass in terms of chlorophyll *a* and *b*, and that of gross primary productivity varied significantly from month to month (Fig 2.10, Fig 2.11).The annual mean gross primary productivity was 7.14 g C/m^2/day, chlorophyll *a*, 220.57 mg/m^3, and chlorophyll *b*, 60.59 mg/m^3. The algal productivity was low during the monsoon months compared to the pre and post monsoon months. No significant correlation could be ascertained between the algal variates and the concentrations of nitrate, ammonia, and phosphate (Table 2.2). The annual mean community respiration was 3.56 g C/m^2/day (Table. 2.1). The rate of community respiration ranged from 1.07 g C/m^2/day to 7.9 g C/m^2/ day (Fig.2.12). A comparison of gross primary productivity and community respiration in Fig.2.13 showed that the rate of respiration is lower than that of gross primary productivity. From February 1994, to November 1994, the respiratory rate remained in the range 1.07 to 3.98 g C/m^2/day whereas the gross primary productivity was in the range 1.74 to 13 g C/m^2/ day except in the month of May 1994, when gross primary productivity was lower than the respiration. Following November 1994 respiratory rate increased and was higher than that of gross primary productivity in January, 1995 and 88.08% of gross primary productivity in February 1995. The highest difference in gross primary productivity and respiration occurred in the month of October when respiration was only 23.23% of gross primary productivity. Throughout the sampling period gross primary productivity was higher than the community respiration except in the months of May 1994 and January 1995.

Phytoplankton Composition

The phytoplankton species composition of the pond during the sampling period is given in Table 2.3. The algal community composed of members of Chlorophyta, Cyanophyta,

Table 2.3. Phyloplankton species composition and seasonal variation in CRL pond during February 1994 to February 1995

Algal species	Months												
	F	M	A	M	J	J	A	S	O	N	D	J	F
Cyanophyta													
Arthrospira sp.	+	+											
Nostoc sp.	+												
Oscillatoria curviceps	+	+		+		+		+				+	+
O. quadripunctulata	++	++	+	+						+	+	+	
Phormidium inundatum	+++	+	++	+				+	+			+	+
Synechococcus p.	+												
Chlorophyta													
Actinastrum hantzschii	+							++	+		+		
Ankistrodesmus falcatus	+	++										+	+
Chlamydomonas sp.							+++	+++	+++	+++	++	+	+
Chlorella pyrenoidosa	++	++	+++	+++		+++	+	+		+	+++	+	+
Chlorococcum infusionum	+	++	++	+			+	+	+				+
Closteriopsis longissima				+									
Eudorina sp.	+	+	+	+							+	+	+
Oocystis pusilla	+	+	+	+		+						+	+
Pandorinamorum	+++	+++	+	+					+			++	
Scenedesmus bijugatus	+		+							+	+	+	
S. carinatus				+									
S. dimorphus	+												
S. quadricauda var. longispina	+	+	+					+	+	+	+	+	
S.q. var. bicaudatus		+											+
S. smithii										+	+		
Volvox aureus	+++	+++	+	++			+		+			+	+
Euglenophyta													
Euglena oxyuris							++	++	+	+	+	+	
Phacus longicauda							++	++	+	+	+	+	+
Trachelomonassimilis-												+	
Bacillariophyceae													
Navicula cuspidata						+	+	+				+	
Nitzschia palea						+	+	+				+	

+++ Dominant ++ Common + Present

Euglenophyta and Bacillariphyceae. *Chlorella pyrenoidosa* was present during 11 out of the 12 months of sampling. *Scenedesmus* had the highest species richness, being represented by six species.

In February 1994, when the sampling was started there was an algal bloom represented by Chlorophycean and Cyanophycean members. *Volvox aureus, Pandorina morum* and *Phormidium inundatum* were the dominant species. *Oscillatoria quadripunctulata* and *Chlorella pyrenoidosa* were common and *C.pyrenoidosa* became dominant in April. This tendency continued till May.

The diatoms, *Nitzschia palea* and *Navicula cuspidata* appeared in July and was present till September. From August to December there was a shift in dominance from *Chlorella-Scenedesmus-Volvox -Pandorina- Eudorina* cluster to that of *Chlamydomonas-Euglena-Phacus*. The waxing and waning of green algal community was reflected in the variation in chlorophyll *b* content as well.

The Chlorophycean members gained dominance from December till February 1995, *P. morum* being the common species present. *N. palea, O. quadripunctulata* and *O.curviceps* were also present.

The similarity index for algal population between different periods of sample collection varied between 0 to 87 (Fig 2.14). The maximum similarity was observed between the months of February and March 1994, and April and May 1994, and the minimum similarity between July 1994, and October 1994.

Fig.2.1 Mean annual variation of euphotic depth in CRL pond

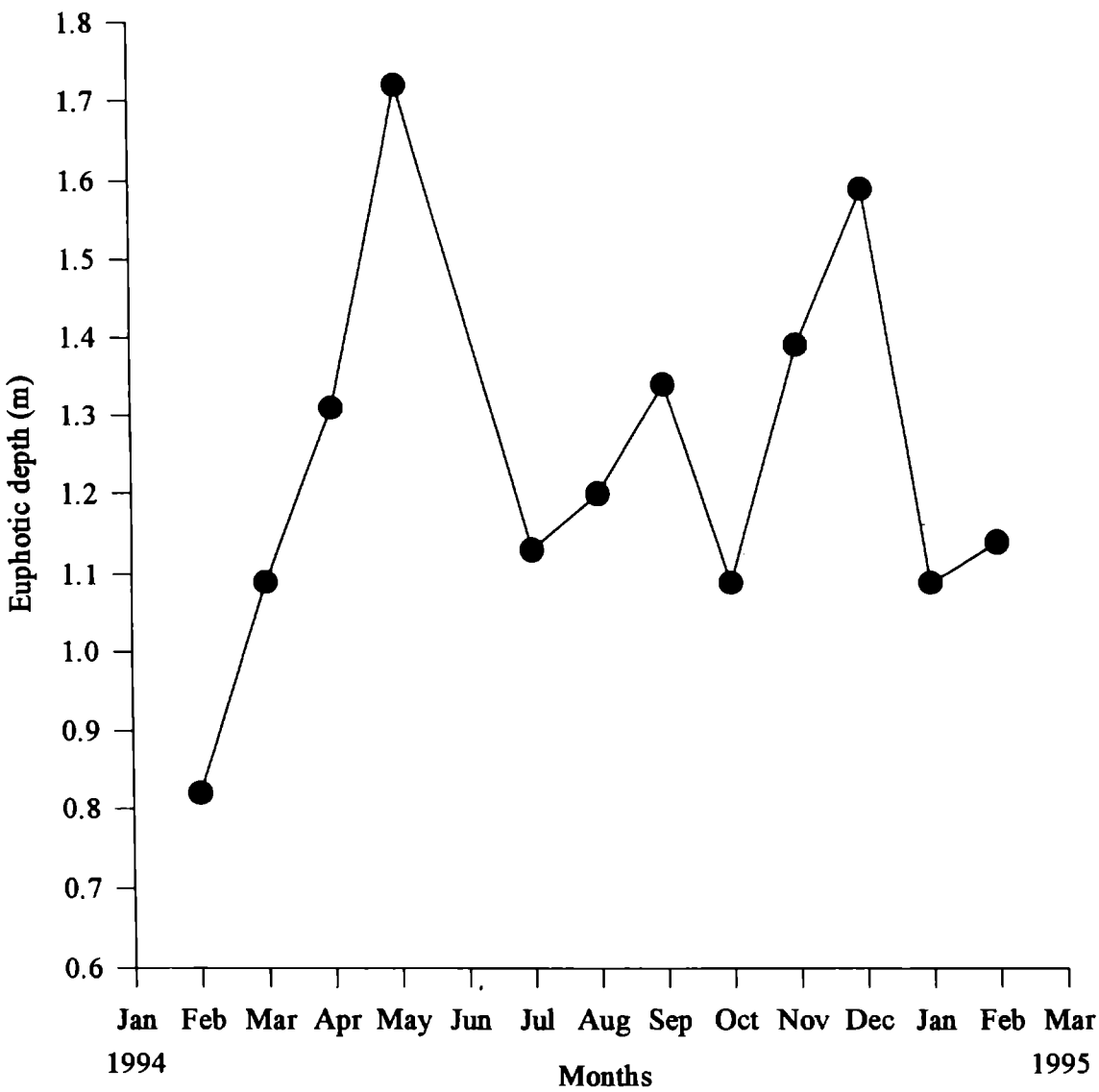


Fig.2.2 Mean annual variation of chlorophyll *a* in CRL pond

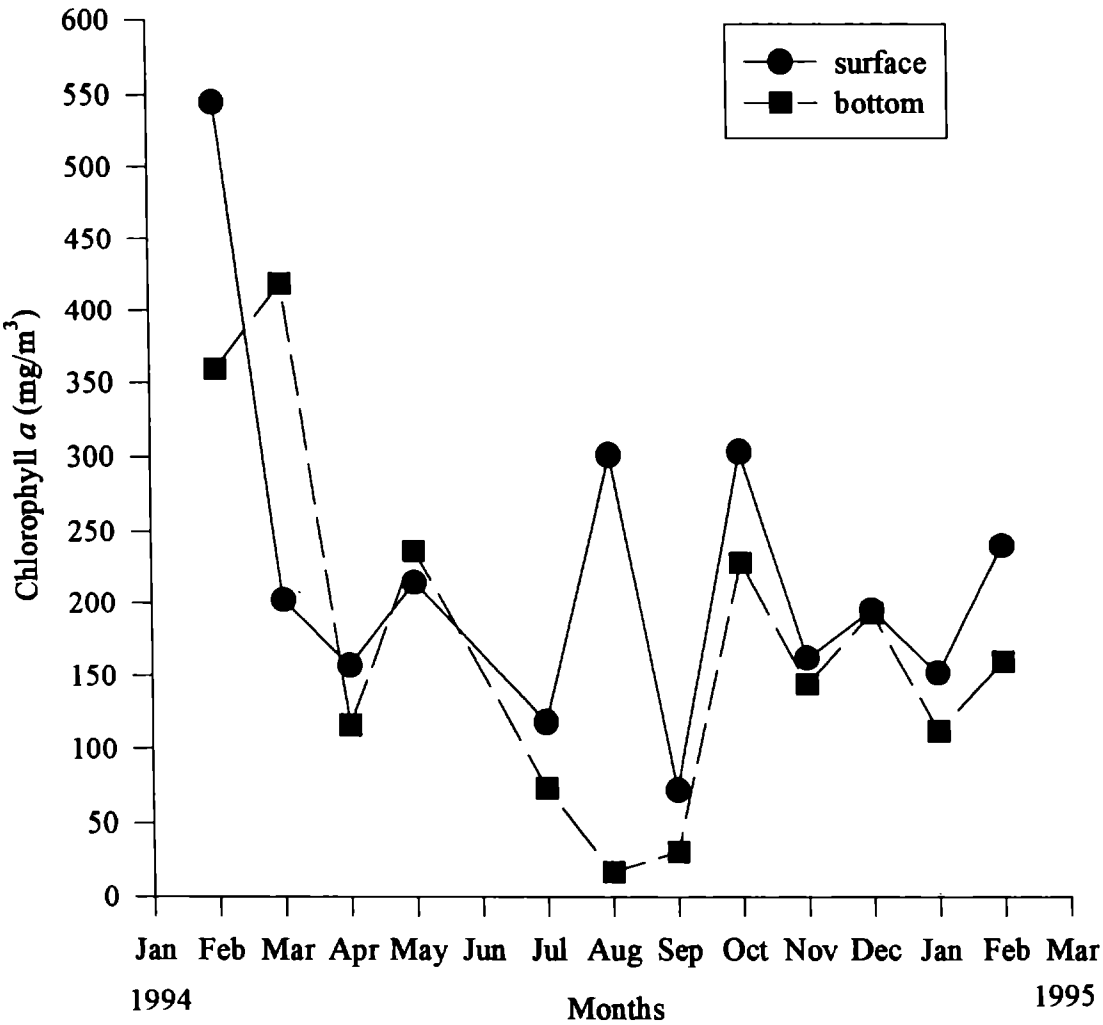


Fig.2.3 Mean annual variation of temperature in CRL pond

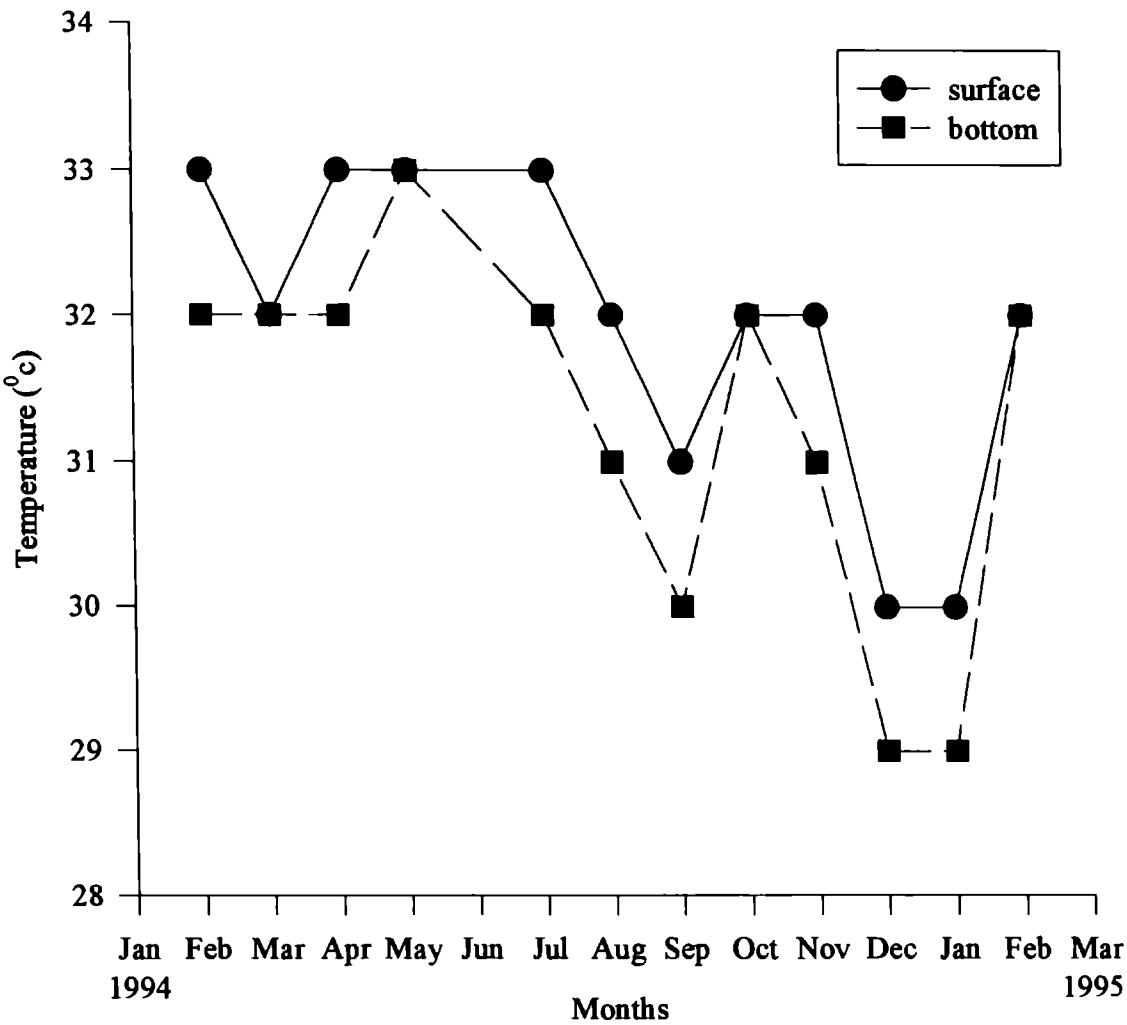


Fig.2.4 Mean annual variation of pH in CRL pond

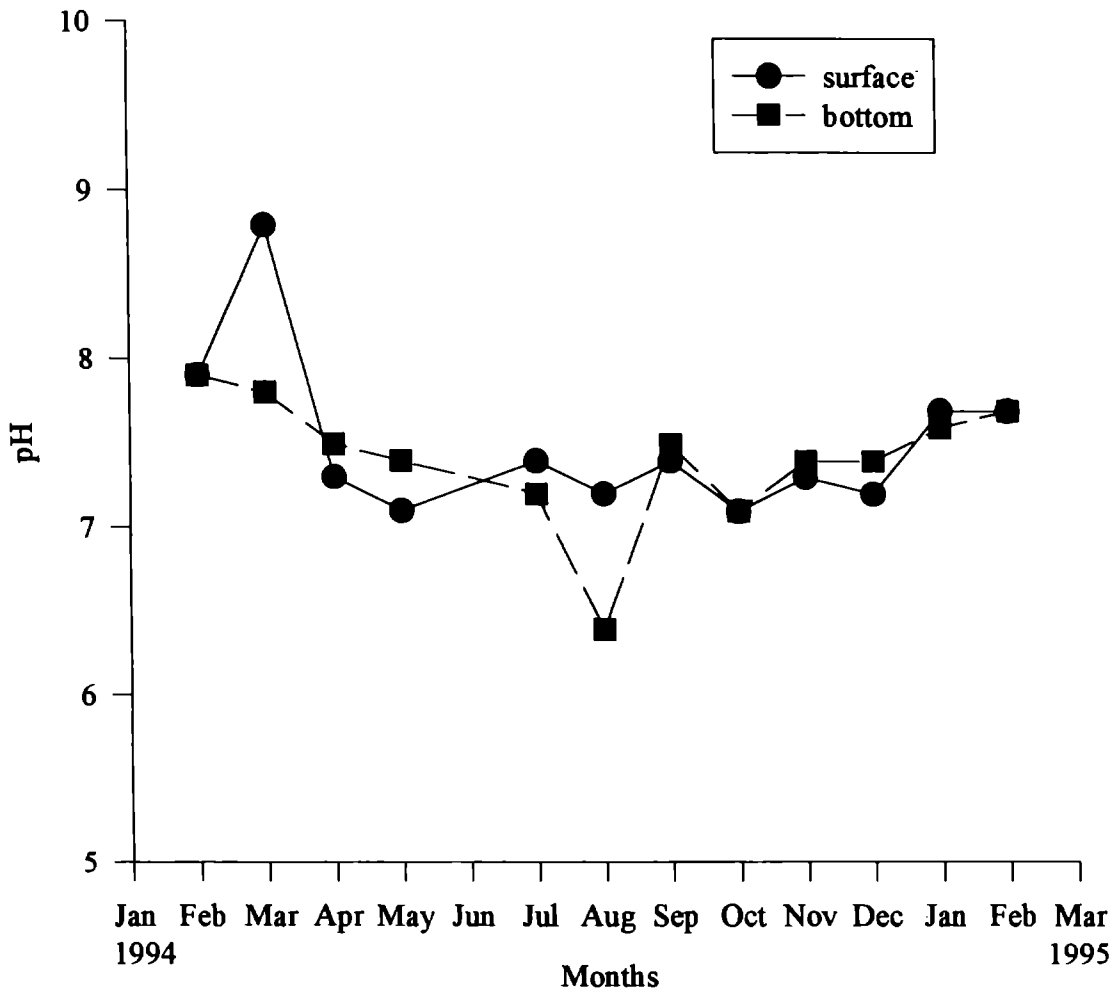


Fig.2.5 Mean annual variation of dissolved oxygen in CRL pond

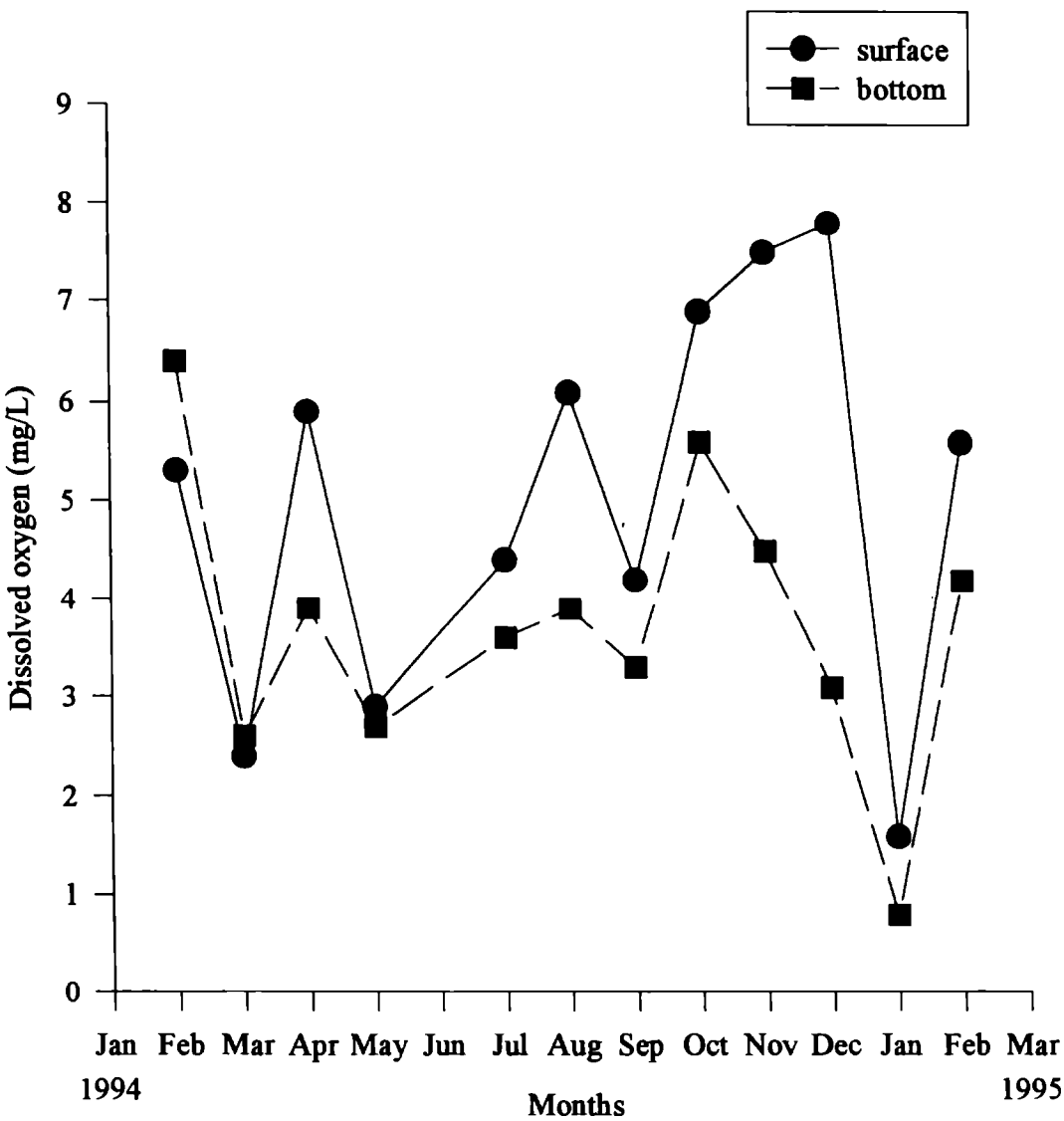


Fig.2.6 Mean annual variation of BOD in CRL pond

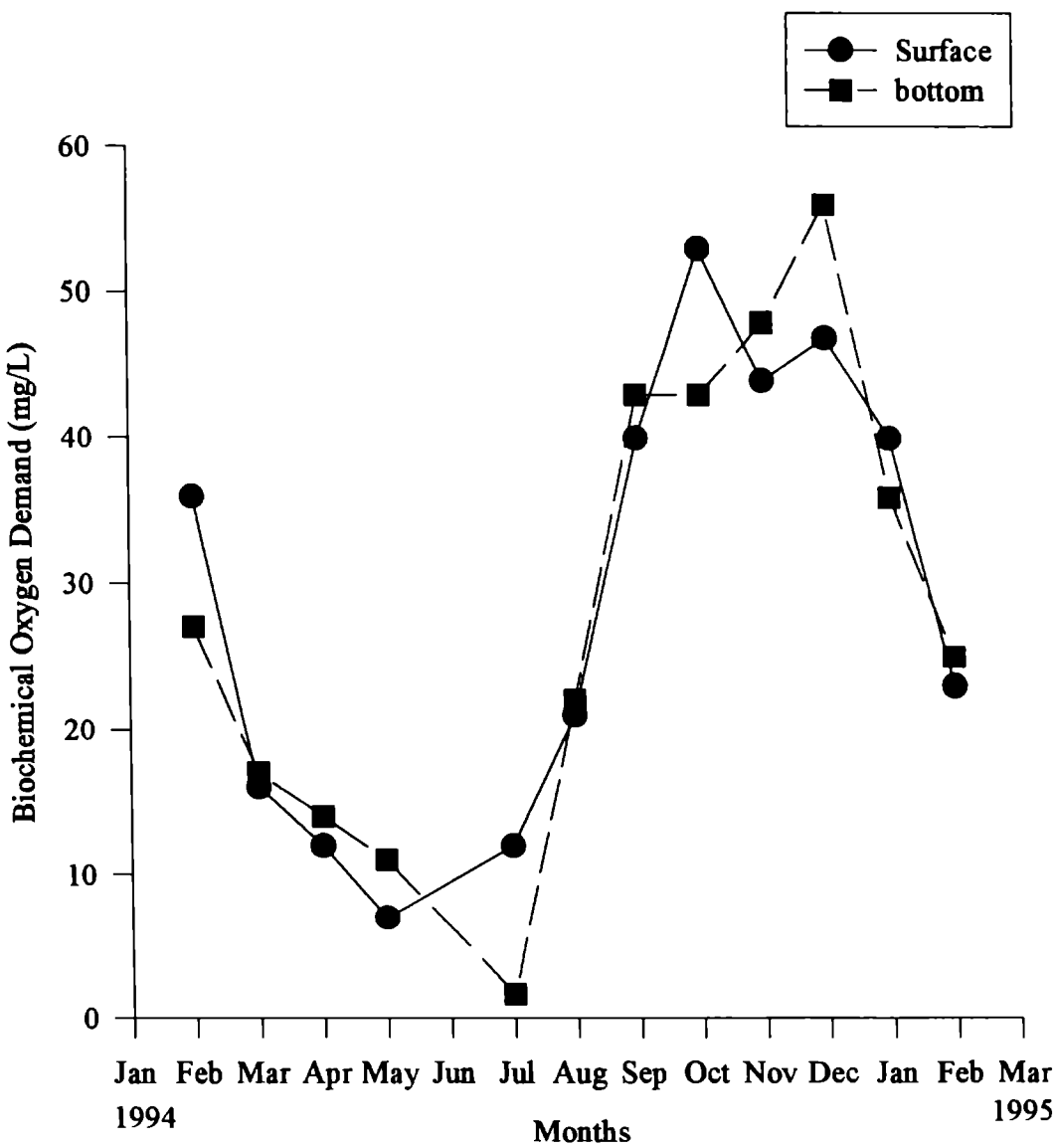


Fig.2.7 Mean annual variation of phosphate in CRL pond

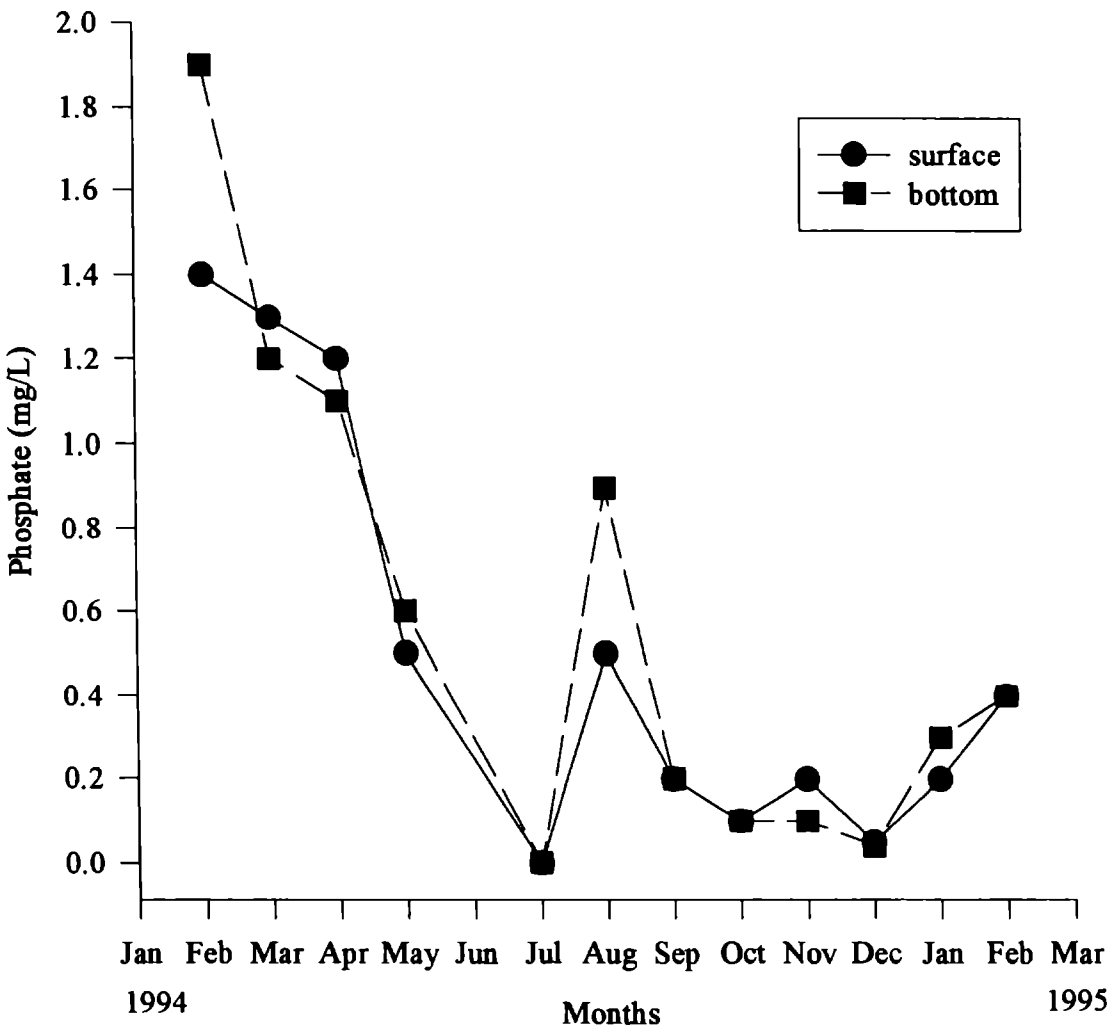


Fig.2.8 Mean annual variation of ammonia in CRL pond

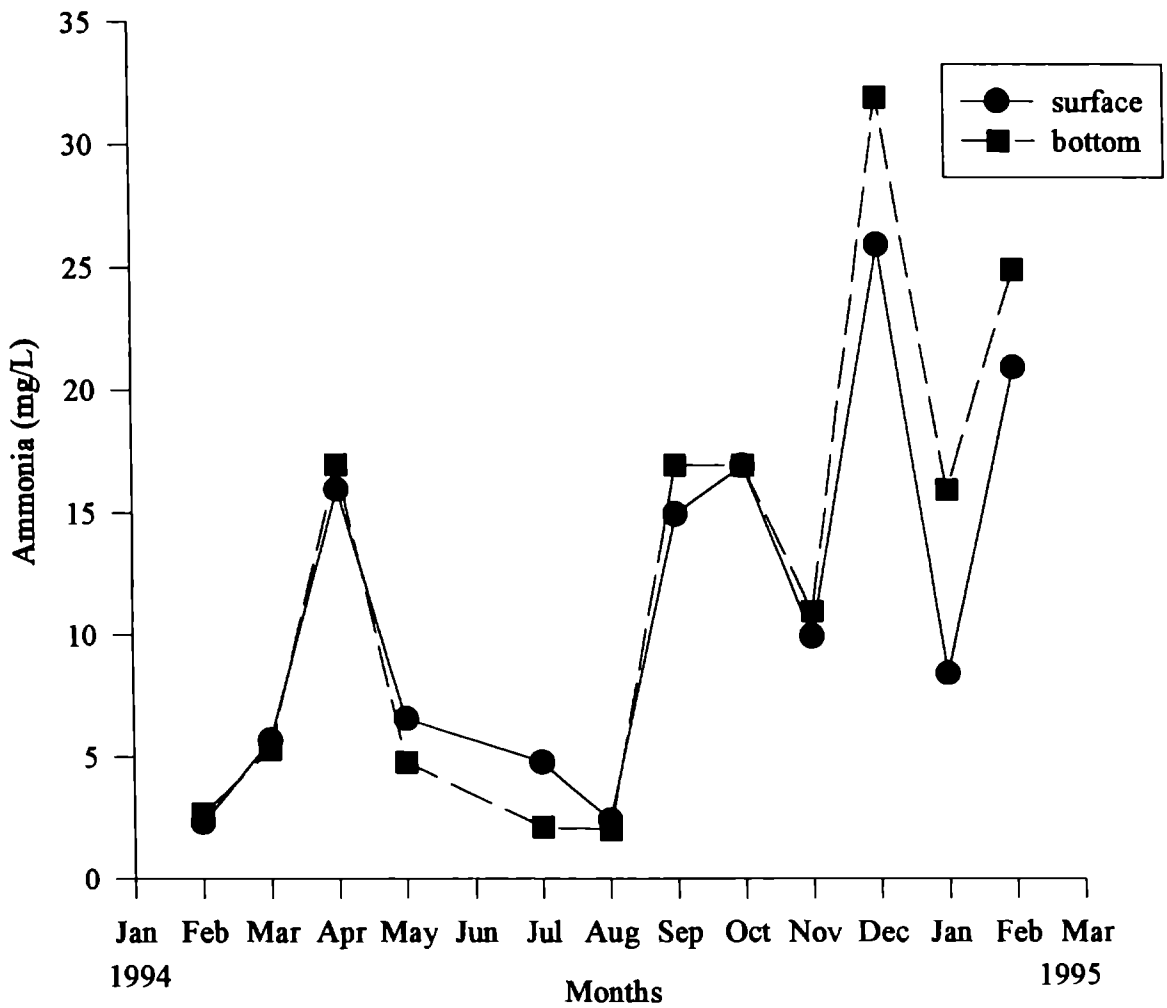


Fig.2.9 Mean annual variation of nitrate in CRL pond

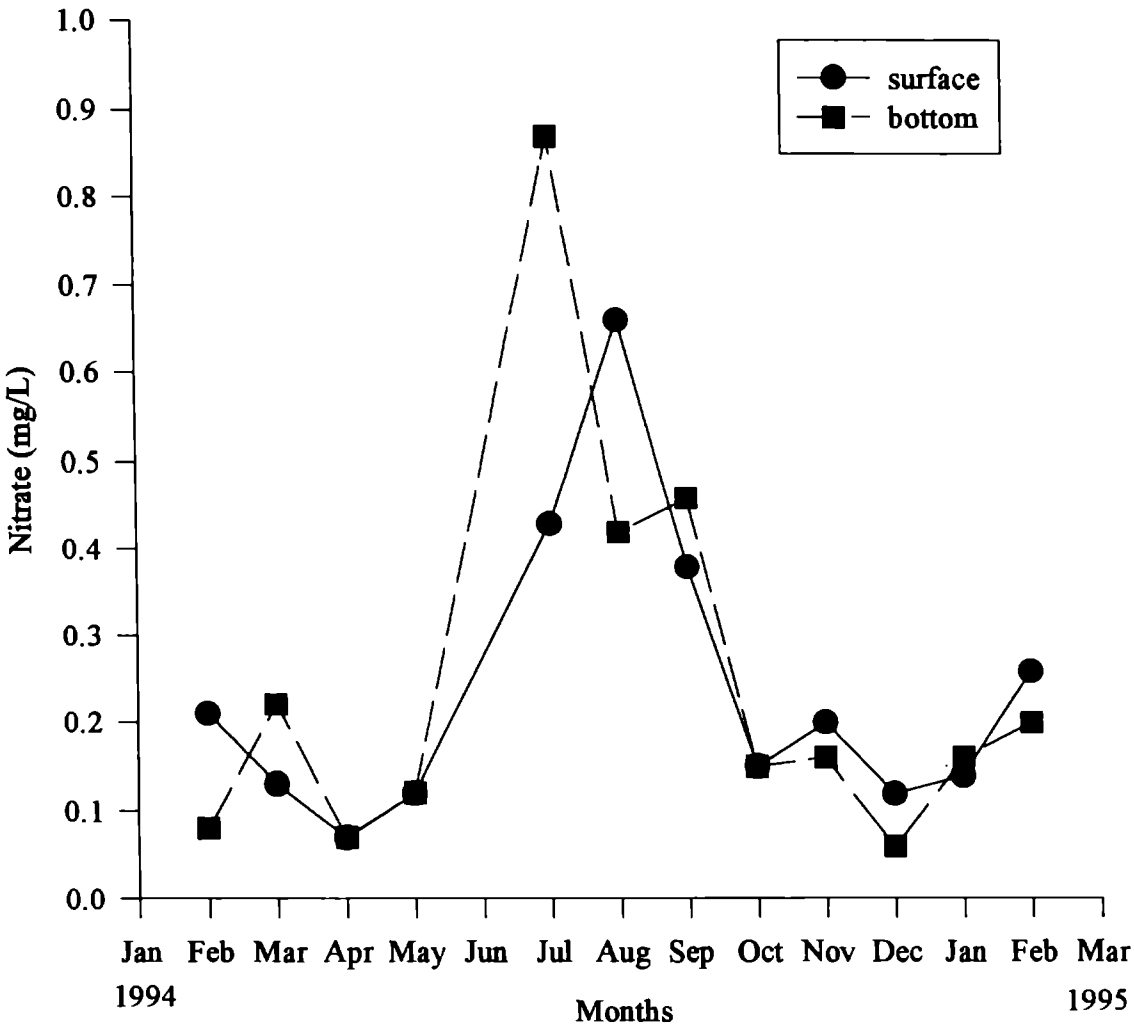


Fig.2.10 Mean annual variation of chlorophyll *b* in CRL pond

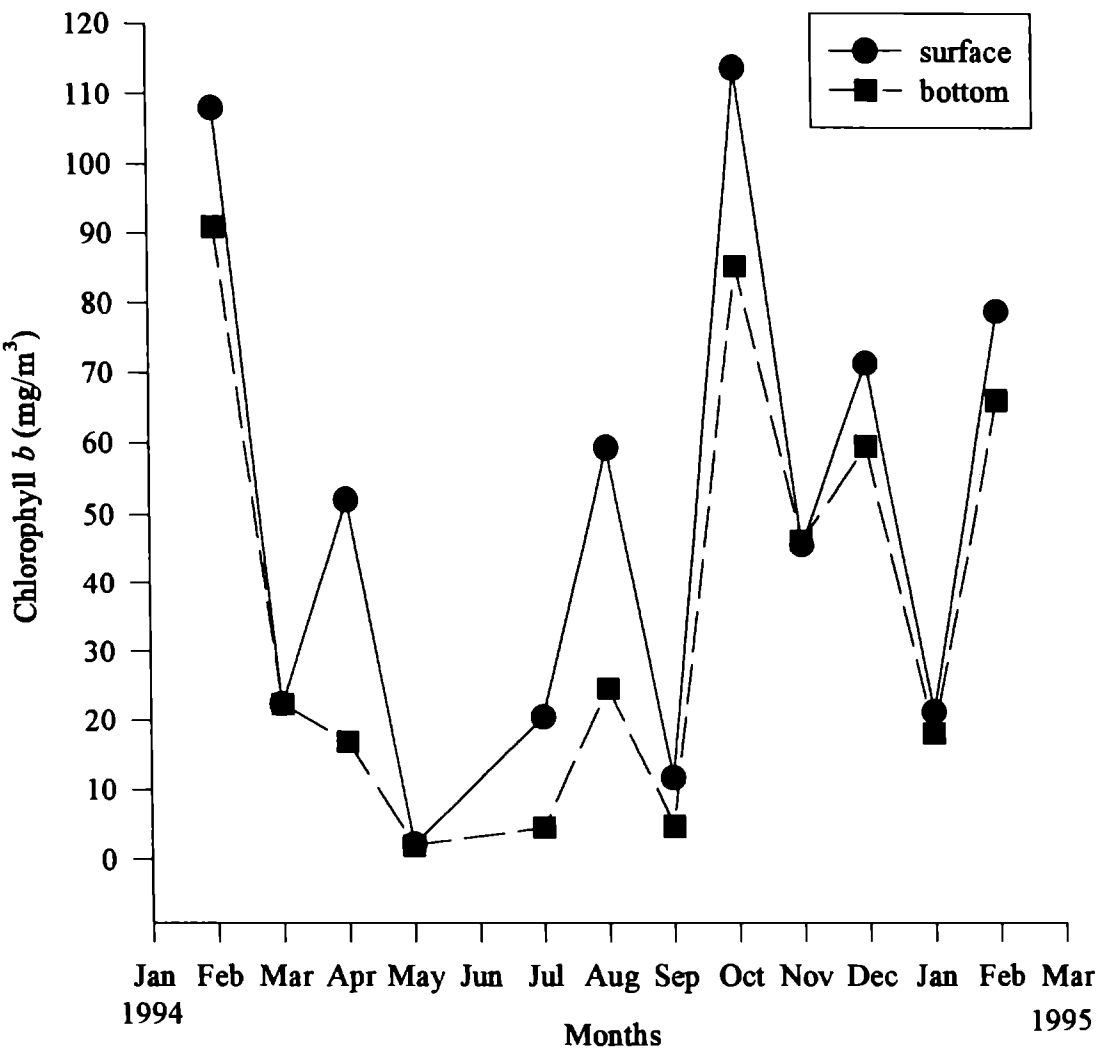


Fig.2.11 Mean annual variation of gross primary productivity in CRL pond

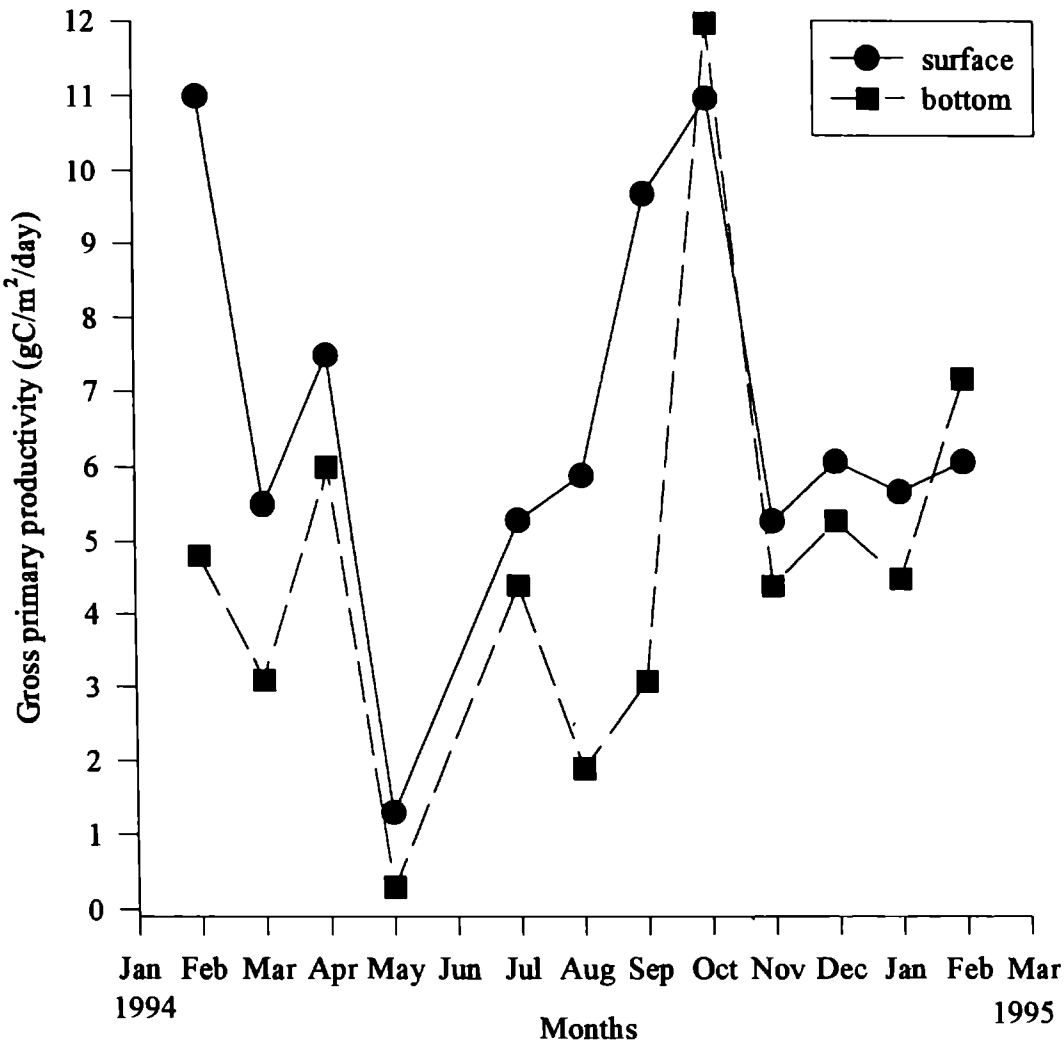


Fig.2.12 Mean annual variation of community respiration in CRL pond

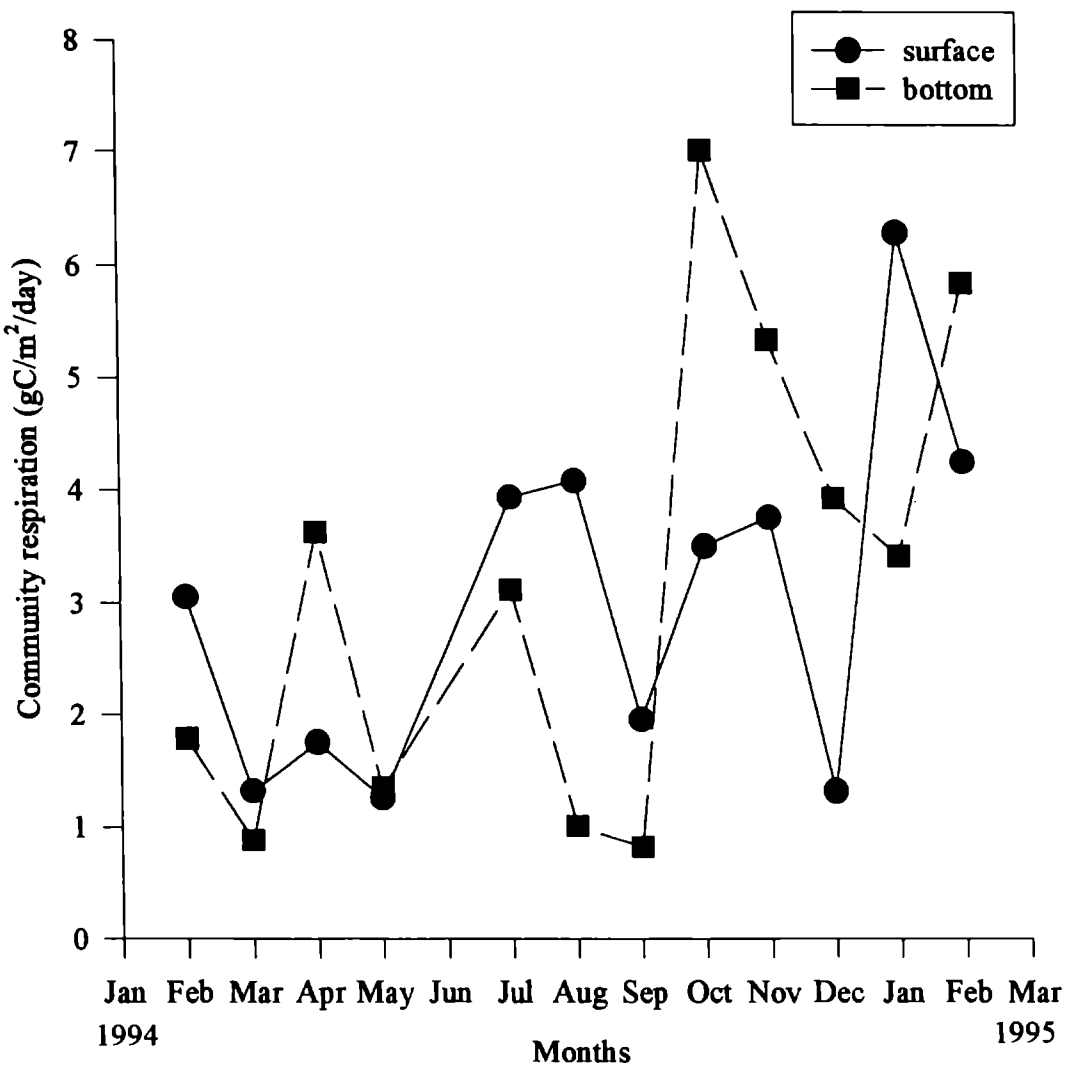


Fig.2.13 Gross primary productivity and community respiration in CRL pond

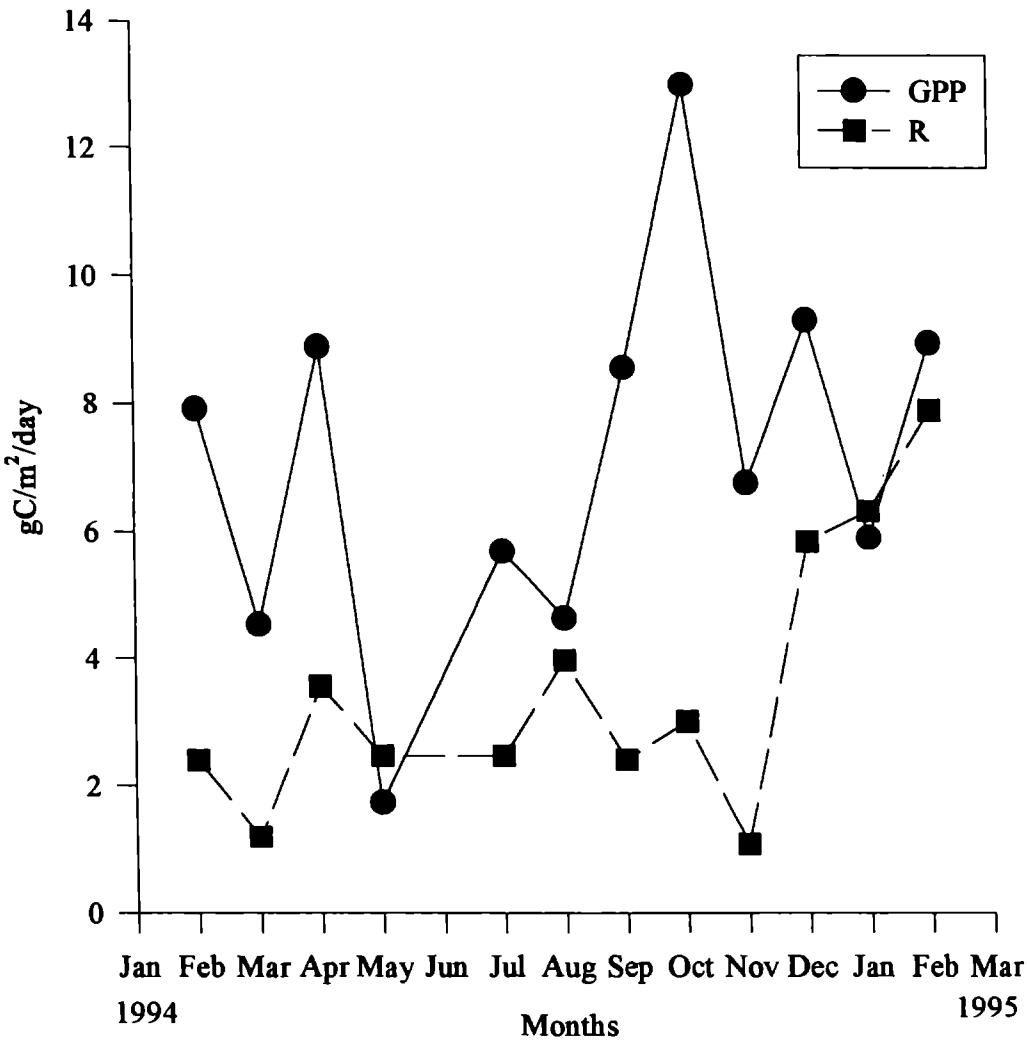
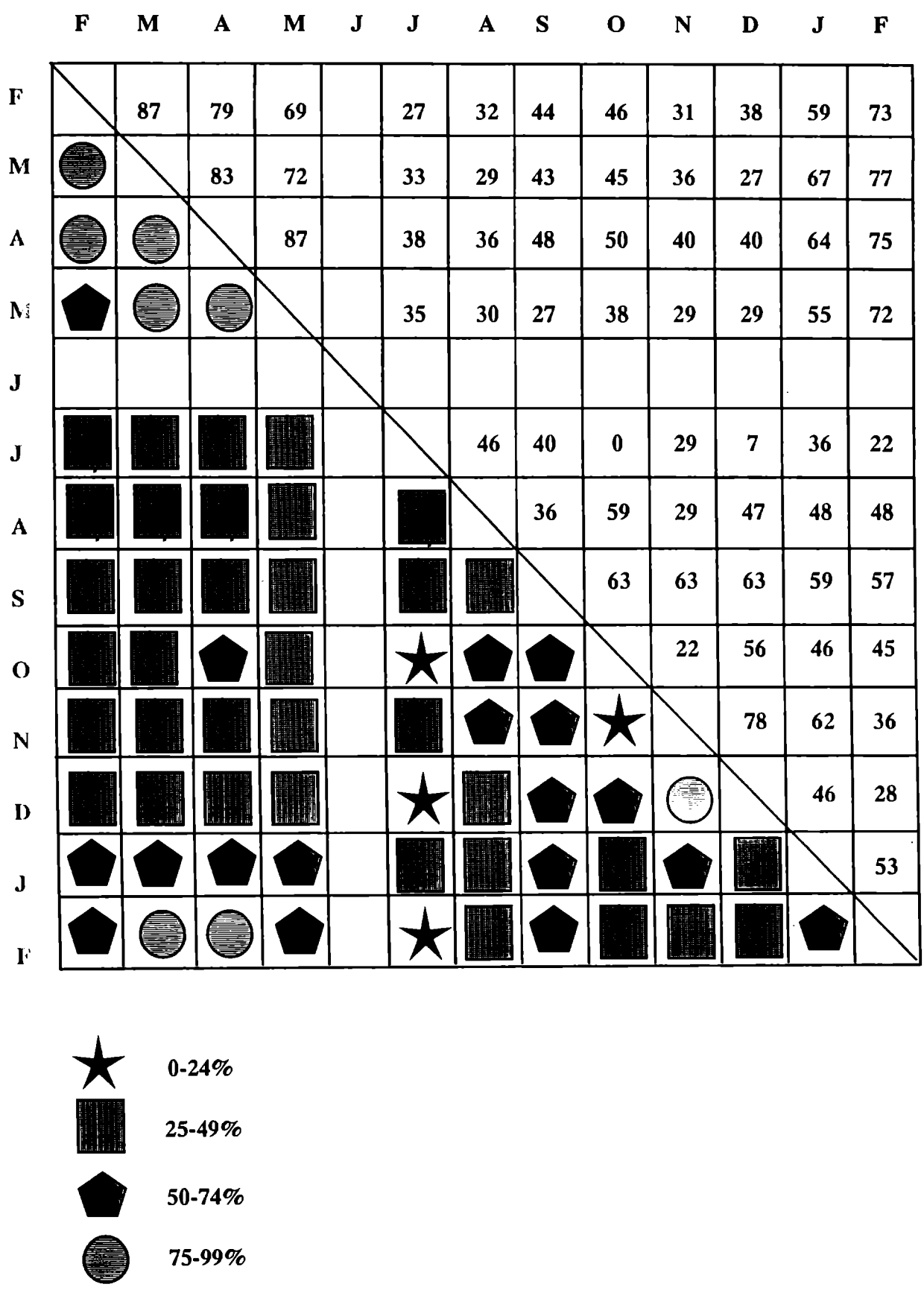


Fig. 2.14 Trellis diagram showing algal similarity index between different sampling period from February 1994 to February 1995.



2.4. DISCUSSION

The assessment of algal population based on chlorophyll and primary productivity revealed that the fire water pond No.2 is an eutrophic system supporting a continuous algal bloom. According to Wetzel (1975) the mean primary productivity of a eutrophic lake is higher than 1000 mg C/m^2/day, and chlorophyll *a* in the range of 10-500 mg/m^3. Depending upon the specific light extinction characteristic of the dominant species, the upper limit for the euphotic content is between 185and 620 mg chlorophyll *a*/m^2 (Reynolds, 1984). Marshall and Peters (1989) defined lakes with chlorophyll value >12 mg/m^3 as eutrophic. The typical features of a eutrophic pond as given by Mason (1990) are total phosphorus >20μg/L, total nitrogen >500μg/L, secchi depth <2.0 m, hypolimnetic dissolved oxygen <10% saturated, and chlorophyll *a* >10μg/L. Depth integrated chlorophyll concentration for oligotrophic to highly productive upwelling regions of oceans based on data from 1971 to 1994 has revealed that the values ranged from 3 to 437 mg chlorophyllm *a*/m^2 (Behrenfeld and Falkowski, 1997). Antoine *et al.*(1996) defined three trophic categories using the average carbon production as oligotrophic (C≤0.1 mg/m^3), mesotrophic (0.1<C≤1mg/ m^3) and eutrophic (C>1mg/m^3).

In the present study, the range of chlorophyll *a* was found to be in the range of 55.66 mg/m^3 to 452.04 mg/m^3, and gross primary productivity in the range of 1.74 g C/m^2/day to 13 g C/m^2/day. These features clearly showed that the pond is of eutrophic nature. Seasonal variation in the primary productivity of the waters in the nearby estuarine system of the CRL is well documented. Generally, there is a decrease in primary production during monsoon months (Nair *et al.*,1975; Pillai *et al.*, 1975). A similar trend was observed in the CRL

pond also. The decrease in the primary productivity during the monsoon season may be due to the dilution of the water and the dispersion of algal cells along the water column.

The concentration of phosphate in the effluent was always within the permissible limits of 5 mg/L. Ammonia concentration increased from February 1994 onwards and was within the permissible limits of 50 mg/L. The nitrate concentration was negligible. The concentration of nitrogen and phosphorus exceeds the typical values quoted for eutrophic ponds. Sakamoto (1966) established that chlorophyll concentration is a function of phosphorus concentration. Patalas (1972) found a relationship between phosphorus loading and chlorophyll concentration of great lakes. But Lake Cameron of Ontario with a phosphate concentration 1.7-2.2 g/m^3/year was not eutrophic due to high flushing rates (Dillon,1975).

The algal variates of CRL pond were not correlated with the concentrations of nitrate, phosphate, and ammonia as revealed by the present study, although algae are generally favoured by high concentrations of nutrients. It is documented that Chlorococcales such as *Scenedesmus* are capable of assimilating organic solutes and may be facultative heterotrophs (Berman *et al* .,1977; Vincent, 1980). Walsh *et al.*(1980) in a study of 23 effluents stated that the algal growth stimulation in complex effluents was not correlated with concentrations of nitrate or phosphate, and the inhibition was not correlated with concentrations of metals or inorganic substances.

The constant algal bloom in the pond, despite the insignificant correlation with the nutrient parameters, suggest that there may be other nutrient sources available to algae. The bioactivity of a complex waste is probably related to interactions among components

with no substance having dominant effect (Walsh *et al.*,1980). The organic effluent from the efinery contain nutrient components which may determine the fate of algal population in the pond.

As in natural freshwater systems, there was a tendency for the seasonal fluctuation of algal flora in the pond. The Chlorophycean members dominated, and the members of Euglenophyta, Cyanophyta, and Bacillariophyceae were present. There was a clear shift in dominance from *Chlorella-Scenedesmus-Volvox -Pandorina-Eudorina* cluster in February to May to that of *Chlamydomonas-Euglena-Phacus* from August to December. The diatoms appeared during monsoon, and blue-green algae were dominant in the premonsoon period along with the green algae. It has been established that toxic chemical stress causes large changes in community structure (Howarth,1991). In aquatic ecosystems new populations can grow very quickly to replace those which are damaged by pollution. Primary production in these systems is controlled from below by nutrients, and the nutrient availability is not greatly affected by the particular toxic stress. The relationship between the nutrients, and the algal community in these systems is not well understood. Sometimes changes in nutrient levels does not alter algal species composition and sometimes it does (Borchardt,1996). The response of the same species to seemingly similar nutrient enrichment conditions differed among studies, and it is suggested that features other than nutrients are more important in determining species composition. It has been found that high algal density results in efficient nutrient removal from primary settled wastewater, but it would lead to self-shading, accumulation of auto-inhibitors and reduction in photosynthetic efficiency (Lau *et al.*,1995).

From the results of the study it is concluded that the effluent holding pond of Cochin Refineries Ltd. is of eutrophic nature. In a eutrophic state, ecosystems become structurally simpler, more dominated by opportunistic species, and more resistant to toxic chemical stress. An ecosystem disturbed either by increased nutrient input or by toxic chemical stress is probably more resistant to further stress from toxic chemicals (Howarth,1991). Thus the algal population in the pond may be more resistant to stress induced by the probable shock load of the effluent. Further, the effluent when discharged into the Chtirapuzha river may promote the growth of algae in the river.

In a similar study of the primary productivity in series of effluent holding ponds at two petroleum refineries, in the USA, Copeland and Dorris (1962) have estimated that the gross primary productivity and community respiration values approached each other near the discharge end of the pond systems indicating that waste stabilisation was attained. The performance of the waste stabilisation ponds arranged in series were found to be efficient in removing nutrients and heavy metals from sewage compared to the single pond (Govindan,1985). The pond system of CRL has only two polishing ponds. The investigation revealed that gross primary productivity of the final pond is higer than the community respiration. The community respiration approached gross primary productivity only twice during the sampling period clearly indicating that the waste stabilisation is not complete in the fire water pond No.2.

The present study has established algal analyses as an effective tools to estimate the efficiency of wastewater treatment systems. Moreover, as the waste stabilisation is not effected completely in the fire water pond No. 2, the wastewater of eutrophic potential is being disharged into the Chitrapuzha river. It is suggested that a series of maturation ponds are required to achieve complete stabilisation of this refinery effluent.

CHAPTER 3

ALGAE IN THE TREATMENT OF REFINERY EFFLUENT -LABORATORY ASSESSMENT

- ★ Introduction
- ★ Materials and Methods
- ★ Culture of Microalgae
  - ◆ Isolation
  - ◆ Purification
  - ◆ Growth kinetics of microalgae in culture
  - ◆ Growth of algae in half-strength medium
- ★ Algal Assays of CRL Effluent
  - ◆ Screening test
  - ◆ Definitive test
  - ◆ Variability of CRL effluent
- ★ Removal of Total Dissolved Solids in the CRL Fire Water Pond No.2
- ★ Phytoplankton Flora of Chitrapuzha River
- ★ Results
- ★ Discussion

CHAPTER 3

ALGAE IN THE TREATMENT OF REFINERY EFFLUENT- LABORATORY ASSESSMENT

3.1. INTRODUCTION

The controlled application of algae in wastewater treatment is one of the challenging areas of algal biotechnology. Rather than being oxygenators of the conventional effluent treatment ponds, algae take up and bioconcentrate a variety of environmental contaminants present in water (Boyle, 1984). Recently algae have been implicated in the degradation of organic contaminants like dyes and pesticides (Jingi and Houtain, 1992; Venketaraman *et al.*,1994). The potential of algae in the absorption of dissolved solids and polluting parameters can be assessed by algal bioassays.

Bioassays were first introduced in the late 1940's and it was in 1970's that these tests were adapted for algal studies. Algal assays are now an indispensable part of test batteries in water pollution monitoring because, firstly algae are the primary producers in the food chain, and secondly they are more sensitive to contaminants than fish or invertebrates (Wong, 1995). Industrial and energy process effluents contain algal toxicants/growth stimulators. Algae in the laboratory culture respond in an easily measurable way to substances that affect primary productivity. The algal growth may be measured directly as change of biomass (cell number or chlorophyll *a*) or in terms of physiological processes within the exposed populations such as the evolution of oxygen, or uptake of carbon dioxide(APHA,1992).

A large number of algal species occurs in fresh and salt water, many of which have been used in assays against pesticides, heavy metals, and other toxicants; only a few have been tested for response to complex wastes. In freshwater toxicity tests *Selenastrum capricornutum* is used extensively to detect the presence of growth inhibitors and stimulators in complex effluents (Miller *et al.*, 1978; Walsh *et al.*, 1980). Other species commonly used and responsive to pollutants include *Chlorella haemosphaera, Ankistrodesmus falcatus, Oscillatoria agardhi, Nitzschia linearis,* and *Euglena gracilis* (Walsh and Merril, 1984). Palmer (1969) listed 60 genera and 80 species of freshwater algae according to the tolerance to pollutants. Although members of each group varied widely in response, green and blue green-algae, generally were most tolerant, but diatoms least tolerant to pollutants.

Most toxicological research with algae have been done with single pollutant such as pesticides and heavy metals, but it is not known whether species most sensitive to single pollutant are equally sensitive to mixtures also (Walsh and Merril, 1984). Most effluents contain organic materials and it is necessary to document more fully the ability of various algal species to grow in such effluents. Therefore specific methods need to be developed for complex industrial wastes to assess the response of individual species, and exploit these species for the biotreatment of effluents. Different algal species can be cultured and tested in the laboratory so that more adaptive species may be isolated for utilisation.

Prior to identification of a species for a biotechnological applications, its characteristics in laboratory culture must be defined to ensure that they do not depart greatly from natural behaviour. The growth of microalgae in axenic cultures follows a characteristic pattern with five phases- a lag phase in which no increase in cell numbers occurs, an exponential phase

in which cell multiplication is rapid, and numbers increase in geometric progression, a phase of declining relative growth, a stationary phase, and death phase (Fogg,1965). Such cultures, on exposure to complex industrial effluents, actually integrate the response over a few generations, and can be used in the assessment of the shockload of the effluent. Similar procedure of algal assays may be employed to assess the water quality of polluted rivers also (Kallqvist,1984; Nandan,1996).

This **chapter** deals with the isolation and identification of pure cultures of algae from the fire water pond No.2 of CRL. The growth pattern of each of the test species isolated were studied in the laboratory. Algal bioassays were performed using the influent and effluent of the fire water pond No.2 to assess the nature of the effluents. The performance of the polishing pond in terms of total dissolved solids removal was assessed. The water samples from different points near the effluent discharge sites along Chitrapuzha river were analysed to assess the effects on the effluent receiving stream.

3.2. MATERIALS AND METHODS

Culture of Microalgae

Isolation

Algae were isolated from water samples collected from the fire water pond No.2 of CRL. The water samples were brought to the laboratory and inoculated into different growth media such as the modified Schreiber solution, Miquel s medium, TMRL medium, and Ward and Parrish medium. The growth media were prepared in GF/C filtered, well aerated, and autoclaved tap water by adding the required quantity of the nutrient stock solutions. The

media were taken in cotton-stoppered sterile Borosilicate conical flasks for the cultivation of algae. The samples collected from fire water pond, were inoculated into these media and were illuminated by cool-white fluorescent lights (5x40 watts) set on a 12:12 L/D cycle at 28±3°C for a week. The samples were observed under the microscope, and those showing algal growth were reinoculated into fresh growth media. Based on the observations, in different growth media, the Ward and Parish medium (Ward and Parrish,1982) was selected for further isolation. As the Ward and Parrish medium did not support the growth of blue-green algae, other media, such as Walnes medium, Gerloff medium and BG II medium were tried for the isolation of blue-green algae. BG 11 medium (Stainer *et al.*,1971) was selected for further isolation of blue-green algae.

Purification

The microalgal populations that developed from the primary inoculum were serially diluted and the dilution culture with minimum contamination was selected for sub-culturing. The serial dilution technique was repeated several times to obtain pure cultures. The almost pure cultures were streaked on to agar plates for further purification. The inoculated agar plates were incubated under the light assembly, and when the cultures established, they were transferred to liquid media. The purification and development of axenic cultures were done under sterile conditions. All the cultures were checked for bacterial growth.

Pure cultures of three algal species could be developed by this procedure. These species were *Chlorella pyrenoidosa, Oocystis pusilla* and *Oscillatoria quadripunctulata.* The description of the species is given below :

Oocystis pusilla

Division Chlorophyta

Class Chlorophyceae

Order Chlorococcales

Family Oocystaceae

Genus *Oocystis*

Species *pusilla*

Cells solitary, elongate-ellipsoid with rounded ends measuring 6-11μm in length. Chromatophores 2-3, almost filling the cells. Cell division by formation of 2-4 autospores. Cell membrane thin, without polar thickenings, distributed in freshwater.

Chlorella pyrenoidosa

Division Chlorophyta

Class Chlorophyceae

Order Chlorococcales

Family Oocystaceae

Genus *Chlorella*

Species *pyrenoidosa*

Cells solitory, spherical. Chloroplast cup-shaped parietal with a distinct pyrenoid. Cells 2.5 - 5μm in diameter.

Oscillatoria quadripunctulata

Division Cyanophyta

Class Cyanophyceae

Order Nostocales

Family Oscillatoriaceae

Genus *Oscillatoria*

Species *quadripunctulata*

Trichomes nearly straight, 1-1.5μm diameter; not constricted at the joints; cells 3.5-5 μm long, contents minutely granular, transverse walls rather obscure marked by a pair of somewhat larger granules on either side.

Growth Kinetics of Microalgae in Culture

The growth pattern of the isolated algal species were determined by measuring the increase in number of cells following growth in culture medium. The single celled *Chlorella pyrenoidosa* and *Oocystis pusilla* were inoculated into 250 mL of Ward and Parrish medium taken in 500 mL sterile conical flasks, at an initial cell density of $1x10^4$ cells/mL, and incubated for 34 days under the light assembly. The experiment was done in triplicate. Growth of the cultures was monitored by haemocytometer cell counts. Subsamples of 5 mL were withdrawn from the flasks every alternate day, fixed in Lugol's iodine, and the cell numbers counted using a haemocytometer under a Carl-Zeiss binocular microscope. A graph was plotted with the logarithm of cell count against the number of days to determine the growth curve.

Oscillatoria quadripunctulata being a filamentous blue-green algae, the absorbance at 620 nm was determined as index of growth instead of cell count. The experiment was

conducted in 500 mL sterile conical flasks containing 250 mL of the BG 11 medium to which 25 mL of the inoculum was added. The cultures were incubated for 34 days, measuring absorbance every alternate day to monitor the growth of the algae. The pattern of growth was elucidated based on a graph with the absorbance along the Y-axis and the days of incubation along the X- axis.

The exponential growth constant k' was calculated after Reynolds (1984) as

$$k' = \ln(Nt_1 / Nt_0)/(t_1 - t_0)$$

where, Nt_0 = cell count at time t_0

Nt_1 = cell count at time t_1

$t_1 - t_0$ = time interval in hours

The generation time t_G was calculated as

t_G = ln 2/k' where k' is the exponential growth constant given above.

The divisions per day(k) during the exponential phase was calculated according to Eppley and Strickland (1968) as follows;

$$k = 3.32/t_1 - t_0 (\log 10\ Nt_1 - \log 10\ Nt_0)$$

where, $t_1 - t_0$ = time interval in days.

Growth of Algae in Half-Strength Medium

The culture media was prepared in half-strength to make the nutrient enrichment to be comparable with the natural conditions where nutrient concentrations may be lower. The half-strength media were prepared by adding half the prescribed amount of nutrients tothe base water. The algae *O.pusilla* and *C.pyrenoidosa* were inoculated into 100 mL of the medium taken in 250 mL sterile conical flasks at an initial cell density of $1x10^4$ cells/ mL. The cultures in full-strength medium were kept as control. The experiment was done in

triplicate, and all the cultures were incubated under the light assembly for eight days as before. The cell count was determined on the fourth, sixth and eighth day. *O.quadripunctulata* was inoculated in 10 mL volume to 100 mL of the half-strength BG 11 medium and incubated as above along with the control. The absorbance of the cultures was measured at 620 nm following inoculation and on the eighth day. The cell yields were compared by Student's *t* test. A probability value of <0.05 was considered to be significant.

Algal Assays of CRL Effluent

Screening Test

The test species, *Oocystis pusilla* was screened to ascertain whether the effluent of CRL was toxic or stimulatory to algal growth. The effluent discharged from the fire water pond No.2 of CRL was collected, in October 1994, brought immediately to the laboratory, filter sterilized and transferred to 500 mL sterilized culture flasks(Borosil) plugged with non-absorbent cotton. A set of three flasks containing 200 mL each of the effluent was inoculated with *O.pusilla* from an exponentially growing culture at an initial cell density $1x10^5$ cells/mL. Another set of three flasks containing the effluent were enriched with the half-strength Ward and Parrish medium and inoculated with *O. pusilla* as before. A similar set containing the growth medium alone was also inoculated with the algae to serve as control. The experimental flasks were incubated for 96 h at 28±2°C under a fluorescent lamp assembly of 5x40 watts.

Samples were withdrawn from the cultures after 96 h, fixed in Lugol's iodine, and the cell counts determined in a haemocytometer. Data were analyzed statistically by an overall

one way analysis of variance and Student's *t* test. A probablity value of <0.05 was cosidered to be significant.

Definitive Test

Effluent samples were collected from two treatment stages of the Effluent Treatment Plant of CRL. The first sample was the discharge from the fire water pond No.1, *i.e.*, the influent to the fire water pond No.2. The second sample was the effluent from the fire water pond No.2. The effluents were filtered through GF/C filter paper (0.45μm)and dilution series of 20, 40, 60, 80, and 100 per cent were prepared using boiled, cooled, aerated freshwater. These test dilutions were taken in 250 mL sterile conical flasks and inoculated with $1x10^5$cells/mL of exponentially growing *O.pusilla* and *C.pyrenoidosa*. A similar inoculum was added to culture flasks containing 100 mL each of half-strength Ward and Parrish medium to serve as control. The test cultures along with the control were incubated for 96 h under the light assembly as in the earlier assays, and the final cell count estimated using a haemocytometer.

The experiment was repeated using *O.quadripunctulata*. The test media were inoculated with 10 mL of the inoculum, and the optical density at 620 nm was measured at the initiation of the experiment, and after 96 h.

The mean and standard deviation of the cell counts of *O.pusilla* and *C.pyrenoidosa* were calculated and expressed as the percentage inhibition / stimulation of growth with respect to the control. The percentage of growth rate depression/stimulation was calculated for *O quadripunctulata* using the equation,

growth depression $\% = 1 - \frac{\mu i}{\mu c} x100$

where, μi = the specific growth rate of the treated culture

μc = specific growth rate of control (Beg *et al.*,1982).

The specific growth rate was calculated by

$$\mu = \ln(n_2/n_1)/(t_2 - t_1)$$

where, μ = specific growth rate

n_1 = absorbance of the culture suspension at time t_1

n_2 = absorbance of the culture suspension at time t_2

A graph was plotted for each test species taking percentage effluent along the X-axis and cell counts/specific growth rate along the Y-axis.

Variability of CRL Effluent

The objective of the experiment was to define the variability in the nature of the refinery effluent. The effluent discharged from CRL was collected in October, November, and December 1994, and May 1995. The variability of the effluent was assayed against the growth of *O.pusilla*. The pure culture of *O. pusilla* was inoculated into the undiluted but filtered effluent samples at an initial cell density of $1x10^5$ cells/ mL, and the cell count was determined after 96 h of incubation. The experimental set up was similar to the previous assays. The percentage growth relative to control was calculated.

Removal of Total Dissolved Solids in the CRL Fire Water Pond No.2

The GF/C filtered influent and effluent of the fire water pond No.2 were analysed initially for total dissolved solids. The estimation of total dissolved solids was done following APHA (1992). The samples in 500 mL volumes were filtered through GF/C filter paper, and the filtrates were transferred to pre-weighed evaporating dishes. These were evaporated

on a water bath and dried in an oven at 180°C for 1 h. The dishes were cooled in a desiccator and weighed. The TDS was determined as the difference in weight.

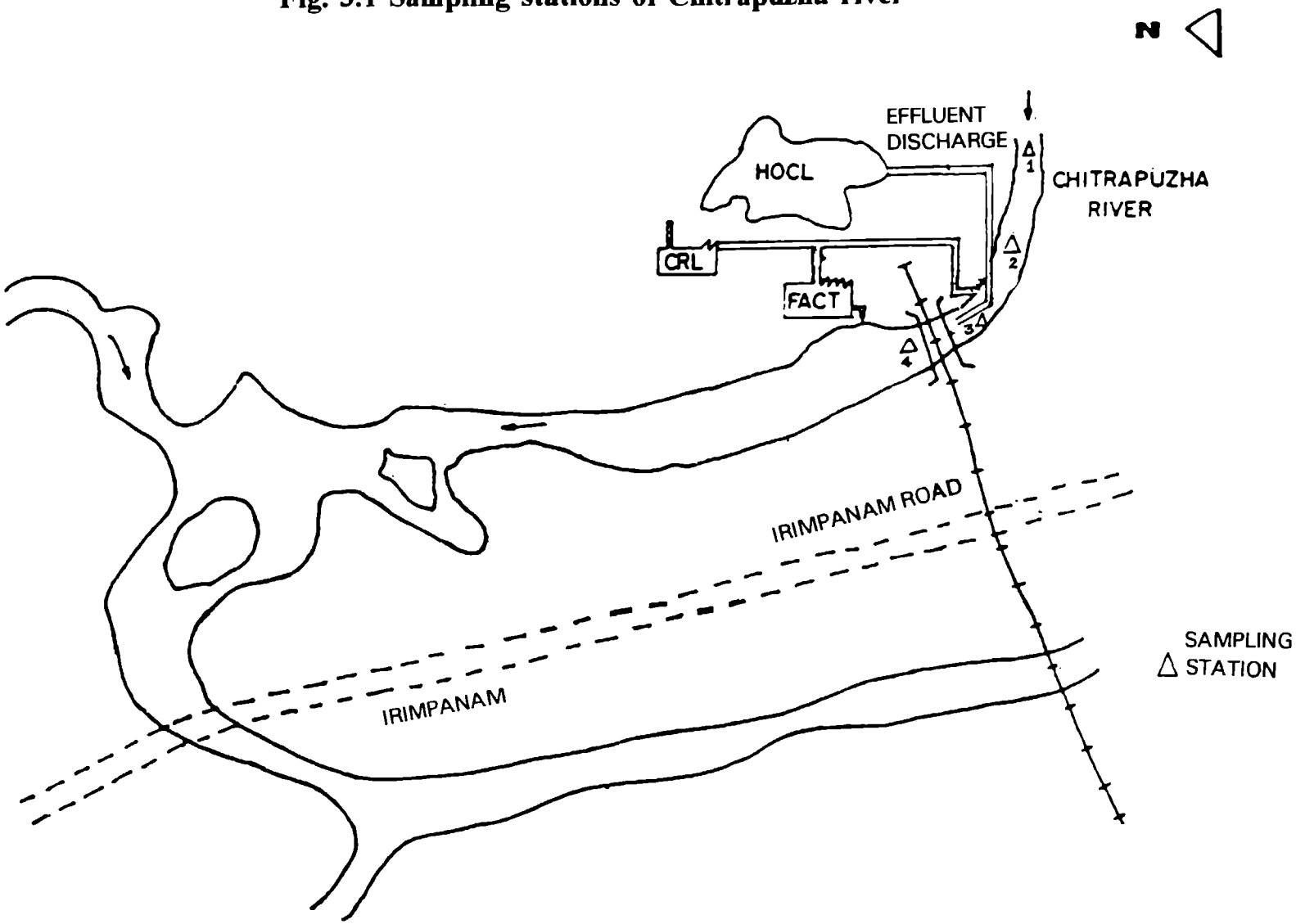
Phytoplankton Flora of Chitrapuzha River

Chitrapuzha river is a tidal tributary of Periyar running a distance of 10 km, and connected to the Cochin backwaters of Vembanad estuary, on either side. During monsoon season the river carries fresh water, and in summer saline water from the backwater incurs on either reaches of the river. The river receives domestic and commercial effluents along its course . Three major industries - Cochin Refineries Ltd., Hindustan Organic Chemicals Ltd., and Fertilsers And Chemicals Travancore Ltd., are located on the banks of the river, and they discharge effluent to the river at Ambalamugal. In addition, it is the route of transport of raw materials, and finished products of industries, as well as cargo by public.

Water samples were collected from the river at four locations(Fig.3.1). The sampling stations No.1 and 2 were located at the upstream of the CRL effluent discharge site The stations 3 and 4 were in the downstream locality of the effluent discharge. Sampling was done in the summer months of 1995.

The water samples were collected using a water sampler, transferred into clean polythene bottles, and brought to the laboratory without delay. The pH of the sample was determined using a pH meter. A sub-sample was fixed in Lugol's iodine, and observed under the microscope. The phytoplankton cells were identified, and the cell counts were determined in a haemocytometer. Chlorophyll *a* was measured as detailed in Chapter 2.

Fig. 3.1 Sampling stations of Chitrapuzha river



3.3. RESULTS

Growth Kinetics of Test Species

The growth pattern of *C.pyrenoidosa, O.pusilla* and *O.quadripunctulata* are given in Figures 3.2 and 3.3. The algae exhibited the typical pattern of growth of the axenic cultures. The lag phase was not very prominent. The exponential growth ceased within 10-12 days. The growth rate of *O.pusilla* and *C. pyrenoidosa* declined after 12 days, and the cell counts were maintained stationary till the 34th day, whereas the growth of *O. quadripunctulata* declined after 18 days, without a distinct stationary phase. The growth rate was highest for *O.pusilla* and lowest for *O.quadripunctulata* (Table 3.1).

Table 3.1. Maximum exponential growth constant, maximum doublings per day and minimum generation time of *Oocystis pusilla, Chlorella pyrenoidosa* and *Oscillatoria quadripunctulata*

Test species	Maximum k'	Maximum k	Minimum t_G
O.pusilla	0.036	1.250	19.25
C.pyrenoidosa	0.020	0.707	33.97
O.quadripunctulata	0.017	0.580	41.5

Growth in Half-Strength Medium

The cell yield of *O.pusilla* and *C.pyrenoidosa* in the half and full- strength media and the test of significance are given in Table 3.2 and 3.3. There was no significant difference in the growth of the two species in the two media tested. Therefore, half-strength medium was selected as the base medium for all algal assays.

Table 3.2. Cell yield of *Oocystis pusilla* expressed as cell count and the student's *t* test following growth in full- strength and half-strength Ward and Parrish medium for eight days

Days	Cell count (x 10^4 cells/mL) $\bar{x} \pm SD$		*t* value	Probability
	Full- strength	Half- strength		
4	3.6±0.69	3.2±0.41	0.94	0.20
6	4.7±0.98	4.2±0.9	0.70	0.26
8	7.5±1.20	7.8±1.0	0.33	0.38

Table 3.3 Cell yield of *Chlorella pyrenoidosa* expressed as cell count and the student's *t* test following growth in full -stength and half-strength Ward and Parrish medium for eight days

Days	Cell count (x 10^4 cells/mL) $\bar{x} \pm SD$		*t* value	Probability
	Full- strength	Half- strength		
4	3.6±0.67	3.2±0.41	0.95	0.20
6	4.8±1.04	4.2±0.90	0.73	0.24
8	7.5±1.20	7.3±1.0	0.33	0.38

The growth of *O.quadripunctulata* was different in the full and half-strength medium. The growth of the cultures in the half-strength medium declined by eighth day, the blue-

green colour faded and the absorbance of the culture was significantly lesser than the control culture in full-strength medium (Table 3.4). Therefore, full-strength medium was selected for all further assays.

Table 3.4.The absorbance at 620nm, and the student's *t* test of *Oscillatoria quadripunctulata* after growth in full-strength and half- strength BG 11 medium for eight days

Days	Absorbance $\bar{x} \pm SD$		*t* value	Probability
	Full- strength	Half- strength		
0	0.03±0.001	0.029±0.00	1.26	0.14
8	0.20±0.010	0.031±0.031	18.37	2.58E-05**

**Significant at 1% level

Algal Assay of CRL Effluent

Screening Test

The results of the screening test are given in Table 3.5. The growth of *O.pusilla* was promoted by the effluent yielding a population of 4.47×10^5 cells/mL in 96 h whereas in control the cell density was 3.33×10^5 cells/mL. The *F* ratio and the probability values revealed that the difference in cell density among treatments was significant at 5% level. Estimation of *t* values showed that there was no difference between the cultures grown in the enriched and unenriched effluent, but there was highly significant difference between the cell yields in the growth medium and the effluent(Table 3.6).

Table 3.5. Four day cell count and ANOVA of *Oocystis pusilla* upon exposure to the effluent from the fire water pond No.2 of CRL. The initial cell density was 1x10^5 cells/mL

Sample	Cell density $\bar{x} \pm SD$ (x10^5 cells/mL)	*F* ratio	Probability
Control	3.33±0.42		
Effluent	4.47±0.27	7.45	0.024*
Enriched effluent	4.32±0.47		

*significant at 5% level

Table 3.6. Student's *t* test between different treatment pairs of control, raw effluent, and enriched effluent estimated by the growth response of *Oocystis pusilla*

Treatment pair	*t* value	Probability
Control/Effluent	3.96	8.31E-03**
Effluent/Enriched effluent	0.50	0.323

** significant at 1% level

Definitive Test

The results of the assay of the influent to the fire water pond No.2 of CRL against thee three test species are summarised in Table 3.7. The growth of *O.pusilla* was inhibited by the influent to fire water pond No.2; the degree of inhibition increased with concentration.

C.pyrenoidosa exhibited stimulation of growth at 20 and 40% influent sample , but growth was inhibited at higher concentrations.

The cell density of *O.pusilla* was reduced by 76% of the control at 20% influent whereas *C. pyrenoidosa* exhibited growth stimulation of 14% at 40%, followed by 56% growth inhibition at 60% effluent. The undiluted influent to the fire water pond No.2, inhibited the growth of *O.pusilla* by 85% and that of *C. pyrenoidosa* by 78% The growth of *O.quadripunctulata* was not affected by 20% dilution of the influent, but at higher concentrations there was growth depression. The percentage inhibition was 70% in the undiluted influent.

Table 3.7. Growth of *Oocystis pusilla*, *Chlorella pyrenoidosa* and *Oscillatoria quadripunctulata* in cultures exposed to the influent to the fire water pond No.2 of CRL for 96 hours

% influent	*O.pusilla* cell density $\bar{x} \pm SD \times 10^5$ cells/mL	% inhibition	*C. pyrenoidosa* cell density $\bar{x} \pm SD \times 10^5$ cells/mL	% inhibition	*O. quadripunctulata* specific growth rate $\mu \pm$ SD	Degree of depression (%)
00	3.03±0.33		3.81±0.17		0.43±0.06	
20	0.72±0.08	76.24	4.25±0.74	11.54*	0.43±0.02	0
40	0.66±0.09	78.22	4.34±2.18	13.91*	0.40±0.01	6.97
60	0.7±0.23	76.9	1.69±0.2	55.65	0.37±0.04	2.32
80	0.45±0.19	85.15	0.81±0.13	78.74	0.32±0.06	25.58
100	0.47±0.13	84.5	0.84±0.11	77.96	0.13±0.02	69.76

* stimulation

The results of the assay of the effluent at the discharge point of fire water pond No.2 are given in Table 3.8. The effluent stimulated the growth of *O.pusilla* ,*C.pyrenoidosa* and *O.quadripunctulata*. The per cent stimulation was highest for *O.pusilla*, followed by *C. pyrenoidosa* and then *O.qudripunctulata*. The graphical representation of the data is given in Figs. 3.4, 3.5 and 3.6. The comparison of results revealed that the influent to the fire water pond No.2 inhibited the algal growth while the effluent stimulated growth.

Table 3.8. Growth of *Oocystis pusilla, Chlorella pyrenoidosa* and *Oscillatoria quadripunctulata* in cultures exposed to the effluent from the fire water pond No.2 of CRL for 96 hours

% Effluent	*O.pusilla* cell density $\bar{x}$±SD x10^5 cells/mL	% stimulation	*C. pyrenoidosa* cell density $\bar{x}$±SD x10^5 cells/mL	% stimulation	*O. quadripunctulata* specific growth rate μ± SD	% stimulation
00	3.03±0.33		3.8±0.17		0.43±0.06	
20	6.59±0.08	117	5.25±0.12	37.8	0.51±0.03	18.60
40	7.89±0.62	160.39	6.82±0.35	80.30	0.50±0.0.01	16.25
60	9.2±0.22	203.63	5.7±0.11	49.6	0.48±0.01	11.62
80	8.85±0.24	192.07	5.48±0.24	43.83	0.51±0.01	18.60
100	9.31±0.4	207.26	6.7±0.05	75.85	0.51±0.06	18.60

Variability of CRL Effluent

The variation in the quality of the effluent discharged by CRL as determined by algal assay is given in Table 3.9. It was found that in three out of four assays, the effluent stimulated the growth of algae. The range of growth stimulation was 132% to 307% of the control. The effluent sample collected in December 1994, inhibited the growth of algae by 48%.

Table 3.9. Cell yield and percentage response of *Oocystis pusilla* exposed to the effluent discharged from CRL at different sampling periods (period of exposure 96 hours, initial cell density 1x10^5 cells/mL)

Sampling period	Cell density (x10^{5}cells/mL)	Percentage of control
Control	3.03	
October,1994	4.47	132.21
November,1994	4.68	154.46
December,1994	1.58	47.85
May,1995	9.30	306.93

Removal of Total Dissolved Solids

The influent to the fire water pond No. 2 had a total dissolved solids content of 602 mg/L and in the effluent it was 572 mg/L. The pond had effected a TDS removal of 4.98% in a retention period of 4 days.

Table 3.10. Observations at sampling stations and analyses of water samples from Chitrapuzha river

Sampling station	Flow	Colour	pH	Cell count $\bar{x} \pm SD$ x10^3 cells/mL	Chlorophyll *a* mg/m^3
1	Slow	Colourless	6.61	6±3.16	8.3
2	Slow	Cloudy	6.81	16.5±3.51	57.8
3	Moderate	Cloudy	7.74	21±6.68	51.4
4	Moderate	Cloudy	7.95	17±4.96	104.8

Table 3.11. Principal algal species of Chitrapuzha river near the effluent discharge sites.

Algal species	Station 1	Station 2	Station 3	Station 4
Actinastı um hantzschii				+
Chlorella pyrenoidosa	+	+	+	+
Chlorococcum infusionum	+		+	+
Closterıopsis longispina				+
Chlamydomonas sp.	+		+	
Euglena sp		+		+
Nostoc sp.			+	
Nitschia palea	+		+	+
Navicula cuspidata	+			
Oocystis pusilla	+	+	+	+
Oscillatoria quadripunctulata			+	+
Pandorina morum				+
Phormidium inundatum			+	
Phacas longicauda		+	+	+
Scenedesmus dimorphus	+		+	+
S. longus vari. *naegeli*				+
S. quadricauda var. *bicaudatus*				+
S.q. var. *longispina*				+
S. bijugatus				+
S. carinatus				+
Volvox aureus				+

+ present - absent

Fig.3.2 Growth curve of *Oocystis pusilla* and *Chlorella pyrenoidosa*

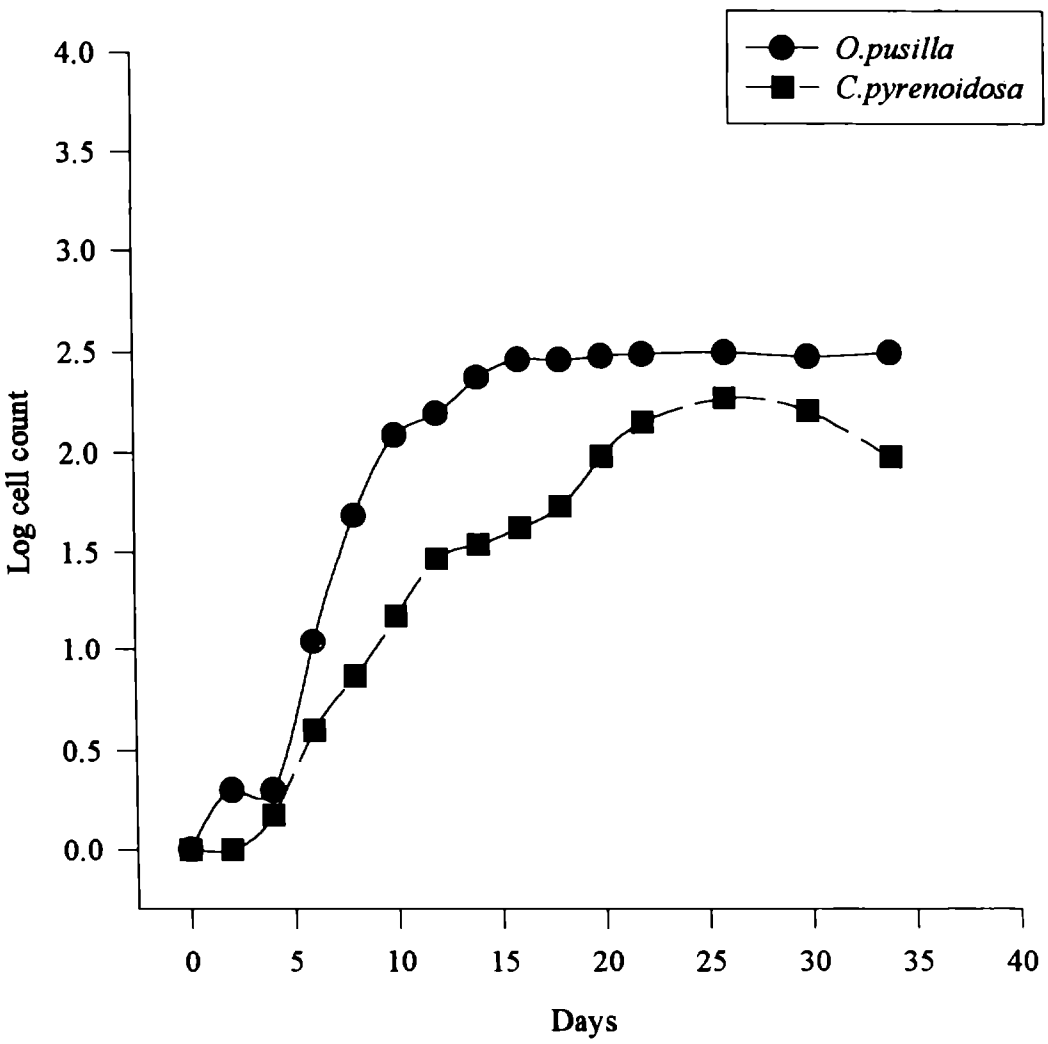


Fig.3.3 Growth curve of *Oscillatoria quadripunctulata*

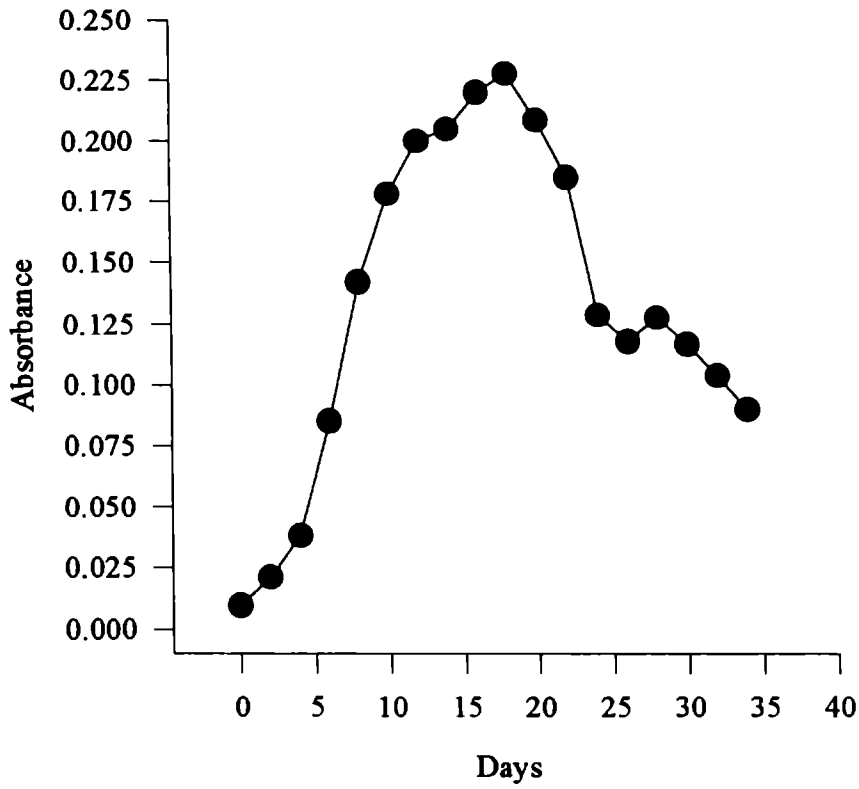


Fig.3.4 Growth pattern of *Oocystis pusilla* in CRL effluent

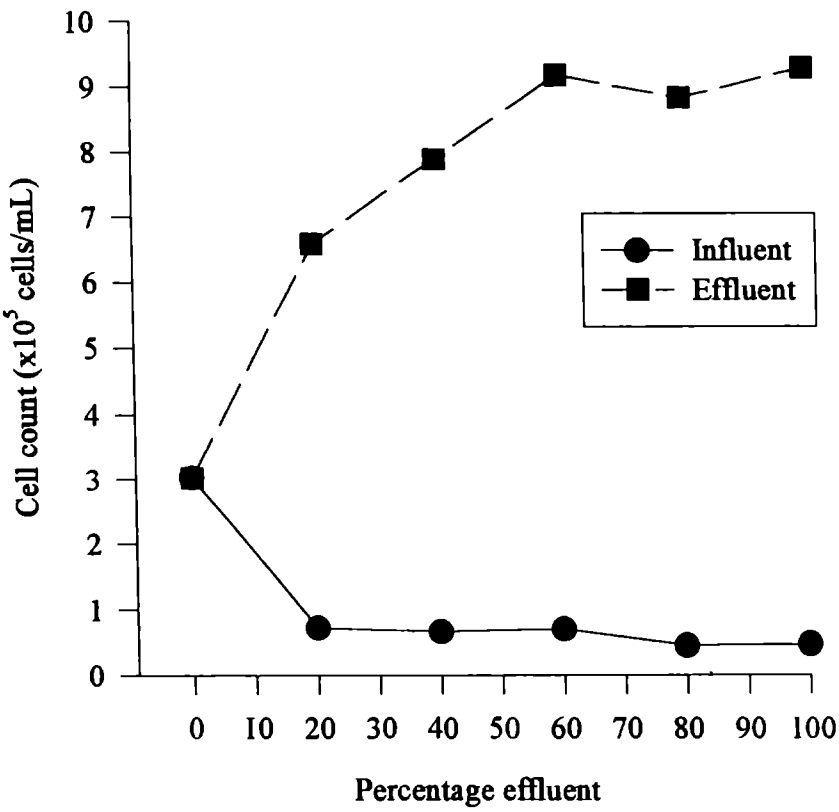


Fig.3.5 Growth pattern of *Chlorella pyrenoidosa* in CRL effluent

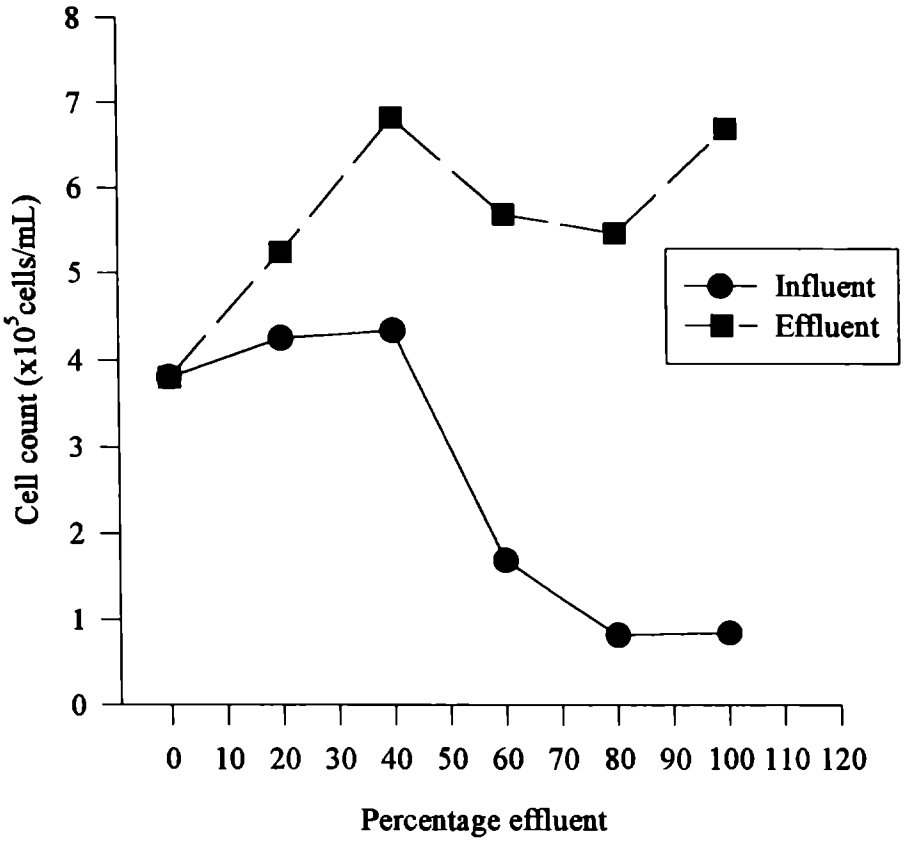
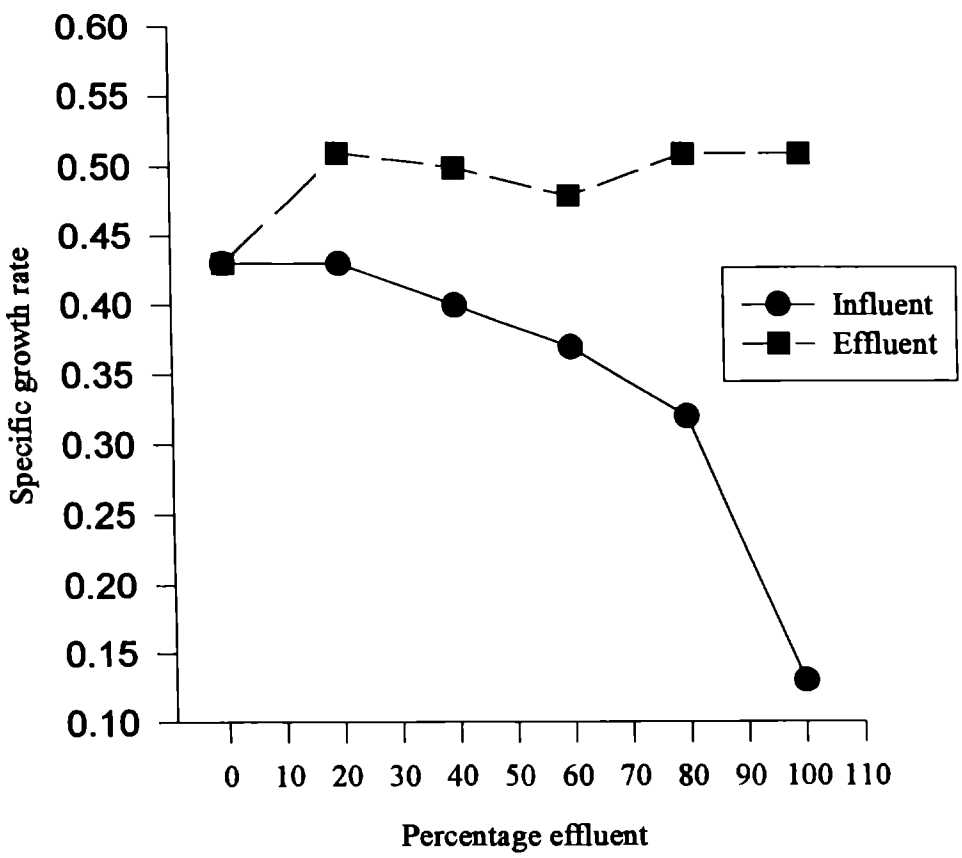


Fig.3.6 Growth pattern of *Oscillatoria quadripunctulata* in CRL effluent



Phytoplankton Flora of Chitrpuzha River

The observations at the sampling stations and the results of the phytoplankton analyses are given in Table 3.10. The river was slow flowing, and the water turbid at the industrial sites. The pH ranged from 6.61 to 7.95. The algal flora was composed of Chlorophyta, Cyanophyta, Euglenophyta and Bacillariophyceae. The phytoplankton count was least at station 1, but increased downstream. The population size ranged from $6x10^6$ cells/L to $21x10^6$cells/L. The chlorophyll *a* content was also observed to be lowest at station 1 compared to the rest of the downstream locations. The concentration of chlorophyll *a* ranged from 8.3 mg/m^3 to 104.8 mg/m^3 The composition of the algal flora is represented in Table 3.11.

3.4. DISCUSSION

The growth patterns of the three test species were defined to ensure that they do not depart from the typical axenic cultures raised in the laboratory. The results confirmed that the isolated species follow the typical growth curve exhibited by axenic cultures. *O. quadripunctulata*, however did not show a stationary phase as the growth declined rapidly after 12 days, which could have been due to nutrient exhaustion. This assumption is substantiated by the low growth of the species in half-strength medium. The half- strength medium supported the growth of *O. pusilla* and *C.pyrenoidosa* suggesting their lower nutrient requirements compared to *O.quadripunctulata*.

The algal assay of the effluent discharged to Chitrapuzha river has shown that it stimulates the growth of algae. This results confirm the observation on very high algal biomass in the fire water pond No. 2 of CRL. Although it was not attempted to fractionate the effluent,

as in previously documented literature, the stimulatory effect of the refinery effluent is due to its organic fraction (Walsh and Garnas, 1979).

The influent to the fire water pond No. 2 inhibited the algal growth. Toxicity/stimulation, as well as structural damages of cells are reported to be caused by a variety of industrial effluents (Walsh *et al.*, 1980 Wong *et al*, 1994, 1995). The toxicity of the influent is reduced in the polishing pond as TDS get reduced by 4.9%. The resultant effluent stimulates the growth of algae.

Transformation of a toxic influent to that of a stimulatory effluent shows that the polishing pond is effective as a detoxifying unit. Phytoplankton is only one component of this system. The detoxification effected in the pond is the net effect of physico-chemical and biological variables. Generally, the organic materials entering a facultative pond are stabilised by bacteria aided by algae as oxygenators. In addition, algae help mineralisation and contribute to food chain (Round, 1984). More recently it is documented that algae play a definite role in the removal it is documented that algae play a definite role in the removal of nutrients (de la Noue *et al.*, 1992; Lau *et al.*, 1995), metabolisation of petroleum aromatic compounds (Subramanian and Uma, 1997), pesticides (Venketaraman *et al.*, 1994), and detoxification of metals (Becker, 1994) from various industrial effluents.

The variable results obtained in the present study point to an essential feature of the Effluent Treatment Plant *i.e.*, the inconsistency of the treatment. The industrial effluents in general, are highly variable due to the inter-pollutant reactions, complexity of the polluting constituents, fluctuating volumes of the effluent generated, and changes in the operating conditions (Calley *et al.*; 1977; Nemerow, 1978).

The eutrophic potential of the CRL effluent was confirmed by the algal assays. Therefore,it is evident that this effluent will promote the growth of algae in the receipient water body. This assumption was confirmed by the analysis of the algal community of the Chitrapuzha river. The algal biomass increased in the down stream of industrial discharges, the chlorophyll concentration was high, and in the eutrophic range. Nair *et al.* (1988) studied the phytoplankton counts and chlorophyll *a* concentration in Chitrapuzha river, and concluded that the maximum phytoplankton production is seen in the site near the effluent discharge area. The river water was nutrient - rich due to the effluent discharge from a fertiliser factory. According to Harikumar *et al.* (1997) the nutrients and heavy metals are heavily distributed in the Chitrapuzha river, and the tolerant phytoplankton species like *Chlorella* sp., *Oscillatoria* sp. and *Nitzschia* sp. dominate in the river. The present investigation conducted in summer, when production is the highest, and washout minimum, showed that there is an increase in algal population in the downstream of industrial discharge sites of the river.

CHAPTER 4

ALGAE IN PETROCHEMICAL EFFLUENT TREATMENT

- ★ Introduction
- ★ Materials and Methods
- ★ Industry Selected
- ★ Effluent Treatment Process of HOC
- ★ Effluent Sampling from HOC
- ★ Analytical Methods
- ★ Algal Assays of HOC Effluent
  - ◆ Screening test
  - ◆ Definitive test
  - ◆ Variability of HOC effluent
- ★ Results
- ★ Analytical Results
- ★ Screening Test
- ★ Definitive Test
- ★ Variability of HOC Effluent
- ★ Discussion

CHAPTER 4

ALGAE IN PETROCHEMICAL EFFLUENT TREATMENT

4.1. INTRODUCTION

The petrochemical industries are expanding fast, and are sources of toxic effluents entering the water courses throughout the world. Petrochemical refers to all chemical compounds which are manufactured from petroleum and natural gas. The products of the industry are categorised as (1) aliphatics (2) cycloaliphatics (3) aromatics and (4) inorganics. The petrochemicals cater public utility as liquid fuels, lubricants, and polymers such as plastics, rubber and fibers. The Indian petrochemical industry which is the major supplier of crucial inputs to large and growing downstream units is presently keeping a high and steady rate of growth. According to Gopalakrishnan (1997), the export of principal petrochemicals have grown during the past four years ending in 1996-1997..

The wastewater from petrochemical industry is categorised to surface run-off, process water and cooling water (Cox,1977). The wastewater characteristics include oils and solvents, high biochemical oxygen demand, suspended solids, aromatics, phenolics, sulphides, halogenated and polycyclic aromatic compounds, and detergents. Different treatement processes are adopted according to the nature of the effluent.

The physico-chemical effluent treatment processes usually employed in petrochemical industries are steam distillation, solvent extraction, activated carbon adsorption, gravity separation, flocculation, sedimentation or flotation , coagulation, and filtration (Chivers,1984).

The common biological treatment processes used for the highly variable petrochemical effluents include trickling filtration, rotating biological discs, activated sludge units, and aerated lagoons. The major process developments in the biological treatment of the effluent include the use of high-rate trickling filters based on plastic media, uprate aerobic activated sludge, and anaerobic treatment.

The general effects of the petrochemical effluents on the aquatic system are oil pollution, chemical pollution, high concentration of suspended solids, and thermal pollution. The pollution of drinking water sources is a major health concern. The phenols, aldehydes, and chlorinated and aromatic hydrocarbons in the effluent are biocides, and very toxic to fish (Chivers,1984). The phenolic compounds are highly toxic to fish and fish food organisms because of the high oxygen demand of the compounds(Train,1979).

The amenability of the toxic petrochemical effluent to biological treatment can be assessed by laboratory studies. The biological treatment of these effluents containing high levels of simple phenols, and aldehydes involve microorganisms which are particularly able to breakdown such compounds. The biological treatment systems are usually inhibited by 75 mg/L phenol, 15 mg/L hydroquinone, 20 mg/L 2-4dinitrophenol and 1 mg/L m-aminophenol (Tolgyessy,1993). Low molecular weight hydrocarbons were found to stimulate or inhibit phytoplankton growth, depending upon the species (Dunstan,1975). Less attention has been paid to the effects of phenolic effluents on algae, in particular on the freshwater algae (Tripathi and Pandey,1990).

The present **chapter** focus on the physico-chemical analyses of the effluent from a petrochemical industry, and the bioassessment of the effluent using three test species of algae.

4.2. MATERIALS AND METHODS

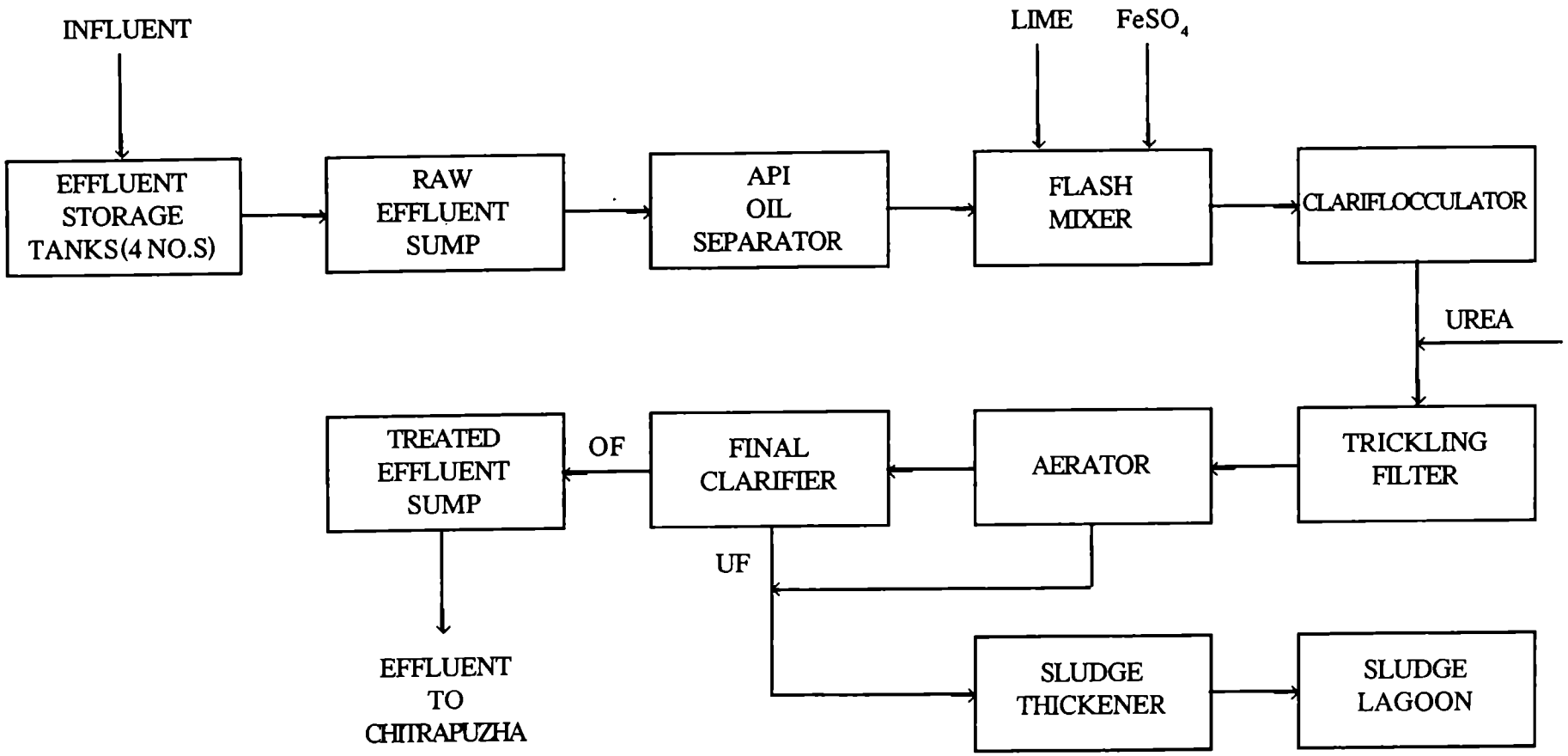
Industry Selected

The petrochemical industry selected for the investigation was Hindustan Organic Chemicals Ltd. (HOC). The HOC is the biggest phenol-acetone complex in India with an installed capacity of 40,000 tonnes per annum of phenol, and 24,600 tonnes per annum of acetone.The phenol complex in HOC consists of three units; propylene unit, cumene unit and phenol unit. The propylene unit consists of fractionating columns where the chemical is purified by the removal of carbonyl sulphide as hydrogen sulphide. Cumene is made in the cumene unit by the alkylation of benzene with propylene in the presence of solid phosphoric acid as a catalyst. In the phenol unit cumene oxidation is carried out in the presence of air in an aqueous emulsion stabilised by alkali in the 8.5-9.5 pH range. The cumene hydroperoxide thus formed is concentrated by evaporation, and fed into two parallel cleavage reactor circuits along with concentrated sulphuric acid catalyst at 55-65^{0}C. The cumene hydroperoxide is cleaved under controlled conditions of temperature and acidity to form phenol, acetone, and byproducts. The organic layer contains 76% cumene, 14% phenol, 18% acetone, and 12% methyl styrene and acetophenone. The mixture is separated in a series of distillation steps.

Effluent Treatment Process of HOC

The effluents from the different process units are collected separately, and mixed in raw effluent sump. The combined effluent is then taken to API oil separator for the removal of oil (Block diagram 2). The effluent is led to the flash mixer where ferrous sulphate and lime are added for the removal of emulsified oil and correction of pH, respectively. The coagulated emulsified oil and the colloidal particles are settled in the clariflocculator. The clarified effluent after being enriched with urea passes to the biological treatment system

Block Diagram 2. Effluent treatment plant of Hindustan Organic Chemicals Ltd.



consisting of trickling filter, activated sludge system, and a final clarifier. The clear effluent from this is collected in a constructed tank from which it is pumped out daily into the Chitrapuzha river about 5 km away from the factory. The total water consumption in HOC is 2250 m^3/day of which 1500 m^3 is used for cooling, 300 m^3 as process water and 450 m^3 for domestic purpose. The total effluent generated is about 700 m^3 / day.

Effluent Sampling from HOC

The effluent samples were collected from the final disposal tank every fortnight during March, April, May, and June 1995. The temperature of effluent was measured at the site itself; the samples for the determination of dissolved oxygen were fixed immediately using manganous sulphate and azide-modified alkali iodide reagent. The rest of the samples were transported to the laboratory in carbuoys for analyses.

Analytical Methods

The samples collected were analysed for pH, dissolved oxygen, biochemical oxygen demand, nitrate, phosphate, ammonia, and chlorophyll following standard methods (APHA,1992) as detailed in **chapter 2**. The phenol was estimated by spectrophotometry (APHA,1992). The GF/C filtered samples in 500 mL volume was distilled and 50 mL of the distillate was used for the analysis. The phenolic compounds react with 4-amino antipyrene and potassium ferricyanide to form coloured dye, the absorbance of which was read at 510 nm.

Algal Assays of HOC Effluent

Screening Test

The test species *Oocystis pusilla* was screened to ascertain whether the HOC effluent was toxic or stimulatory to algal growth. Effluent was collected from the final effluent holding

tank , brought immediately to the laboratory, filter sterilized and transferred to 500 mL sterilized culture flasks (Borosil) plugged with non-absorbant cotton. A set of three flasks containing 200 mL each of the effluent was inoculated with *O.pusilla* from an exponentially growing culture at an initial cell density $1x10^5$cells/mL. Another set containing the effluent was enriched with Ward and Parrish medium and were inoculated as before. A similar set containing the culture medium alone was also inoculated with algae to serve as control. The experimental flasks were incubated for 96 h at 28±2°C under the fluorescent lamp assembly of 5x40 watts. The final cell yield was estimated using a haemocytometer after fixation in Lugol's iodine. The cell yields of the different sets of cultures were compared using ANOVA and student's *t* test. A probability value of ≤ 0.05 was considered to be significant.

Definitive Test

The dilution series of the GF/C filtered effluent were prepared in the range of 20, 40, 60, 80 and 100% using filtered, boiled, cooled and aerated fresh water. These dilutions were taken in 250 mL volume in sterile conical flasks and inoculated with $1x10^5$ cells/mL of *O.pusilla* and *C. pyrenoidosa*. The test cultures along with the control were incubated for 96 h and the final cell count was estimated as before. The experiment was done in triplicate.

The experiment was repeated with *O.quadripunctulata*. The volume of the test media was 100 mL, into which 10 mL of the inoculum were added. The optical density at 620 nm was measured at the initiation of the experiment, and after 96 h of incubation.

The mean and standard deviation of the replicate cell counts/specific growth rate were calculated, and expressed as the percentage inhibition/ degree of depression as explained in **chapter 3**.

A graph was plotted with the percentage of waste along the X-axis and the numerical cell density/growth rate along the Y-axis.

Variability of HOC Effluent

The variability in the effect of HOC effluent collected during different months was assayed against *O.pusilla* inoculated at an initial cell density of $1x10^5$ cells/ mL. The cell density of the test algae after 96 h of incubation was calculated, and expressed as the percentage inhibition.

4.3. RESULTS

Analytical Results

The results of the analysis of the physico-chemical properties of the HOC effluent are given in Table 4.1.The effluent was alkaline; the pH range from 8.35 to 8.75. The range of dissolved oxygen was 5.86 to 8.95 mg/L, and the biochemical oxygen demand 35.4 to 72.43 mg/L. Nitrate, phosphate, and ammonia ranged from 0.073 to 2.46 mg/L, 0.008 to 5.69 mg/L and 0.011 to 8.4 mg/L respectively. The phenol content was between 0.43 mg/L and 1.38mg/L.

Table 4.1 Analytical results of the HOC effluent

Parameter	March 1995	April 1995	May 1995	June 1995
Temperature ^{0}C	32	33	32	31
pH	8.35	8.73	8.43	8.75
Dissolved oxygen (mg/L)	5.86	6.43	7.32	8.95
BOD (mg/L)	72.43	39.8	43.6	35.4
Nitrate (mg/L)	0.87	1.38	0.073	2.46
Phosphate (mg/L)	0.60	1.17	0.008	5.69
Ammonia (mg/L)	8.40	0.0385	4.32	0.011
Phenol (mg/L)	0.52	0.43	0.78	1.38

Screening Test

The results of the screening tests are given in Tables 4.2 and 4.3. The growth of *O.pusilla* was inhibited by the effluent. The cell yield obtained after 96 h exposure to the effluent was 0.28×10^5 cells/mL. In the maintenance medium kept as control the cell density was 3.03×10^5 cells/ mL after four days of growth. The *F* ratio and probability values revealed that the difference in the cell density among treatments was highly significant. Estimation of the *t* values (Table 4.2.) showed that there was no significant difference between the cultures grown in the enriched and unriched effluent. There was highly significant difference between the growth in the control medium and in the effluent.

Table 4.2. Cell count and ANOVA of *Oocystis pusilla* after growth in the HOC effluent for 96 hours

Sample	Cell count ($\times 10^5$ cells/mL) $\bar{x} \pm SD$	*F* ratio	Probability
Control	3.03±0.33		
Effluent	0.28±0.17	125.32	1.28E-04**
Enriched effluent	0.47±0.16		

**significant at 1% level

Table 4.3. Student's *t* test between different treatment pairs of control, raw effluent and enriched effluent estimated by the growth response of *Oocystis pusilla*

Treatment pair	*t* value	Probability
Control/Effluent	12.65	1.123E-04**
Effluent/Enriched effluent	1.38	0.121

**significant at 1% level

Definitive Test

The results of the assay of the effluent of HOC using the three test species of algae are represented in Table 4.4, and Fig.4.1, and Fig.4.2. The effluent inhibited the growth rate of all the three species.The percentage inhibition of growth of *O.pusilla* in 20% of the effluent

Table 4.4. Growth of *Oocystis pusilla*, *Chlorella pyrenoidosa* and *Oscillatoria quadripunctulata* in cultures exposed to the effluent of HOC for 96 hours

%	*O.pusilla*		*C. pyrenoidosa*		*O. quadripunctulata*	
inffluent	cell density $\bar{x} \pm SD \times 10^5$ cells/mL	% inhibition	cell density $\bar{x} \pm SD \times 10^5$ cells/mL	% inhibition	specific growth rate $\mu \pm$ SD	Degree of depression (%)
0	3.03±0.33		3.81±0.17		0.43±0.06	
20	1.17±0.13	61.39	1.78±0.14	53.28	0.40±0.04	6.98
40	1.33±0.59	56.11	1.66±0.4	56.43	0.38±0.29	10.93
60	1.33±0.59	56.11	1.26±0.06	66.90	0.37±0.02	14.88
80	1.46±0.49	51.95	1.31±0.23	65.88	0.29±0.09	31.63
100	0.69±0.33	77.36	1.27±0.007	66.66	0.26±0.12	39

Fig.4.1 Growth of *Oocystis pusilla* and *Chlorella pyrenoidosa* in HOC effluent

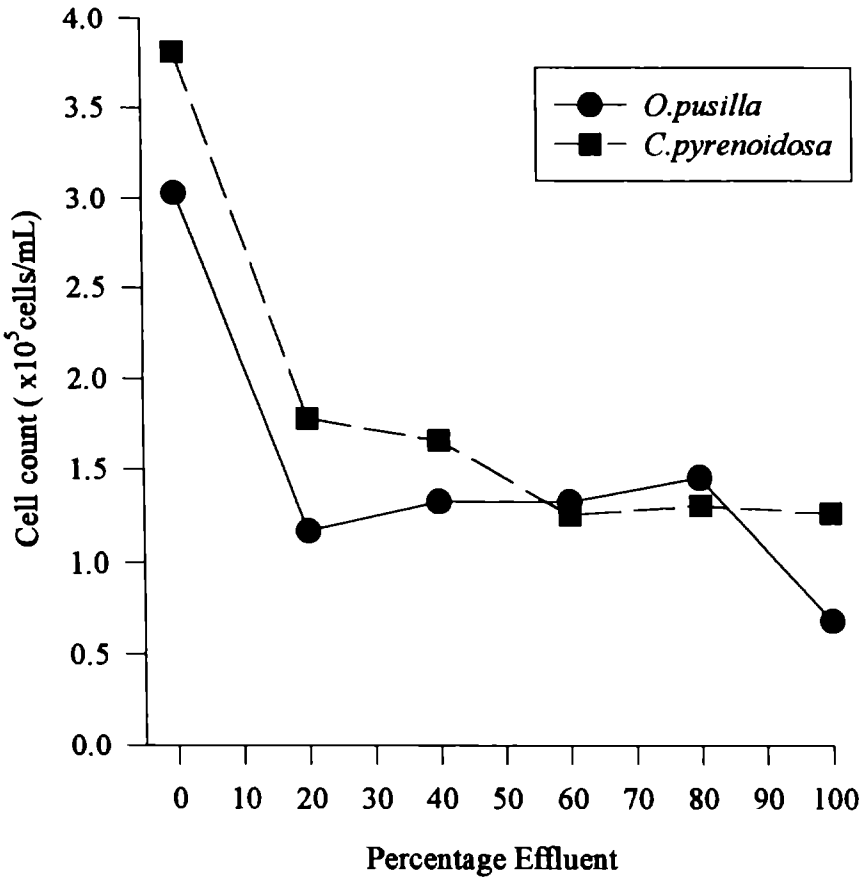
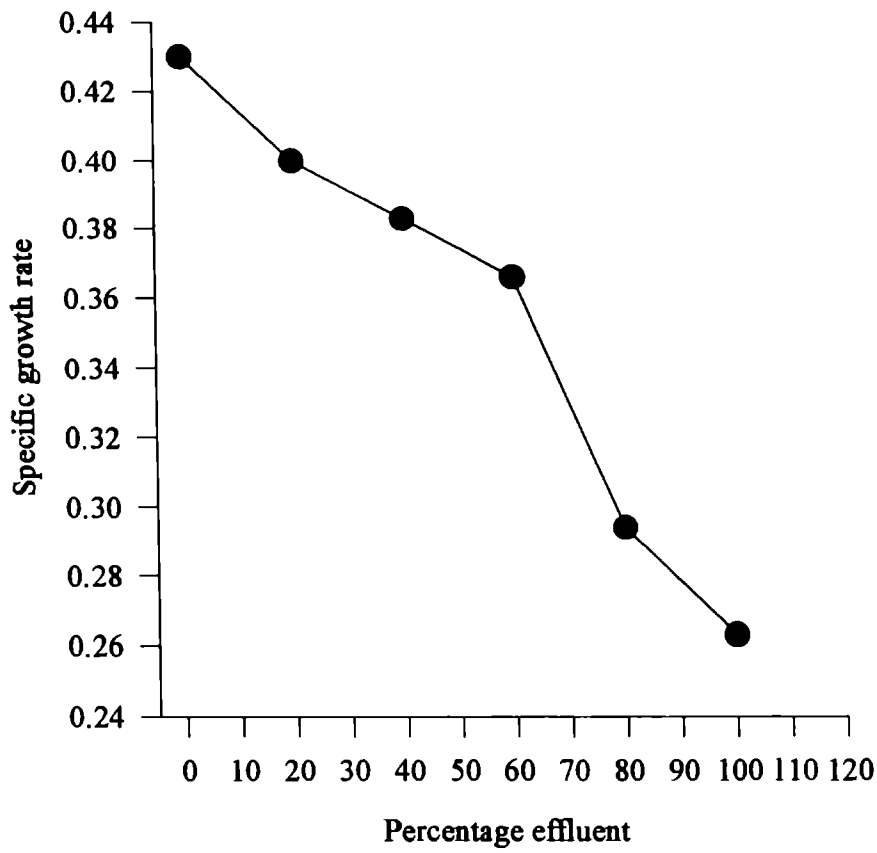


Fig.4.2 Growth of *Oscillatoria quadripunctulata* in HOC effluent



was 61% while in the 100% effluent it was 77% . The percentage inhibition of growth of *C.pyrenoidosa* in 20% effluent was 53% and in 100% effluent 67%. The degree of growth depression of *O. quadripunctulata* in the 100% effluent was only 39%.

Variability of HOC Effluent

The variability in the effect of the effluent collected during different months assayed by the growth of *O.pusilla* is given in Table 4.5. The growth of the test species was inhibited in all cases. The percentage depression varied from 51 to 91%.

Table 4.5. Cell yield and percentage inhibition of *Oocystis pusilla* in HOC effluent sampled in four consecutive months. Initial cell count was $1x10^5$ cells/mL

Sampling month	96 h Cell count/mL	Percentage inhibition
Control	$3.03x10^5$	
March 1995	$1.50x10^5$	50.5
April 1995	$0.68x10^5$	77.56
May 1995	$0.28x10^5$	90.92
June 1995	$0.69x10^5$	77.23

4.4. DISCUSSION

The physico-chemical analysis of the effluent showed that the composition of effluent is variable, and does not always conform to the permissible limits of discharge. The BOD exceeded the permissible limit of 30 mg/L in three out of four samplings. The effluent was alkaline, and the temperature was between 31 and 33°C.

As evaluated by the algal assays the effluent was toxic to algae. The effluent did not support growth even after enrichment. The growth of *O. pusilla* was suppressed by 77% ; *C.pyrenoidosa* by 67%, and *O.quadripunctulata* by 39%. According to Walsh and Merrill (1984) suppression of growth after 48 or 72 h is the best indicator of toxicity. The observed toxicity may be traced to, and explained by the bioavailable contaminants absorbed by algal cells. In a complex industrial effluent containing a mixture of organic, and inorganic compounds, it is not possible to relate the toxicity to any particular factor. Wong *et al.*(1995) reported that the stress effect of organic toxicants on the fine structure of algae is the abnormal build up of large starch grains, and mass destruction of organelles. The interference with oxidative phosphorylation, photosynthesis, respiration, and protein and nucleic acid synthesis occurred in *Chlorella* cells exposed to toxicants such as DDT or PCP (Fedtke1982; Hoagland and Duke,1982).

From March 1995 to June 1995 the toxicities estimated ranged between 51% and 91% . If these results can be considered as an indication of bioactivity of this effluent, then it poses a threat to the existence of the biota in the receiving and downstream waters. However, it is most likely that algae under field conditions exposed to wastes over protracted periods of time are likely to adapt either physiologically or genetically to toxicants. Moreover as, Payne and Hall (1979) have stated, if an algal population in the field is reduced by a waste at 5% of the density before exposure, it will recover its original density after some unspecified period of time. This is substantiated by the observation in Chitrapuzha (chapter 3) that in spite of a toxic discharge from HOC , the river is abundant in phytoplankton community. Being a slow flowing stream, a true phytoplankton community, well adapted to the microhabitat has

developed and is maintaining itself by active reproduction. Even though the algae have seemingly no role in the treatment of HOC effluent, they may be utilised as an indicator of the environmental hazard predictor of the effluent. Moreover, a properly adapted strain holds chances for being used to absorb organic contaminants.

CHAPTER 5

ALGAE IN PHENOL AND PHENOLIC EFFLUENT-GROWTH AND ABSORPTION

- ★ Introduction
- ★ Materials and Methods
  - ★ Effect of Phenol on the Growth of Algae
  - ★ Acclimation of Algae to Phenol
  - ★ Absorption of Phenol
  - ★ Acclimated Algae in Petrochemical Effluent Treatment
- ★ Results
- ★ Discussion

CHAPTER 4

ALGAE IN PHENOL AND PHENOLIC EFFLUENT-GROWTH AND ABSORPTION

5.1. INTRODUCTION

Phenols are organic chemicals consisting of a basic benzene ring and one or more hydroxyl groups. These molecules are both natural, and anthropogenic xenobiotics. The commercial application of phenol includes the production of resins, nylons, plasticizers, dyes, pesticides, gasoline additives etc. The wastewater from fossil fuel refining process, phenol manufacturing plants, pharmaceuticals and a variety of other industries contain phenol. The highest reported concentrations of phenols are from the effluents of petroleum and petrochemical industries (Moore and Ramamoorthy, 1984). Phenol comes under European Economic Community Black List and the US Environmental Protection Agency's list of priority pollutants (Mc Eldowney *et al.*,1993). The Indian Standard prescribe a phenol concentration of 2μg/L as the guideline concentration for drinking water, and 1mg/L for industrial effluent discharges to inland surface water (ISI,1982a, 1982b).

Activated sludge process is one of the most widely accepted biological system for the treatment of phenolic wastes. Mason (1990) reported that toxic levels of phenol in aerobic treament systems range from 50-100 mg/L. Tolgyessy (1993) observed that the biological tratment system was inhibited at 75 mg/L phenol, 15 mg/L hydroquinone, 20 mg/L 2-4 dinitrophenol, and 1 mg/L m-aminophenol.

Biotransformation of phenol is effected by certain bacteria, yeasts and fungi. It has been well established that *Pseudomonas putida* and many other bacterial species can degrade phenol(Zache and Rehm, 1989). Yeasts such as *Trichosporon cutaneuum* was also reported to degrade phenol (Huang and Tseng, 1996). Contribution of bacteria to the overall rate of degradation is affected by phenol concentration, temperature, sunlight, presence of nutrients and other pollutants, and bacterial abundance. The number of bacteria capable of utilising phenol is usually a small percentage of the total population (WHO,1994). The cyanobacteria are capable of metabolising petroleum aromatic compounds as elucidated by Narro (1987). According to Megharaj *et al.*(1991) *Nostoc linckia* can degrade phenol.

Phenols have a highly variable effect on aquatic plants. Some compounds may actually stimulate the growth at low concentrations, whereas, others are consistently toxic (Moore and Ramamoorthy, 1984). A major problem associated with phenolic compounds is their organoleptic properties in water and fish flesh. Phenol in drinking water forms persistent odour producing compounds on chlorination (Train, 1979). The USEPA (1987) reported that the acute and chronic toxicities of phenol to freshwater aquatic life occurs at concentration as low as 10.2 mg/L and 2.56 mg/L respectively. The sparse exposure data do not allow the evaluation of the risk from phenol to either aquatic or terrestrial ecosystems. In view of the derived environmental concern level for water it is assumed that aquatic organisms may be at risk in any surface or sea water contaminated with phenol (WHO,1994).

The aim of the present study was to investigate the growth response of algae *Oocystis pusilla*, *Chlorella pyrenoidosa*, and *Oscillatoria quadripunctulata* to phenol and to explore the possibility of utilising these species in the treatment of a petrochemical effluent.

5.2. MATERIALS AND METHODS

Effect of Phenol on the Growth of Algae

The three test species, *Oocystis pusilla*, *Chlorella pyrenoidosa,* and *Oscillatoria quadripunctulata* were exposed to different concentrations of phenol to assess the effect on growth and survival of the species.

Algal assays were conducted simultaneously in two concentration series of phenol. The test series were (a) 0.1, 1, 2, 4,and 5 mg/L phenol, and (b) 10, 25, 50, 75, and 100 mg/L phenol. The test concentrations were added to the respective culture media and inoculated with exponential growing cultures. The assays were done in triplicate Control sets of cultures were raised in the culture medium free of phenol. The procedure adopted and the experimental conditions were similar to the previous assays (Chapter 3). The effect of phenol on the growth of the algae was measured on the fourth and the eighth day in terms of cell number/absorbance. The dose effects were interpreted by ANOVA test. An F ratio with probability value ≤ 0.05 was considered significant.

Acclimation Algae to Phenol

The objective of the assay was to evaluate the effect of continued exposure of *O. pusilla*, *C. pyrenoidosa*, and *O. quadripunctulata* to phenol, and to develop tolerant strains. The cells of *O. pusilla* and *C. pyrenoidosa* exposed to 4 mg/L were centrifuged after 4 days growth, and inoculated to test media of 7 and 10 mg/L phenol. The cultures were incubated for 8 days as in the previous experiment. The growth was measured on the fourth and eighth day as cell count, chlorophyll *a* and productivity, and compared with the control sets of unexposed cultures. The eighth day cultures were centrifuged and resuspended in fresh

culture medium to check the viability of the cells. The assays were repeated by successively inoculating the cells to higher concentrations of phenol in the order 12, 15, 17, 20, 22, and 25 mg/L.

The cultures of *O.quadripunctulata* growing in 5 mg/L were inoculated to successively higher concentrations of phenol up to 25 mg/L as in the above assays. Growth was measured in terms of absorbance at 620 nm, chlorophyll *a* and productivity on the fourth and eighth days of incubation. The results were interpreted in comparison with that of the control.

Absorption of Phenol

Phenol was added to the growth media at 25 mg/L and inoculated with the test species of acclimated and non-acclimated algae. The cultures were incubated for 96 hours. Subsamples were withdrawn, and filtered to estimate the phenol concentration at time intervals of 10 minutes , 1 h, 2 h, 3 h, 4 h, 5 h, 24 h, 48 h, and 96 h of exposure. The phenol was estimated by 4-amino antipyrene method as detailed in Chapter 4.

Acclimated Algae in Petrochemical Effluent Treatment

Algal assays were conducted to check whether the phenol acclimated strains of algae can grow in the petrochemical effluent, and reduce the total dissolved solids thereby lessening the toxicity of the effluent. The effluent was filtered through Whatman GF/C filter paper. The cells from the exponential growing phenol acclimated cultures of *O. pusilla* and *C.pyrenoidosa* were inoculated into 1000 mL of the effluent taken in 2 litre sterile conical flasks at an initial cell density of 1×10^4 cells/mL. The cultures along with the effluent control were incubated for eight days as in earlier experiments. The growth was estimated as cell count and chlorophyll *a*. The experiment was done in triplicate. The total dissolved solids was

estimated on the fourth, eighth, and twenty fourth day of incubation of the algae.The experiment was repeated with *O.quadripunctulata*. The growth index followed was measurement of absorbance and chlorophyll *a*.

5.3. RESULTS

Effect of Phenol on the Growth of Algae

The results of the exposure of the three algal species to the first test series of phenol is given in Table 5.1. The cell density of *O. pusilla* in the control culture was 3.16 x10^4 cells/mL on the fourth day while that of the culture in 4mg/L phenol was 4.07x10^4 cells/mL. The corresponding cell counts on the eighth day was 7.15 x10^4 cells/mL and 4.4x10^4cells/mL,

Table 5.1. Cell count (x10^5 cells/mL) of *Oocystis pusilla* and *Chlorella pyrenoidosa* and increase in absorbance at 620 nm of *Oscillatoria quadripunctulata* following exposure to phenol

Phenol mg/L	*O. pusilla* cell density $\bar{x} \pm SD$		*C. pyrenoidosa* cell density $\bar{x} \pm SD$		*O. quadripunctulata* absorbance $\bar{x} \pm SD$	
	4 days	8 days	4 days	8 days	4 days	8 days
00	3.16±0.4	7.15±1.00	3.25±0.26	16.25±0.39	0.04±0.01	0.14±0.02
0.1	2.76±0.71	7.11±0.87	3.85±0.00	14.33±0.22		
1	3.56±0.34	7.14±0.36	3.75±0.28	8.42±0.12		
2	3.43±0.2	3.86±0.31	3.95±0.00	10.73±0.19	0.077±.0.002	0.25±0.02
4	4.07±0.65	4.40±0.86	3.41±0.86	10.79±0.12		
5					0.164±0.004	0.31±0.012

respectively. The inhibition of growth was evident at ≥ 2 mg/L phenol (Fig. 5.1.). As revealed by the analysis of variance there was no significant difference in the growth rate of *O. pusilla* on exposure to phenol up to 4 mg/L within the first four days. However, strongly significant depression of growth occurred in a period of eight days at phenol levels ≥ 2 mg/L (Table 5.2a and 5.2b).

Table 5.2a. Analysis of variance of *Oocystis pusilla* exposed to phenol for 4 days

Source	Sum of squares	DF	Mean square	*F* ratio	Probability
Treatment	2.867	4	0.717	2.962	0.0894
Block	0.128	2	0.064	0.26	0.775
Error	1.936	8	0.242		
Total	4.930	14			

Table 5.2b. Analysis of variance of *Oocystis pusilla* exposed to phenol for 8 days

Source	Sum of squares	DF	Mean square	*F* ratio	Probability
Treatment	38.205	4	9.557	15.824	7.215E-04**
Block	0.603	2	0.302	0.500	0.6246
Error	4.83	18	0.604		
Total	43.636	14			

**significant at 1% level

The growth of *Chlorella pyrenoidosa* was stimulated by the test doses of phenol on the fourth day (Table 5.1, Table 5.3a). The cell density at 4 mg/L phenol was 3.41 $x10^4$ cells/mL compared to the control value of $3.25x10^4$ cells/mL. On the eighth day the cell density declined from $16.25x10^4$ cells/mL in the control culture to $10.79x10^4$ cells/mL in cultures exposed to 4 mg/L. The decline was highly significant (Table 5.3 b). The graphical representation of the data is given in Fig 5.2.

Table 5.3a Analysis of variance of *Chlorella pyrenoidosa* exposed to phenol for 4 days.

Source	Sum of squares	DF	Mean square	*F* ratio	Probability
Treatment	1.095	4	0.274	5.889	0.0164*
Block	0.057	2	0.028	0.61	0.567
Error	0.371	8	0.046		
Total	1.523	14			

*significant at 5% level

Table 5.3b. Analysis of variance of *Chlorella pyrenoidosa* exposed to phenol for 8 days.

Source	Sum of squares	DF	Mean square	*F* ratio	Probability
Treatment	117.82	4	29.455	448.623	1.933E-09**
Block	1.21133E-03	2	6.0667E-049	2400E-03	0.9908
Error	0.525	8	0.066		
Total	118.347	14			

**significant at 1% level

The growth of *Oscillatoria quadripunctulata* was stimulated by the test doses of phenol as revealed by the increased absorbance both on the fourth and eighth day of exposure (Table 5.1, Fig 5.3). The absorbance increased on the eighth day to 0.31 at 5 mg/L compared to the control value of 0.14. The results of ANOVA showed that there was a significant increase in the absorbance following exposure to phenol (Table 5.4a, 5.4b).

Table 5.4a Analysis of variance of *Oscillatoria quadripunctulata* exposed to phenol for 4 days.

Source	Sum of squares	DF	Mean square	*F* ratio	Probability
Between	0.024	2	0.012	303.925	9.338E-07**
Within	2.4000E-04	6	4.000E-05		
Total	0.025	8			

**significant at 1% level

Table 5.4b. Analysis of variance of *Oscillatoria quadripunctulata* exposed to phenol for 8days.

Source	Sum of squares	DF	Mean square	*F* ratio	Probability
Between	0.046	2	0.023	73-004	6.14E-05**
Within	1.8880E-03	6	3.1467E-07		
Total	0.048	8			

**significant at 1% level

The cell density of the three test species exposed to the higher test levels of phenol is given in Table 5.5.

The cells of *C. pyrenoidosa* showed a decreased growth at all phenol concentrations compared to the control (Fig 5.4). The mean cell density of the control culture was $9x10^4$ cells/mL on the fourth day and at 100 mg/L it was reduced to 2.67×10^4 cells/mL. The tendency was retained on the eighth day with the control value $50.93x10^4$ cells/mL, against $28.33x10^4$ cells/mL at 100 mg/L phenol. The analysis of variance showed that the difference between the control and the phenol treated cultures was highly significant on the fourth and eighth day. Similar results were obtained with *O. pusilla* also (Table 5.5, Fig 5.5).

Table 5.5. Cell count ($x10^4$ cells/mL) of *Oocystis pusilla* and *Chlorella pyrenoidosa* and increase in absorbance at 620 nm of *Oscillatoria quadripunctulata* after 8 day exposure to phenol

Phenol mg/L	*O. pusilla* cell density $\bar{x} \pm SD$		*C. pyrenoidosa* cell density $\bar{x} \pm SD$		*O. quadripunctulata* absorbance $\bar{x} \pm SD$	
	4 days	8 days	4 days	8 days	4 days	8 days
0.00	6.50±1.73	16.42±0.05	9±0.87	50.93±6.72	0.043±0.005	0.175±0.022
10	5±2.29	13.12±0.22	5.33±0.29	22.25±8.83	0.086±0.007	0.183±0005
25	6±0.05	12.96±1.66	6.67±2.36	29.67±7.75	0.081±0.002	0.162±0.014
50	4.33±202	13.35±1.88	3.33±2.2	26.07±5.56	0.044±0.01	0.13±0.007
75	2±0.87	10.76±0.76	1.67±0.29	22.67±5.53	0.027± 0.01	0.109±0.002
100	1.67±0.28	10.33±0.6	2.67±0.29	28.33±2.92	0.029± 0.00	0.082±0.003
F ratio	4.887	10.096	10.681	10.490	57.7	33.69
Probability	0.016*	1.61E-03**	9.270E-04**	9.966E-04**	4.744E-07**	6.029E-06**

* significant at 5% level

** significant at 1% level

The growth of *O. quadripunctulata* was stimulated up to 25 mg/L phenol following 96 h exposure. Inhibition of growth was evident at higher levels of phenol and on prolonged exposure (Fig 5.6). There was significant difference in the absorbance of the cultures among the test doses and the control (Table 5.5).

Adaptation of Algae to Phenol Upon Successive Exposure

The results of the exposure of the three test species to 7 and 10 mg/L phenol following growth in the lower test doses are given in Table 5.6. *O. pusilla* cells were observed to grow in 7 and 10 mg/L phenol. On the eighth day there was a slight reduction in growth especially

Table 5.6. Cell count/absorbance, chlorophyll *a* and productivity of *Oocystis pusilla*, *Chlorella pyrenoidosa* and *Oscillatoria quadripunctulata* expressed as percentage of control on exposure to 7 and 10 mg/L phenol for 8 days

Test Species	Phenol mg/L	Cell count/ Absorbance		Chlorophyll *a*		Productivity	Cell count after resuspension in phenol free medium	
		4 days	8 days	4 days	8 days	4 days	8 days	8 days
O. pusilla	7	226	106	189	189	194	108	90
	10	235	55	182	190	190	73	67
O. pyrenoidose	7	58	65	77	83		77	92
	10	34	52	31	50		39	100
O. quadripunctulata	7	98	110	105	116	171	132	84
	10	100	123	102	153	188	132	66

at 10 mg/L. The cells were found to be viable on resuspension in the phenol free media; the cell yield was 90% and 67% compared to the control. *C. pyrenoidosa* cells showed growth

depression both on the fourth and eighth day on exposure to phenol. Upon resuspension in the phenol free medium the cells remained viable with percentage cell yield 92% and 100% with respect to the control. The exposure of *O. quadripunctulata* to phenol showed a growth stimulation both on the fourth and eighth day, and the viability was retained in 7 and 10 mg/L phenol exposed cells on resuspension in phenol free medium.

The growth of the three test species on successive exposure to phenol up to 25 mg/L is given in figures 5.7, 5.8, and 5.9. On inoculation to successively higher concentrations of phenol, the cell count, chlorophyll *a* and productivity of *O. pusilla* was nearly the same as or higher than the control. The growth was higher than the control in *C. pyrenoidosa* both on the fourth and eighth day. The acclimation of *O. quadripunctulata* to higher concentrations of phenol showed that the acclimated cells were having the same growth rate, chlorophyll *a* and productivity as that of the control.

Absorption of Phenol

The concentration of phenol in the control (uninoculated test medium) decreased to 21.52 mg/L in 10 minutes and to 21.45 mg/L in 5h. The concentration was 21.1 mg/L after 24 h. By the 48th h the concentration came down to 0.39 mg/L. It was 0.2 mg/L when sampled after 96 h. (Fig. 5.1).

The non-acclimated culture of *O.pusilla* and *C. pyrenoidosa* did not grow in the phenolic medium, and therefore the phenol absorption was not monitored in this experimental set. The acclimated culture of *O. pusilla* and *C. pyrenoidosa* could grow in the phenolic medium, and reduce the phenol content to 17 mg/L in 24 h. By the 48th h the phenol concentration in the test media inoculated with *C. pyrenoidosa* decreased to 0.14 mg/L and that in *O. pusilla* got reduced to 2.29 mg/L. The non-acclimated *O. quadripunctulata* grew

in the test medium and reduced the phenol content to 16.74 mg/L and 3.96 mg/L within 5 h and 24 h respectively. The acclimated of *O. quadripunctulata* reduced phenol concentration to 14.59 mg/L in 5 h and to 4.75 mg/L in 24 h. The phenol concentration decreased to 0.52 mg/L in 96 h. A comparison of the three species as given in Fig.5.10 reveal that the maximum phenol removal was effected by *O. quadrtipunctulata* effecting 33 - 42% reduction in 5 h and 81-84% in 24 h irrespective of whether acclimated or not.

Phenol Acclimated Algae in Petrochemical Effluent Treatment

The acclimated culture of the the three species thrived in the effluent of Hindustan Organic Chemicals Ltd., whereas the growth of the non-acclimated cultures was depressed (Table 5.7). The TDS of the effluent was initially 411mg/L. Upon retention for 24 days, there was apparently no change in the TDS concentration in the control. The effluent inoculated with *O.pusilla* and *C. pyrenoidosa* also did not show any significant change in the TDS. When inoculated with *O. quadripunctulata*, the TDS reduced to 277 mg/L within 96 h. However, the amount of TDS increased and reached near the control value after 24 days of incubation (Fig.5.11).

Table 5.7. Cell yield (as % of control) of acclimated and non-acclimated *Oocystis pusilla*, *Chlorella pyrenoidosa* and *Oscillatoria quadripunctulata* after growth in HOC effluent for 96 h

Test species	Cell yield / Absorbance % of control		Acclimated culture	
	Non-acclimated	Acclimated	Productivity g C/L/h	Chlorophyll *a* µg/L
O. pusilla	22.64	148.51	0.585	7.096
C. pyrenoidosa	33.33	144.36	0.540	15.088
O. quadripunctulata	61.12	139.39	0.648	71.99

Fig.5.1 Effect of phenol on the growth of *Oocystis pusilla*

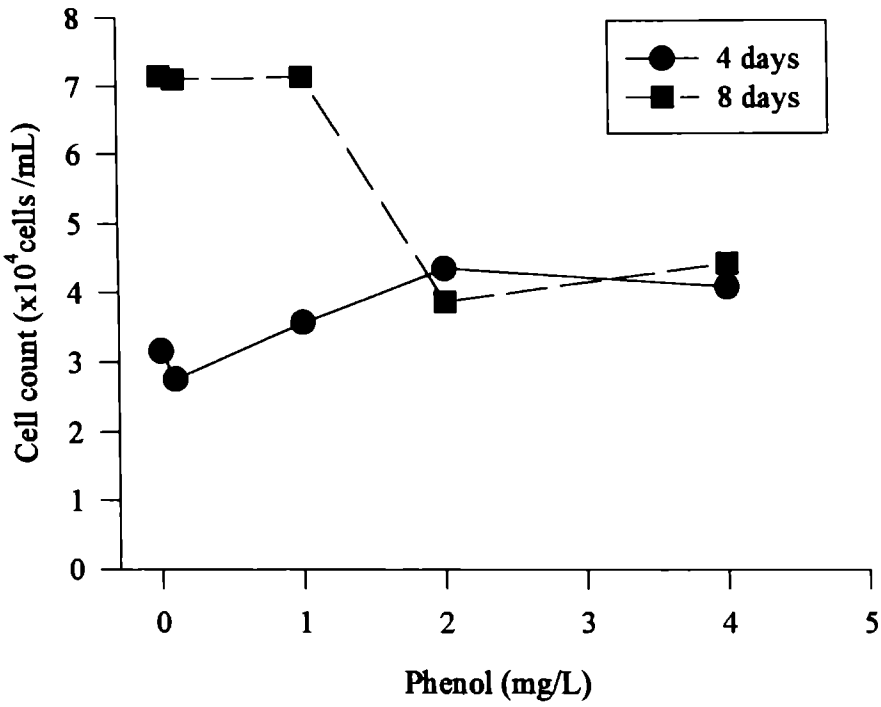


Fig.5.2 Effect of phenol on the growth of *Chlorella pyrenoidosa*

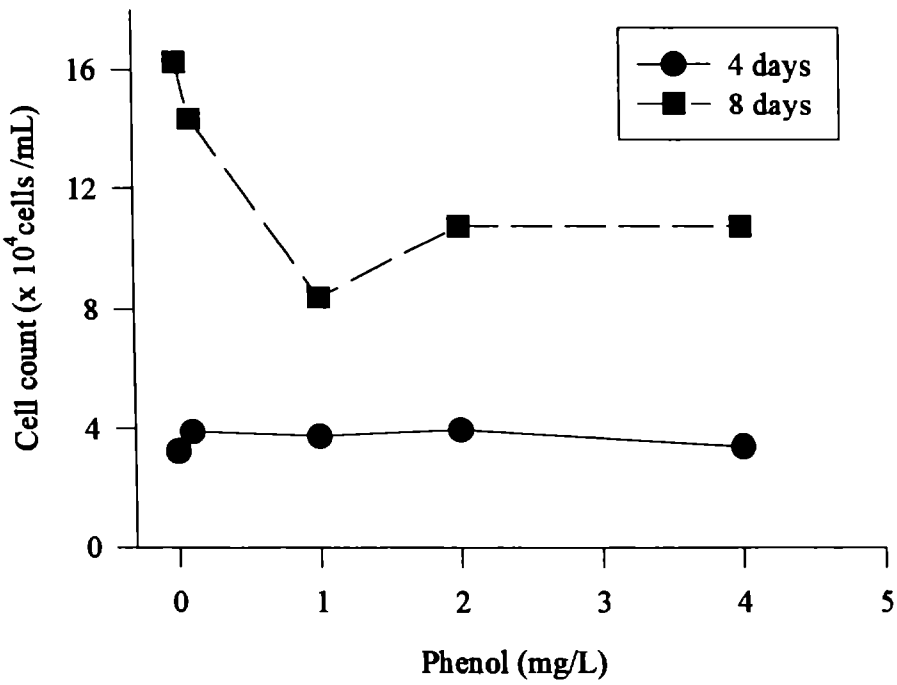


Fig.5.3 Effect of phenol on the growth of *Oscillatoria quadripunctulata*

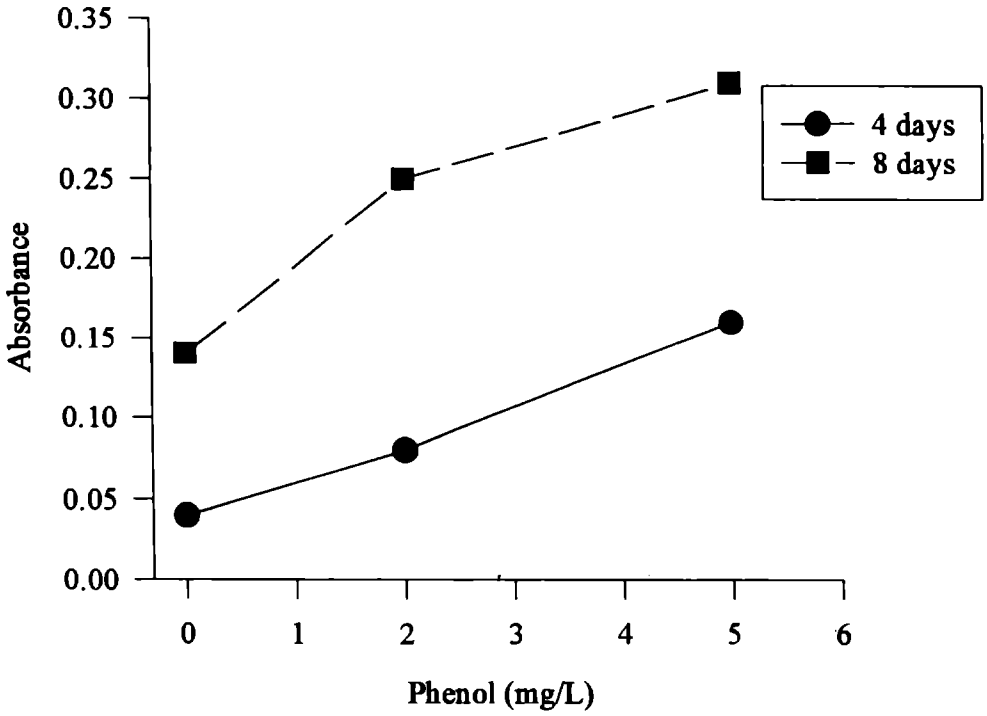


Fig.5.4 Effect of phenol on the growth of *Chlorella pyrenoidosa*

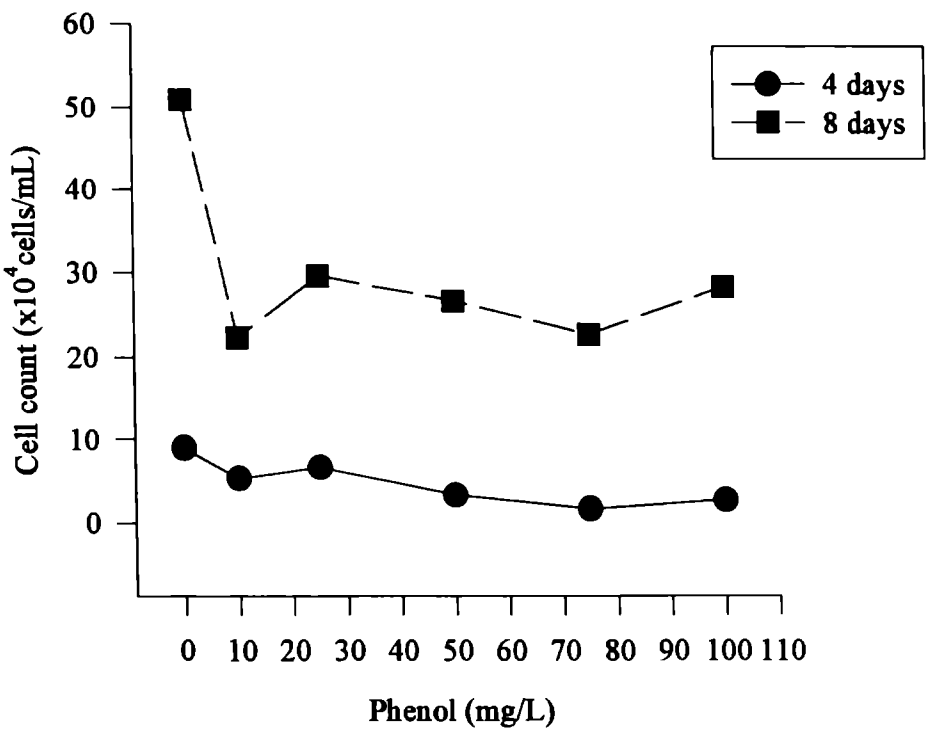


Fig.5.5 Effect of phenol on the growth of *Oocystis pusilla*

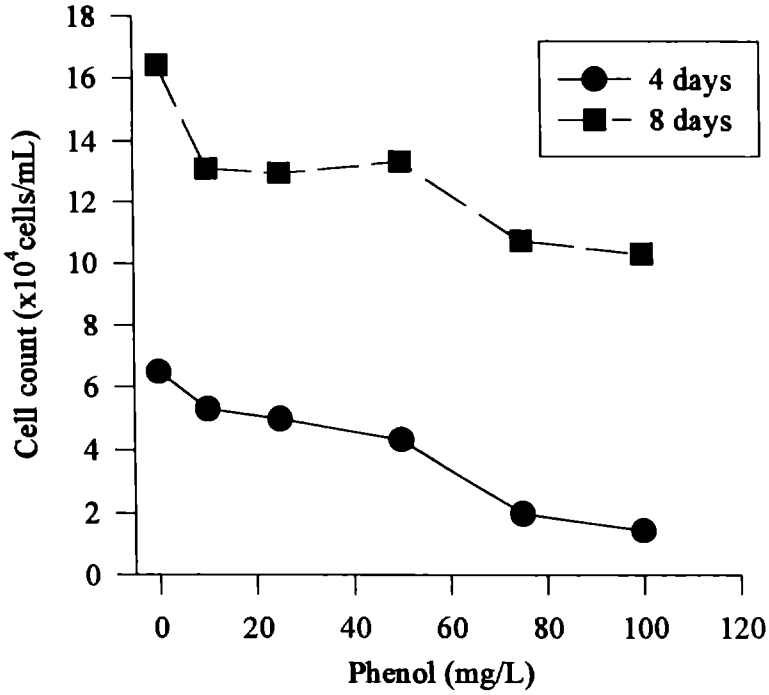


Fig.5.6 Effect of phenol on the growth of *Oscillatoria quadripunctulata*

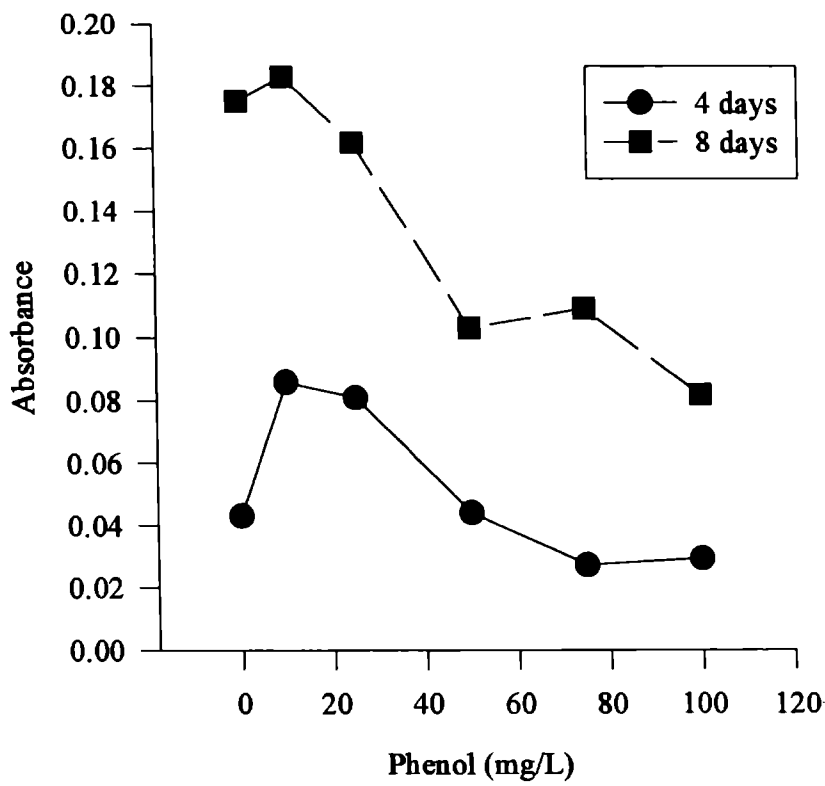


Fig.5.7 Acclimation of *Oocystis pusilla* to phenol

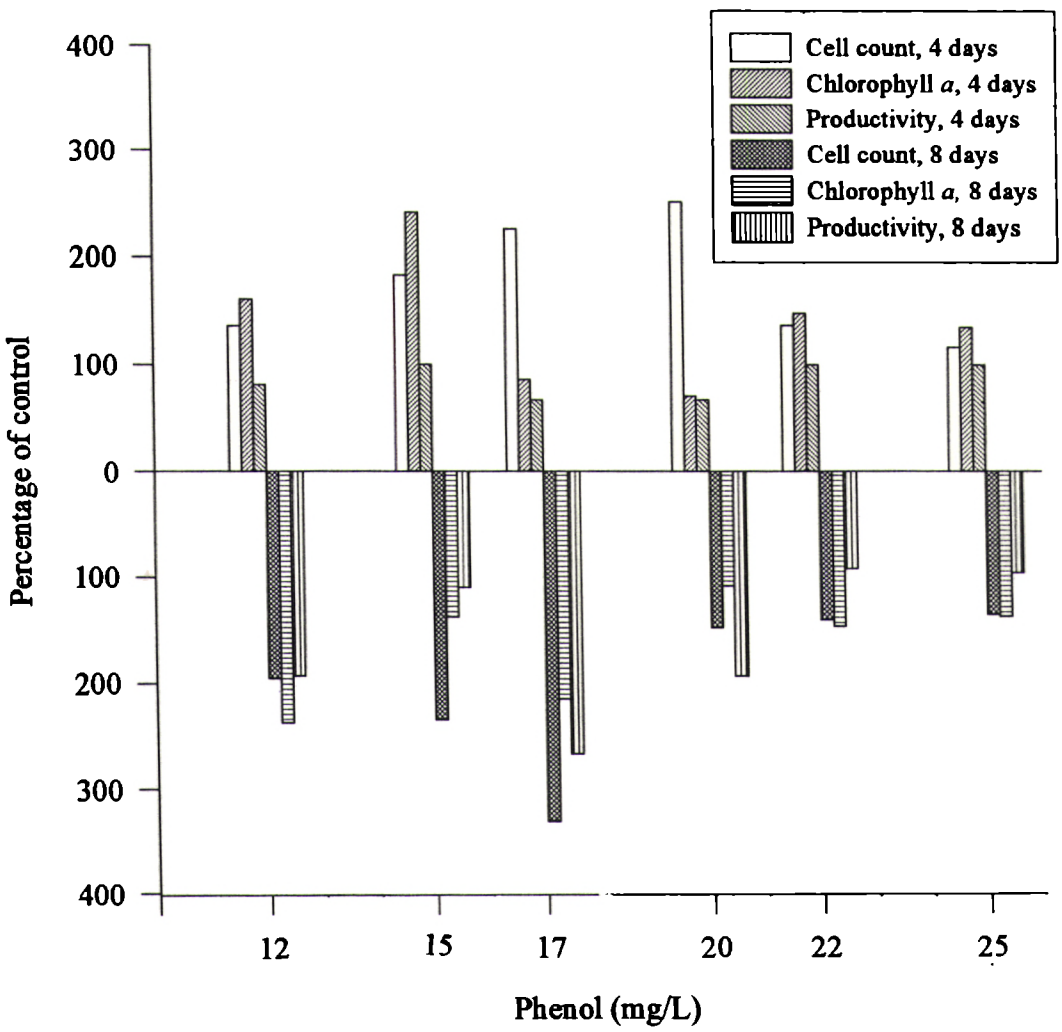


Fig.5.8 Acclimation of *Chlorella pyrenoidosa* to phenol

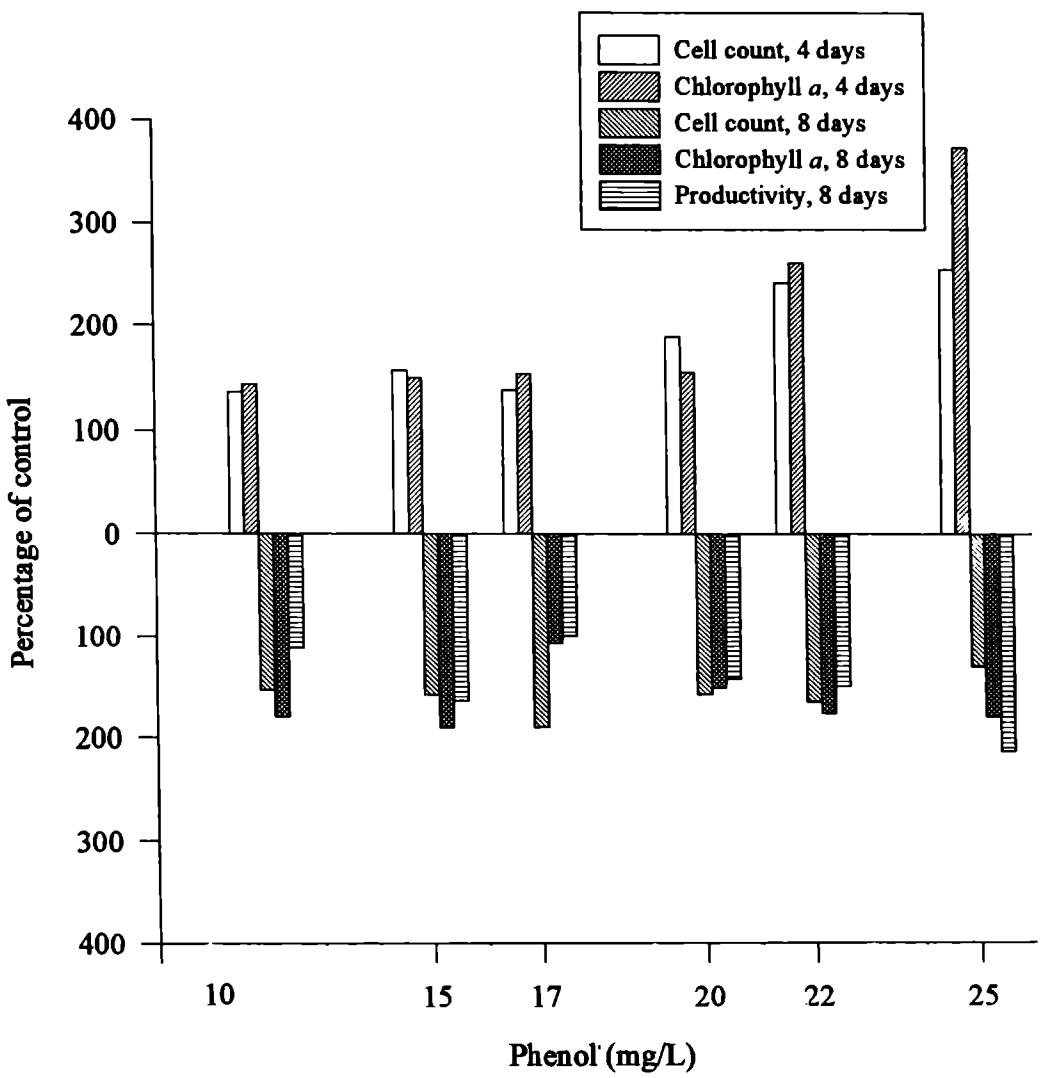


Fig.5.9 Acclimation of *Oscillatoria quadripunctulata* to phenol

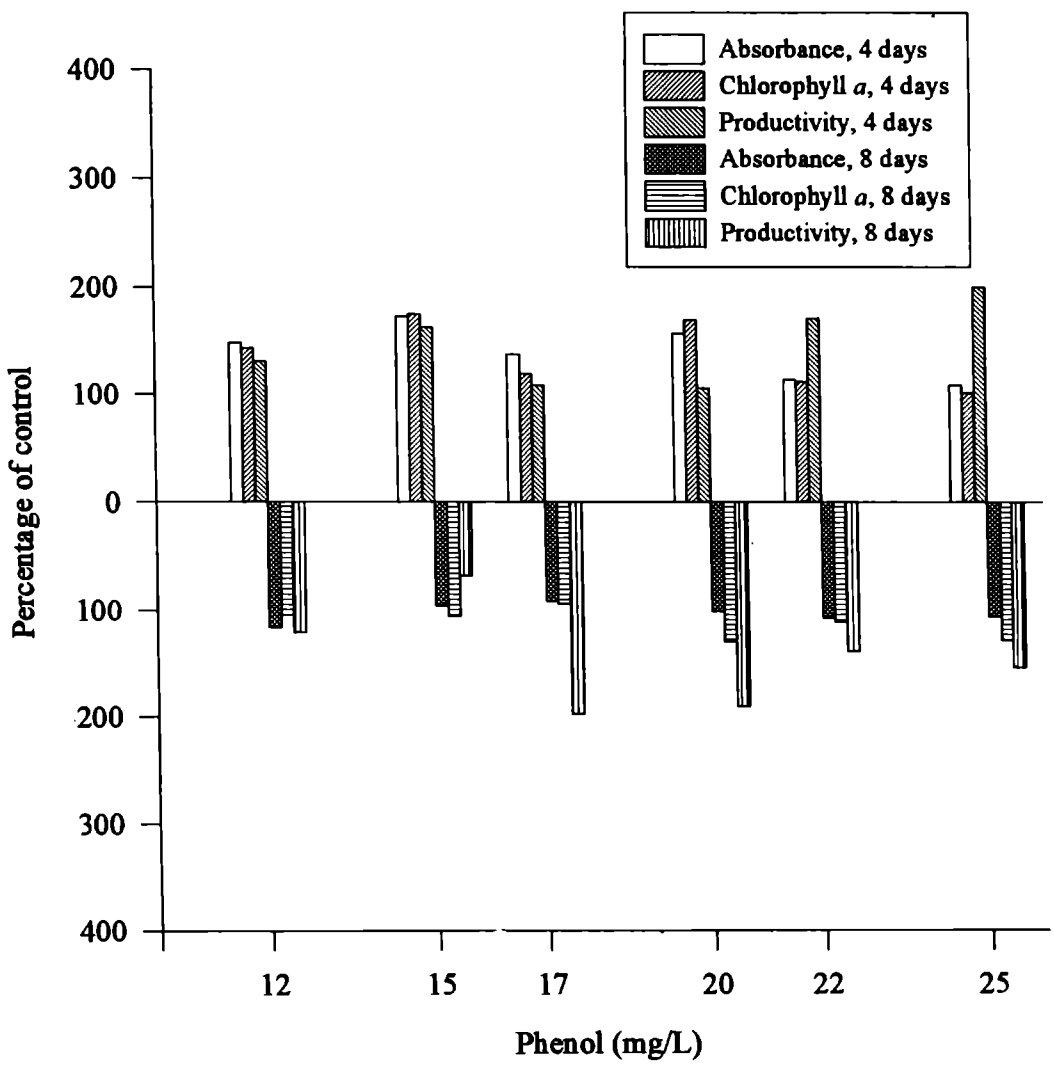


Fig.5.10 Phenol removal from the growth medium by the acclimated algae

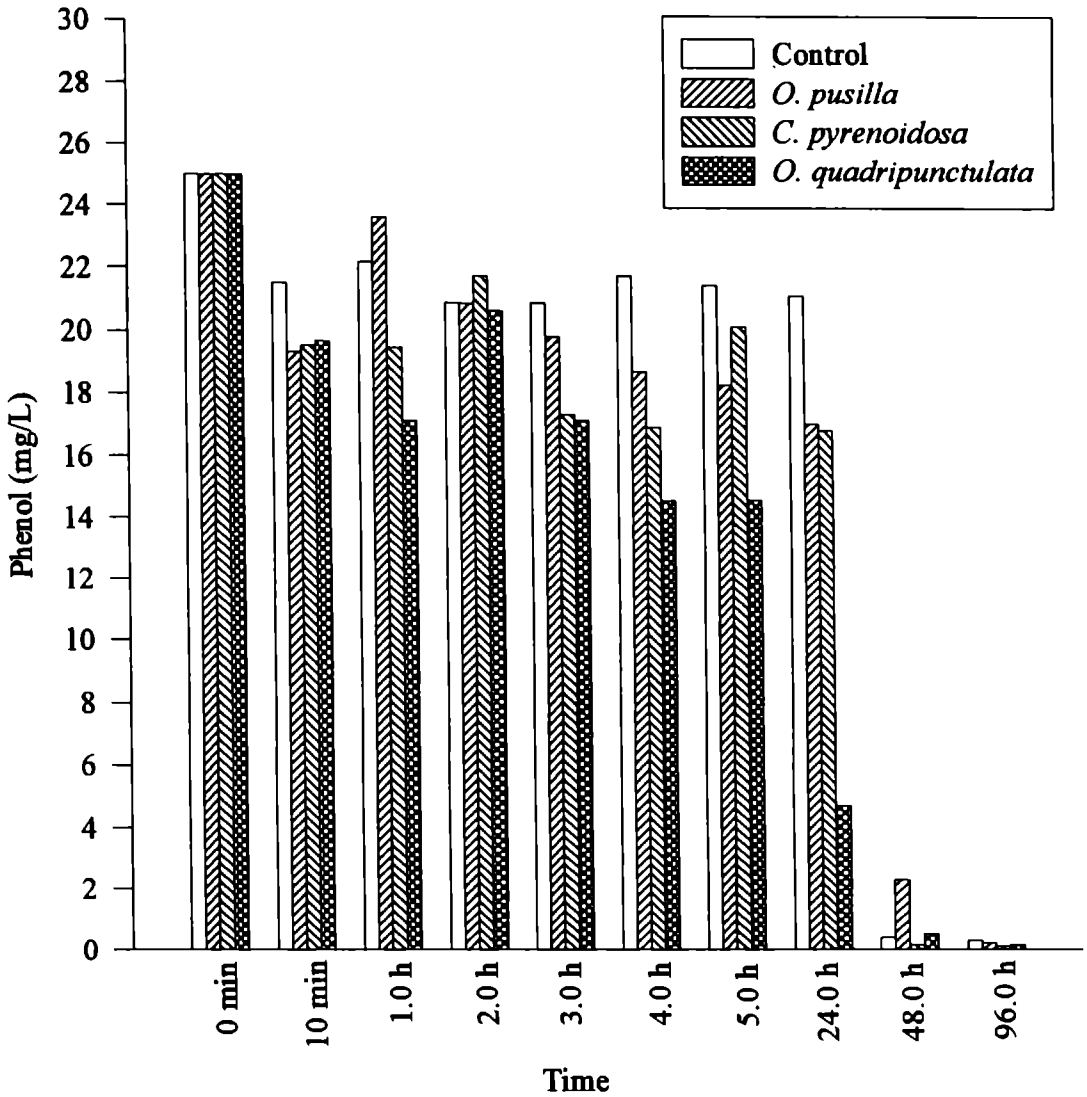
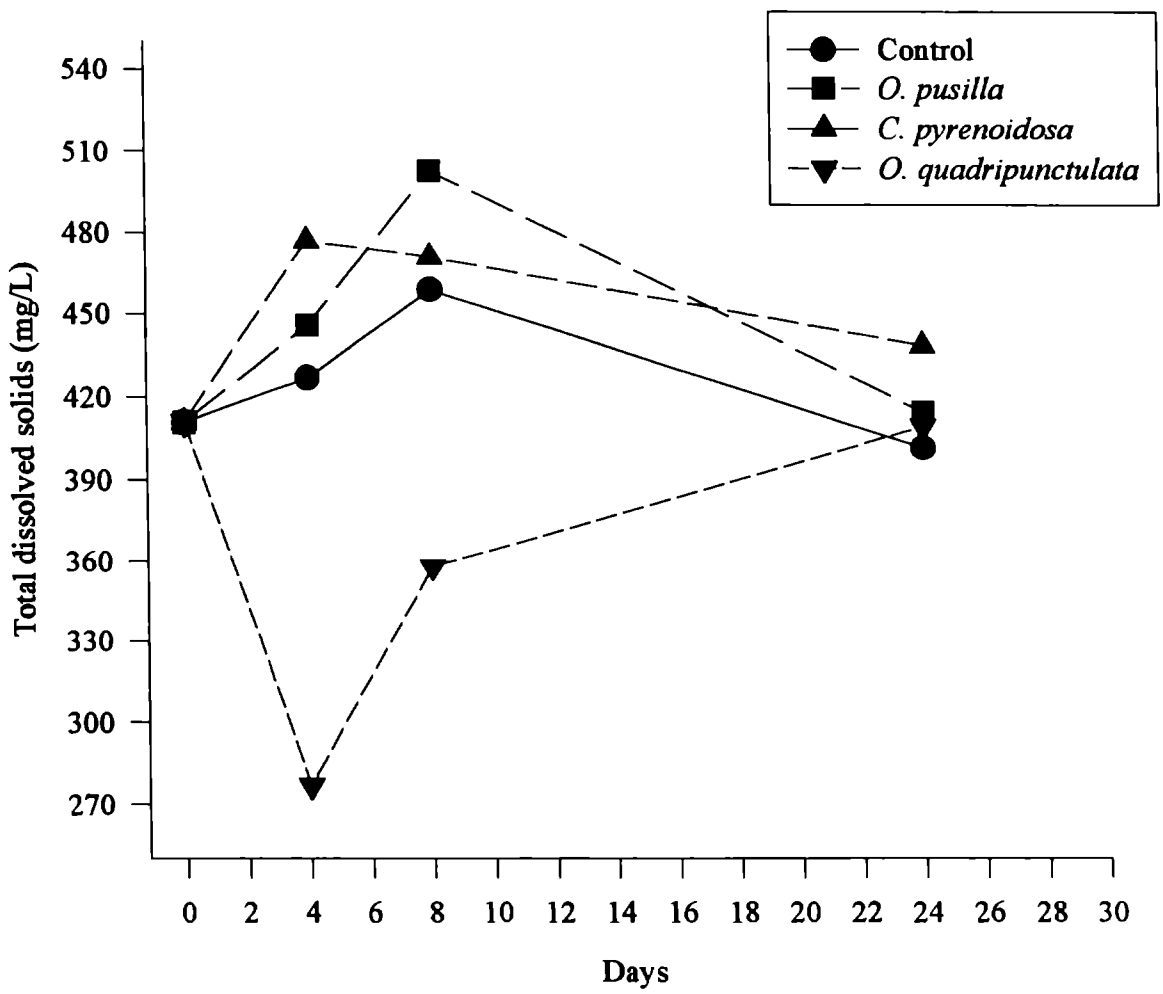


Fig.5.11 Effect of algal growth on TDS removal



5.4. DISCUSSION

The toxicity of a chemical compound at the organism level depends upon characteristics of the exposed species as well as on the fate and behaviour of the compounds. Phenol is an aromatic compound sparingly soluble in water, capable of being volatalised, and oxidised .

In the present investigation, phenol was found to stimulate the growth of the test organism at low test doses following 96 h exposure, whereas prolonged exposure, and higher doses were inhibitory to the algae. Comparing the three species it may be stated that phenol is most toxic to *C. pyrenoidosa* effecting a significant depression in growth at 1 mg/L in eight days of exposure. The growth of *O. pusilla* was significantly affected only ≥ 2 mg/L. In a similar study on the differential response of green algal species to solvents *C. ellipsoidea* was reported to be the most sensitive species , the growth being inhibited at 0.05%, 0.1% and 0.2% phenol concentrations (Tadros *et al.*, 1994). Acute toxicity level of phenol to *C.pyrenoidosa* was reported to be 10-30 mg/L in terms of 96 h LC_{50} (Moore and Ramamoorthy, 1984). Tissler and Zagore (1995) observed that *Scenedesmus quadricauda* had only a low sensitivity to phenol. The 24 h EC_{50} value was 403 mg/L. Shigoeka *et al.*, (1988) also indicated high EC_{50} values for *Selenastrum capricornutum*, and *Chlorella vulgaris* following 4 day growth in phenol.

Among the three species tested, *O. quadripunctulata* was the most tolerant to phenol. The growth inhibition occurred only at ≥ 25 mg/L. It may be remembered that *O. quadripunctulata* was the most tolerant species to HOC effluent as the degree of growth inhibition was only at 39%. Many blue-green algae are reported to metabolise phenols. *Oscillatoria* sp., strain JCM can oxidise biphenyl (Cernigla, 1980). A marine cyanobacterium *Phormidium valderianum* BDU 3050 was able to degrade phenol completely at 100 mg/mL by its intracellular oxidase and laccase enzymes and this organism was pointed for use in phenolic

effluent treatment (Subramanian and Uma, 1997). The toxicity threshold value for *Microcystis aeruginosa* is 6 mg/L phenol (WHO, 1994). Difference in toxicity levels for different species of algae has been explained variously by previous authors. Heterotrophic capability of *Euglena gracilis* compared with the lack of it in *Scenedesmus quadricauda* was put forward as a possible cause of the differential response of these two species to oils (Shales *et al.*,1989). This does not explain the differential sensitivity of *O. pusilla* and *C. pyrenoidosa*. Perhaps the difference in toxicity level depends on the life history strategies of each species.

The acclimation experiments showed that both *O. pusilla* and *C. pyrenoidosa* which were inhibited by low doses of phenol could be made to grow in phenolic medium by acclimation. In a study on the acclimation of algal communities to zinc toxicity it was concluded that the acclimation of the test organism to a toxicant has an important effect on its response to toxicity (Wang, 1986). The tolerance of algae to phenol on repeated exposure is species specific, the mechanism involved may be either storage or metabolic utilisation.

The phenol acclimated algae were tested for their potential to remove phenol from the medium. It was found that *O. quadripunctulata* cultures could effect a drastic drop in phenol concentration by the 24th h, a decrease in phenol level almost 5 times less than that of the control. Phenols are reported to react in sunlit -natural water *via* reaction with photochemically produced hydroxyl, and peroxyl radicals; typical half-lives are reported to be 100 and 192 h respectively (WHO, 1994). Therefore the drastic reduction in phenol in 24 h would have been due to the absorption by *O. quadripunctulata*. Moreover *O. pusilla* and *C.pyrenoidosa* didnot exhibit this phenomenon. The results of the study confirm that *O. quadripunctulata* is the most tolerant species and the removal of phenol from the medium can be effected in 24 h. Cyanobacteria is more prone to heterotrophic growth, and is established to metabolise aromatic compounds through ring hydroxylation, o-methylation, side-chain hydroxylation acetylation

and formylation (Subramanian and Uma, 1997). It may be assumed that *O. quadripunctulata* uses phenol as a source of carbon.

The phenol acclimated cultures of the algae assayed against the effluent of Hindustan Organic Chemicals Ltd. were found to grow, despite the toxicity it had on unacclimated cultures. In a study on the degradation of azodyes by algae it was found that the unacclimated algae display a lower azoreductase activity compared with the acclimated algae. The azoreductase enzyme of *Chlorella vulgaris* is an induced enzyme, and the substrate can act as a kind of inducer (Jingi and Houtain,1992). Species of *Oscillatoria* were also reported to degrade dyes from wastewaters. In another study on the use of cyanobacterial mats containing *Oscillatoria* species conditioned in the laboratory to tolerate elevated aqueous concentrations of 100 mg/L zinc and manganese, they were able to remove these metals from waters experimentally contaminated with metal levels, similar to those found in mine drainage (Bender *et al.*,1994).

The studies on using algae in the treatment of rubber effluent revealed that using retention times of 2-10 days did not effect any marked improvement in BOD and TDS. The COD and suspended solids increased with increasing retention time (Abbasi,1987). In the present study *O. quadripunctulata* was found to be efficient in the removal of total dissolved solids whereas *O. pusilla* and *C. pyrenoidosa* were not as effective. However, reduction of TDS by *O. quadripunctulata* was dependent on the period of exposure. Maximum reduction was effected on the 4th day, after which *O. quadripunctulata* seem to have released subbstances, may be as a result of the declining stage of the culture. This essentially points to a problem in algal treatment systems i.e., during the period of scenescence, the algae are likely to relaease dissolved substances increasing the TDS content of the effluent. Therefore retention time is significant in algae - based treatment systems.

CHAPTER 6

SUMMARY AND CONCLUSION

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The perturbation of the aquatic environment by various pollutants, especially those from industries, emphasise the need for development of specific treatment options based on physico-chemical and biological methods. The ability of organisms to respond to the environmental stress factors, and to adapt their physiology in order to maintain their competitiveness finds application in the assessment, and abatement of pollution.As a representative group of the lower trophic level, algae are important in indicating the environmental contamination levels over time. Moreover, recent implications of the biodegradative capacity of the primarily autotrophic algae, points to the importance of ecology, isolation, identification and genetic characterisation of algal species prone to mixotrophy and heterotrophy.

The present investigation on the algal ecology of the oil refinery effluent holding pond of Cochin Refineries Ltd., establishes that phytoplankton community can be used as an effective tool in assessing the trophic status and the efficiency of treatment systems. Based on the evaluation of community function, the effluent of Cochin Refineries Ltd. was proved to be of eutrophic potential. The pond effluent supported a substantial algal bloom throughout the year as indicated by the chlorophyll *a* concentration ranging from 55.66 mg/m^3 to 452.04 mg/m^3. The algae composed of chiefly members of Chlorococcales are often associated

with waters polluted with organic effluents. The gross primary productivity was in the range 1.74 g C/m^2 /day to 13 g C/m^2 /day. Throughout the sampling period, the gross primary productivity was higher than the community respiration. Gross primary productivity and community respiration approached each other only twice during the entire sampling period. These observations lead to the conclusion that the waste stabilisation is not complete in the fire water pond No.2 and a series of ponds are required to achieve complete stabilisation.

The algal assay approaches in assessing the quality of the treated refinery effluent confirmed the eutrophic nature of the effluent from Cochin Refineries Ltd. The isolation of algal species with versatility in degrading specific pollutants is the first approach in algae-based treatment systems. The three algal species isolated were *Oocystis pusilla*, *Chlorella pyrenoidosa*, and *Oscillatoria quadripunctulata*. The influent to the fire water pond No.2 inhibited the growth of the test species while the effluent from the fire water pond No.2 stimulated growth. The percentage growth stimulation of *O. pusilla*, *C. pyrenoidosa* and *O. quadripunctulata* in the effluent was 207.26%, 75.85% and 18.6% respectively. This indicated that the fire water pond No. 2 was effective as a detoxifying unit. The detoxification process in the pond is the net effect of physico-chemical and biological variables. The recent reports on degradation of environmental contaminants by algae points to the probable additional role of algae in detoxifying the effluent. The eutrophic potential of the effluent is further substantiated by the abundant algal growth in the Chitrapuzha river, which is slow flowing stream into which the effluent of CRL is discharged. The chlorophyll concentrations in the river ranged from 8.3 mg/m^3 to 104.8 mg/m^3 The variability in the properties of the industrial effluents is established by the algal assays against the effluents generated during different periods. The range of growth stimulation was 132% to 307% of the control, while

the growth was inhibited during one sampling. Thus, the algae proved to be excellent monitors of the efficiency of the effluent treatment systems.

The analyses of the effluent from Hindustan Organic Chemicals Ltd. showed that the effluent is toxic to the test species of algae. The nutrient addition did not promote the growth of algae, clearly indicating the toxic nature of the effluent. Of the three species tested, *O.quadripunctulata* was least inhibited by the effluent, the degree of depression being only 39%. The percentage growth depression of *O. pusilla* and *C. pyrenoidosa* in the undiluted effluent were 77.36% and 66.66% respectively. The toxicity exhibited in the laboratory cultures is likely to be reflected in the receiving and downstream waters of the HOC effluent. However, it was found that in spite of the toxic discharge from HOC,Chitrapuzha river is rich in phytoplankton flora. It should be remembered that the river at this point receives the discharges of three major industries and sewage run off. Therefore, the water quality cannot be attributed to any particular factor, but is the result of complex interaction between different compounds. May be true phytoplankton community, well adapted to the microhabitat has developed in the river,and is maintaining itself by active reproduction. Therefore, a properly adapted strain holds chances for the use in the absorption of organic contaminants.

It has been found that many organisms can metabolise toxic chemicals once they are adapted to the particular toxicant. Exposure of the three test species to phenol showed that *O. pusilla* and *C. pyrenoidosa* were sensitive to phenol while *O.quadripunctulata* was the most tolerant species. Phenol is most toxic to *C. pyrenoidosa* effecting a growth depression at 1 mg/L on the eighth day of exposure. The growth of *O. pusilla* was significantly affected only at $\geq$ 2 mg/L. The growth inhibition of *O.quadripunctulata* was only at $\geq$ 25 mg/L. The two green algal species originally sensitive to phenol developed tolerance on successive

addition of phenol to the medium upto a concentration of 25 mg/L. Of the three species, *O. quadripunctulata* was found to effect a phenol reduction of 33 - 42% in 5 h. The phenol concentration in the growth medium was reduced by 81 - 84% in 24 h by *O.quadripunctulata.* It should be mentioned that after 96 h of exposure, the phenol content in the control as well as the algal treated growth media reduced to the range 0.38% to 1.32%. The present research establishes *O. quadripunctulata* as a promising organism for the biotreatment of phenolic effluents.

The phenol acclimated cultures could grow in the petrochemical effluent. The capacity of the species to remove total dissolved solids was assessed, and *O. quadripunctulata* was found to remove TDS content by the fourth day, the percentage removal being 33%. But the concentration of TDS increased after 4 days, thus indicating the importance of retention time in biological treatment systems.

The present investigation proved *O. quadripunctulata* as a versatile biotreatment organism. But mere phenotypic adaptation is not enough for application in effluent treatment systems. Techniques of screening for genetically resistant strains of *O. quadripunctulata* and other blue-green algae that offer faster growth rate, tolerance to extreme conditions, and efficient removal of toxic ions are required. With new vistas opening up in Algal Biotechnology for pollution abatement, an ecofriendly and potent tool is now available as the results with *O. quadripunctulata* culture indicates.

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