Water Quality Management - Application of Flocculants and Algicides

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Certificate

This is to certify that this thesis entitled, **"Water quality management–Application of flocculants and algicides"** is an authentic record of the research work carried out by Ms. Subitha Prabakaran O. P under our joint guidance and supervision at 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 in Environmental Biotechnology and no part of this work has previously formed the basis for the award of any other degree/diploma of this University or of any other University.

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DECLARATION

I hereby declare that the thesis entitled, **"Water quality management–Application of flocculants and algicides"** is an authentic record of the research work carried out by me under the joint guidance and supervision of Dr. A. Mohandas, Emeritus Professor, School of Environmental Studies, Cochin University of Science and Technology, and Dr. Ammini Joseph, Reader, 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 in Environmental Biotechnology and no part of this work has previously formed the basis for the award of any other degree, diploma, fellowship or associateship of this University or any other University.

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Contents

Chapter 1

An Introduction to Water Quality Management

Contents

Water is a renewable resource sustained by the hydrological cycle. The hydrological cycle and services are of prime concern as they move through and define the environment and biology of our planet. The history of human civilisation is replete with the ways humans have learned to manipulate and use the hydrological services. Hydrological services are a diverse group, categorised into five broad groups (Brauman, 2007). They are:

- improvement of extractive water supply,
- improvement of in-stream water supply,
- water damage mitigation,
- provision of water related cultural services,
- provision of water-associated supporting services.

Extractive water uses include municipal, agricultural, commercial, industrial, and thermoelectric power use. *In situ* uses include hydropower generation, water recreation and transportation, as well as freshwater fish production. Globally, freshwater withdrawals have been estimated at 35 % of accessible runoff and in-stream uses estimated at about 19% of runoff, though these diversions are not distributed uniformly worldwide (Oki & Kanae, 2006). Water damage mitigation is a regulating service; which includes ecosystem mitigation of flood damage, sedimentation of water bodies, saltwater intrusion into groundwater, and dryland salinisation. Cultural hydrologic services include spiritual uses, aesthetic appreciation, and tourism. The water-related supporting services of terrestrial ecosystems are wide ranging and include the provision of water for plant growth and to create habitats for aquatic organisms. Trade-offs are inherent in the supply of hydrologic services. Under various scenarios of quantity, quality, location, and timing of flow, some services will be improved at the expense of others.

1.1 World water distribution

Water is vital in all components of the environment. Water covers about 70% of earth's surface. It occurs in all spheres of the environment-in the oceans as a vast reservoir of saltwater, on land as surface water in lakes and rivers, underground as groundwater, in the atmosphere as water vapour, in the polar icecaps as solid ice, and in many segments of the anthrosphere such as in boilers or municipal water distribution systems. Water is an essential part of all living systems and is the medium from which life evolved and in which life exists. The relative water distribution of earth (Mckinney & Schoch, 2003) is given below as percentage.

Salt water	97.4
Ice caps & glaciers	1.98
Ground water	0.59
Lakes	0.007
Soil moisture	0.005
Atmosphere, rivers	
Plants, animals	0.001

As human populations and their economies grow, water uses tend to increase although the amount of freshwater in the world remains roughly the same. The amount economically available for human use is only 0.007% of the total, or about 13,500 km^3, which is about 2300 m^3 per person—a 37 % drop since 1970 (Wolf, 2007).

1.2 Water quality degradation

Availability of freshwater of adequate quantity and quality is a prerequisite for sustainable development. Water pollution and wasteful use of freshwater threaten development projects and make water treatment essential in order to produce safe potable water. Discharge of toxic chemicals, over-pumping of aquifers, long-range atmospheric transport of pollutants and

contamination of water bodies with substances that promote algal growth are some of today's major causes of water quality degradation. Some problems have been present for a long time but have only recently reached a critical level, while others are newly emerging. To understand the effects of water pollution and technology applied in its control, it is useful to classify pollutants. Based on their environmental and health effects they are classified into:

- Disease causing agents or pathogens, including bacteria, viruses, protozoa and parasites,
- Oxygen demanding organic wastes,
- Water-soluble inorganic chemicals, including acids, salts, and metals,
- Inorganic plant nutrients (nitrates and phosphates) from fertilizers,
- Organic chemicals, including oil, gasoline, plastics, pesticides, solvents and detergents,
- Water-soluble radioactive isotopes,
- Sediment or suspended matter (particulates) from soil and other solids,
- Heat from the cooling of industrial and power plants,
- Oil from petrochemical industry, leakage from pipes and accidental spillage, and
- Genetic pollution caused by the introduction of exotic species.

To a limited extent, streams and rivers have the ability to assimilate biodegradable wastes. Thus, they can recover the effects of pollution naturally, without significant or permanent environmental damage. The capacity for self purification depends on the strength and volume of pollutants and on the stream discharge or flow rate.

1.3 Water on international agenda

The urge to protect hydrosphere resources has been on the main agenda of international conventions and national policies. UN conference on water, 1977 was the first initiative to protect hydrological resources. Following it, water is on international agenda with a number of water conservation and management conferences, polices and action plans. Major international programmes and conferences on pollution prevention, conservation and management programmes and conferences are listed below (www.worldwatercouncil.org).

- UN Conference on Water, Mar del Plata (1977) defined water as a common good and highlighted the right of people to have access to drinking water regardless of the development stage and socio-economic situation.
- International Drinking Water Supply and Sanitation Decade (launched by the UN) 1981- 1990, raised its goal as to "Provide every person with access to water of safe quality and adequate quantity, along with basic sanitary facilities, by 1990."
- Global consultation on Safe Water and Sanitation for the 1990's, New Delhi - organised by UNDP appealed to all nations for concerted action to enable people to obtain two of the most basic human needs - safe drinking water and environmental sanitation.
- In 1992 the International Conference on Water and the Environment held in Dublin, Ireland, produced four key principles to guide policies for water and sustainable development.
- UN Conference on Environment and Development (UNCED Earth Summit, Rio de Janeiro, 1992) action plan-Agenda 21 encouraged the global management of fresh water and the integration of sectoral water plans and programmes within the framework of national economic and social policy.

- UN Conference on Human Settlements (Habitat II), Istanbul (1996) advocated the necessity to provide adequate quantities of safe water.
- The priorities of the First World Water Forum, Marrakech (1997) were water sanitation, shared water management, ecosystem conservation, gender equality, and efficient use of water.
- Second World Water Forum, La Hague (2000) emphasised the need for better governance and an integrated water resources management through sharing water resources and governing water wisely.
- United Nations Millennium Declaration (2000) defined its development goal as: "Halve, by 2015, the proportion of people who are unable to reach or to afford safe drinking water."
- International Conference on Freshwater, Bonn (2001) recognised water as a key to sustainable development.
- According to the report published by third world water forum (2003) significant progress has been made since the second world water forum and it is possible to meet the water challenges.
- The International Decade for Action "Water for Life" 2005 to 2015 (launched by the UN) aims to promote efforts to fulfil international commitments made on water and water-related issues by 2015.
- Fourth World Water Forum (Mexico), held in 2006 declared its theme as "Local Actions for a Global Challenge", through participation of local actors.

1.4 Water quality management

Water quality management is the practice of planning, developing, distribution and optimum utilization of water resources under defined water polices and regulations, for the protection of water's quality for various beneficial uses, for the provision of adequate wastewater collection, treatment, and disposal for municipalities and industries, and for the activities

that might create water quality problems. The sustainable management of water quality has policy, technical, institutional and financial components.

Water quality is the physical, chemical and biological characteristics of water in relationship to a set of standards. Water quality standards are limits on the amount of physical, chemical or microbiological impurities allowed in water that is intended for a particular use. Water quality requirements or objectives can be usefully determined only in terms of suitability for a purpose or purposes, or in relation to the control of defined impacts on water quality. Water quality standards are created for different types of water bodies and water body locations as per desired uses. The primary uses considered for such characterization are parameters which relate to drinking water, safety of human contact, and for health of ecosystems. Water quality objectives aim at supporting and protecting designated uses of freshwater, i.e. its use for drinking-water supply, livestock watering, irrigation, fisheries, recreation or other purposes, while supporting and maintaining aquatic life and/or the functioning of aquatic ecosystems. Water quality objectives provide the basis for pollution control regulations and for carrying out specific measures for the prevention, control or reduction of water pollution and other adverse impacts on aquatic ecosystems (Tebbutt, 1998).

Water quality criteria for raw water, used for drinking-water treatment and supply usually depend on the potential of different methods of raw water treatment to reduce the concentration of water contaminants to the level set by drinking-water criteria. Drinking water treatment can range from simple physical treatment and disinfection, to chemical treatment and disinfection, to intensive physical and chemical treatment.

Water quality criteria for irrigation water generally take into account, amongst other factors, such characteristics as crop tolerance to salinity, sodium concentration and phytotoxic trace elements.

Livestock may be affected by poor quality water causing death, sickness or impaired growth. Variables of concern include nitrates, sulphates, total dissolved solids (salinity), a number of metals and organic micro pollutants such as pesticides. In addition, toxic algae and pathogens in water can present problems. Some substances, or their degradation products, present in water used for livestock may occasionally be transmitted to humans.

Water quality criteria for recreational purpose are used to assess the safety of water to be used for swimming and other water-sport activities. The primary concern is to protect human health, by preventing water pollution from faecal material or from contamination by microorganisms that could cause gastro-intestinal illness, ear, eye or skin infections. Criteria are therefore usually set for indicators of faecal pollution, such as faecal coliforms and pathogens.

Water quality criteria for the protection of aquatic life may take into account only physico-chemical parameters which tend to define water quality that protects and maintains aquatic life, ideally in all its forms and life stages, or they may consider the whole aquatic ecosystem. Water quality parameters of concern are traditionally dissolved oxygen (because it may cause fish kills at low concentrations) as well as phosphates, ammonium and nitrate (because they may cause significant changes in community structure if released into aquatic ecosystems in excessive amounts). Heavy metals and many synthetic chemicals can also be ingested and absorbed by organisms and, if they are not metabolised or excreted, they may bioaccumulate in the tissues of the organisms. Some pollutants can also cause carcinogenic, reproductive and developmental effects.

Water quality criteria for commercial and sports fishing take into account, in particular, the bioaccumulation of contaminants through successive levels of the food chain and their possible biomagnification in higher trophic levels, which can make fish unsuitable for human consumption. They are

established at such a concentration that bioaccumulation and biomagnification of any given substance cannot lead to concentrations exceeding fish consumption criteria, i.e. criteria indicating the maximum content of a substance in fish for human consumption that will not be harmful (Helmer & Hespanhol, 1997).

In India, five water quality classes have been designated (A-E) on the basis of the water quality requirements for a particular use (Trivedy, 2004).

Water quality classification	Usage
Class A	Drinking water source without conventional treatment but after disinfection.
Class B	organised outdoor bathing.
Class C	Drinking water source with conventional treatment followed by disinfection.
Class D	Maintain aquatic life (i.e. propagation of wildlife and fisheries).
Class E	irrigation, industrial cooling and controlled waste disposal.

1.5 Management tools

Water quality management provides an integrated approach to the protection, improvement and sustainable use of rivers, lakes, estuaries, coastal waters and groundwater. The main aims are to prevent further deterioration, protect and enhance the status of aquatic ecosystems and associated wetlands, to promote the sustainable consumption of water; to reduce pollution of waters and to reduce the effects of floods and droughts (Pahl-Wostl *et al*., 2008; Vigil, 2003). The objective of water quality management is to balance the interests of users with the development of the

resource, while at the same time improving and preserving environmental quality. Prior to management, present and potential future beneficial uses of water body have to be identified. In water management, decisions are based on the comparison of water quality data with criteria and standards. Technical feasibility and risk assessment are taken into account before setting criteria and standards. Hydrometric and water quality surveys are conducted as a part of standard setting (Tebbutt, 1998).

Monitoring, as a practical activity, provides the essential information which is required for an assessment of water quality, especially for describing water resources and identifying actual and emerging problems of water pollution, formulating plans and setting priorities for water quality management, developing and implementing water quality management programmes, evaluating the effectiveness of management actions. However, assessments require additional information, such as an understanding of the hydro- dynamics of a water body, information on geochemical, atmospheric and anthropogenic influences and the correct approaches for analysis and interpretation of the data generated during monitoring (Bartram & Ballance ,1996).

1.6 Management of eutrophication

Eutrophication of surface waters is generally considered as a matter for environmental concern. It is the process of nutrient enrichment and gradual filling in of enclosed water body. As it is a natural process it can be thought of as an inevitable and continual ageing of an enclosed water body. The natural process of eutrophication, from the oligotrophic through senescent stages takes thousands of years. Cultural eutrophication is the acceleration and hastening of the natural ageing process because of human activity in the drainage basin of the water body (Nathanson, 2003).

Accelerated nutrient inputs directly affect the population growth of primary producers-phytoplankton, macrophytes and periphyton. Excessive growth of algae increases the amount of organic matter settling to the bottom.

Eutrophication linked changes also affects taxonomic composition, competitive strategy and biodiversity. The increase in oxygen consumption by the settling biomass can lead to oxygen depletion and changes in community structure or death of the benthic fauna. Increased growth and dominance of fast growing filamentous macroalgae in shallow sheltered areas are yet another effect of nutrient overload, which will change the coastal ecosystem, increase the risk of local oxygen depletion, and reduce biodiversity and nurseries for fish (Sigee, 2005; Nathanson, 2003; Wetzel, 2001).

In reality the ecosystems get out of equilibrium with respect to the utilisation values as well. If the lentic water body is a source of drinking water, its value may be greatly impaired because phytoplankton rapidly clogs water filters, and may cause a foul taste. Toxic blooms may cause injury to human health. Eutrophic system is unappealing for swimming, boating and sporting (Wright, 2005).

Various methods for managing algal blooms through physical, chemical (Barroin & Feuillade, 1986; Tucker & Lloyd, 1987; Hawkins & Griffiths, 1987; Oliveira-Filho *et al*., 2004) or biological means (Sigee *et al*., 1999; Jeong *et al*., 2003) do exist. The methods available for reducing algal crops will only succeed if their purpose and effects are understood and their ecological targets are clear.

The repression of eutrophication by reducing nitrogen and phosphorus contents of the water system is the ideal preventive strategy. For the implementation of this long term strategy understanding of the water body, especially interaction of phytoplankton with major plant nutrients and their cycling is of great significance.

However, as an immediate corrective approach, these are not considered practical as it often takes long period of time to take effect. *In situ* treatment of algal blooms to achieve immediate effect, as well as to

maintain long term good water quality is more of an essential option. However chemical treatments have shortcomings, foremost of which is the generation of secondary pollutants and it has undesirable effects on non target organisms. Here comes the use of organisms or compounds having biological origin.

The vast and devastating effect of eutrophication on environment made it a persistent and one of the most challenging problems. Increasing severity of the eutrophication problem necessitates the need to control algal bloom in a sustainable way, as it threatens the environment and livelihood of populations.

Taking into consideration of the aforementioned reasons it was thought worthwhile to design a set of experiments for the evaluation of trophic status of a lentic water body and *ex situ* nutrient enrichment, and to study the interaction of phytoplankton to nutrient enrichment in a eutrophic body, and also to develop an appropriate microbial technology to control algal bloom. This technology precisely consisted of isolation and purification of microorganism associated with declining algal bloom. Besides, another experiment was designed to promote microalgal flocculation by chitosan as an environmental friendly *ex situ* algal removal technique.

The thesis is divided into 5 chapters commencing with an introduction as chapter 1. The chapters 2, 3 and 4 are in the form of scientific papers with an introduction, materials and methods, followed by results, discussion and a list of references. The fifth and final chapter gives a conclusion based on the results of the study, and a summary of the whole work.

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Chapter 2

Nutrient Control and Water Quality

Contents

2.1 Introduction

The understanding of the dynamics of the eutrophication process in the tropical environment is often limited by inadequate literature about the tropical water bodies (Huszar *et al*., 2006). The characteristics of tropical aquatic systems indicate the existence of very complex relationship between the presence of nutrients and primary productivity. Some specific features of tropical lakes such as high recycling rates, elevated primary production during the whole year, high nutrient assimilation, high settling velocity of nutrients, intense organic matter decomposition, and high grazing rate lead to the establishment of a very dynamic ecosystem (Sperling, 1997). Depletion of dissolved oxygen in the hypolimnion occurs in many tropical lakes regardless of trophic status. Higher year-round light and temperature can lead to less seasonality in development of algal blooms and to more efficient recycling of nutrients than in temperate lakes. The tropical lakes and reservoirs, mainly those situated in urban areas, are generally subjected to severe eutrophication problems mainly due to a lack of infrastructure regarding sewage collection and treatment (Sperling, 1997).

Recent studies on the eutrophication in tropical lakes revealed that lakes throughout the area are commonly undergoing the process of eutrophication: some are facing hypertrophication, and some lakes even approaching to hypertrophic level. In a study to examine the relationship between total phosphorus (TP) and chlorophyll (Chl) from 192 lakes of tropical and subtropical origin Huszar *et al*. (2006) observed that 15% of the systems studied were oligotrophic (< 3µg/LChl- *a*), 35% mesotrophic, and 50% eutrophic (> 11 µg/LChl- *a*). Twenty five percentage of the latter have average annual chlorophyll concentrations greater than 50 µg/LChl- *a* suggesting hypereutrophic conditions. Increased algal blooms, depletion of dissolved oxygen, frequent large-scale mortalities of fish, decline of endemic fish species, predominance in the fishery by introduced species, changes in other

biotic species composition, proliferation of water hyacinth, and increasing health risks also manifest the ecological degradation in Lake Victoria (Lungayia *et al*., 2001). The lakes and reservoirs all over India without exception are in varying degrees of environmental degradation. The degradation is mainly due to eutrophication from domestic and industrial effluents and agricultural practices. Gupta (2006) reported that pollution from uncontrolled domestic sewage and industrial pollution causes substantial algal blooms, dissolved oxygen depletion in subsurface waters, and fish kills in Hussain Sagar Lake, India. Extensive encroachment for intensive agriculture, usage of chemical fertilizers and fish feeds, and flow of municipal sewage and industrial waste in to the lake Kolleru, India affected it adversely and has led to eutrophication (Pattanaik *et al*., 2008).

Trophic status of lentic freshwater body

Nutrients play an important role in the health and functioning of aquatic ecosystems. Globally, nitrogen and phosphorus are the two elements that immediately limit, in a Liebig sense, the growth of photosynthetic organisms (Borchardt, 1996). Nitrogen generally limits overall productivity in the marine system.

Phosphorus limitation occurs most often in freshwater system, in environments of intermediate salinities, and along the coasts during periods of high freshwater input (Wetzel, 2001). Silicon could also become a more generally limiting nutrient particularly for diatom growth and appears to be more spatially and temporally variable. Although total algal biomass is not limited by silica availability, the algal community composition, inter specific competition and succession can be altered markedly (Yoshie & Yamanaka, 2005).

Systematic evaluation of the role of nutrient limitation in tropical lakes is not possible because too few of the wide variety of tropical lakes have been examined. East African lakes have received relatively more attention than other lakes. Nitrogen limitation may be widespread because of the low nitrate

concentrations and moderate to high phosphorus concentrations common in eastern African lakes. However, nitrogen to phosphorus ratios and uptake rates of radioactive phosphorus provide strong evidence for phosphorus limitation in some Kenyan lakes. In South American tropical floodplain lakes, seasonal and regional differences in the relative importance of nitrogen or phosphorus limitation occur. Concentrations of both total nitrogen and total phosphorus in South American reservoirs correlate with chlorophyll. Physiological assays and enrichment experiments carried out in Lake Titicaca, in Boliva and Peru provide good evidence for adequate phosphorus supply, while increased algal growth after nitrogen additions occurred in Lake Valencia, Venezuela. (www.unep.or.jp).

As per the report of Zhao (2004) in China 80% lake and reservoir eutrophication is restricted by phosphorus, about 10% lake and reservoir eutrophication is relative to nitrogen, and the rest 10% to other factors. Fisher *et al*. (1995) suggested that nutrient limitation in tropical areas as inferred from nutrient additions, physiological indicators, or dissolved N:P ratios do not show uniform N or P limitation but rather show seasonal and between system variance. An 8-year investigation on the changes of nutrients and phytoplankton chlorophyll-*a* in lake Taihu, China by Chen *et al*. (2003) revealed large spatial heterogeneity and revealed that nitrogen was not a limiting factor in the lake. Arcifa *et al*. (1995) in a review of experimental nutrient enrichment studies in 10 Brazilian lakes and reservoirs did not find clear cut N limitation, rather limitation varied both between systems and seasonally within single systems.

Natural standing waters range from ultra oligotrophic to eutrophic, with progressive increase in productivity and related parameters. Oligotrophic and eutrophic waters are part of a continuum in terms of water quality, and it is convenient to recognize five main groups - hypertrophic, eutrophic, mesotrophic, oligotrophic, and ultra-oligotrophic in descending order of

enrichment and productivity (Sigee, 2005). Organization for Economic Cooperation and Development (OECD), provides specific criteria for temperate lakes in terms of the mean annual values of total phosphorus, chlorophyll-a, and Secchi depth. The OECD classification is based upon a regression model, which observed the relationships between phosphorus and algal densities in various deep upland lakes during the nineteen seventies. On this scheme, for example, the mean annual concentration of total phosphorus ranges from 4 µg/L - 10 µg/L for oligotrophic lakes, and 35 µg/L - 100 µg/L for eutrophic lakes. Mean concentration of chlorophyll in surface oligotrophic water is in the range 1 µg/L - 2.5 µg/L and in eutrophic waters it is in the range 8 µg/L - 25 µg/L. Oligotrophic lakes have a mean annual value of secchi depth in the range 12m - 6m and if it is in the range 3m - 1.5m water body is classified as eutrophic (OECD, 1982).

The trophic state of lakes and reservoirs under both nutrient limiting and non nutrient limited conditions are expressed as Trophic State Index (TSI) based on the phytoplankton biomass (Carlson, 1977; Carlson & Simpson, 1996).

$$TSI\ (SD) = 60 - 14.41\ In(SD)$$

$$TSI\ (CHL) = 9.81\ In(CHL) + 30.6$$

$$TSI\ (TP) = 14.43 In(TP) + 4.15$$

SD = secchi disc transparency (m)

CHL = Chlorophyll pigment concentrations (mg/m^{-3})

TP = Total phosphorus (mg/m^{-3})

TSI values of < 30 are common among lakes and reservoirs of classical oligotrophy and values from 50 -70 corresponds to classical eutrophy. Hyper-eutrophic conditions are common at TSI values of > 70. The TSI changes in some lakes over an annual period especially during the periods of intensive

zooplankton grazing and with difference in non-algal turbidity and when there is a reduction in the availability of nutrients seasonally (Wetzel, 2001).

Elemental cellular stoichiometries of natural phytoplankton communities and seston can reflect the type and extent of nutrient limitation and availability (Wetzel, 2001). Red field ratio (C:N:P) of 106:16:1 (or 41:7.2:1 by weight) among marine plankton is generally supported by numerous studies (Hecky *et al*., 1993). The variation in this ratio is small, usually < 20%. It has been attributed to the relatively nutrient sufficient conditions of marine plankton. But these particulate composition ratios in lakes have been coupled to various physiological conditions such as rates of growth and productivity, nutrient conditions etc. (Sterner and Elser, 2002). Stoichiometric ratios are approximate indicators of relative nutrient limitations and it will be severe in case of C : N (μmol μmol^{-1}) > 14.6; N:P (μmol μmol^{-1}) > 26, C : P (μmol μmol^{-1}) > 258, Si : P > 100, C : Chl*a* (μmol μg^{-1}) > 8.3 and alkaline phosphatase activity (AP) :Chl*a* (μmol μg/h) > 0.005 (Wetzel, 2001). Streams, shallow lakes and reservoirs with short residence times have C: P ratios < 350 and N : P ratios < 26, whereas lakes with longer residence times (> 6 month) differentiate from their inflows typically with C:P > 400 and N:P > 30. Tropical lakes tend to have relatively high C:N ratios, indicative of potential nitrogen limitations although the number of lakes sampled were relatively small (Hecky *et al*., 1993).

Hydrological aspects in eutrophication

Although nutrients are often limiting for algal growth, other factors might be just as important (Reynolds, 1989). For example, light may limit algal growth in water containing high levels of suspended matter or algal cell concentrations. Light is scattered by particles and absorbed by algal pigments and dissolved organic matter (Bleiker & Schanz, 1997). Other environmental factors such as flushing rate, water temperature, *p*H and water hardness also influence growth rates and the composition of algal communities (Scholten *et al*., 2005). However, since nutrients form the

basis of ecosystem production, nutrient availability is the key factor affecting the functional role of algae as basic producers in the food web. Resuspension of inorganic sediment particles is mainly caused by wave action and occasionally, by foraging fish (Ogilvie & Mitchell, 1998). The dynamics of nutrients are also determined by the depth and stratification of lakes. In deep lakes, thermal stratification occurs during winter and summer. Nutrients present in the epilimnion will be transported to hypolimnion. The turn over in autumn and spring makes the nutrients from mineralised material in the hypolimnion available to the epilimnion. There is no stratification in shallow lakes but there is a continuous exchange of material between sediment and water. The mineralization rate of sediment and the subsequent release of nutrients will increase due to higher temperature in summer (Jeppesen *et al*., 1997). In the drier tropics, the hydrological changes linked to climatic change and especially rainfall has had consequences for biological communities in shallow lakes (Kalk *et al*., 1979).

Nutrient uptake by algae under eutrophic condition

The primary productivity which forms the basis of aquatic food webs flourishes during eutrophication. Algae can respond quickly and opportunistically to increases in nutrient availability (Scholten *et al*., 2005). However, the capacity of individual algae to store nutrients is limited. It is the population that retains the nutrients, but only during the growing season. Decaying algae release nutrients and the following season all nutrients must be reacquired. The algal biomass density is the net result of algal production and algal losses due to sedimentation and grazing by zooplankton or other secondary producers.

Nutrients and Algal Communities

Major changes in the seasonal succession of phytoplankton in lakes are related to changes in availability of phosphorus, nitrogen and silica. Although variability is large, the general patterns of seasonal succession of

phytoplanktonic biomass are reasonably constant from year to year, if the drainage basin and lake are not perturbed.

In addition to a general increase in phytoplankton biomass, eutrophication also affects taxonomic composition, competitive strategy (Sommer, 1989; Valiela, 1993), and biodiversity. Species composition varies considerably in relation to water quality. Desmids and chrysophytes, for example, tend to be characteristic of low-nutrient waters, while colonial blue-greens, chlorococcales, and centric diatoms occur as dominant forms in more nutrient-rich habitats (Wetzel, 2001).

Oligotrophic sites tend to be dominated over much of the growing season by unicellular phytoplankton such as nanoplanktonic diatoms and picoplanktonic blue-greens. These r-selected algae are adapted for rapid exploitation of nutrient resources under limiting conditions. In mesotrophic and eutrophic lakes, r-selected species is a particular feature of the clear-water phase, with K-selected species (large unicells and colonial blue-greens) forming dominant blooms over the major growth phase. At highest nutrient levels (hypertrophic lakes and ponds), there is a reversion to small, unicellular, rapidly growing species. These nanoplanktonic organisms form dense blooms of diatoms and green algae as soon as adequate light is available. An analysis of phytoplankton data in European lakes (Schreurs, 1992) shows that cyanobacteria dominate lakes with relatively low fractions of soluble reactive phosphorous (SRP), while green algae dominate systems with higher SRP. Blue-green dominance increases with total N concentrations. On the basis of total P, cyanobacteria dominate moderate classes (100 mgm^{-3}– 800 mgm^{-3}), while green algae dominate at higher levels (>800 mgm^{-3}). Lakes with low nutrient concentrations encompass a significant representation of flagellates in their phytoplankton communities. In an enclosure experiment to investigate factors affecting the dominance of blue-green algae shows that with addition

of CO_2 and available nitrogen there occurred a shift in dominance from blue green algae to green algae (Shapiro, 1990).

Laboratory aquatic microcosms in phytoplankton studies

Water quality is the result of a complex interaction of biological components within environmental variation. A thorough understanding of these interactions is essential in effective water management. Laboratory aquatic microcosm systems can be effective experimental tools for the quantitative description of mineralization (Wilhm, 1970), nutrient recycling, community metabolism and succession (Romanuk *et al*., 2006). Application of the microcosm approach to the aquatic systems not only furthers our understanding of the ecological processes but also facilitates experimental manipulations that are not easily controlled in the natural system. Microcosms should be small enough to be established in laboratories and manipulated easily; they should also be simpler compared to the degree of organisation of the natural system, so as to permit a level of comprehension which is not possible at the real world level. In addition, microcosm systems as physical models should be reliable experimental tools so that the community structure and dynamics of such a system should give good replication.

The response of phytoplankton and zooplankton to experimental alteration of nitrate and phosphorus levels in outdoor enclosures was investigated by Nandini and Rao (2000). A study of three food web structures in microcosms was investigated by Olsen *et al*. (2002) with the intention to study the effect of phosphorus limitation inhibition on dissolved organic carbon consumption in aquatic microbial food webs. Koch *et al*. (2004) used laboratory bioassays with light and nutrient amendments to assess resource limitation of phytoplankton in three Midwestern U.S. rivers, each characterized by different water regulation regimes. Piehler *et al.* (2004) carried out a series of nutrient bioassays to assess the relationship between increased inorganic nutrient concentration and phytoplankton community

structure and function. The total nitrogen, phosphorus, biomass, *p*H, dissolved oxygen and temperature of three eutrophic waters were investigated in the rapid-growth season of phytoplankton by Yeguang *et al*. (2006). Effect of bacteria in stoichiometry and nutrient limitation of phytoplankton was studied (Danger *et al*., 2007). Small-scale, short-term bioassays involving separate *in vitro* additions of nitrogen and phosphorus was carried out by Loureiro *et al*. (2008) to study primary production.

Objectives

The interaction of phytoplankton with major plant nutrients and their cycling is of great significance in the sustainable management of phytoplankton blooming. The suppression of eutrophication by reducing nitrogen and phosphorus contents of the water system is the ideal preventive strategy.

In this investigation the phytoplankton biomass and the trophic status of a natural pond was evaluated. The chances of its developing a persistent bloom was evaluated in microcosm experiments conducted in the laboratory in view of regulating nutrient input as a means of controlling algal blooms.

2.2 Materials and methods

Water and sediment samples were collected from an excavated pond in Kochi, during the summer months when the water level was minimal. The water samples were analysed for chlorophyll *a*, total phosphorus, total nitrogen and *p*H. The sediment samples were analysed to determine the *p*H, total nitrogen, total phosphorus and total organic carbon. A portion of water sample fixed in Lugol's iodine was observed under the microscope to ascertain the composition of phytoplankton community.

2.2.1 Experimental design and culture conditions of microcosms

The pond ecosystem was simulated in fifteen litre glass tank micro-ecosystems. The sediment removed from the pond was laid on the bottom of

fifteen litre glass tanks with a surface area of 0.1 m^2 at a thickness of 5 cm. Ten litres of pond water were added to each of the tanks. The tanks were enriched with nutrients (NH_4Cl and K_2HPO_4) such that the resultant N : P was 8 : 1, 30 : 1 and 90 : 1. The unmodified (unenriched) microcosm was taken as control to represent the natural trophic state of the pond. The pond water had a N:P value of 4:1. Three replicate tanks were maintained for each. All tanks were exposed to sunlight. The ambient temperature was 30 ± 3^0 C. The water and sediment samples were withdrawn from each tank for 28 days with a time-step of seven days. The samples were analysed for chlorophyll a, total phosphorus, total nitrogen and total organic carbon.

2.2.2 Analytical work

The digestion and distillation of water sample for total nitrogen were done according to Radojevic and Bashkin, (1999). The water sample for total nitrogen determination was first boiled with NaOH to expel ammonia. Sodium hydroxide-thiosulphate reagent was added to it after cooling. It was then distilled with H_2SO_4 and catalyst $CuSO_4$. The distillate was collected in boric acid and analysed by indophenol blue colourimetric method for ammonium ion (Solorzano, 1969).

A known volume of water sample was digested with persulphate and its phosphorus was determined by ascorbic acid method to determine total phosphorus (Standard methods, 1999). The pH of water sample was measured using a digital *p*H meter.

Total nitrogen was calculated from concentration of NH_3-N of the sediment sample. Ammonium distillate collected in boric acid after digestion with sulphuric acid containing potassium sulphate in kjeldahl flask was analysed by indophenol blue colorimetric method for ammonium ion (Solorzano, 1969).

Soil sample ignited at 550^0 was cooled and extracted with H_2SO_4 to determine the total phosphorus content of sediment. Total phosphorus content

of the extracted sample was determined by ascorbic acid method spectrophotometrically (Standard methods, 1999).

Organic carbon was estimated by potassium dichromate back titration. Distilled water was added to pre weighed wet sediment sample at 2:1 ratio and stirred vigorously for one hour. After 30 minutes settling *p*H of the supernatant was measured using a *p*H meter.

Planktonic algae were concentrated from the water sample by filtration through membrane filters of pore size 0.45µm. Chlorophyll *a* was extracted from the algal concentrate in to 90 % aqueous acetone and the absorbance of the extract was determined with a spectrophotometer. The concentration of chlorophyll *a* was computed using the trichromatic equation,

chlorophyll $a = 11.85(OD664) - 1.54(OD647) - 0.08(OD630)$ (APHA, 1999)

2.3 Observations

The pond water community of phytoplankton was dominated by green algae. Diatoms were also present. The most abundant genera were *Chlorella, Selanastrum, Pediastrum*, *Scenedesmus* and *Cyclotella*. The amount of chlorophyll *a* was estimated to be 13.43 ± 0.01 µg/L. The total nitrogen content of water was 392 ± 0.02 µg/L and total phosphorus 92 ± 0.13 µg/L. The *p*H of the water sample was 7.7. Over an incubation period of 28 days, there was increase in phytoplankton productivity as measured through chlorophyll *a*. The observations are detailed below.

- The unmodified microcosms that had originally an N:P of 4:1 produced 18.39 ± 0.05 µg/L of chlorophyll *a* in seven days which increased to 55.42 ± 0.01 µg/L by 28^{th} day. During this period the total nitrogen in the water reduced slightly whereas total phosphorus showed much more reduction changing the N:P to 6:1(Fig. 2.1).

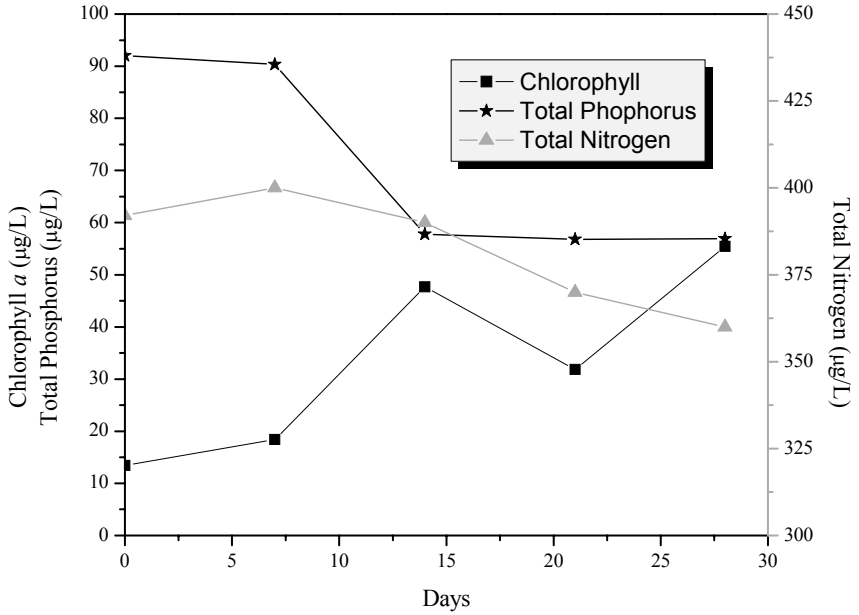

Fig 2.1 Temporal variability of chlorophyll *a*, total phosphorus and total nitrogen of water samples from N:P = 4:1 microcosm during the four week study.

Sediment analysis showed that total nitrogen decreased over time while total phosphorus had increasing tendency as also total organic carbon (Table 2.1). The results indicate that there is sediment release of nitrogen, which could have been absorbed by the algae, but phosphorus added into the sediment by biomass settling and adsorption has more residence time. The natural concentration of nitrogen and phosphorus is sufficient to support the algal bloom for at least a month even at the initial level of 4:1.

Table 2.1 Temporal variability of total nitrogen, total phosphorus and total organic carbon of soil samples in N:P = 4:1 microcosm during the four week study

Period (Days)	TN(g/Kg)	TP(mg/Kg)	TOC (g/Kg)
7	0.93 ± 0.00	0.56 ± 0.01	2.24 ± 0.15
14	0.82 ± 0.01	0.68 ± 0.01	10.82 ± 0.48
21	0.65 ± 0.01	0.68 ± 0.01	22.09 ± 0.40
28	0.58 ± 0.01	0.65 ± 0.00	26.53 ± 0.17

- The microcosm enrichment at N:P = 8:1 produced a sudden induction of algal growth, pushing up the chlorophyll *a* to 48.03 ± 0.02 µg/L in a week's time; but tending to decline after three weeks. The maximum biomass was observed on 21st day with a chlorophyll *a* concentration of 74.65 ± 0.02 µg/L (Fig. 2.2). The dominant genera were *Pediastrum*, *Chlorella* and *Scenedesmus*. Compared to the control microcosm, the concentration of both nitrogen and phosphorus was high and therefore it was congenial to greater biomass build up; but nitrogen limitation could have occurred beyond three weeks.

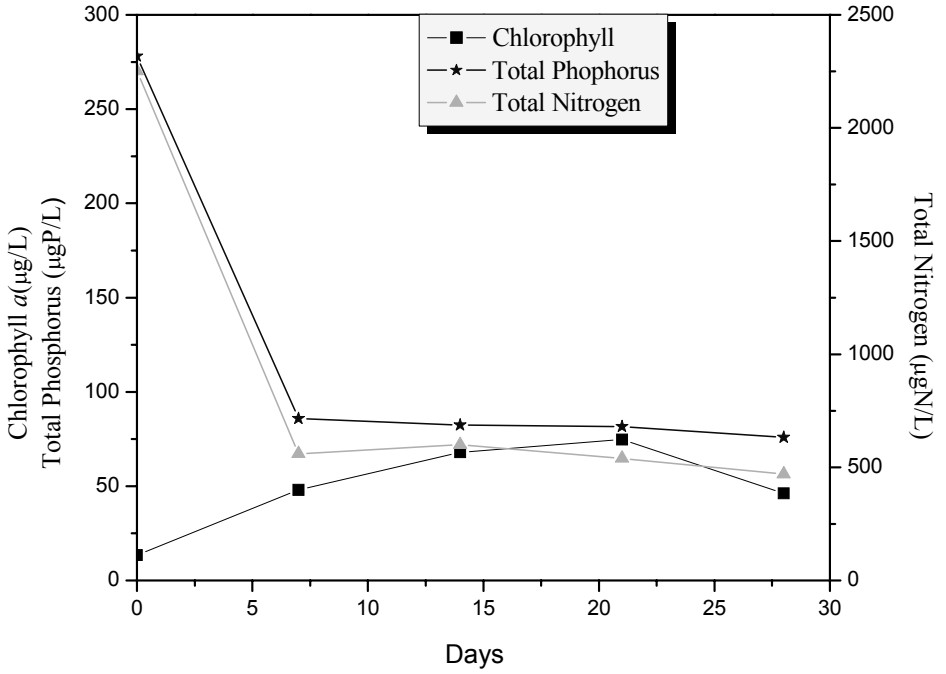

Fig 2.2 Temporal variability of chlorophyll *a*, total phosphorus and total nitrogen of water samples from N:P = 8:1 microcosm during the four weeks study.

The sediment analysis showed decrease in total nitrogen and an increase in total organic carbon and total phosphorus as in control (Table 2.2).

Table 2.2 Temporal variability of total nitrogen, total phosphorus and total organic carbon of soil samples in N:P = 8:1 microcosm during the four week study

Period (Days)	TN(g/Kg)	TP(mg/Kg)	TOC (g/Kg)
7	1.29 ± 0.04	0.57 ± 0.00	13.78 ± 0.38
14	1.24 ± 0.05	0.74 ± 0.01	17.73 ± 0.90
21	1.07 ± 0.04	0.61 ± 0.01	23.98 ± 0.33
28	0.66 ± 0.00	0.58 ± 0.00	28.31 ± 0.74

- The enrichment systems with N:P = 30:1 had slow bloom induction, as the chlorophyll *a* values showed a lag phage upto 3 weeks. But the bloom outburst was exponential between the third and fourth week so that the 28th day chlorophyll *a* was 107.42 ± 0.01 µg/L. The trend of utilisation of nitrogen and phosphorus was similar (Fig. 2.3). Excessive nitrogen in the system did not induce the bloom. Therefore it is the N: P ratio that is important in the induction of bloom.

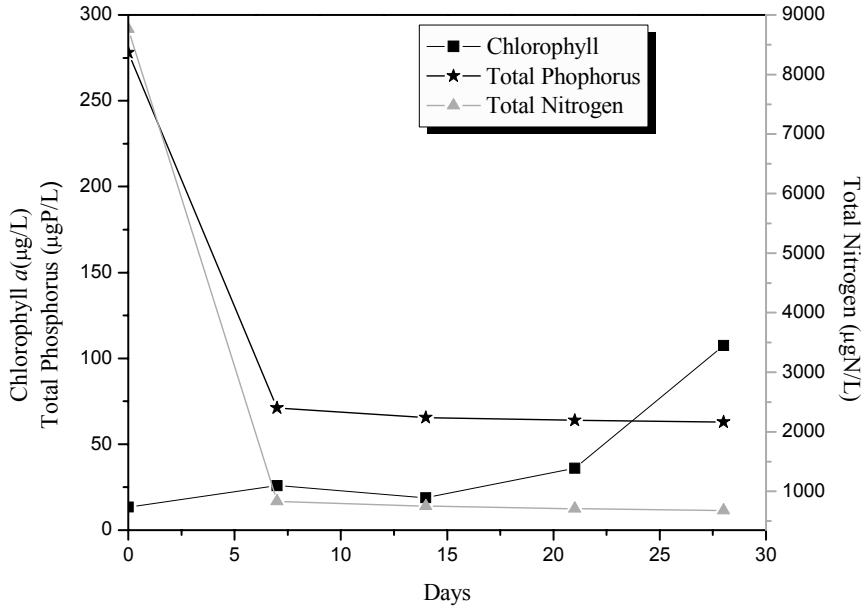

Fig 2.3 Temporal variability of chlorophyll *a*, total phosphorus and total nitrogen of water samples from N:P = 30:1 microcosm during the four week study

It was observed that the N: P on 28th day is 11:1 which is much closer to the Redfield ratio. The dominant genera in this microcosm were *Selenastrum, Scenedesmus, Pediastrum* and *Chlorella*. Sediment analysis showed a decrease in nitrogen development of the bloom and an increase in phosphorus and total organic carbon (Table 2.3.).

Table 2.3 Temporal variability of total nitrogen, total phosphorus and total organic carbon of soil samples in N: P = 30:1 microcosm during the four week study

Period (Days)	TN(g/Kg)	TP(mg/Kg)	TOC (g/Kg)
7	2.10 ± 0.073	0.53 ± 0.003	19.96 ± 1.33
14	1.95 ± 0.021	0.61 ± 0.006	19.97 ± 0.70
21	1.55 ± 0.018	0.61 ± 0.005	18.52 ± 0.55
28	1.18 ± 0.016	0.71 ± 0.004	22.33 ± 0.57

- The enriched system at N: P = 90:1, had the same N content as the previous one, but phosphorus was only 1/3 and the resultant algal biomass on 28th day was low i.e. 73.83 µg/L.

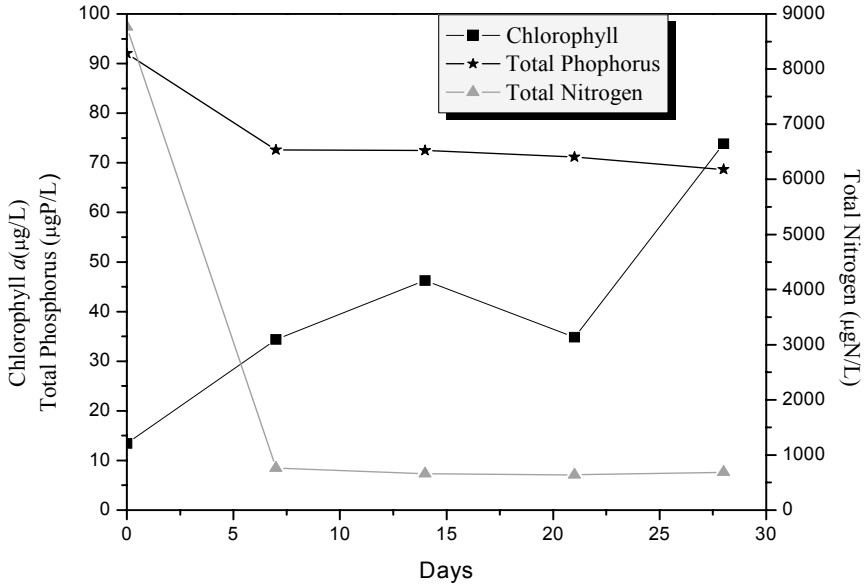

Fig 2.4 Temporal variability of chlorophyll *a*, total phosphorus and total nitrogen of water samples from N:P = 90:1 microcosm during the four weeks study.

Dominant genera in this microcosm were *Pediastrum, Scenedusmus* and *Cyclotella*. Nitrogen was in far excess of P and therefore it is phosphorus limitations that have retarded algal biomass production. The results of sediment analysis are given in Table 2.4.

Table 2.4 Temporal variability of total nitrogen and total phosphorus and total organic carbon of soil samples in N:P = 90:1 microcosm during the four week study

Period (Days)	TN(g/Kg)	TP(mg/Kg)	TOC (g/Kg)
7	1.96 ± 0.010	0.57 ± 0.005	5.55 ± 0.11
14	1.74 ± 0.050	0.77 ± 0.019	15.58 ± 0.38
21	1.44 ± 0.015	0.65 ± 0.003	28.69 ± 0.90
28	1.38 ± 0.005	0.67 ± 0.005	32.16 ± 0.97

2.4 Discussion

The assessment of algal population based on chlorophyll revealed the eutrophic nature of the pond selected for the study. The concentration of chlorophyll *a* in eutrophic lentic water bodies is widely reported to be in the range 8 - 25 µg/L (Sigee, 2005)

The total phosphorus content of the pond was 92 ± 0.13 µg/L. According to Porcella *et al*. (1980) total phosphorus content > 25 µg/L is the threshold value for eutrophication. Sigee (2005) describes total phosphorus in the range 35 – 100 µg/L as an index of eutrophic state of temperate freshwater lakes. Richardson *et al*. (2007) reported that a surface water mean total phosphorus threshold concentration exceeding 15 µg/L causes ecological imbalance.

Another important parameter described in the trophic status of water body is total nitrogen. Eutrophic criterion of total nitrogen as per Mason (2002) is > 500 µg/L. As per Wetzel (2001) total nitrogen of mesoeutrophic lake is the range 500 – 1100 µg/L. The total nitrogen content of the natural

pond studied had a value of 392 ± 0.02 µg/L. Total nitrogen content of the present study matched the oligomesotrophic criterion (250 - 600 µg/L) as described in Wetzel (2001).

Excessive nutrients enter the water bodies from point sources such as from sewage, industry and non point sources like surface run off, irrigation water etc. Nitrogen and phosphorus input and enrichment in water are the primary factors held for eutrophication. According to Zhao (2004) 80% of the lakes and reservoirs eutrophication in China is due to phosphorus limitation, about 10% controlled by nitrogen and rest 10% by other factors. In many ecosystems, the phytoplankton biomass is affected by the concentration of nitrogen and phosphorus (Cloern, 2001; Bledsoe *et al*., 2004; Reynolds, 2006). In the present investigation, it was observed that when nitrogen was in excess, the biomass was related to phosphorus concentration. The ratio of N:P in the water body have been used as an indicator of predicting the likely development and persistence of algal blooms. In freshwaters, if N:P is over 20:1, phosphorus is considered to be the limiting element; if N:P is below 10:1, nitrogen is considered to be the limiting element. If the ratio is between 10:1 and 20:1, the limiting element becomes uncertain (Yeguang *et al*., 2006). Lin *et al*. (2008) have stated that many tropical and subtropical lakes are nitrogen limited. This agrees with the present observation where the pond water has an N:P of 4:1. According to Yang *et al*. (2008) red tides are induced when total nitrogen reaches 300 µg/L and phosphorus reaches 20 µg/L. Therefore, the pond in the present investigation is eutrophic and potent enough to promote phytoplankton bloom; but sustenance of bloom for long term is doubtful as the system is nitrogen limited.

Additional input of nutrients into the system changed the pattern of induction and sustenance of the algal bloom. At 8:1, there occurred induction of bloom but the bloom declined after three weeks, probably due to nitrogen limitation. Under same concentration of phosphorus, when the level of

nitrogen was increased, the bloom persisted beyond three week in fact, the curve was exponential between third and fourth week producing 107 µg/L Chl *a* on the 28th day. The same trend was observed when the concentration of phosphorus was lowered keeping the high nitrogen level constant, although the biomass attained was less. Therefore, it is derived that by further input of nitrogen the system will only result in sustained algal bloom, but the concentration of phosphorus will limit the biomass i.e. in such systems the final biomass build up depends on the concentration of phosphorus. In nitrogen limited systems bloom induction is quick, but soon nitrogen starvation leads to decline of the bloom. Therefore, there is less chance of persisting blooms in nitrogen limited systems.

In this investigation, based on the nutrient studies it is evident that an N:P of 8:1 can induce a sudden bloom, but for persistence of the bloom, higher N:P is required. The threshold couldn't be defined.

Therefore, what are the options for control of algal blooms in an enclosed water body? Definitely it is reduction of the nitrogen and phosphorus inputs, together with monitoring of the ratio of nitrogen and phosphorus. The sediment absorption and release, the temperature and hydrodynamics are important factors to be studied.

The pond under investigation although nitrogen limited, has sufficient growth of phytoplankton as to classify it in eutrophic category based on chlorophyll *a*, total nitrogen and total phosphorus. The source of nutrients is traced to surface run off i.e. non point pollution. Controlling inputs of nutrients will not be a practical solution in such systems. When similar pond and lake waters are used for drinking purpose after treatment or for industrial use interference of the algal bloom on the treatment efficiency is a major problem. Hence, effective methods of algal control in the intake waters are required. Therefore, the existing algal bloom control methods are reviewed and alternate methods are probed further in this investigation.

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Chapter 3

Algicidal Control of Algal Growth

Contents

3.1 Introduction

Eutrophication of freshwater environments and concomitant occurrence of algal blooms have received great deal of attention due to their significant adverse impacts on water quality, deoxygenation of underlying water, their toxicity, foul odour and resulting decrease in aesthetic quality (Wetzel, 2001). Short- term strategy to eliminate or reduce algal bloom generally involves chemical or biological *in situ* treatment. The usage of chemicals, most probably copper salts, was the conventional way of algal elimination from fresh water bodies (Hawkins & Griffiths, 1987; Meador *et al*., 1998; Han *et al.*, 2001; Oliveira-Filho *et al*., 2004). Copper treatments can result in potentially high levels of Cu in the surface waters and sediments (Hullebusch *et al*., 2003). Another major drawback of chemical treatments is that they are non selective and adversely affect a variety of non-target aquatic species. (Meepagala *et al*., 2005). Toxicity data provided by Oliveira-Filho *et al*. (2004) confirm that planktonic crustaceae and algae are extremely susceptible to increases in free copper levels in water bodies. Since phytoplanktonic and zooplanktonic organisms form the basis of aquatic food webs, increased levels of bioavailable copper can dramatically affect freshwater ecosystems. Herbicides such as diuron, simazine, atrazine and the like block the electron flow in photosystem II and are known to inhibit the growth of algae especially *Microcystis*. But the xenobiotic nature of these herbicides seems to prevent its further use as algicides.

An increasing number of laboratory studies and field application have shown that plant mulch with allelopathic activity could also be used to control aquatic weeds and algal growth. Barley straw has long been known for its algal control property in freshwater systems (Newman & Barrett, 1993; Everall & Lees, 1997; Caffey & Monahan, 1999; Terlizzi *et al*., 2002; Brownlee *et al*., 2003). It was extensively used in the British Isles lakes, potable water reservoirs, canals, and streams and was proved successful

(Welch *et al*., 1990). But the results were varying in North America and the reasons for this variation remain unclear (Boylan & Morris, 2003). The factors that must be properly addressed for the success of barley straw for freshwater algal control include adequate straw dosage, starting treatment well in advance of bloom development, proper positioning of the straw in the body of water, adequate aeration of the straw and adequate water circulation.

Aqueous extract of *Lantana camara* leaves was reported to eliminate *Eichhornia crassipes* and *Microcystis aeruginosa* (Kong *et al*., 2006). The ethyl acetate extract of the roots *Ruta graveolens* was studied for algicidal activity in blue-green alga, *Oscillatoria perornata*, a pest in commercial catfish (Meepagala *et al*., 2005). Mulderij *et al*. (2006) in a laboratory microcosm experiment showed a significantly lower phytoplankton biomass in treatments with Water soldier, *Stratiotes aloides* L. exudates .Park *et al*. (2006) reported that rice straw extract exhibited an inhibitory effect on *M. aeruginosa*.

Biological control of algae represents a potential short term measure to reduce the population or prevent the build up of nuisance algal populations. This involves more fundamental alterations to the lake ecosystem when compared to nutrient limitation and biomanipulation. This alteration is invariably temporary, with reversion of the ecosystem to its original state within a relatively short period of time. A wide range of microorganisms can be used as biological control agents, fungi and viruses, protozoa, actinomycetes, and bacteria.

Fungi commonly involved in parasitic associations with microalgae are uniflagellated Chytridiomycetes, usually called chytrids (Ibelings *et al*., 2004). To a lesser extent, biflagellated forms belonging to the Oomycetes and other types of fungi have also been observed to be algicidal (Mountfort *et al*., 1996; Elbrächter & Schnepf, 1998). Fungal infection causes high mortality of freshwater diatom populations, influencing succession of phytoplankton

assemblages (Donk, 1989). Effectiveness of fungus as algal control agent is often limited by high specificity and difficulties in large-scale culturing.

Viruses are potentially very useful as biological agents as they have rapid generation time and high burst. LPP-1 cyanophage was reported to have algicidal activity (Manage *et al*., 1999). The high degree of host specificity, occurrence of resistant host mutants and effects of environmental factors all contribute to the complexity and unpredictability of cyanobacterial/phage interactions in the field applications. Another difficulty associated with the usage of cyanophages as biological agents in the lake environments are the problems related with the large amounts of active inoculum (Sigee, 1999).

Within aquatic ecosystems protozoans have an important role in the reduction of phytoplankton populations by predation (Scholten *et al*., 2005). Important protozoans reported to consume algae as food source are ciliate *Nassula (*Canter *et al*., 1990), the flagellate *Ochromonas* (Cole & Wynne, 1974) and the amoebae *Acanthamoeba* (Wright *et al*., 1981) and *Mayorella* (Laybourne-Parry, 1992). The effectiveness of protozoans as biocontrol agents will depend upon a number of factors – including protozoan growth and grazing rates, predation specificity, cyanobacterial growth rates and predation rates by higher organisms (Scholten *et al*., 2005). Actinomycetes gave good results as potent algicides in the laboratory experiments (Yamamoto *et al.,* 1998) but limited in environment. This may be due to its sinking out of epilimnion (Sigee, 2005)

Allelopathic interactions among algae can be exploited to control unwanted algae. Growth inhibition of *Dunaliella salina, Platymonas elliptica, Chlorella vulgaris , Nitzschia closterium, Chaetoceros muelleri, Chaetoceros gracilis, Nitzschia closterium minutissima, Phaeodactylum tricornutum* and *Isochrysis galbana* by a growth inhibitor (1-[hydroxyl-diethyl malonate]-isopropyl dodecenoic acid, $C_{22}H_{38}O_7$) from the culture (cell-free filtrates) of *Isochrysis galbana* was investigated by Yingying *et al*. (2008).

Aquatic bacteria associated with declining algal blooms are considered as a promising biological agent to control algal blooms (Daft *et al*., 1985; Yamamoto *et al.*, 1993; Manage *et al*., 2000; Kang *et al*., 2007)**.** The strains included *Alcaligenes* (Manage *et al*., 2000), *Alteromonas* (Imai *et al*., 1998), *Bacillus* (Nakamura *et al*., 2003; Mu e*t al*., 2007), *Cytophaga* (Imai *et al*., 2001), *Flavobacterium* (Fukami *et al*., 1997), *Flexibacter* (Sallal, 1994), *Micrococcus* (Kim *et al*., 2008), *Myxobacterium* (Shilo, 1970), *Pseudomonas* (Wang *et al*., 2005), and *Xanthomonas* (Walker & Higginbotham, 2000). The seven attributes which are considered for a potential bacterial biological control agent of bloom-forming phytoplankton are adaptability to variations in physical conditions, ability to search or trap, capacity and ability to multiply, prey consumption, ability to survive low prey densities, wide host range, and the ability to respond to changes in the host (Daft *et al*., 1985). In general, algicidal bacteria are effective through either a direct (Lovejoy *et al.,* 1998; Doucette *et al*., 1999; Kim *et al*., 2008) or an indirect attack (Kodani *et al*., 2002; Ahn *et al*., 2003; Wang *et al*., 2005). The use of a bacterium as algicide in natural environment depends on inoculation rates, host range of organisms, responses of non-target species, methods to produce, stabilize, and formulate inoculum, and the effects of *p*H, temperature, and other environmental parameters on efficiency.

Several studies have demonstrated that extracellular substances such as hydroxylamine (Berger *et al*., 1979); phenazines (Dakhama *et al*., 1993); aminophenol (Yamamoto *et al*., 1998); rhamnolipids (Wang *et al*., 2005); protease (Lee *et al*., 2000); bacillamide (Jeong *et al*., 2003), Surfactin (Ahn *et al*., 2003); and sophorolipid (Sun *et al*., 2004) are algicidal in nature.

Commercial application of algicides is, however, limited to copper compounds especially copper sulphate as in products such as clearigate and cutrine-plus. Therefore, the aim of this investigation is to find out an environmentally safe algicide of microbial origin.

Objectives of the present study include:

- Screening, isolation and characterisation of micro-organisms of algicidal property.
- To check the efficiency of micro-organisms as algicides and its host range
- Extraction of algicidal compound

3.2 Materials and methods

3.2.1 Screening of algicidal microbes

Water sample from a pond with degraded algal bloom was filtered through 0.8µm nucleopore membrane filters. Filtrate was inoculated to nutrient broth in order to enrich microbes in the water sample. Nutrient broth together with filtrate was incubated at 28 ± 1^0 C for 48 hours.

- **Algal assay**

Synechococcus elongatus was grown in BG-11 medium in borosilicate culture flasks in triplicate at an inoculum density of 10^4 cells/mL. A sample of 5 mL of the 48 hour grown microbial consortium was added to 95 mL cultures of *S.elongatus* in logarithmic growth phase. Algal cultures amended with microbial consortium and incubated at 28 ± 1^0 C were kept under the light intensity of 1500 lux with 12h light: 12h dark photo-cycle. After a week, algal growth status was observed visually and microscopically and compared with the controls. Similarly, screening for algicidal micro organisms was done in the cultures of chlorophycean members, in logarithmic growth phase of *Chlorella pyrenoidosa*, *Oocystis pusilla*, and *Scenedesmus quadricauda* grown in Ward and Parish medium at a cell density of 10^4 cells/mL. Cyanophycean filamentous forms *Oscillatoria acuminata and Oscillatoria subbrevis*, which were grown in BG-11 medium at an inoculum density of 10 mg dry weight /L, were also taken for the algal assay in a similar way as of *S.elongatus.*

3.2.2 Rapid detection of algicidal activity by disc diffusion assay

- **Development of Colony forming units**

In order to obtain single colonies for rapid algicidal detection by disc diffusion assay, the above microbial consortia was inoculated on nutrient agar plates by pour plate method. The plates were then incubated at 28 ± 1^0 C for 48 hours so that distinct CFU were obtained.

- **Development of algal lawn**

The culture of *S.elongatus* in exponential growth phase was concentrated by centrifugation (3500 rpm, 10 min.). Concentrated *S.elongatus* culture was taken for preparation of algal lawns. Algal lawns for disc diffusion assay was prepared as double layer BG-11 agar plates, with 2% agar medium at the bottom of the Petri plate and 1.2% agar medium incorporated with concentrated *S.elongatus* on the top. Prepared algal plates incubated for two days were kept under the light intensity of 1500 lux with 12h light: 12h dark photo-cycle at 28 ± 1^0 C. Similarly, double layer Ward and Parish agar plates were prepared with concentrated *C. pyrenoidosa*, *O. pusilla* and *S. quadricauda*. In order to prepare double layer agar plates of *O.acuminata* and *O.subbrevis*, long filaments were broken into shorter ones in a mortar and pestle by gentle maceration. The broken short filaments were then incorporated to 1.2 % BG-11 medium and plates for disc diffusion assay were prepared in a similar fashion for *S.elongatus*.

- **Disc diffusion assay**

Replicate cores (0.5 cm diameter) of single bacterial colonies (CFU) with distinct morphologies were cut from one day old bacterial agar plates and inverted onto lawns of *S.elongatus* so that antagonist and algae were in direct contact. Cores cut from un- inoculated plates were used as control. Preparations were incubated at room temperature, under the light intensity of 1500 lux with 12h light: 12h dark photo-cycle and examined daily for four days, any yellowing, or clearing, of the treatment compared to control being

recorded as positive. Confirmation of algicidal activity was also ensured in double layer agar plates of *C. pyrenoidosa*, *O. pusilla, S. quadricauda*, *O.acuminata* and *O.subbrevis* in a similar style done in *S.elongatus.*

3.2.3 Isolation of antagonistic bacteria from PFU and its algicidal activity

Bacteria that formed plaques around the core in the previous assay were inoculated on to nutrient agar plates (2%), incubated in dark for 2 days at 28 ± 1 ^{0} C and purified by repeated streaking onto nutrient agar plates. Two algicidal bacteria were isolated in this manner. Once purified the bacteria were axenically maintained in the dark on the nutrient agar plates at 4^{0} C. Algicidal activity of two isolated bacteria were compared by the algal disc diffusion assay on *S.elongatus, O.acuminata* and *C.pyrenoidosa* cultures. The algicidal activity was measured in terms of the diameter of the lyses zone.

3.2.4 Identification of bacterial isolate

Generic level identification of the isolates was carried out following Oliver (1982) and demarcated into species following Alsina and Blanch (1994). The tests carried out were Gram staining, motility, growth at $4^{0}$and 41^{0}C, cytochrome oxidase (Kovac's), oxidation/fermentation of glucose, catalase, production of arginine dihydrolase, ornithine and lysine decarboxylase, Voges-Proskaeur reaction, citrate utilization, NO_3 reduction, and production of gelatinase, amylase and lipase.

- **Gram Staining**

According to the method recommended by Christian Gram a thin emulsion of the culture with sterile saline was made on the clean glass slide. The emulsion was dried and heat fixed. The bacterial film was flooded with crystal violet for 1 min. and then washed gently with distilled water. The slide was flooded with mordant, Lugol's iodine solution and allowed to stand for one min. and washed with water. Decolourizing agent was poured on it and allowed to stand for 30 sec. and slide was rinsed in gentle running water and kept for drying. The counter stain saffranine was flooded on the smear and

allowed to stand for one min. The slide was washed, air dried and observed under microscope having oil immersion objective.

- **Motility test**

Motility of the isolate was tested by using soft agar. Medium was poured into tubes and autoclaved at 121^0 for 15 min. It was inoculated by stabbing the agar deep at the centre and incubated at 28 ± 1^0C. Motile strains were observed to have diffuse growth into medium away from the stab line.

- **Growth at 4^0 and 41^0 C**

Growth at 4^0 and 41^0C was tested by inoculating the organism on nutrient agar plates. Overnight cultures were inoculated and incubated at 4^0C and 41^0 C for 48 hours. Growth was detected by visual observance.

- **Cytochrome oxidase (Kovac's)**

Presence of cytochrome oxidase was detected according to the method recommended by Kovacs' (1956). The organism was freshly grown on nutrient agar. A platinum loop was used to pick a colony and make a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned violet within 10 sec, indicating the formation of indophenol.

- **Oxidation/Fermentation of glucose**

The media used was Marine Oxidation Fermentation (MOF) Medium (Himedia) as per manufacturer's recommendations. Twenty two g of the medium and 15 g agar were added to 1000 ml distilled water and sterilized by autoclaving at 121°C for 15 min. To the above basal medium, 1% glucose was added and 4 mL aliquots were transferred into sterile tubes which were then autoclaved at 110°C for 10 min. The tubes were laid out to slants and after drying, the cultures were inoculated by stab and the streaked on the slant. The tubes were incubated at 28 ± 1°C and results were recorded as follows:

O – oxidation (yellow colouration in the slant alone)

F – fermentative (yellow coloration throughout the tube)

(F) – fermentation with gas production

A – alkaline reaction (no colour change in the tube)

- **Catalase**

An overnight culture on Zobell's agar slants was used for this test. A thick smear of the test organism was made from the culture on a clean glass slide and it was flooded with 3 % hydrogen peroxide (H_2O_2). Immediate formation of gas bubbles indicates the liberation of oxygen which was considered as positive reaction.

- **Production of arginine dihydrolase**

The basal medium prepared in distilled water without L (+) arginine hydrochloride was heated to dissolve the solids. *p*H was adjusted and sterilized by autoclaving at 15 lbs for 15 min. L (+) argnine hydrochloride was added and dispensed as 3 mL aliquots in sterilized culture tubes. The medium in the culture tubes were overlaid with liquid paraffin to a height of 5 mm and autoclaved again at 10 lbs for 10 min. As control, tubes containing only basal medium overlaid with liquid paraffin were included.

The test organisms were stab inoculated into the medium through the liquid paraffin layer. Colour changes were recorded after incubation at 28±1°C for upto 7 day; the colour change from yellowish orange to red being positive.

- **Production of Ornithine and Lysine decarboxylase**

One percent of the L-aminoacid [L(+) Lysine dihydrochloride or L(+) Ornithine monohydrochloride] was incorporated in Falkow's medium (Falkow, 1958). The basal medium prepared in distilled water without L (+) Lysine dihydrochloride or L (+) Ornithine monohydrochloride was heated to dissolve the solids. *p*H was adjusted to 6.7 and sterilized by autoclaving at 15 lbs for 15 min. L(+) Lysine dihydrochloride or L(+) Ornithine monohydrochloride was added

and dispensed as 3 mL aliquots in sterilized culture tubes. The medium in the culture tubes was overlaid with liquid paraffin to a height of 5 mm and autoclaved again at 10 lbs for 10 min. As control, tubes containing only basal medium overlaid with liquid paraffin were included.

The test organisms were stab inoculated into the medium through the liquid paraffin layer. Colour changes were recorded after incubation at 28 ± 1^0C for upto 7 days. A subsequent change from yellow to violet or purple in the tests indicates the presence of alkaline degradation products.

- **Voges-Proskaeur reaction**

To a 48 h old culture, 0.5 ml 6% α-naphthol and 0.5ml 16% KOH solution were added, agitated thoroughly and maintained for 2 h. A positive result was indicated by the development of pale pink colour. It appears normally within 5 min. but may not reach maximum red colour intensity for about an hour. Negative tubes were re-examined and confirmed after long period.

- **Citrate utilization**

Simmon's citrate agar (Himedia), was used as per manufacturer's recommendations. The medium was melted and dispensed in 4 ml aliquots into tubes and autoclaved at 121°C for 15 min. and laid out to form slants. They were inoculated with 24 - 48 h old cultures and incubated at 28 ± 1°C for 24 h. Utilization of citrate was established if the alkaline colour (blue) appeared after 2-3 d of incubation.

- **Reduction of Nitrate**

Test for reduction of nitrate was carried out by inoculating isolate into suitable nutrient broth medium containing 0.1 %(w/v) KNO_3. The cultures were incubated until good growth was obtained. Presence of nitrite was determined by adding 0.5 mL of reagent A, (containing 1 g sulphalinic acid in 100 mL 5N glacial acetic acid) followed by 0.5 mL of reagent B (containing

0.6 g Dimethyl alpha-naphthylamine in 5 N glacial acetic acid) to 5 mL of culture. Development of a red colour indicates the nitrate reduction capability of organism.

- **Hydrolytic potential of isolates**

(a) Production of Gelatinase

The medium was melted, poured into tubes and autoclaved at 121°C for 15 min. A 24 h old culture was inoculated and incubated at 28 ± 1°C for 3-5 d. Un-inoculated tubes were maintained as control. After the incubation period the tubes were kept at 4ºC for 6 h. The tubes were inverted to observe for liquefaction of gelatin while the control remains solid.

(b) Production of Amylase

Medium was autoclaved at 121°C for 15 min. and dispensed into sterile Petri plates and single line streak of the organism was made across the centre of the plate. After four days of incubation at 28±1ºC, the plates were flooded with Gram's iodine. Hydrolysis of starch was detected by the formation of clear zone around the colony. The colour of the zones is dependent on the degree of hydrolysis of the starch, when it was hydrolyzed to the stage of dextrin, then the zones were reddish brown, and when the breakdown had gone further, they were colourless.

(c) Production of Lipase

The medium was prepared by blending Tributyrin mechanically into the nutrient broth to form a stable emulsion. Agar was then added to it and sterilised at 121^0 for 15 min. and poured into sterile plates. A 24 h old culture was spot inoculated onto the plates and incubated at 28±1ºC for 7 days. A zone of clearance was observed in the plates of positive sample.

3.2.5 Bacterial cell density and algicidal efficiency- Co-culture assay

The algicidal effect of bacterium on the growth of *S. elognatus, C. pyrenoidosa* & *O. acuminata,* in exponential growth phase was investigated.

Algal cultures of each species in respective algal medium were appropriately diluted (10×10^4cells/mL) with the same medium and 95 mL were inoculated into duplicate 250 mL Erlenmeyer flasks.

The bacterium was incubated in King's B liquid medium at 30 ^{0}C overnight. When the cell density reached 1 to 2×10^8cells/mL, the culture was centrifuged (3500 rpm, 10 min), washed twice with sterilized respective algal medium and 5 mL aliquots were inoculated into algal cultures at final concentrations of 1 to 2×10^4 cells/mL, 10^5 cells/mL, 10^6 cells/mL 10^7 cells /mL and 10^8 cells/mL. Duplicate control cultures were prepared by adding 5 mL of respective algal medium to 95 mL algal culture.

The flasks were incubated at room temperature under the light intensity of 1500 lux with a 12h light: 12h dark - photo-cycle for 20 days. The cell density of the algal species was determined on alternate days using a haemocytometer under a microscope. Algal biomass as (dry weight) was taken into account in case of *O.acuminata* to check the relation between algicidal effectiveness and bacterial density.

3.2.6 Extractability of Algicidal metabolite

Cultures of antagonistic bacteria (3-5 days old) were centrifuged at 10,000 *g* at 4 °C for twenty min. The supernatant was filtered through a series of filters including glass microfiber filter (GF/C, Whatman), cellulose acetate membrane (0.45 µm) and PVDF membrane (0.22 µm), and used for further analysis. The filtrate was extracted with different solvents such as chloroform, petroleum ether, hexane, methanol, ethyl acetate, and a mixture of chloroform : methanol (2:1) to recover algicidal compound. The extraction was done by mixing two parts of cell free supernatant with one part of the solvent in a separating funnel. The solvent layer was allowed to separate and drained off subsequently. Twenty µL of the solvent fraction were impregnated on sterile filter paper disc and allowed to dry for 30 minutes. The process was repeated to get 40 µL extract impregnated per disc and dried in vacuum desiccator for 24 hours. Control discs were kept with

solvents alone. The discs were placed on algal lawn of *S.elongatus* prepared as described in section 3.2.1 and incubated. Development of clear zones was observed, and the diameter of the lysis zone was measured after 96 hours.

3.2.7 Chloroform extract of algicidal metabolite – an alternate method

The bacterial strain was cultured on King's B agar for 72 hours at 28 ± 1^{o}C by continuous streaking. After 72 hours macroscopic bacterial growth on the agar was removed. The agar was cut into pieces and extracted with chloroform over three hours by shaking in a rotary shaker. It was filter sterilized through 0.2µm filter paper (Kerr *et al*., 1999). Algicidal effectiveness of chloroform extract of cell free filtrate in different algal species was checked after 96 h incubation by the disc diffusion assay. The algicidal activity of the extract on different species of blue green and green algae was compared.

3.2.8 Algal assay of chloroform extract of the isolate- estimation of EC_{50} and EC_{90}

The experiment was done in two steps: range finding and definitive test.

- **The range finding test**

The *S.elongatus* cultures were inoculated at the cell density of 10^4 cells/mL to BG -11 medium in borosilicate culture flasks in triplicate amended with chloroform extract at concentrations of 10 mg/L, 100 mg/L, 500 mg/L and 1000 mg/L. At the end of 96 h incubation (12/12 light photo period) aliquots were drawn from uniformly shaken culture and cells were counted directly in a haemocytometer.

- **The definitive test**

Definitive test was conducted in a similar way as range finding test. The concentration of algicidal extracts was fixed on the basis of the results of range finding test. Two series of extract concentrations were taken to determine EC_{50} and EC_{90}. Logarithmic scale concentrations of 1 mg/L, 1.8 mg/L, 3.2 mg/L, 5.6 mg/L and 10 mg/L of chloroform extract were taken to find out EC_{50}. A Concentration ranging from 10 mg/L to 100 mg/L was used to find out EC_{90}. The total cell

count after 96 h was enumerated in haemocytometer. The results were compared with the control.

The pure cultures of *C.pyrenoidosa* were also assayed through range finding and definitive test procedures followed for *S.elongatus*. Definitive test for EC_{50} was checked in the logarithmic scale of concentration between 10 mg/L and 100 mg/L. EC_{90} of chloroform extract in *C.pyrenoidosa* was done at concentrations of 100 mg/L, 180 mg/L, 320 mg/L, 560 mg/L, and 1000 mg/L

- **Statistical analysis**

The data were analysed statistically to quantify and predict the probable effects of the chloroform extract of the isolate on algae. The algicidal effectiveness of various doses of chloroform extract was compared by ANOVA assuming the null hypothesis that all groups are equal i.e., the various concentration of algicidal chloroform extract had the same effect on the organisms. Further Dunnet's test was done in cases where significant differences were observed, to identify the test concentrations that produced significant effect than control. The significant differences among the treatment concentrations were brought out by the Tukey's multiple comparison tests. The data analysis was done with the software TOXSTAT. EC_{50} and EC_{90} were computed by probit analysis (SPSS software.)

3.2.10 Fractionation of chloroform extract of *Pseudomonas* SEB 8

- **Wave length scan**

The chloroform extract was scanned in the UV-Visible spectrophotometer in the wavelength range 200nm - 700 nm.

- **Fractionation**

The chloroform extract was concentrated under vacuum and was spotted on TLC plates (silica gel G) having 0.2 mm thickness. Different solvent systems used for separation of fractions were, chloroform: methanol (1:1); ethyl acetate: acetic

acid: water (3: 2: 1); and acetone: benzene: water (92: 30: 8). The R_f values were measured.

3.2.10 Algicidal activity of separated fractions on TLC plates

For testing the anti algal property of separated fractions, four different spots from the TLC plates were scrapped off and dissolved in 30% alcohol and centrifuged at 10,000 *g* for 15 minutes at 4^0 C. Antialgal activity of each fraction was tested by disc diffusion assay as depicted earlier on *S.elongatus* lawns.

3.3 Result

3.3.1 Algicidal activity of microbial consortia

S.elongatus culture inoculated with microbial consortium turned pale brown upon incubation for a week while the control remained reddish- brown in colour. In microscopic examination of *S.elongatus* disintegrated cells were observed, and its cell size appeared diminished and the cells were more or less spherical (Fig 3.1).

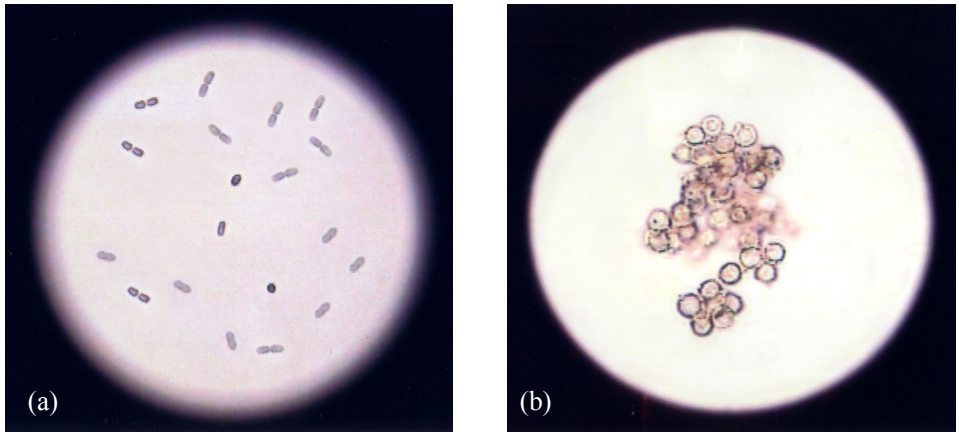

Fig. 3.1 Microphotograph of *S.elongatus* (a) Control (x 400), (b) *S.elongaus* exposed to microbial consortium (x1000)

The unicellular green algal cultures *C.pyrenoidosa*, *O.pusilla* and *S. quadricauda*, after one week incubation with microbial consortium, turned yellowish green in colour. Similar microscopic results were observed with the

treatment to *C.pyrenoidosa*, *O.pusilla* and *S. quadricauda* but the rate of decrease and disintegration was lesser than those seen in *S.elongatus*.

The algal biomass of *O.acuminata* and *O.subbrevis*, which was blue green in colour initially, turned yellowish following one week growth in the treated cultures. In microscopic observation *O.acuminata* and *O.subbrevis* filaments were found to be depigmented and disintegrated. Shorter filaments were seen in experimentals when compared with the controls (Fig 3.2, 3.3).

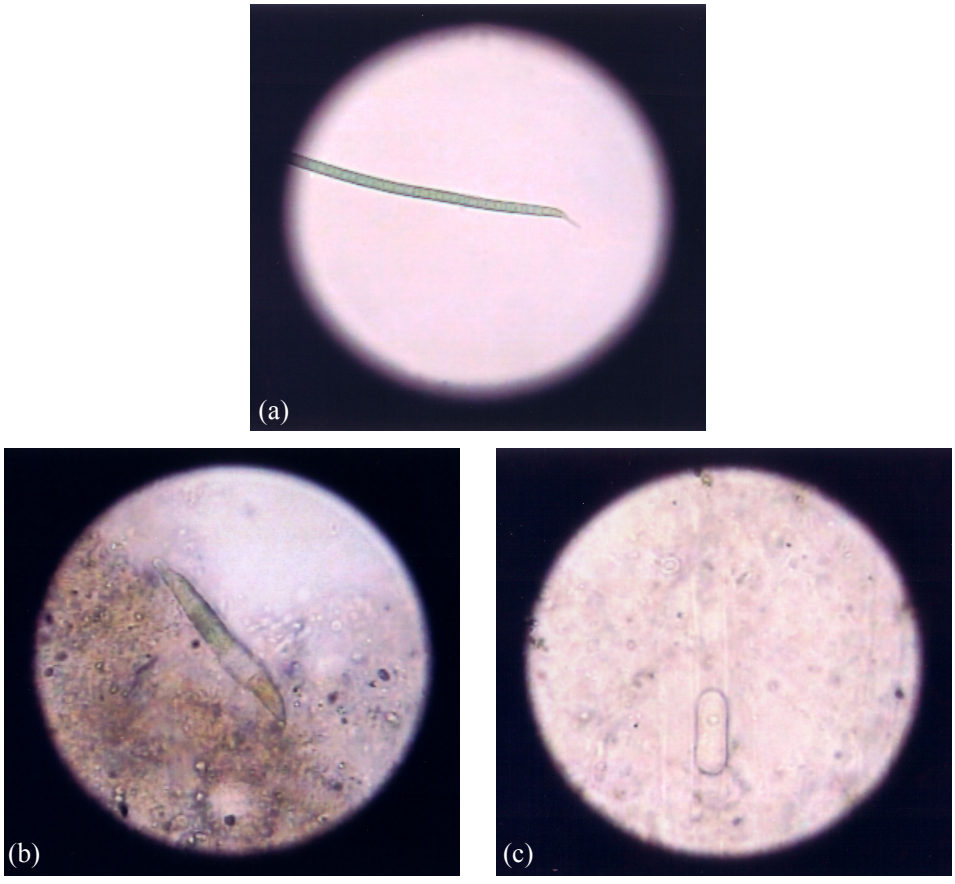

Fig. 3.2 Microphotograph of *O.acuminata* (a) control (x 400), (b) and (c) *O.acuminata* exposed to microbial consortium (x 400).

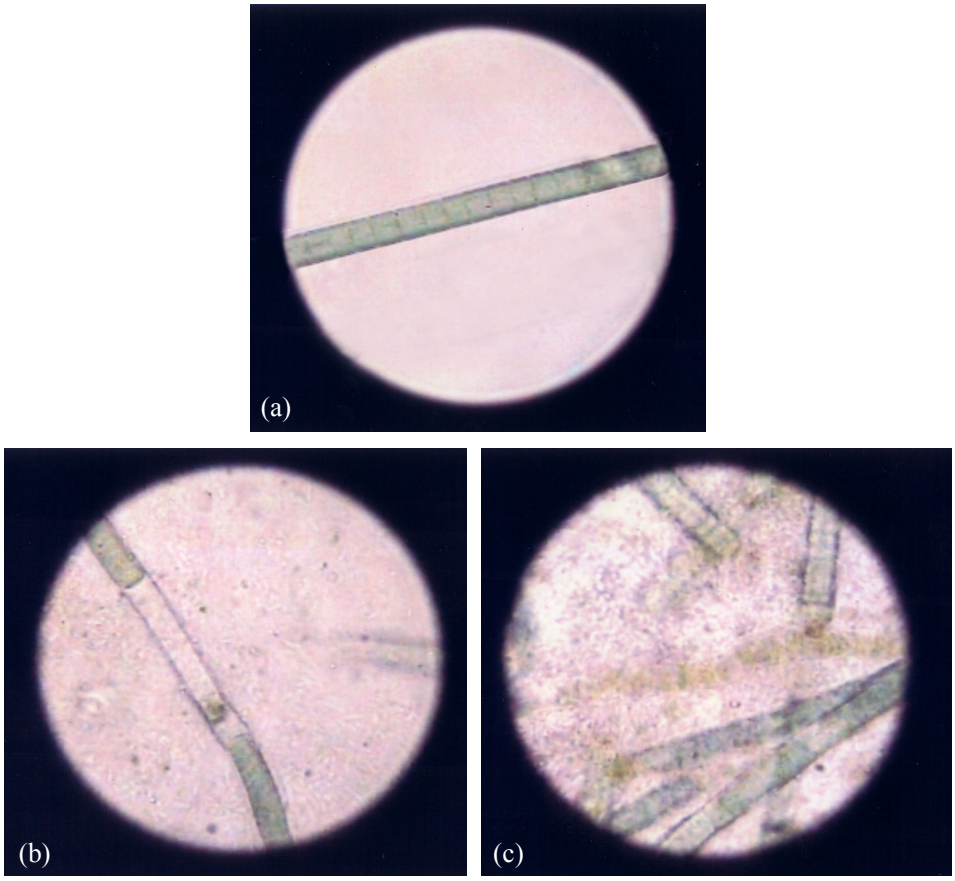

Fig 3.3 Microphotograph of *O.subbrevis* (a) control(x 400), (b) and (c) *O.subbrevis* to microbial consortium (x 400)

The visual and microscopic examination of all algal cultures revealed the presence of algicidal micro organisms in the enriched consortium. This assumption was confirmed by disc diffusion assay.

Disc diffusion assay

The microbial consortium was resolved into eighteen distinct CFUs. Upon disc diffusion assay, two of these CFUs exhibited algicidal activity (Table 3.1) as observed by plaque formation. Intense halo was observed in blue green lawns while green algal lawns developed moderate halo when exposed to CFU 1. The antagonistic activity of CFU 9 was comparatively weak, but uniform throughout all species tested.

Table 3.1 Visual observation of algae lytic activity based on PFU after 96 hour (based on comparative scale)

CFU	Visual observation of algae lytic activity					
	A	**B**	**C**	**D**	**E**	**F**
1	++	++	+++	+++	++	+++
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	+	+	+	+	+	+
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-

A = *C.pyrenoidosa*, B = *O. pusilla*, C = *O.acuminata*, D = *O.subbrevis*, E = *S. quadricauda*, F = *S.elongatus*. (+ Weak halo, ++ moderate halo and +++ intense halo)

3.3.2 Isolation of bacteria from PFU and its algicidal efficiency

The CFU 1 and CFU 9 of the experiment were purified further and algal diffusion assay was performed. The results confirmed algicidal effect of both

CFU 1 and CFU 9 on *S.elongatus*, *O.acuminata*, *C.pyrenoidosa* and in the logarithmic growth phase. The effect of CFU 1 was very pronounced when compared to the CFU 2 (Fig.3.4). In case of *C.pyrenoidosa* the algicidal effect was less than that on the other two cyanophycean members. Algal plates examined under the microscope revealed the complete destruction of cells in the halo zone. Depigmented and disintegrated cells were observed in the margins of the halo zone. Due to its pronounced algicidal efficiency CFU 1 was picked for identification and further experiments.

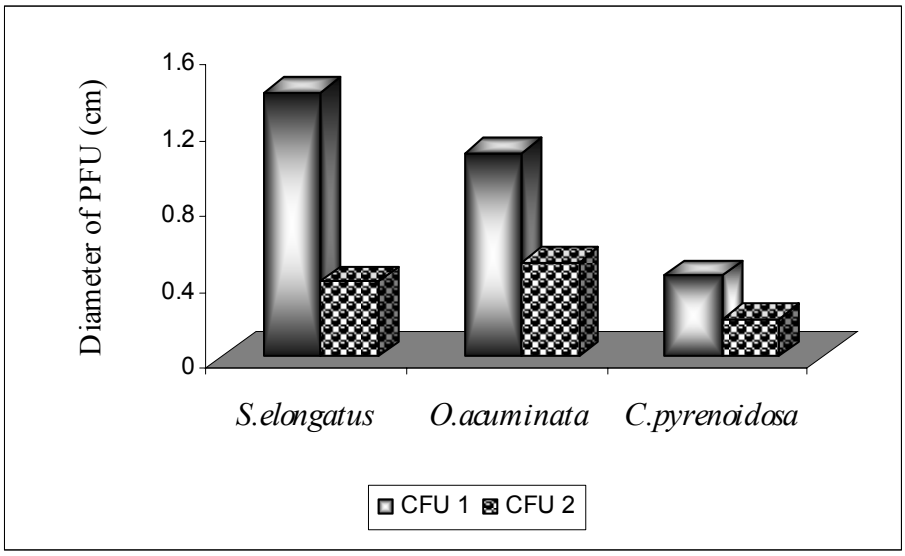

Fig. 3.4 Algicidal efficiency of two bacterial isolates

3.3.3 Identification of the algicidal bacterium

Based on the colony morphology and biochemical characteristics tabulated in Table 3.2 the isolate CFU 1 was identified as *Pseudomonas*. The isolate resembles *Pseudomonas*. The isolate is hereafter referred to as *Pseudomonas* SEB 8. The growth of *Pseudomonas* SEB 8 in nutrient and King'B medium was compared. It was seen that bacterium favoured King's B medium. So King's B medium was used for further experiments.

Table 3.2 Biochemical characteristics of isolated algicidal bacterium

Property	Result
Colony morphology	Translucent, green pigmented colony, margin serrate, elevation flat
Gram staining	Gram negative rods
Motility	Motile
Growth at 41^0	positive
Growth at 4^0	negative
Cytochrome oxidase	positive
Marine oxidative fermentation	Oxidative
Catalase	Positive
Arginine dihydrolase	positive
Lysine decarboxylase	negative
Ornithine decarboxylase	negative
Voges-Proskauer	Positive
Citrate utilization	Positive
NO_3 reduction	positive
Gelatin hydrolysis	positive
Starch hydrolysis	negative
Lipid hydrolysis	positive

3.3.4 Bacterial cell density and algicidal effect - Co-culture assay

The cell density of *S.elongatus* and *C.pyrenoidosa* declined at all bacterial inoculum concentration compared to the control. The control cell density reached 120 x 10^4 cells/mL in *S.elongatus,* and 250 x 10^4cells/mL in *C.pyrenoidosa* in 20 days but all the bacterial treated cultures of algae continued to be in the lag phase. There was no difference in the algal cell counts between the treatment densities of microbial inoculum (Fig 3.5 and Fig 3.6).

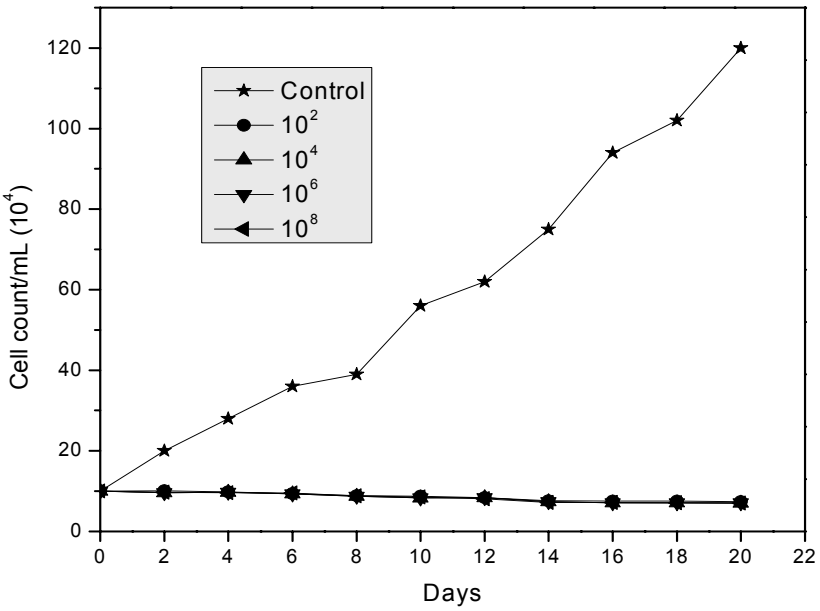

Fig. 3.5 Growth curve of *S.elongatus* co-cultured with *Pseudomonas* SEB 8 (density levels 10^2- 10^8)

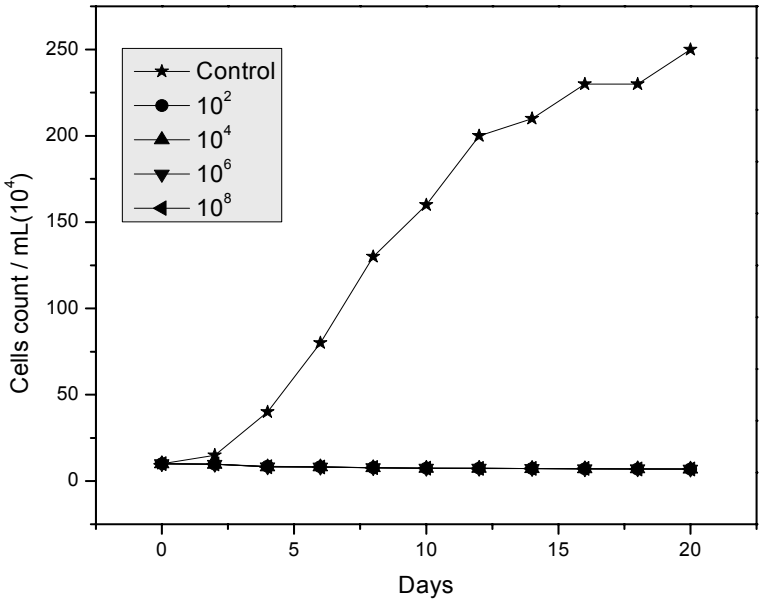

Fig.3.6 Growth curve of *C.pyrenoidosa* co-cultured with *Pseudomonas* SEB 8 (density levels 10^2- 10^8).

Similar results were observed with the biomass of *O.acuminata* (Fig 3.7). Algicidal activity was seen through out the period of observation.

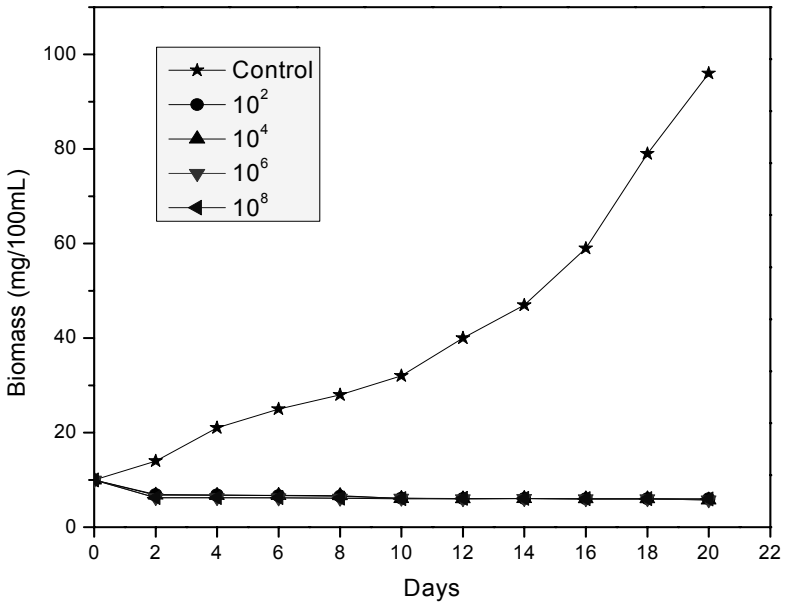

Fig. 3.7 Growth curve of *O.acuminata* co-cultured with *Pseudomonas* SEB 8 (density levels 10^2- 10^8)

3.3.5 Extractability and Algicidal efficiency of extra cellular metabolites

On extracting cell-free filtrate of *Pseudomonas* SEB 8 with five organic solvents and one mixture, algicidal activity was expressed in chloroform, chloroform : methanol and petroleum ether. There was no activity in ethyl acetate, hexane and methanol fractions assessed by disc diffusion assay in *S.elongatus*. The chloroform extract produced the most distinct and largest clear zone (Fig 3.8, 3.9).

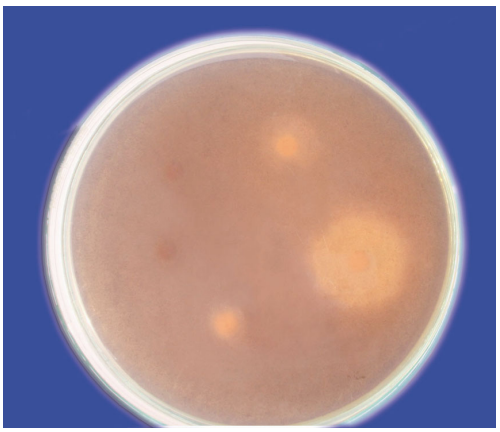

Fig 3.8 Disc diffusion assay in *S.elongatus* (chloroform extract)

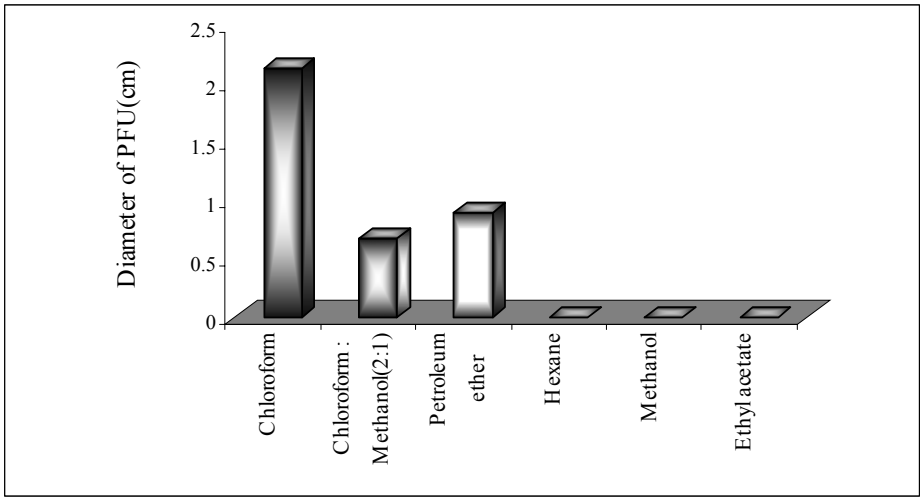

Fig 3.9 Algicidal efficiency of extra cellular metabolites in organic solvents expressed as diameter of plaque forming unit.

3.3.6 Comparison of algicidal activity of chloroform extract of *Pseudomonas* SEB 8

- Plaque forming units were formed within 24 h of the experiment in all Cyanophycean members, *S.elongatus, O.acuminata* and *O.subbrevis*. Maximum algicidal activity was observed with *O.acuminata* i.e. PFU diameter 3.2 cm, followed by *O.subbrevis* (2.8 cm) and in *S.elongatus* (1.48 cm) after 24 h. The filaments of *O.acumianta* and *O.subbrevis* were most susceptible (Fig. 3.10).
- None of Chlorophycean species was susceptible to chloroform extract in the first day, although algicidal activity was observed after 24 h. Maximum algicidal activity was observed with the green algae *O.pusilla*, which had a lysis zone of 1.5 cm diameter after 96 hours (Fig 3.11). The pattern of algicidal effectiveness was similar in *C.pyrenoidosa* and *S. quadricauda*. Algicidal efficiency in Chlorophycean members was lesser when compared to the Cyanophycean species tested.

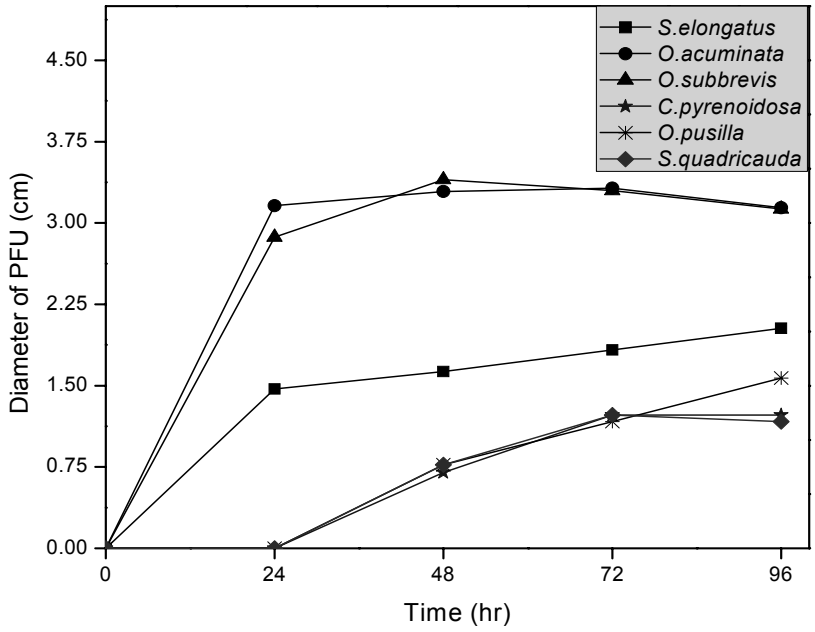

Fig.3.10 Comparison of algicidal efficiency of chloroform extract of *Pseudomonas* SEB 8

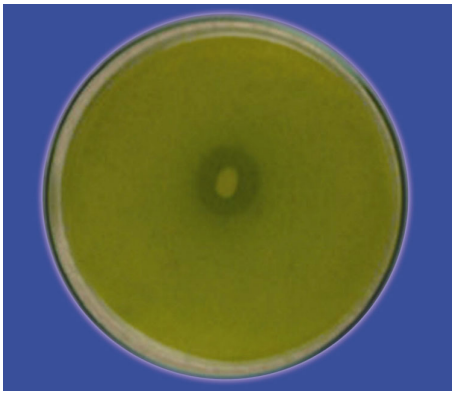

Fig 3.11 Disc diffusion assay in *O.pusilla*

3.3.7 The algal assay of chloroform extract

- **Range finding test on *S.elongatus***

The chloroform extract inhibited the growth of *S.elongatus*. There was about 77 % reduction of growth as measured by cell count of the cultures with the minimum concentration tested, i.e. 10 mg/L in the range finding test. The cell count ranged from 26.177 x 10^4 to 0.867 x 10^4 in the control and treatments (Table 3.3).

Table 3.3 Toxicity study on *S.elongatus* with *Pseudomonas* SEB 8 extract

Con(mg/L)	Cell count Cells/ mL	SD	% Inhibition
Control	26.177	1.218	0
10	6.133	0.611	77
100	0.377	0.108	99
500	0.800	0.200	97
1000	0.867	0.115	97

Definitive test (determination of EC_{50}) on *S.elongatus*

The concentrations of *Pseudomonas* SEB 8 extract chosen for definitive test (LC_{90}) were between 1 mg/L and 10 mg/L. F test carried out on cell count gave F value of 915.32 rejecting the null hypothesis that all test groups are equal.

Table 3.4 Results of definitive test on *S.elongatus* exposed to *Pseudomonas* SEB 8 extract

Summary statistics					
Gp	**Con (mg/L)**	**Cell count [b]**	**SD**	**% Inhibition**	**ANOVA**
1	Control	26.469	0.352		F = 915.32
2	1	13.757	0.080	48	Critical F value = 3.11
3	1.8	10.627	0.142	60	(0.05,5,12)
4	3.2	7.377	0.051	72	Since F > Critical F
5	5.6	7.377	0.051	72	REJECT Ho:All groups equal
	10	6.261	0.124	76	
Dunnets test		t stat	Sig		Dunnett table value = 2.50 (1 Tailed Value, P=0.05, df=12,5)
		91.426	*		
		113.937	*		
		137.311	*		
		137.311	*		
		145.335	*		
Tukey's		Gp	Group 6 5 4 3 2 1		*= significant difference (p=0.05) . = no significant difference Tukey value (6,12) = 4.75
		6	\		
		5	* \		
		4	* . \		
		3	* * * \		
		2	* * * * \		
		1	* * * * * \		

Gp = Group; Con = Concentration
b - Cell count/mL

Dunnets test had shown that all groups are significantly different from the control. The multiple comparisons done by Tukey method pointed out that the toxicity of the extract increases significantly with the dosage from 1 mg/L to 10 mg/L (Table 3.4). LC_{50} was found to be 0.89 mg/L

- **Definitive test (determination of EC_{90}) on *S.elongatus***

The concentrations of *Pseudomonas* SEB 8 extract chosen for definitive test (LC_{90}) were between 10 mg/L and 100 mg/L. There was successive growth reduction with increasing dosage. The highest dose tested, 100 mg/L, inhibited cell counts by 99 %. The test results were statistically significant (Table 3.5) and growth inhibition dose dependent. EC_{90} was found to be 24.82 mg/L.

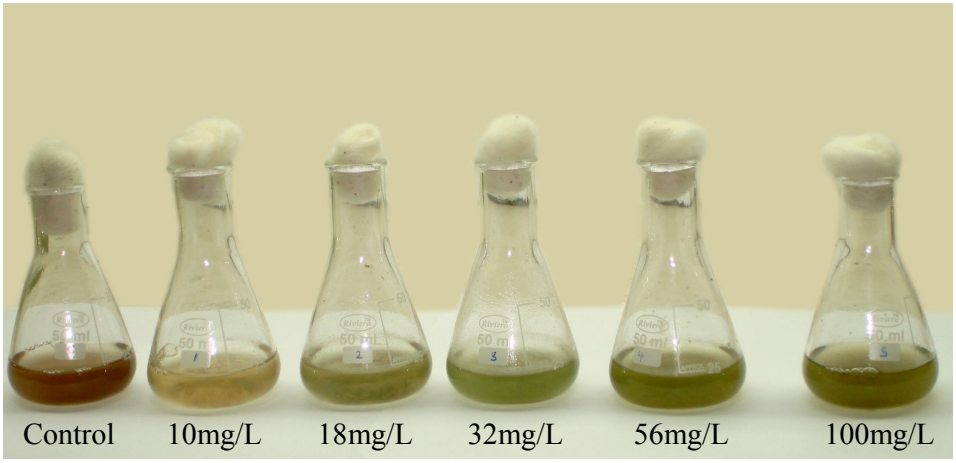

Fig 3.12 Definitive test of *S.elongatus* exposed to *Pseudomonas* SEB 8 chloroform extract

Table 3.5 Results of definitive test on *S.elongatus* exposed to *Pseudomonas* SEB 8 extract

Summary statistics					
Gp	**Con (mg/L)**	**Cell count**	**SD**	**% Inhibition**	**ANOVA**
1	Control	26.469	0.352	0	F= 10611.929
2	10	6.261	0.124	76	Critical F value = 3.11
3	18	5.193	0.163	80	(0.05,5,12)
4	32	1.347	0.059	95	Since F > Critical F
5	56	0.923	0.035	97	REJECT Ho: All groups
6	100	0.380	0.008	99	equal
Dunnets test		t stat		Sig	Dunnett table value = 2.50 (1 Tailed Value, P=0.05, df=12,5)
		147.907		*	
		155.722		*	
		183.877		*	
		186.975		*	
		190.952		*	
Tukey's		Gp		Group 6 5 4 3 2 1	* = significant difference (p=0.05) . = no significant difference Tukey value (6,12) = 4.75
		6		\	
		5		* \	
		4		* . \	
		3		* * * \	
		2		* * * * \	
		1		* * * * *\	

- **Range finding test of *C.pyrenoidosa* exposed to *Pseudomonas* extract**

C.pyrenoidosa on a similar experiment with chloroform extract gave 21.877 x 10^4 cells/mL in control cultures. Growth was inhibited in the test cultures at different degrees depending upon the test concentrations. There was 51 % inhibition of growth measured as cell count, at 10 mg/L. The highest dose tested, 1000 mg/L, inhibited cell density measured as cell count by 71 % (Table 3.6).

Table 3.6 Range finding test of *C.pyrenoidosa* exposed to *Pseudomonas* SEB 8 extract

Con (mg/L)	Cell count	SD	% Inhibition
Control	21.877	0.146	0
10	10.757	1.521	51
100	7.137	0.374	67
500	6.897	0.335	68
1000	6.247	0.631	71

- **Definitive test (determination of EC_{50}) on *C.pyrenoidosa***

In the definitive test involving algicidal chloroform extract at 10 mg/L to 100 mg/L suppressed the growth of the *C.pyrenoidosa*. The test dose for 50% growth inhibition (EC_{50}) was estimated to be 9.44 mg/L. ANOVA on cell density and Dunnets test showed that the test effects were significant. Tukey method of multiple comparisons showed that there was no significant variation among the dosages 10 mg/L, 18 mg/L and 32 mg/L (Table 3.7). As observed in the range finding test, the highest dose of 1000 mg/L produced only 71% reduction of growth. Hence, EC_{90} was not estimated.

Table 3.7 Results of definitive test on *C.pyrenoidosa* exposed to *Pseudomonas* SEB 8 extract

Summary statistics					
Gp	**Con (mg/L)**	**Cell count**	**SD**	**% Inhibition**	**ANOVA**
1	Control	21.877	0.146	0	
2	10	10.757	1.521	51	F= 167.678
3	18	10.143	0.631	54	Critical F value = 3.11
4	32	9.073	0.501	59	(0.05,5,12)
5	56	7.443	0.326	66	Since F > Critical F
6	100	7.137	0.374	67	REJECT Ho:All groups equal
Dunnets test		t stat	Sig		Dunnett table value = 2.50 (1 Tailed Value, P=0.05, df=12,5)
		18.568	*		
		19.592	*		
		21.379	*		
		24.100	*		
		24.612	*		
Tukey's		Gp	Group 6 5 4 3 2 1		* = significant difference (p=0.05) . = no significant difference Tukey value (6,12) = 4.75
		6	\		
		5	. \		
		4	. . \		
		3	* * . \		
		2	* * . . \		
		1	* * * * *\		

3.3.8 UV/Vis spectrum of *Pseudomonas* SEB 8 chloroform extract

Chloroform extract was scanned in UV/Visible range 200-700 nm. Nine peaks were observed in the range 200- 400 nm. A minor peak occurred at 700 nm (Fig. 3.13)

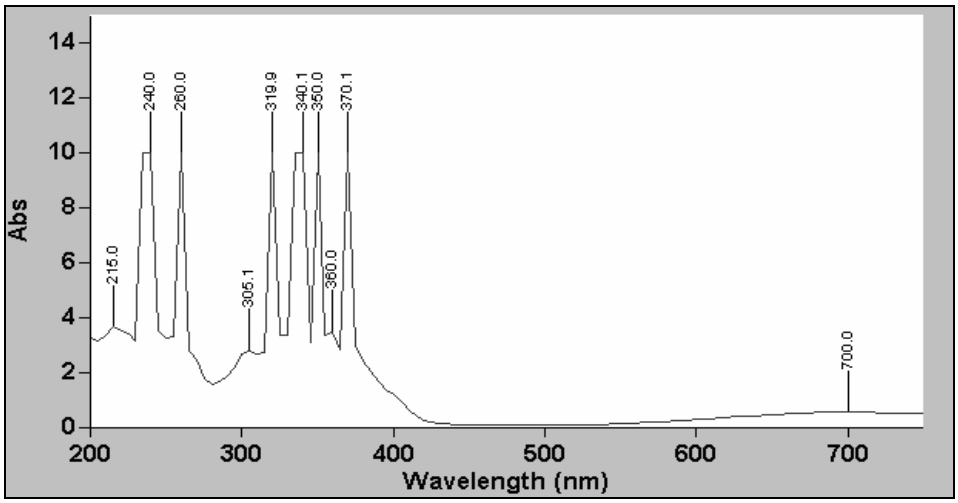

Fig 3.13 Chloroform extract of *Pseudomonas* culture supernatant

3.3.9 Fractionation of chloroform extract of *Pseudomonas* SEB 8

The solvent systems chloroform: methanol (1:1) and ethyl acetate: acetic acid: water (3: 2: 1) did not effect separation of fractions. The solvent mixture acetone: benzene: water (92: 30: 8) partitioned the extract into four pigment bands - copper blue, light purple, leaf green and yellowish green (Fig 3.14). Yellowish green fraction was found near the solvent front. Compound distance and R_f value is given table 3.8.

Table 3.8 Thin layer chromatographic separation of chloroform extract

Pigment band	Compound distance from the origin (cm)	R_f value
Copper blue	7.83	0.58
Light purple	9.27	0.69
Leaf green	10.73	0.80
Yellowish green	11.33	0.84

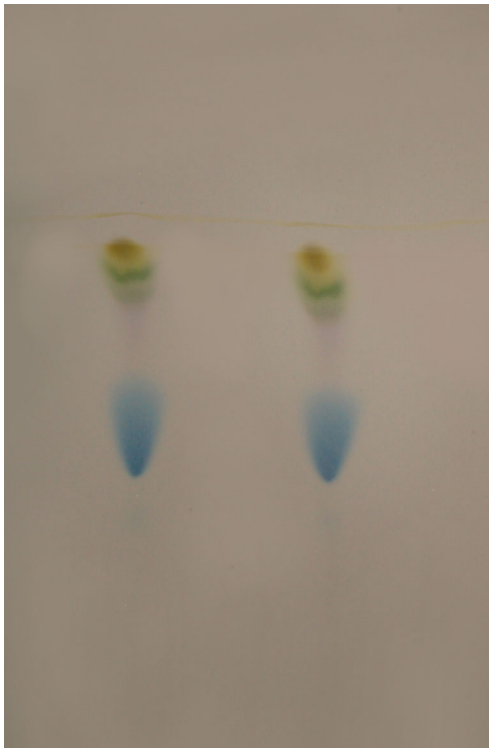

Fig 3.14 Thin layer chromatogram of *Pseudomonas* SEB 8 chloroform extract eluted in a solvent mixture of acetone: benzene: water mixture.

Each of the fractions was redissolved in ethanol and the absorption spectrum measured in UV/Vis spectrometer. Two peaks at 230 nm and 320 nm were observed in blue pigment (Fig. 3.15). Purple pigment had absorption peaks at 250 and 360 nm (Fig 3.16). One major peak at 245 nm and four minor peaks at 205, 230, 330 and 365 were there for the leaf green fraction. Four peaks were observed in yellowish green pigment at 220 nm, 245 nm, 260 nm and 365 nm (Fig 3.18).

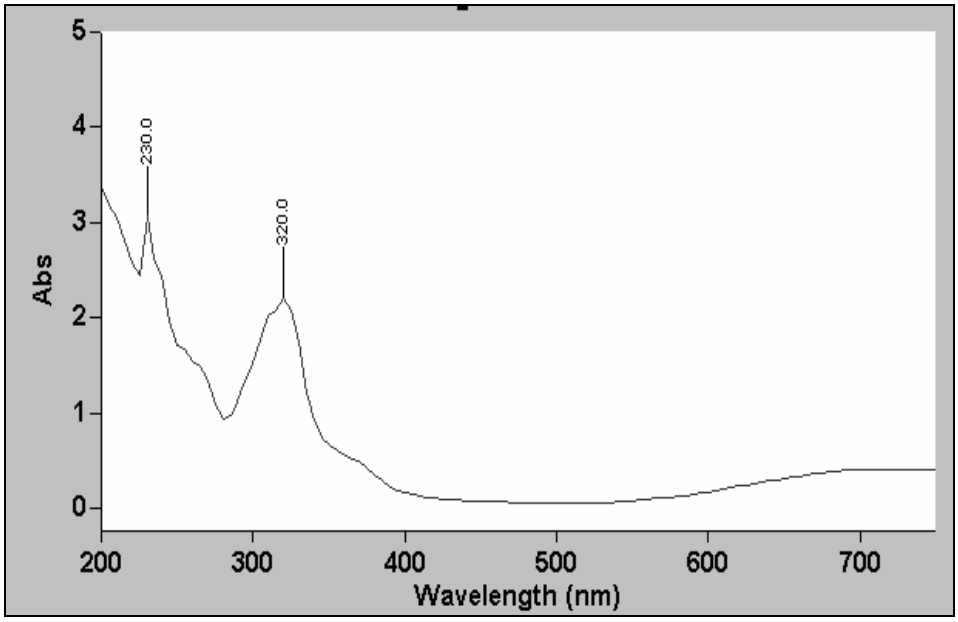

Fig 3.15 UV/Vis spectrum of blue coloured fraction

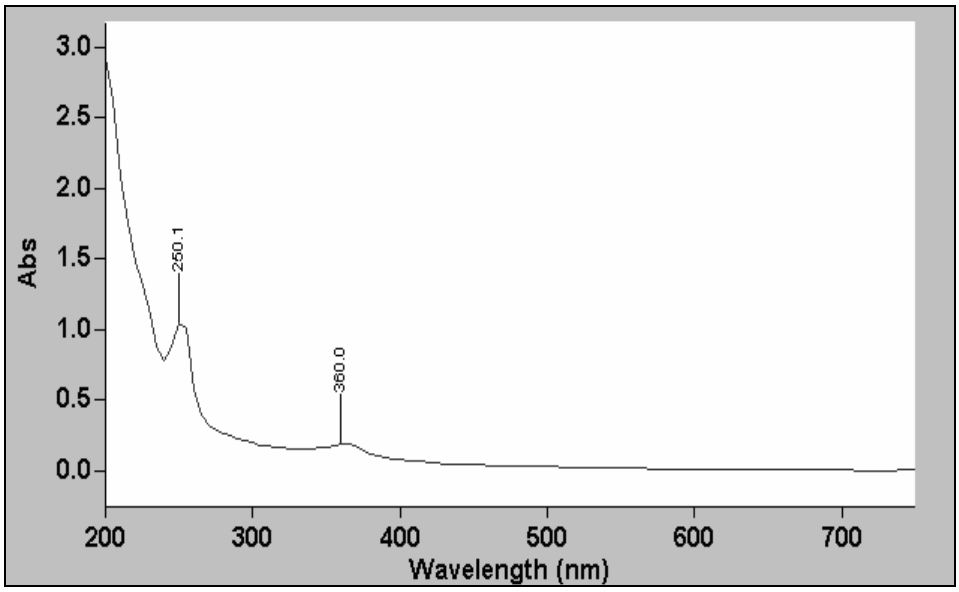

Fig 3.16 UV/Vis spectrum of purple pigment fraction

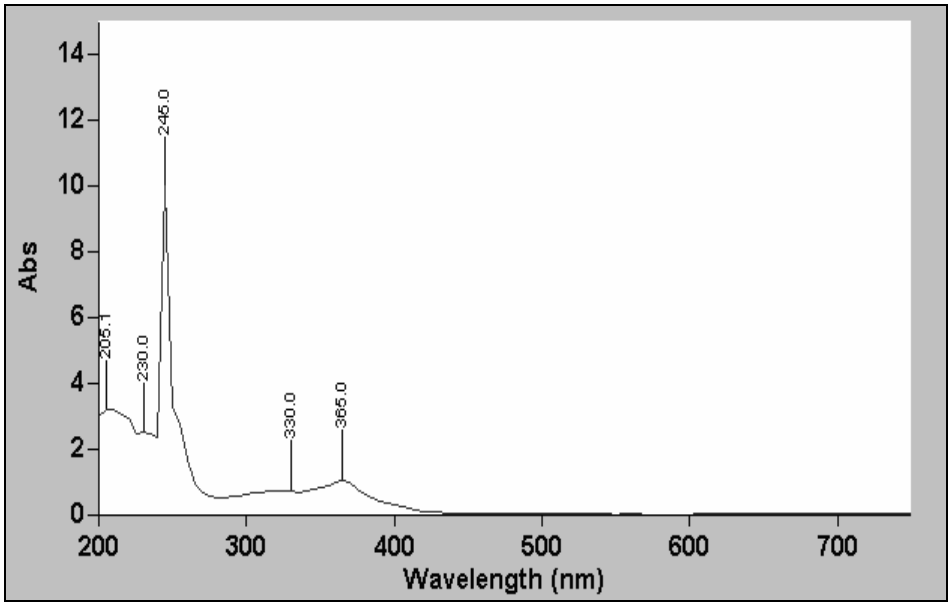

Fig 3.17 UV/Vis spectrum of green pigment fraction

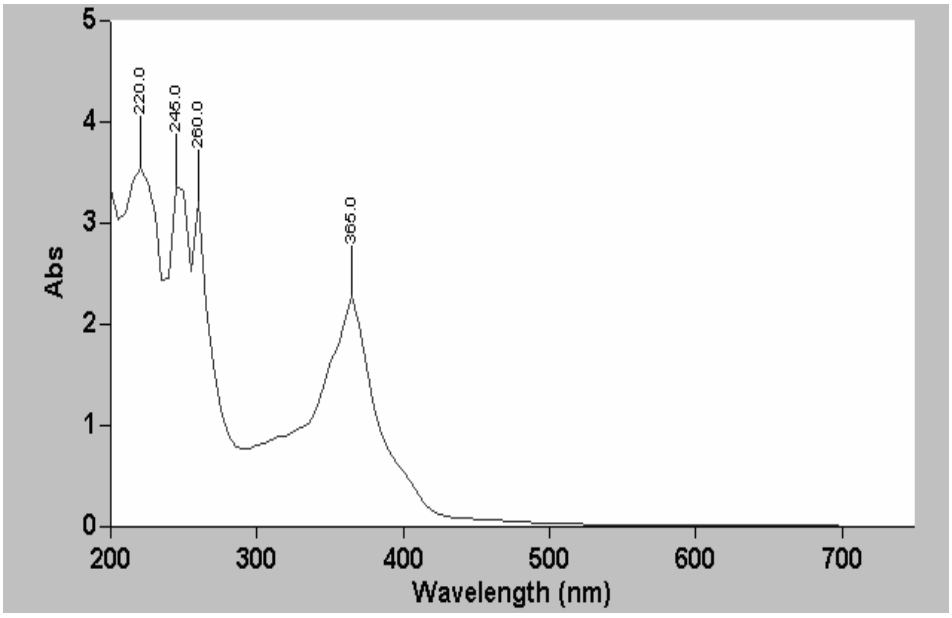

Fig 3.18 UV/Vis spectrum of yellowish green pigment fraction

3.3.10 Algicidal efficiency of separated fractions

There was no algicidal activity for copper blue, light purple and leaf green pigment fractions separated on the TLC. The yellowish green pigment separated near the solvent front showed strong algicidal activity in *S.elongatus* disc diffusion assay (Fig 3.19). The plaque was formed within 24 h and the halo was intense with a diameter of 3.5 cm.

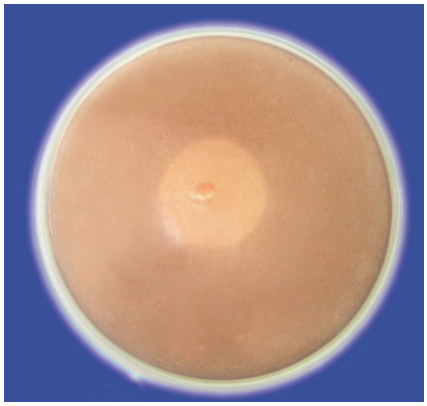

Fig.3.19 *S.elongatus* agar plates showing algicidal activity of yellowish green pigment.

3.4 Discussion

Bacteria associated with declining algal blooms play an important role in the fate of bloom. In this study bacteria associated with declining bloom were screened to check the effect on blue-green and green algae. Results of preliminary screening revealed the presence of algicidal activity and it was confirmed by rapid disc diffusion assay. Two CFU's were found to be algicidal in nature and its efficiency was compared. Organism with better algicidal efficiency was selected for the further study. Based on the morphology and biochemical characteristics the isolate is identified as *Pseudomonas*. A few strains of *Pseudomonas* are reported to have lytic activity against cyanobacteria (Yamamoto *et al*., 1993). Kodani *et al*. (2002) isolated nine algicidal *Pseudomonas* strains and selected *Pseudomonas* sp. K44-1 for its marked algicidal activity. *Pseudomonas putida*

was reported as algicidal bacteria especially against diatom *Stephanodiscus hantzschii* and blue green alga *Microcystis aeuriginosa* by Kang *et al*. (2007).

Study on the bacterial cell density on algicidal effectiveness depicts the low requirement of bacteria as inoculum, i.e. 10^2 cell /mL. In order to check whether bacteria is directly attacking algae or any secondary metabolites of algicidal nature are acting, bacterial filtrate was extracted with organic solvents such as chloroform, methanol, petroleum ether, ethyl acetate, hexane and a mixture of chloroform : methanol. Cell free filtrate of *Pseudomonas* extracts in chloroform, petroleum ether and chloroform : methanol mixture exhibited algicidal activity. The effect was more pronounced in chloroform extract. Methanol extract of *Pseudomonas* sp. K44-1 showed well marked algicidal activity as per the study of Kodani *et al*. (2002).

Host range of chloroform extract was studied in *S.elongatus*, *S.quadricauda, C.pyrenoidosa*, *O.accuminata, O.subbrevis* and *O.pusilla*. All algal species were susceptible to chloroform extract of *Pseudomonas*. Efficiency was less in chlorophycean members and it was effective only after 24 hours. Dakhama *et al.* (1993) reported that *Pseudomonas aeruginosa* strongly inhibited the growth of green microalgae and cyanobacteria. The EC_{50} and EC_{90} of chloroform extract were studied in *S.elongatus* and *C.pyrenoidosa*. The EC_{50} in *C.pyrenoidosa* (9.44 mg/L) is much higher than that of *S.elongatus,* being 0.88 mg/L. The highest concentration dosed gave an inhibitory effect only upto to 71% in *C.pyrenoidosa*. A 90% of the *S.elongatus* cells perished with the addition of 24.82 mg/L chloroform extract. Green algal species is reported to induce polysaccharide production to protect it against oxidative stress caused by toxins (Mohamed, 2008). Nature of the cell wall and defensive strategy such as induced polysaccharide production may be the reason for less inhibitory effect in green algal cells.

The concentrated chloroform extract was partitioned in a solvent mixture of acetone, benzene and water into four coloured pigment bands, copper blue, light

purple, leaf green and yellow green. Scanning was performed for each pigment partitioned in silica TLC plates by re-dissolving in ethanol. The algicidal fraction yellow green pigment had absorption at 220, 245, 260 and 365 nm. The peaks at 245 nm and 365nm coincided with the peak of leaf-green fraction. It may be assumed that the algicidal activity could be due to the compounds absorbing at 220 nm and 260 nm. . Dakhama *et al*. (1993) has stated that among phenazine pigments of *Pseudomonas* pyocyanine had no effect on algal growth whereas 1-hydroxyphenazine and oxychlororaphine showed strong antialgal activity. Pure crystals of both Phenazine -1-carboxylic acid (PCA) and 1- Hydroxyphenazine appear as yellow while pyocyanin is a blue coloured compound (Fernandez & Pizarro, 1997). The compound identified on TLC plate to have algicidal activity is yellow green in colour.

Based on the present observation and the existing literature available it may be assumed that the present compound could be of any phenazine or mixture of phenazine-1-carboxylic acid, 1 hydroxyphenazine and phenazine-1- carboxamide. The phenazines are a group of heterocyclic nitrogen-containing secondary metabolites synthesized by some strains of *Pseudomonas* spp. and a few other bacterial genera, most of which possess broad –spectrum antibiotic activity towards bacteria, fungi, plant and animal tissues. Phenazine-1-carboxylic acid (PCA), Pyocyanin (PYO), Phenazine-1- carboxamide or oxychlororaphine (PCN), 1-Hydroxyphenazine, 2-Hydroxyphenazine-1-carboxylic acid, 2 - Hydroxyphenazine, 1,6-Dihydroxy- 5,10-N-dioxide (iodinin) and D-Alanyl griseoluteic acid (AGA) are some common phenazines of bacterial origin(Mavrodi *et al*., 2002). Most phenazines produced by *Pseudomonas* spp. are simple carboxy- and hydroxyl substituted derivatives, but their physical properties, and consequently their antibiotic activity, differ according to the nature and position of substituents on the heterocyclic ring. Phenazine antibiotics synthesized by *Pseudomonas* spp. are products of a conserved pathway responsible for assembly of the tricyclic phenazine core to yield phenazine-1-carboxylic acid (PCA), the common precursor of strain-specific modifying

enzymes that introduce additional functional groups on the heterocyclic ring. These substituents are largely responsible for differences in the physical and chemical properties of the individual phenazines and hence, their biological activity. The ability of phenazines to undergo redox transformations resulting in the formation of reactive oxygen species and oxidative stress is an underlying theme in their interactions with other organisms (Mavrodi *et al*., 2006)

As revealed by this study these compounds may be useful for controlling algal blooms. In conclusion *Pseudomonas* sp. isolated in the present study may provide a better strategy to reduce/ control freshwater algal bloom. Further work is recommended to determine its application in lakes and reservoirs for water quality management.

The phenazine compound produced by the isolate of *Pseudomonas* SEB 8 has far reaching implications in the selective control of cyanobacterial blooms often develop in aquaculture ponds. In several aquacultural systems in India during the middle of the culture cyanobacteria multiply beyond control and elevate *pH* to above 10. The alkaline *pH* triggers a cascade of events including elevated ammonia toxicity, dissolution of phosphate and further blooming. After a period of time the bloom crashes leading to oxygen depletion, ammonia toxicity and death of the rearing stock. In this context the aquaculture sector looks for a biological agent which can be effectively used against cyanobacterial bloom. The phenazine compound detected is promising in the direction provided sufficient attempts are made to mass produce the compound. Another line of work can be initiated with this as the starting material.

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Chapter 4

Removal of Algae by Flocculation

Contents

4.1 Introduction

4.1.1 Algal Removal

Eutrophication related algal problems are gaining world wide attention in water treatment. The major algae-related problems in conventional water treatment are unpleasant tastes, odours, and filter clogging. Increased disinfection by-product (DBP) concentration, and microbial re-growth in distribution systems are other algae related problems. The production of toxins by some algae is also of concern in the drinking water industry (Gray, 2005).

Controlling or harvesting suspended algae is always a major bottleneck in water treatment and biomass recovery. Algal removal operations as function of treatment technique include micro straining, centrifugation, flocculation in combination with sedimentation or flotation, rapid/slow sand filtration, oxidation and direct filtration (Petrusevski, 1996). Over the years, these methods have been followed to remove algae with varying degrees of success. Significant shortcomings are still there for all existing methods. An associated problem with any type of filtration is fast filter clogging. There is chance of oxidation by-products which may harm living beings in case of oxidation process. Process optimization of operation is the hurdle in case of coagulation and related processes.

Based on the size, fresh water phytoplanktons are divided into four major groups (Lee, 2008). They are picoplankton (0.2 to 2 μm), nanoplankton (2 to 20 μm), microplankton (20 μm to 200 μm), and macroplankton (>200 μm). The smaller size of phytoplankton causes difficulty in water treatment as it passes through the filters and when present in large concentrations it rapidly clogs filters. Algal properties having influence on water treatment include size, shape, mobility, cell surface characteristics, ability to produce extracellular organic matter and low density associated with vacuole formation. Even some species of algae may show a significant different behaviour as a function of growth stage or difference in the composition of its

suspending aqueous medium (Petrusevski *et al.*, 1993). The characteristics of dominant species in the water body affect the efficiency of treatment mainly.

Mobility allows phytoplankton to be in a suitable environment by responding to external stimuli like gravitational, chemical and thermal gradients. Mobility may be of active (mucilage extrusion, cilia and flagella) and inactive (buoyancy) type (Sigee, 2005). Several types of algal motility may interfere with water treatment processes. Motile organisms have the ability to free themselves and move through the coagulated suspension.

Extracellular organic matter from algae may act as coagulant aid or may hinder coagulation depending on the species of algae and extracellular concentrations (Bernhardt & Calsen, 1991). Extracellular organic matter can be a threat to public health as a trihalomethane precursor (Petrusevski *et al.*, 1996).

Prior research has shown that algae are negatively charged (Bernhardt & Calsen, 1991). Negative algal surface charge hinders algal agglomeration and in case of filtration it inhibits algal adherence onto the filtering material (Petrusevski *et al.*, 1996). The zetapotential and charge density at a constant *p*Hvaried from genus and even varied between different species of the same genus (Ives, 1959).

4.1.2 Flocculation of suspended algae

Flocculation is reported to be cheap, simple, and volume independent method for concentrating algal cells when compared to other existing methods. Algal removal by coagulation and sedimentation can be economical options for the mitigation of filter clogging problems in conventional water treatment plants as it requires little or no capital investment. Conventional flocculants are mineral additives (ferric chloride, aluminium chloride, aluminium sulphate, poly aluminium chloride), or synthetic polymers (such as polyacrylamide). Numerous studies have evaluated the effectiveness of trivalent metal salts, inorganic polymers and organic polymers for algal

coagulation, (Ives,1959; Tenney *et al*,. 1969; Tilton *et al.,* 1972; Lubián, 1989; Jun *et al.*, 2001; Strand *et al.,* 2003; Yan & Jameson, 2004; Knuckey *et al*., 2006).

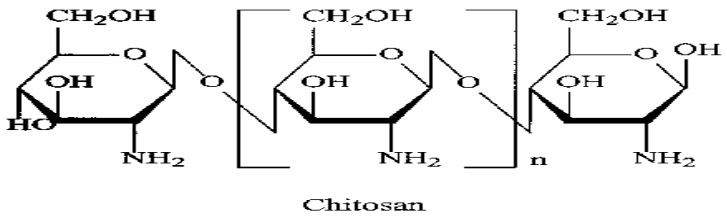
Multivalent metal salts are effective flocculants or coagulants. The commonly used salts include ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$) and ferric sulphate ($Fe_2(SO_4)_3$). Coagulation efficiency of metal ions increases with increasing ionic charge. Multivalent metal salts, such as alum, have been widely used to flocculate algal biomass in wastewater treatment processes (McGarry, 1970; Moraine *et al*., 1980; Lincoln, 1985). Alum is an effective flocculant for *S.quadricauda* and *Chlorella* (Golueke & Oswald,1965). However, flocculation by metal salts may be unacceptable if biomass is to be used in aquaculture and other related applications. Polyferric sulfate (PFS) is observed to be a better flocculant compared to the more traditional non-polymerized metal salt flocculants (Jiang *et al*., 1993). Flocculation with pre- polymerized metal salts is said to produce flocs that are easily dewatered and effective over a wider *p*H range than non-polymerized salts. An alternative to using metal salts is the use of cationic polymers (Tenney *et al.*, 1969).

Most of the conventional flocculants have several environmental consequences. An increase in aluminium concentration has been cited as a possible cause of Alzheimer's disease. Aluminium (Al) is clearly a powerful neurotoxicant. Flaten (2001) had reviewed on the epidemiological evidence linking aluminium and Alzheimer's disease and observed that nine out of thirteen published cases have statistically significant positive relations. Production of large volumes of sludge is a disposal problem in the case of alum as reported by Pan *et al*. (1999). Dispersion of acrylamide oligomers may cause health hazards (Roussy *et al.*, 2004). Page *et al*. (2003) depicts the presence of non coagulable organic matter especially polysaccharide compounds with a higher hydrophilic character after alum treatment, indicating that these components are refractory to alum treatment.

Cationic polymers doses between 1 mg/L and 10 mg/L can induce flocculation of freshwater algae; however, high salinity of the marine environment can inhibit flocculation by polyelectrolytes (Bilanovic *et al.*, 1988). Auto flocculation produced by modifying the culture medium, has been investigated for algal cultures in both fresh and seawater systems (Suh *et al*., 1997; Lee *et al*., 1998). The bacterium *Paenibacillus* sp. AM49 is known to produce a bioflocculant that has proved effective for harvesting *Chlorella vulgaris* (Hee-Mock *et al.*, 2001). Aqueous extract of dry seeds of *Moringa oleifera* act as effective coagulants or as coagulant aid for water and wastewater treatment (Ndabigengesere & Narasiah, 1998; Okuda *et al*., 2001; Katayon *et al.,* 2006; Bhatia *et al*., 2007; Kwaambwa & Maikokera*,* 2008). Other natural coagulants include extracts of *Prosopis juliflora* and *Cactus latifaria* (Diaz *et al*., 1999) and Okra and Nirmali seeds (Samawi & Shokralla, 1996).

Therefore, alternative flocculants have been considered for environmental application. Comparison among studies are difficult to make because of differences in model or natural water composition, algae type, strength of coagulant stock solution and the choice of coagulant *p*H An ideal flocculant should be inexpensive, nontoxic, and effective in low concentration. In addition, the flocculant should be selected so that further downstream processing is not adversely affected by its use. Bio- macromolecules may be of great interest since they are natural products characterized by their environment friendly behaviour. Among these biopolymers, chitosan may be considered as an emblematic material to be used for coagulation-flocculation.

Chitosan is a partially deacetylated polymer of acetyl glucosamine. Chitosan is prepared from chitin by alkaline de-N- acetylation. Chitin is found in a wide range of natural sources such as crustaceans, fungi, insects, annelids, molluscs, coelenterata etc. However, chitosan is manufactured only from crustaceans, primarily because large amount of crustacean exoskeleton is available as by- product from food processing industry.



Chitosan

Fig.4.1 Composition of Chitosan

The most important and significant developments in chitin and chitosan technology are in the medical and environmental applications. Chitosan is well known as a complexing agent for many metal ions (Guibal, 2004, Gerente *et al*., 2007), phenolic compounds (Popa *et al.*, 2000), natural and synthetic polyanions (Peniche & Arguelles-Monal, 2001; Guibal, 2004) and pesticides (Yoshizuka *et al*., 2000). It was previously reported that chitosan is a non-toxic biodegradable polymer with moderate bacteriostatic effect (Koide, 1998). All these properties make chitosan very attractive for applications in water treatment. Another benefit of chitosan application is a possibility to use chitosan containing sludge formed during water treatment in production of fertilizers or additives to animal feeding mixtures (Bratskaya *et al*., 2004).

The largest single use of chitosan is the clarification of waste and effluent water (Onsoyen & Skaugrud, 1990). Chitosan has been studied for use as a coagulant or flocculant for a wide variety of suspensions including the following: fish processing (Guerrero *et al*., 1998), food industry (Savant & Torres, 2000), silt in river water, (Divakaran & nd Pillai, 2002a), latex particles (Ashmore & Hearn, 2000), microorganisms (Strand *et al.,* 2002, Barany & Szepesszentgyfrgyi, 2004; Zou *et al.*, 2006) and mineral colloids (Roussy *et al*., 2004).

Chitosan is reported as algal flocculant by a few authors (Lavoie & Noue, 1983; Lubián, 1989; Divakaran & Pillai, 2002b; Grima *et al.*, 2003). Chitosan is required in low dosages in freshwater but its flocculating power is reduced in salt water. Optimum flocculation dose of chitosan varies greatly,

optimum flocculation of *Tetraselmis chui, Thalassiosira pseudonana* and *Isochrysis* sp. has been observed at a chitosan dosage of 40 mgL^{-1} and in contrast 150 mgL^{-1} was required for optimum flocculation of *Chaetoceros muellari.* Heasman *et al.* (2000) did not observe any consistent correlation between the taxonomic group of the algae and the quantity of chitosan needed for optimal flocculation. In view of the variable results reported it is attempted to verify the use of chitosan as flocculant in water treatment to meet water quality standards.

Objectives of the study

- To check the effectiveness of chitosan as an algal flocculant and compare it with conventional flocculant alum.
- To check the effect of algal *p*H, turbidity and species on flocculation by chitosan
- To compare biochemical composition of algae after centrifugation and flocculation as a concentration technique.
- To check whether chitosan has algistatic or algicidal property

4.2 Materials and methods

4.2.1 Development of algal cultures

- **Development of algal stock cultures**

Pure cultures of *C. pyrenoidosa*, *S. elongatus* and *S. quadricauda* were obtained from the culture collection of the School of Environmental Studies, CUSAT. The algae were grown in one litre Borosil culture flasks aseptically. *C. pyrenoidosa* and *S.quadricauda* were cultured in Ward and Parish medium (Ward & Parrish, 1982) and *S. elongatus* was cultured in BG 11 medium (Stainer *et al.*, 1971). Cultures were illuminated by a bank of day light fluorescent lamps to stimulate photosynthesis. This light source imparted an intensity of 1500 lux to the culture surface on a 12 h light and 12 h dark cycle.

- **Development of test cultures**

The stock cultures of the three species were inoculated into filtered tap water enriched with respective algal medium in 10 L glass tanks and illuminated as before. The inoculum size was controlled to yield cell densities in the range 10^3 to 10^6 cell/mL in a growth period of 10 days. These cultures were used for flocculation studies. A natural bloom of fresh water algae was induced by inoculating pond water sample to filtered water sample dosed with garden nutrient mix.

4.2.2 Flocculation of algae

- **Preparation of chitosan solution**

Chitosan used in this study was obtained from M/s South India Sea Foods, Kochi, Kerala, India. It was extracted from crustacean exoskeletons, had an average molecular weight of 180 kDa, and was 80% deacetylated. Different concentrations of chitosan were prepared by dissolving in 10 mL (0.1 N) HCL and and making up to 100 mL with distilled water.

- **Jar test**

The jar test method was used for the study of coagulation- flocculation. The well mixed algal cultures were taken in one litre beaker. The *p*H of the algal suspension was measured and adjusted to a fixed value (depending upon the experiments) using dilute hydrochloric acid solution (0.1 N). Then it was dosed with flocculant; flash mixed for 1 minute, flocculated at 40 rpm for 30 minutes, and settled for 40 minutes. All jar tests were performed at an ambient temperature of 30 ± 2^0 C. The performance of the flocculants was visually evaluated *in situ* for dose-response characteristics in respect to size of the floc, and by turbidity. The *p*H of the supernatant was determined after flocculation. Turbidity of algal suspension was measured using Nephelometer calibrated as per standard procedure (Standard methods, 1999). *p*H values of the suspensions were measured using digital *p*H meter. Flocculation was carried out on a six spindle multiple stirrer unit with stainless steel paddles.

All experiments were performed with three replicates and mean value was taken for quantification.

4.2.3 Comparison of flocculants

This experiment was designed to evaluate and compare flocculation efficiency of a conventional flocculant alum, and chitosan which has a GRAS (generally regarded as safe) status. Flocculation efficiency of both flocculants was tested in *S. elongatus* culture having turbidity range 20-25 NTU. The initial *p*H of the culture was 10.5. The ten -day old culture of *S. elongatus* was transferred to one litre beaker for jar test. The *p*H was adjusted to 7.0 by adding hydrochloric acid. One hundred mg chitosan powder was accurately weighed and dissolved in 100 mL distilled water to obtain a solution containing 1.0 mg/mL of solution. Alum was prepared by adding 1.0 g alum to 1000 mL distilled water. Alum was added to the cultures in the beaker at concentrations 20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L and 100 mg/L to determine the range of dose limits for effective flocculation. The flocculation efficiency was computed based on turbidity estimation of the supernatant. Turbidity was measured in nephelometer and expressed in NTU.

In a second set of experiment, chitosan was added to *S. elongatus* cultures at concentrations 5 mg/L, 10 mg/L, 20 mg/L, 40 mg/L, 60 mg/L, and 80 mg/L. Flocculation efficiency was determined as before. The results of the two sets of experiments were compared based on the flocculation efficiency and resultant *p*H.

4.2.4 Sludge volume index of algal cultures flocculated by chitosan and alum

The sludge volume index is the volume in milliliters occupied by 1 g of suspension after 30 minutes settling. *S. elongatus* with a turbidity of 20-25 NTU was dosed with chitosan at a concentration of 5 mg/L and flash mixed for 1 minute, flocculated at 40 rpm for 30 minutes. *S. elongatus*-floc suspension was gently stirred and it was then transferred to 1 L graduated

measuring cylinder and allowed to settle for 30 minutes. The volume occupied by the flocculated algal mass was measured. Suspended solids of the S. *elongatus*-floc suspension were determined by gravimetric method. A well-mixed suspension was filtered through a weighed glass-fiber filter and the residue retained on the filter was dried to a constant weight at 105°C. The increase in weight of the filter was expressed as the total suspended solids. The same experiment was repeated with alum as flocculant at dose of 20 mg/L. The sludge volume index was calculated as:

$$\text{SVI} = \frac{\text{settled sludge volume (mL/L)} \times 1000}{\text{Suspended solids (mg/ L)}}$$

Same experiment was repeated with *C.pyrenoidosa* and natural bloom of same turbidity and at a chitosan concentration of 10 mg/L and an alum dosage of 20 mg/L.

4.2.5 Standardisation of measurement parameter of algal flocculation

In order to find out better flocculation efficiency parameter *S. elongatus* was flocculated with chitosan at 5 mg/L. *C. pyrenoidosa* culture and Natural bloom were flocculated with chitosan at concentration of 10 mg/L by jar test experiment 4.2.2. The turbidity of algal cultures was 20- 25 NTU. The algal turbidity and chlorophyll *a* of supernatant of the *C.pyrenoidosa*, *S.elongatus* and natural bloom were measured before and after flocculation and compared. Chlorophyll *a* was extracted from the algal concentrate into 90 % aqueous acetone solution and the absorbance of the extract was determined with a spectrophotometer. The concentration of chlorophyll *a* was computed using the trichromatic equation (APHA, 1999)

$$\text{Chlorophyll } a = 11.85(\text{OD664}) - 1.54(\text{OD647}) - 0.08(\text{OD630})$$

4.2.6 Effect of *p*H on flocculation by chitosan

The test cultures of *C. pyrenoidosa* and *S. elongatus* having turbidity range 20 – 25 NTU and with *p*H 9.8 were transferred to jar test beakers.

*p*H was adjusted at three levels i.e. 7, 8 and 9. Chitosan was added at 5 mg/L, 10 mg/L, 20 mg/L, 40 mg/L, 60 mg/L and 80 mg/L at each *p*H level. The flocculation efficiency was estimated based on turbidity measurements.

The experiment was repeated under post *p*H correction i.e. the test cultures were inoculated with chitosan at 5 mg/L, 10 mg/L, 20 mg/L, 40 mg/L, 60 mg/L and 80 mg/L, well mixed and the *p*H was adjusted to 7, 8 and 9. The effect of *p*H adjustment prior to, and after addition of chitosan was analysed. The results were analysed by two way analysis of variance to elucidate the interference of *p*H with chitosan flocculation efficiency.

4.2.7 Effect of turbidity and algal species

Test cultures of *S. elongatus* and *C. pyrenoidosa* and natural bloom at turbidity levels of 20 - 25 NTU, and 30 - 35 NTU were taken in beakers. The *p*H was adjusted to 7. Chitosan was added to each at concentrations 5 and 10 mg/L. The jar test was performed and flocculation efficiency was determined as before.

4.2.8 Biochemical composition of flocculated and centrifuged algal biomass

Algal cultures of *C.pyrenoidosa*, *S.elongatus* and *S.quadricauda* in exponential growth phase were harvested by flocculation with chitosan and by centrifugation. Harvested biomass was then dried at 60^0C in hot air oven. Dried sample was then powdered in glass mortar and kept in desiccator over silica gel.

- **Determination of total carbohydrate by anthrone method**

Fifty mg of the dried algal biomass was weighed out into a clean glass tube and hydrolyzed by keeping it in a boiling water bath for 3 hours with 5 mL of 2.5 N HCl and cooled to room temperature. It was neutralised with solid Na_2CO_3 until the effervescence ceased. The supernatant was collected by centrifuging and solution was made up to 10 mL. Three different samples were treated in the same manner. Four mL of anthrone reagent were added to 1 mL

of the supernatant, then heated for 8 minutes in a boiling water bath, cooled rapidly and read the green colour at 630 nm in spectrophotometer (Hedge and Hofreiter, 1962). The amount of total carbohydrate present in the samples was calculated and expressed as mg/100 mg of the sample.

- **Determination of total protein by organic nitrogen estimation**

Total organic nitrogen of dried algal biomass was determined in CHNS analyser. The determination in CHNS was based on Isotope Ratio Mass spectroscopy (IRMS). Quantitative combustion was carried out by oxygen jet injection directly at the sample. Exactly 5 mg of the algal sample were fed to the digestion chamber of the instrument. The gases were pre-separated in the elemental analyzer and injected into mass spectrometer by continuous flow procedure. Digestion temperature was kept at 950^0C. Injection of reference gases was also performed automatically. Total protein content of the algal sample was calculated by multiplying total organic nitrogen by a factor 6.25 (Jones, 1931).

- **Estimation of lipid by sulphophosphovanillin method**

A sample of five hundred mg of dried algal biomass was taken in a homogeniser and added 10 mL of Chloroform- Methanol mixture (2:1) and mixed well. The homogenate was filtered through Whatman No: 1 filter paper, added 2mL of 0.9% Nacl solution and shaken well. Each of this was transferred to a small separating funnel and allowed to stand overnight at 4^0. The lower phase of the biphasic layer, which contains all the lipids, was removed and the volume was adjusted to 10 mL by the addition of chloroform. A 0.5 mL sample of the above extract was measured into clean test tube and allowed to dry in a vacuum desiccator over silica gel. Then added 0.5 mL of concentrated H_2SO_4 and mixed well. After plugging with non absorbent cotton it was heated in a boiling water bath for 10 minutes, and then cooled to room temperature. A 0.2 mL sample of the acid digest was taken in a separate clean glass tube and added 5 mL of Vanillin reagent (0.1 gm vanillin dissolved

in 50 mL 80% Orthophosphoric acid). The mixture was mixed well in a cyclomixer and allowed to stand for 30 minutes. The absorbance was measured at 520 nm (Barnes and Blackstock,1973). Concentration of total lipid is expressed as mg/100 mg of the sample.

4.2.9 Chitosan- algal assay

- **Plate assay**

Algal lawn of *C.pyrenoidosa*, *S.elongatus* and *S.quadricauda* was prepared by double layer method. Sterile Petri plates were laid with a lower layer of algal medium with 2 % agar. An upper layer algal medium with 1.2% agar incorporated with desired algae was prepared by adding concentrated algal biomass into molten algal medium. Preparations were incubated at room temperature, under the light intensity of 1500 lux with 12h light: 12h dark photo-cycle for 2 days. Different concentrations of chitosan were prepared by dissolving in 10 mL 0.1 N HCl and making up to 100 mL with distilled water. After mixing properly, a 20 µL sample of the Chitosan solution was impregnated on sterile filter paper disc and allowed to dry for 30 minutes. The process was repeated to get 40 µL extract impregnated per disc and dried in vacuum desiccator for 24 hours. Control discs were kept with distilled water alone. The impregnated discs were placed on the algal lawn. The algal plates were examined daily for 5 days, and any yellowing, or clearing zone in the treated lawns compared to control.

- **Liquid culture assay**

The algal cultures of *C.pyrenoidosa*, *S.elongatus* and *S.quadricauda* were inoculated at a cell density of 10^4 cells to respective algal medium in borosilicate culture flasks, in triplicate and amended with chitosan solution at 2.5 mg/L, 5 mg/L, 10 mg/L, 20 mg/L and 40 mg/L concentrations. Cultures were illuminated by a bank of day light fluorescent lamps of an intensity of 1500 lux to the culture surface on a 12 h light and 12 h dark cycle. At the end of 96 hour incubation (12/12 light cycle) total algal cell count was enumerated

in a haemocytometer. Duplicate control cultures in respective algal medium were also kept.

4.3 Results

4.3.1 Comparison of flocculants

Alum effected 83 % to 100 % flocculation of *S. elongatus* in the range 20 mg/L -100 mg/L. The highest settling of algae occurred at 100 mg/L giving a clear supernatant. The *p*H of the supernatant was 5.8. The flocculation efficiency is likely to decline at < 20 mg/L as per the trend observed (Fig. 4.2). In the range 20 to 100 mg/L, alum treatment reduced the *p*H of the supernatant medium to 5.5-6.0, which is not advisable in water quality management. Chitosan effected 99 % flocculation at 5 mg/L which successively decreased as the concentration increased (fig 4.2). The *p*H remained nearly unchanged at $\underline{\sim}$ 7 following treatment.

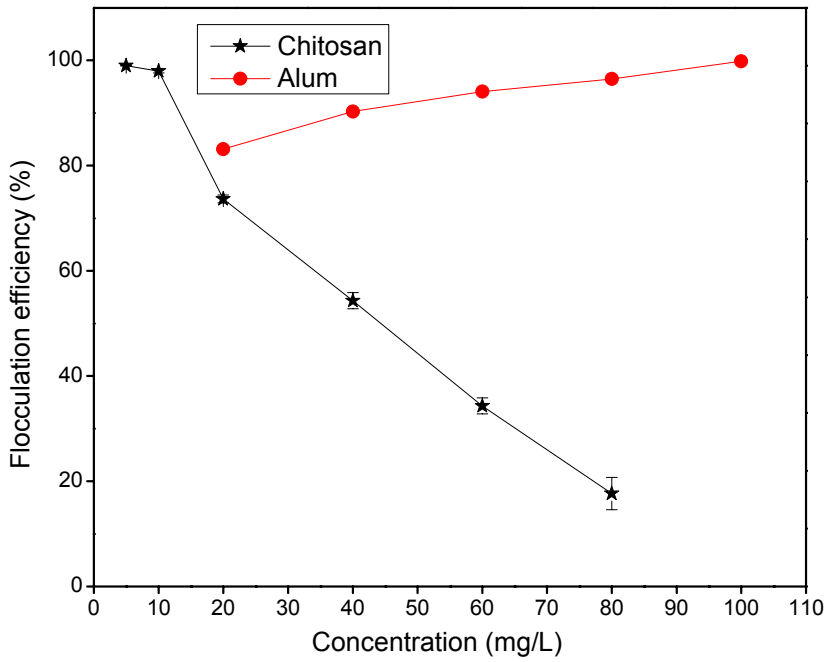


Fig.4.2 Flocculation efficiency of *S.elongatus* (algal turbidity 20-25NTU) by Alum and chitosan treatment.

4.3.2 Sludge volume index (SVI) of algal biomass flocculated by chitosan and alum

Table 4.1 comparison of sludge volume index between chitosan and alum in three different algae

Algal Species	Sludge Volume Index (mL/L)	
	Chitosan	Alum
S.elongatus	60.38 ± 0.17	132.5 ± 2.95
C.pyrenoidosa	72.33 ± 0.35	124.03 ± 1.40
S.quadricauda	86.46 ± 0.45	155.73 ± 0.90

The results of SVI are expressed in the Table 4.1. SVI for algal species flocculated with chitosan falling in the range 60 mL/L -87 mL/L. The range was high with alum-algae sludge (124 mL/L - 156 mL/L).

4.3.3 Comparison of measurement parameters of algal flocculation

Flocculation efficiency of chitosan as flocculant was checked by two parameters-turbidity and chlorophyll *a* of the supernatant. The results are expressed in Table 4.2. The flocculation efficiency based on turbidity and chlorophyll *a* of both, the pure cultures *S.elongatus* and *C.pyrenoida* and natural bloom, were nearly same. Therefore, turbidity was taken as measurement parameter for further experiments.

Table 4.2 Comparison of filtrate turbidity and Chlorophyll *a* following chitosan flocculation

Algal Species	Flocculation Efficiency (%)	
	Filtrate Turbidity	Chlorophyll*a*
S. elongatus	99.2 ± 0.10	99.1 ± 0.05
C.pyrenoidosa	98.1 ± 0.06	98.1 ± 0.08
Natural bloom	96.47 ± 0.15	96.2 ± 0.05

4.3.4 Effect of *p*H on flocculation by chitosan

Flocculation efficiency of chitosan in *C.pyrenoidosa* and *S.elongatus* was *p*H dependent. In case of *C.pyrenoidosa* it can be observed that the most effective flocculation with minimum chitosan requirement i.e. 10 mg/L was obtained in neutral *p*H (Fig. 4.3) At *p*H 8 and 9 maximum efficiency was obtained with 20 mg/L. At all *p*H levels flocculation efficiency decreased as the concentration increased beyond 20 mg/L.

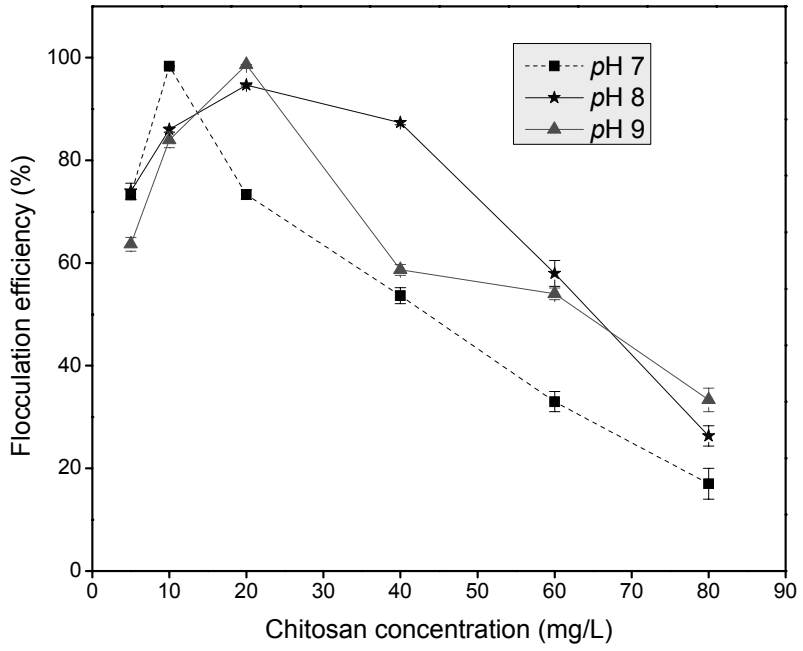


Fig.4.3 Flocculation efficiency of Chitosan at 7, 8 & 9 *p*H (pre-treatment *p*H correction) in *C.pyrenoidosa* (Algal Turbidity 20-25 NTU)

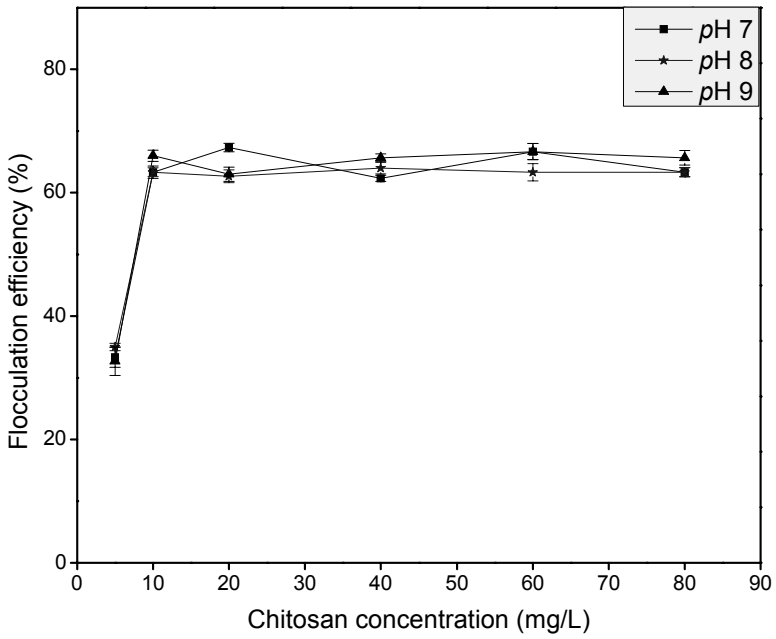


Fig. 4.4 Flocculation efficiency of Chitosan at 7, 8 & 9 *p*H (post *p*H correction) in *C.pyrenoidosa* (Algal Turbidity 20-25 NTU)

Effect of *p*H on flocculation efficiency in *C.pyrenoidosa*, was found to be significant as $p < 0.005$ ($p = 1.36 \times 10^{-23}$). Similarly, effect of chitosan concentration on flocculation efficiency was also statistically different ($p = 3.32 \times 10^{-44}$) Combined effect of *p*H and chitosan concentration on flocculation efficiency also have a p value of 1.95×10^{-26}, thus rejecting the hypothesis - all groups are equal (Table 4.3)

Table 4.3 ANOVA on data of flocculation Efficiency (20-25 algal turbidity) at different chitosan concentration and *p*H in *C.pyrenoidosa*

Source of Variation	SS	df	MS	F	F crit	P-value
*p*H	1489.371	2	744.6857	317.4128	3.259446	1.36E-23
Chitosan	27598.07	5	5519.614	2352.665	2.477169	3.32E-44
Interaction	3609.881	10	360.9881	153.8666	2.106054	1.95E-26

Post *p*H correction (i.e., *p*H correction after chitosan addition) could effect flocculation up to 67 % only in all *p*H levels tested (Fig 4.4). The flocculation efficiency trend was similar for three *p*H levels tested. Flocculation efficiency increased up to a concentration of 10 mg/L, then the efficiency remained unchanged up to the highest concentration tested i.e. 80 mg/L. Statistical interaction between *p*H variation and concentration variation was provided by statistical analysis (Table 4.4). ANOVA gave *p* value of 1.54×10^{-6} indicating a significant difference between *p*H and concentration interaction. The significant difference among *p*H ($p < 0.005$) was evident as given in table 4.4.

Table 4.4 ANOVA on data of flocculation Efficiency (20-25 algal turbidity) at different chitosan concentration and *p*H in *C.pyrenoidosa* with post *p*H correction

Source of Variation	SS	df	MS	F	F crit	P-value
*p*H	14.79788	2	7.398941	5.782213	3.259446	0.006643
Chitosan	7092.87	5	1418.574	1108.604	2.477169	2.37E-38
Interaction	100.8335	10	10.08335	7.880057	2.106054	1.54E-06

S.elongatus required only 5 mg/L chitosan for 99 % flocculation efficiency at *p*H 7. Chitosan concentration of 10 mg/L was required at *p*H 8 and at *p*H 9, 20 mg/L were needed for maximum flocculation efficiency (Fig 4.5). A decreasing trend of similar fashion with increasing concentration was observed in *S.elongatus* as in *C.pyrenoidosa*.

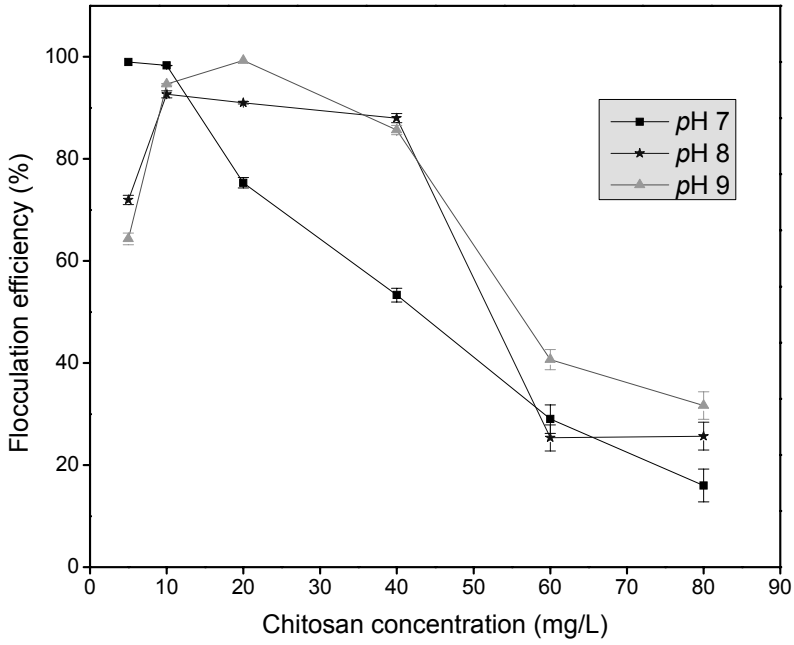


Fig 4.5 Flocculation efficiency (%) of chitosan at different *p*H (pre-treatment *p*H correction) in *S. elongatus* culture (Algal turbidity 20-25 NTU)

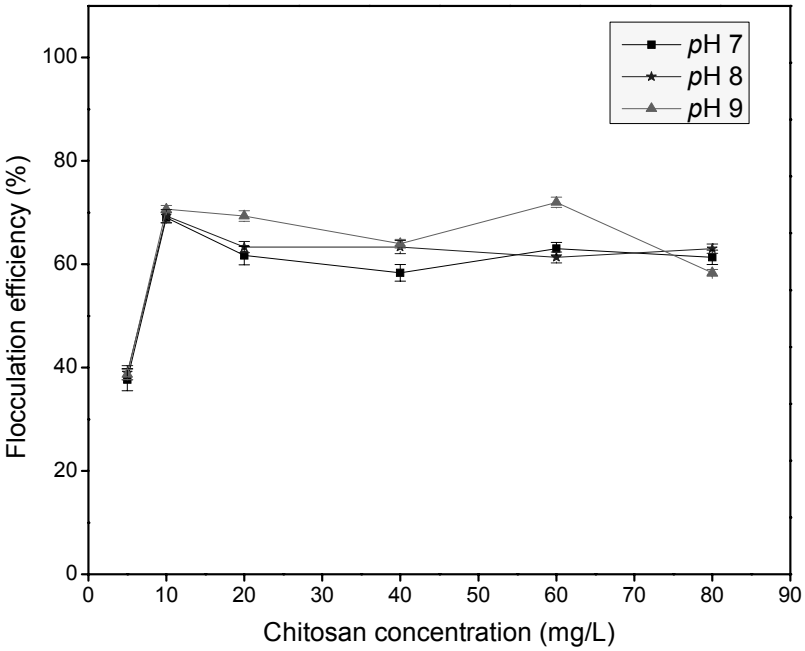


Fig 4.6 Flocculation efficiency (%) of chitosan with post *p*H correction in *S. elongatus bloom* (Algal turbidity 20-25 NTU)

The significant interaction between Chitosan concentration and *p*H in flocculation efficiency was provided by statistical analysis- Two factor ANOVA, i.e. a probability value 3.7 x 10^{-28}, rejecting the null hypothesis that there is no interaction between *p*H and concentrations (Table 4.5).

Table 4.5 ANOVA on data of flocculation Efficiency (20-25 algal turbidity) at different chitosan concentration and *p*H in *S.elongatus*

Source of Variation	SS	df	MS	F	F crit	P-value
*p*H	509.3472	2	254.6736	90.58622	3.259446	8.93E-15
Concentration	40626.34	5	8125.269	2890.121	2.477169	8.27E-46
Interaction	5422.531	10	542.2531	192.8769	2.106054	3.7E-28

In 'post *p*H correction' experiments maximum flocculation efficiency obtained was up to 66 % (Fig 4.6). ANOVA on data of flocculation efficiency at different Chitosan concentration and *p*H showed significant interaction with a probability value of 4.58 x 10^{-11} (Table 4.6).

Table 4.6 ANOVA on data of flocculation Efficiency (20-25 algal turbidity) at different chitosan concentration and *p*H in *S.elongatus* with post *p*H correction

Source of Variation	SS	df	MS	F	F crit	P-value
*p*H	123.9982	2	61.99912	40.60004	3.259446	5.93E-10
Concentration	5525.511	5	1105.102	723.6746	2.477169	4.78E-35
Interaction	270.4374	10	27.04374	17.70956	2.106054	4.58E-11

4.3.5 Effect of turbidity and algal species

The effect of algal turbidity on flocculation was compared at 5 mg/L and 10 mg/L chitosan concentration in three algal species. The results were compared using t- test. At low turbidity (20-25 NTU) the culture of *C.pyrenoidosa* and natural bloom behaved similar i.e. the flocculation

increased with concentration of chitosan. The flocculation efficiency of *S.elongatus* was similar at 5 and 10 mg/L chitosan. At high cell density (turbidity 30-35 NTU) overall flocculation efficiency decreased at respective chitosan concentration (Table 4.7, 4.8, 4.9).

Table 4.7 Turbidity related flocculation efficiency (%) of chitosan in *C. pyrenoidosa*

Chitosan (mg/L)	Turbidity	mean	t stat	t critical	Probability
5	20-25 NTU	75.33± 1.53	2.287	2.132	0.0421
	30-35 NTU	72.73±1.27			
10	20-25 NTU	98.33± 0.31	18.862	2.132	2.3266E-05
	30-35 NTU	83.41±1.29			

Table 4.8 Turbidity related flocculation efficiency (%) of chitosan in *S.elongatus*

Chitosan (mg/L)	Turbidity	mean	t stat	t critical	Probability
5	20-25 NTU	99.29±0.05	40.901	2.132	1.0677E-06
	30-35 NTU	74.82±1.01			
10	20-25 NTU	98.36±0.34	14.331	2.132	6.8879E-05
	30-35 NTU	93.37±0.41			

Table 4.9 Turbidity related flocculation efficiency (%) of chitosan in Natural bloom

Chitosan (mg/L)	Turbidity	mean	t stat	t critical	Probability
5	20-25 NTU	74.82±1.01	9.270	2.132	0.00038
	30-35 NTU	66.24± 1.10			
10	20-25 NTU	93.44±0.41	17.612	2.132	2.3266E-05
	30-35 NTU	85.76± 0.70			

Table 4.10 Differential response of the species to flocculation efficiency (turbidity 20 – 25)

Source of Variation	SS	df	MS	F	F crit	P-value
Between Groups	162.613	2	81.307	116.919	5.143	1.57E-05
Within Groups	4.172	6	0.695			
Total	166.786	8				

Table 4.11 Differential response of the species to flocculation efficiency (turbidity 30 – 35)

Source of Variation	SS	df	MS	F	F crit	P-value
Between Groups	46.822	2	23.411	95.485	5.143	2.83 E-05
Within Groups	1.471	6	0.245			
Total	48.293	8				

4.3.6 Biochemical composition of flocculated and centrifuged algal biomass

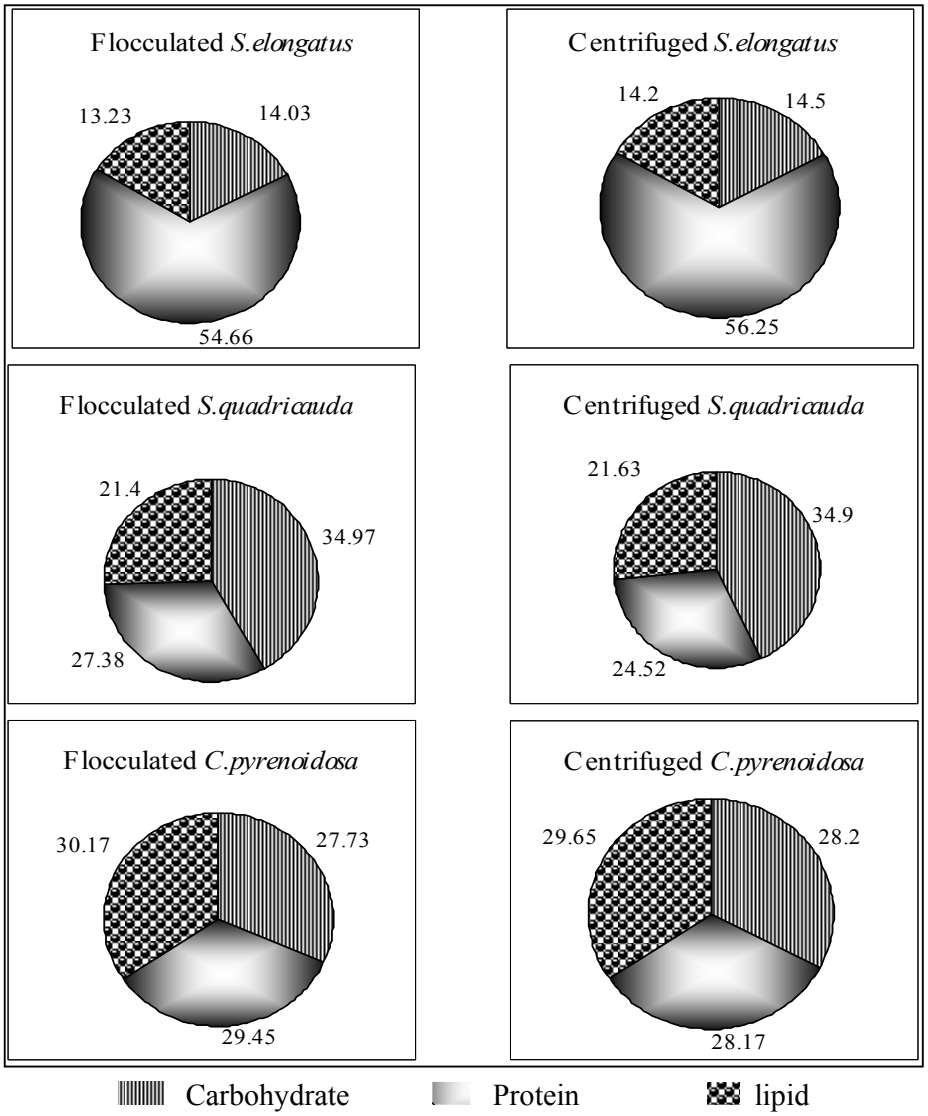


Fig 4.7 Biochemical composition of flocculated and centrifuged algal biomass

The biochemical compositions (protein, carbohydrate, lipids and ash) of three species of freshwater algae were characterized. Interspecies comparisons showed that the carbohydrate content of *S.quadricauda* was greater than that of the other two species. *S.quadricauda* had the same value ,34.66 ± 0.26, for both flocculated as well as centrifuged mass. Probability value from the t- test gave a value higher than 0.05 and hence there is no significant difference between carbohydrate levels of both flocculated and centrifuged biomass (Table 4.11).

Table 4.11 Results of biochemical comparison – carbohydrate

Algae	Concentration method	Mean (mg %)	t stat	t critical	Probability
S.elongatus	Flocculated	14.57 ± 0.04	0.572	2.776	0.598
	centrifuged	14.55 ± 0.05			
S.quadricauda	Flocculated	34.66 ± 0.26	0	2.776	0.5
	Centrifuged	34.66 ± 0.25			
C.pyrenoidosa	Flocculated	28.08 ± 0.54	0.564	3.182	0.306
	centrifuged	27.87 ± 0.35			

S.elongatus, S.quadricauda and *C.pyrenoidosa* were checked for protein concentration in the flocculated and centrifuged mass. The results are shown in the Table 4.12. *S.elongatus* had a higher protein content among the three species but the t- test gave a probability > 0.05 accepting the hypothesis all groups are equal.

Table 4.12 Results of biochemical comparison- protein

Algae	Concentration method	Mean (mg %)	t -stat	t critical	Probability
S.elongatus	Flocculated	54.800± 0.766	0.800	3.182	0.241
	centrifuged	55.490 ± 1.282			
S.quadricauda	Flocculated	26.977 ± 1.036	1.094	2.776	0.168
	centrifuged	26.167 ± 0.757			
C.pyrenoidosa	Flocculated	29.203 ± 0.525	1.999	3.182	0.070
	centrifuged	28.493 ± 0.320			

Lipid content of the three species used in the experiment was checked and results are given in the Table 4.13. *S.quadricauda* has lipid content higher than that of *S.elongatus*, the value being 21.73 ± 0.35 and 21.70 ± 0.24 in the flocculated and centrifuged mass. *C.pyrenoidosa* has the highest lipid content among the three species tested, flocculated mass has a value of 30.14 ± 0.37 and 29.88 ± 0.21 for the centrifuged mass. The t- test between flocculated and centrifuged mass gave probability > 0.05 accepting the hypothesis that there is no difference in the lipid content of both groups.

Table 4.13 Results of biochemical comparison- lipid

Algae	Concentration method	Mean (mg %)	t stat	t critical	Probability
S.elongatus	Flocculated	13.93 ± 0.08	-0.346	2.776	0.373
	centrifuged	13.95 ± 0.09			
S.quadricauda	Flocculated	21.73 ± 0.35	0.150	2.776	0.444
	centrifuged	21.70 ± 0.24			
C.pyrenoidosa	Flocculated	30.14 ± 0.37	1.059	3.182	0.184
	centrifuged	29.88 ± 0.21			

4.3.7 Chitosan-algal assay

- **Plate assay**

There was no plaque formation or yellowing in any of the chitosan impregnated algal lawns. This showed that chitosan did not inhibit algal growth.

- **Liquid culture assay**

Algal growth was observed visually by the development of colour and turbidity in all the test cultures of *C.pyrenoidosa*, *S.elongatus* and *S.quadriquada*, but the algae didn't form homogenous suspension as in the control cultures; instead clumps of algae were formed and settled at the

bottom. Upon microscopic examination, it was found that the cells were intact, but stuck together

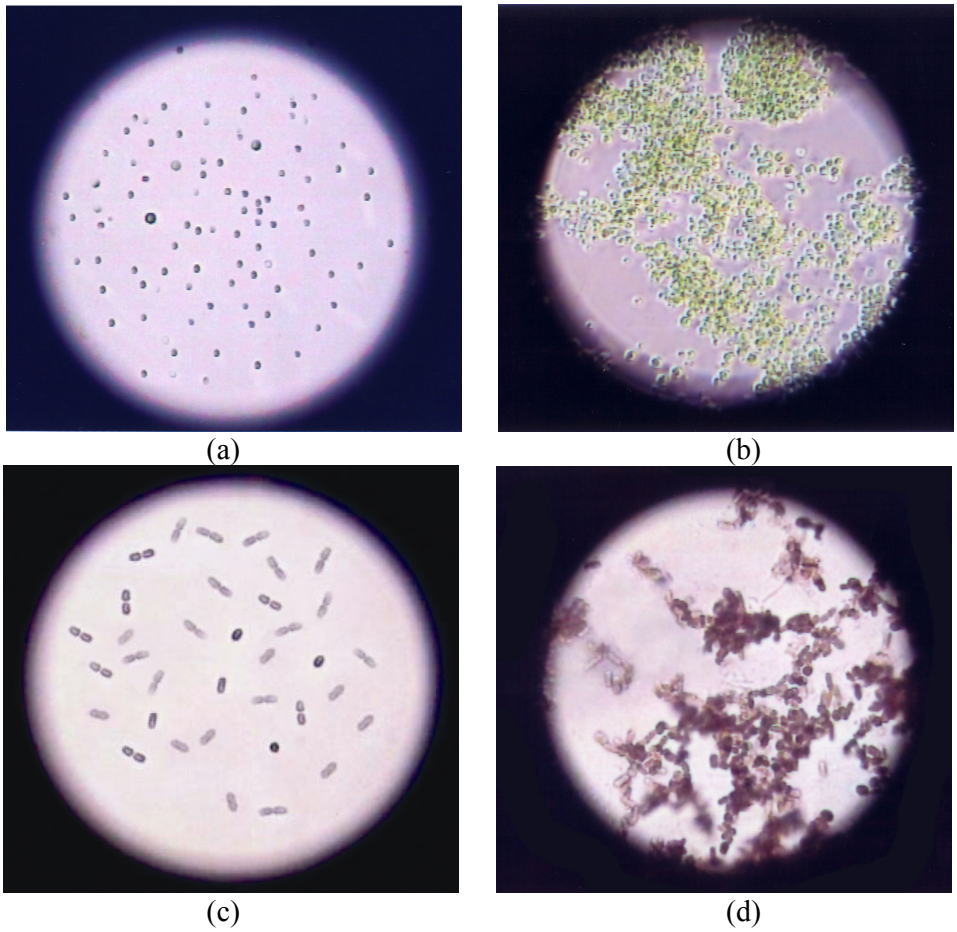


Fig 4.8 Microphotograph of algal floc after chitosan flocculation (a) *C.pyrenoidosa* (b) *C.pyrenoidosa* floc (c) *S.elongatus* (d) *S.elongatus* floc

In all cases except control, algae tend to remain the clumps even after gentle shaking it remain settled at the bottom of the culture flask. So cell count was not a reliable measurement. Visual and microscopic observation of algal flocs was taken into account to check whether chitosan has any effect on algal cells and it growth. The observation under the microscope showed that the algal cells were intact after flocculation, but stuck together in massive clumps at all chitosan concentrations.

4.4 Discussion

The results of the experiments conducted to evaluate the efficiency of alum and chitosan confirmed the better efficiency of chitosan at minimum concentration of 5 mg/L, when compared to a high concentration of 80 mg/L of alum. Earlier reports had suggested that chitosan can effectively flocculate algal species at 5 mg/L to 200 mg/L. Divakaran and Pillai (2002b) reported a 90 % turbidity removal at 5 mg/L of chitosan in an algal suspension having a turbidity of 30 NTU. These results have a similarity with the trend observed in this study. A 96–98% reduction in the number of suspended cells (*Euglena gracilis* culture) was obtained in the cultures with 200 mg/L of chitosan at *p*H 7.5 in the study of Gualtieri *et al*. (1988). For a *p*H range of 6·0–9·0, a mixed culture of Chlorophyceae dominated by *Chlorella* sp. in a high-rate algal pond (HRAP) fed with dilute pig-waste obtained a flocculation efficiency of 95–100% at 20 mgL^{-1} chitosan (Buelna *et al*.,1990).

A high coagulation demand in case of alum may be due the chelate complex formation between extracellular organic matter/cellular organic matter of alga and alum. Pivokonsky *et al*. (2006) and Auvray *et al*. (2006) gave an experimental evidence to the effect of algogenic organic matter in coagulation which in turn produce coagulation demand. Auvray *et al*. (2006) emphasized that both extracellular organic matter (EOM) and cellular organic matter (COM) disturbed the flocculation of suspended kaolin with PACl (polyaluminium chloride), and proteins in COM were identified as possible inhibitory substances for the coagulation with PACl. These proteins could consume PACl in the coagulation process due to the formation of chelate complexes between these inhibitory proteins and the coagulant. The consumption of alum by cyanobacterial proteins could be one of the important causes of the increase in coagulant demand.

The *p*H of the supernatant after flocculation in case of chitosan remains unchanged and that of alum treated get reduced to 5.89. This also favours the usage of chitosan as lower *p*H is not advisable in water quality management.

Flocculation efficiency of chitosan is reported as *p*H dependent. Most of the studies of this nature were done in mineral particles (Roussy *et al*., 2004). The effect of *p*H in flocculation of colloids of biological origin was investigated by very few authors (Strand *et al*., 2003 and Divakaran and Pillai, 2002 b). The *p*H dependency of chitosan flocculation was studied at different *p*H of 7, 8 and 9. Neutral and alkaline *p*H was selected for the study because the *p*H of algal blooms is always high. The flocculation efficiency at three *p*H was studied at six chitosan concentrations ranging from 5 mg/L – 80 mg/L. The result of *p*H dependency on flocculation showed that the best flocculant concentrations varied with the *p*H. At neutral *p*H the most effective flocculation occurred with a minimum chitosan concentration of 10 mg/L in *C.pyrenoidosa* and 5 mg/L in *S.elongatus*. At *p*H 8 and 9 maximum efficiency was observed with 20 mg/L in both species. These observations give a proof for *p*H dependency of chitosan flocculation and most effective flocculation with minimum chitosan requirement was observed at neutral *p*H. Another series of experiment was done to find out whether there is any difference in flocculation, if *p*H is changed after chitosan addition. The flocculation efficiency gets reduced in all *p*H levels tested.

The effect of turbidity and algal species was checked in another series of tests. As concentration of suspension increased the efficiency decreased. According to Tenney *et* al. (1969) a definite stoichiometric relationship exists between algal cell concentration and requisite cationic polymer dosage for optimal flocculation. The efficiency was higher in *S.elongatus* when compared to *C.pyrenoidosa* and a natural bloom dominated by green algae. This difference in flocculation efficiency may be due to algal cell size and concentration.

The presence of algae in source waters not only causes problems in the treatment process, but also complicates the treatment and disposal of sludge or further processes in algal recovery. Because of incorporation of algae, water treatment plant sludge becomes more complex and behaves like a mixture of inorganic and organic sludge. Sludge dewatering can be influenced by many factors such as particle size distribution, shape, specific surface area, density, particle charge, bound water content, *p*H, and organic content. Pan *et al.* (1999) studied the dewatering characteristics of 'algae containing alum sludge'. The presence of organic materials in the source water produces smaller flocs with more water content, resulting in poor dewaterability of alum sludge. . The alum-algae flocs in the present study are also small and the sludge volume index for different alum-algae sludge was in the range of 124 mL/L - 157 mL/L. The SVI for 'chitosan-algae' floc was in the range 60 mL/L – 89 mL/L and flocs were larger and feathery when compared to alum-algae sludge. A normal sludge with good settling characteristics generally has a SVI of less than 100 mL/L (Standard methods, 1999)

Concentrating and storing algae in a moist form provide high nutritional value (D'Souza *et al.*, 2002; Heasman *et al.*,2000; Knuckey *et al*., 2006). Moist algal concentrates can potentially provide a more cost-effective alternative to fresh algal cultures as well as simplify hatchery procedures. Centrifugation is the conventional method for algal concentration, especially in hatcheries. Algal concentrates produced by centrifugation have been fed to bivalves and prawn larvae with promising results (D'Souza *et al.*, 2000; Heasman *et al.*,2000; Robert *et al.*, 2001). Centrifugation of algal cells is an energy demanding one and is volume dependent. (Knuckey *et al*., 2006). An alternative to centrifugation is flocculation. For an algal floc to be an acceptable aquaculture feed it must be nontoxic and it should also be capable of being de-flocculated to release the trapped cells for planktonic feeders such as oysters and larval prawns.

The results of present study reveal that there is no change in the biochemical composition of the three algal species in flocculated and centrifuged algal biomass. This data favour that chitosan flocculation for algal removal for various purposes. The experiments by Knuckey *et al*., (2006) demonstrated a proof-of-concept for a commercial application of algal concentrates prepared by flocculation, especially for use at a remote nursery without on-site mass-algal culture facilities. The process was rapid, simple and inexpensive, and relatively cost neutral with increasing volume when compared to concentration by centrifugation.

Chitosan assay revealed that it is not algicidal in nature. Algal cells remained intact, but as clumps throughout the assay. The polymer adsorption is the first necessary step in flocculation and stabilization. The type and amount of adsorbed polymer and the conformation of the adsorbed layer will determine whether flocculation or stabilization will occur and if so by what mechanism (Somasundarana *et al*., 2005). The mechanism of chemically induced algal flocculation by cationic polymers, is interpreted in terms of bridging phenomenon between the discrete algal cells and linearly extended polymer chains, forming a three dimensional matrix that is capable of subsiding under quiescent conditions (Tenney *et al.*, 1969) The combination of flocculation and adsorption data of Strand *et al*. (2003)on the interaction between chitosans and bacterial suspensions clearly showed that charge neutralization was not the main flocculation mechanism and emphasized bridging as one dominating mechanism for flocculation. Therefore, chitosan flocculation may be used as an *ex-situ* water quality management tool; but process optimisation of chitosan flocculation is complicated.

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Chapter 5

Summary and Conclusions

Eutrophication is a persistent and one of the most challenging environmental problems in the world. Once a water body is eutrophicated, it will lose its primary functions and subsequently influence sustainable development of economy and society. In many instances, eutrophicated water bodies have been rendered unsafe for human consumption as well as for other activities such as irrigation and industrial needs. The degradation of water quality can, in effect, contribute to water scarcity as it limits its availability for both human use and the ecosystem. Eutrophication control in a sustainable way is fast becoming a necessity as it threatens the security and livelihood of the population and the environment.

Reduction of limiting nutrients, especially nitrogen and phosphorus is the ideal preventive strategy to curb eutrophication. Assessment of the interaction of phytoplankton with variation in nitrogen and phosphorus content has to be best studied for the implementation of this ideal preventive strategy. The present study of a trophic status evaluation of a water body and an *ex situ* nutrient enrichment microcosm experiment was undertaken with the intention of studying the trophic status and its interaction with phytoplankton. Many of the recent reports have stated that several of the tropical and subtropical lakes are nitrogen limited. According to this study a N:P ratio of 8:1 can induce a sudden algal bloom, but for the persistence of algal bloom a higher N:P is required. Nitrogen sources to the water body concerned are of non-point origin, and it is difficult to curb the inputs of nutrients. Hence, alternate environment friendly methods to control or reduce algal blooms were probed.

Short term strategy to control or reduce algal bloom is *in situ* chemical or biological treatment. Conventional copper compounds treatment, has several drawbacks and is not an environmental friendly option. Biological control of algal bloom gained attention as it is nature's way to tide over the problem. *In vitro* antagonism of *Pseudomonas* sp. isolated in this study against blue-green and green algae points to the possibility of organism or its secondary metabolites as a viable alternative to the conventional chemical treatment. Algicidal activity of cell-free supernatant extracted in chloroform gave a clear picture that secondary metabolites are responsible for antagonism. Algicidal compound removed blue green algae almost completely and green algae upto 77%. The chloroform extract was further purified by TLC and its UV/Vis spectrum was examined to know more about algicidal compound. Major absorption peaks are in UV range and showed similarity to phenazine antibiotics. The existing literature gives an assumption that the algicidal compound extracted could be of any phenazine or mixture of phenazine-1-carboxylic acid, 1-hydroxyphenazine and phenazine-1-carboxamide. *Pseudomonas* sp. isolated in this study may provide a better strategy to reduce/ control freshwater algal bloom. However, more work has to be carried out to ascertain the right dosage and mode of application for the management of algae in water bodies. The effect of algicidal compound on other algal species, and aquatic organisms, mode of action of algicidal compound in algae at phenotypic and genotypic level, persistence of algicidal activity at varying environmental conditions, optimising cultural conditions, media designing, species level identification of *Pseudomonas* SEB 8, and further purification and characterisation of algicidal compound are important work to be done before environmental application.

Algal removal operations in conventional water treatment and biomass recovery need special attention as the technique must be safe and sustainable for downstream processing and consumption. Flocculation is reported as a promising technique as it is cheap, simple, volume independent, and having economical option when compared to other existing ones. Conventional

chemical flocculants have several environmental consequences and thus it is not a sustainable option. Chitosan is an emblematic material from this point of view as it is non toxic and effective at low concentration. So, effectiveness of chitosan as an algal flocculant was examined and compared with alum. Results of the present investigation have confirmed the effectiveness of chitosan at low concentration at neutral *p*H. Process optimization is reported as the major impediment in the success of flocculation as algal removing technique. Therefore, effects of algal *p*H, turbidity and species on flocculation were studied. Optimum flocculation concentration was observed to be varying with *p*H, turbidity and algal species. Better flocculation efficiency at minimum concentration of chitosan was obtained at *p*H 7, and it decreased with increased algal turbidity. The efficiency was higher in *S.elongatus* when compared to *C.pyrenoidosa*. Comparison of flocculation and centrifugation as treatment technique for algal biomass recovery revealed that there is no difference in algal biochemical composition after treatment. This examination depicts the added advantage of using chitosan as an algal flocculant, and 'chitosan–algal flocculation' can highlight as emblematic material for *ex situ* water quality management tool for algal control or removal.