INVESTIGATIONS ON *PHAEOCYSTIS* SP., A HARMFUL BLOOM-FORMING ALGA ISOLATED FROM COCHIN ESTUARY

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Ph.D. Thesis under the Faculty of Marine Sciences

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Certificate

This is to certify that the thesis entitled "**Investigations on *Phaeocystis* sp., a Harmful Bloom-Forming Alga Isolated from Cochin Estuary"** is an authentic record of research work carried out by **Mrs. Sini, P. J.** under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** in **Marine Biology** and no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.

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Declaration

I hereby do declare that the thesis entitled, "**Investigations on *Phaeocystis* sp., a Harmful Bloom-Forming Alga Isolated from Cochin Estuary"** is an authentic record of research work done by me under the supervision and guidance of **Prof (Dr) A. V. Saramma**, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology for the degree of **Doctor of Philosophy** in **Marine Biology** and that no part thereof has been presented before for the award of any other degree in any university.

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Sini. P.J.

To

My dear parents, husband and son....

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Simi P.J

CONTENTS

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Abbreviations

^{0}C	:	degree Celsius
ANOVA	:	Analysis of Variance
cells/ml	:	cells per millilitre
cm	:	centimetre
et al.	:	Co-authors
g	:	gram
g/L	:	gram per litre
GF/F	:	Glass Fibre Filter
HA	:	Harmful Algae
HAB	:	Harmful Algal Bloom
hrs	:	hours
m	:	metre
mg/g	:	milligram per gram
min	:	minutes
ml	:	millilitre
ml/L	:	millilitre per Litre
mm	:	millimetre
mM	:	millimolar
nm	:	nanometre
ppt	:	parts per thousand
sec	:	seconds
sp.	:	species
sq. Km	:	square kilometre
UV	:	Ultra violet
Vis	:	Visible
µg/L	:	microgram per litre
µg/ml	:	microgram per millilitre
µl	:	microlitre
µm	:	micrometer
µM	:	micromolar
µmol/L	:	micromoles per litre

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Chapter 1

GENERAL INTRODUCTION

Oceans covering about 71% of earth's surface contain more than 5000 species of planktonic microscopic algae, the phytoplankton, which form the base of the marine food chain and produce roughly 50% of the oxygen we inhale. The algae are thallophytes that have chlorophyll *a* as their primary photosynthetic pigment and lack a sterile covering of cells around the reproductive cells (Lee, 2008). Algae can be aquatic or sub-aerial, when they are exposed to the atmosphere rather than being submerged in water. Aquatic algae are found almost anywhere from freshwater springs to salt lakes, with tolerance for a broad range of pH, temperature, turbidity, dissolved O_2 and CO_2 concentration. Planktonic algae are mostly unicellular species, living suspended throughout the lighted regions of all water bodies including under ice in polar areas. Benthic algae are found attached to the bottom or living within sediments or grow attached on stones (epilithic), on mud or sand (epipelic), on other algae or plants (epiphytic), or on animals (epizoic). The size of algae ranges from picoplankton, 0.2–2.0mm in diameter to giant kelps with fronds reaching up to 60m in length (Barsanti and Gualtieri, 2006).

The size of the algae, ecology and colonial habitats, cellular structure, levels of organization and morphology, photosynthetic pigments, reserve and structural polysaccharides and type of life history reflect the varied evolutionary origin of this heterogeneous assemblage of organisms, including both prokaryotic and eukaryotic species. In most

habitats they function as the primary producers in the food chain, producing organic material from sunlight, carbon dioxide, and water. They form a major source of food for zooplankton, fishes, and via the food chain, ultimately for human beings. Besides forming the basic food source for the food chains, they also liberate oxygen through photosynthesis that is necessary for the metabolism of the consumer organisms and thus accounts for the production of a major fraction of the world's oxygen. Unfortunately some algae can cause nuisance, through fish kills, intoxication of shellfish, and water discolorations. The high biomass developments of these algae are designated as "Harmful Algal Blooms" (HAB).

Harmful Algae

The term "Harmful Algae" (HA) is used in a broad sense, referring to algae that can cause a variety of deleterious effects on aquatic ecosystems, including negative aesthetic effects such as beach fouling, oxygen deficiency, clogging of fish gills and poisoning of various organisms. A direct effect of some Harmful Algal Blooms (HAB) can be oxygen deficiency in deep waters, which in turn, causes mass mortality of benthic animals and fish kills (Graneli *et al.*, 1989). Of the known 5,000 phytoplankton species, known HAB species comprise only about 300 species that can cause water discoloration and out of it only 80 species produce potent toxins (Hallegraeff, 2003). During a bloom, there could be ten million algal cells in a litre of seawater (GEOHAB, 2001). Blooms have very high population density, but the potential of the bloom species to inflict harm is influenced by regional, seasonal and species-specific characteristics. Thus both a very high and low biomass bloom can cause harmful effects (Smayda, 1997). For some HAB species, even low cell

density (i.e. a few hundred cells/L of seawater) may be hazardous to organisms and public health, and can cause environmental damage.

The HAB species are broadly classified into two groups- the toxin producers, which can contaminate seafood or kill fish and the high biomass producers, which can cause mortalities of marine life after reaching dense concentrations. Some HAB species have characteristics of both groups (GEOHAB, 2001). Generally, the toxin producers may cause haemolytic, hepatotoxic, osmoregulatory effects and other unspecified toxicity to kill animals that ingest these species. Furthermore, after digestion, the biotoxin from phytoplankton may accumulate in tissues of shellfish, which will lead to toxin accumulation in mammals and other higher levels in the food chain. The high-biomass producers may cause physical damage by virtue of gill clogging or oxygen-depletion during decay of the dead cells which can affect fish, shellfish, and marine mammals of shallow bays or seas.

The HAB species that are cosmopolitan in distribution are euryhaline and eurythermal in nature like *Alexandrium ostenfeldii*, while other taxa are restricted to warm waters e.g *Alexandrium tamiyavanichii*. Several cold water species show a unipolar distribution, being restricted to either the northern or the southern hemisphere. The warm tropical waters serve as a barrier for the dispersal of cold water species. Some species show a bipolar distribution that may have arisen after dispersion via deep cold waters at the equator, atmospheric circulation, or via biological vectors such as birds etc. (Lundholm and Moestrup, 2006).

Major taxa of Harmful Algae

HAB species are present in the majority of phytoplankton taxa studied. The major group in this context is dinoflagellates, and about 185 species are

harmful in nature and 60 species produce potent toxins. The important genera that include harmful algae are *Alexandrium, Gymnodinium, Ceratium, Dinophysis, Gyrodinium, Prorocentrum, Protoperidinium, Pfiesteria, Peridinium, Pyrodinium, Heterocapsa, Amphidinium* and *Cochlodinium*. They produce saxitoxins, brevetoxins, ciguatoxins, diarrhetic toxins etc. and allelochemicals. Under class Prymnesiophyceae, the important genera of harmful algae are *Chrysochromulina, Prymnesium* and *Phaeocystis,* that produce toxins which are cytotoxic, neurotoxic and icthyotoxic in nature and compounds with antibacterial and allelopathic activity. The class Raphidophyceae contains the harmful algae *Chattonella, Fibrocapsa* and *Heterosigma*. They mainly produce neurotoxins and free fatty acids causing fish kills and generate reactive oxygen species responsible for gill tissue injury and mucus production. Most diatoms are known to cause harm either by physical means, by causing oxygen depletion or by the production of phycotoxin. The genus *Pseudo-nitzschia* is known to produce a neurotoxin domoic acid. The other genera under diatoms that include toxic algae are *Amphora* and *Nitzschia*. Under the cyanobacteria the important genera that contain harmful species include *Microcystis, Oscillatoria, Planktothrix, Anabaena, Aphanisomenon, Calothrix, Cylindrospermopsis, Lyngbya, Nodularia, Nostoc, Scytonema* and *Trichodesmium*. The destructive brown tides are caused by the pelagophytes *Aureococcus anophagefferens* and *Aureoumbra lagunensis* (Graneli and Turner, 2006).

Harmful Algal Toxins

Harmful algal toxins appear to be involved primarily in allelopathy, being released in the dissolved state into seawater and causing deleterious effects on other competitor phytoplankton species (Graneli and Hansen, 2006). They are secondary metabolites that are only coincidentally toxic

and are primarily associated with other processes such as nitrogen storage, nucleic acid biosynthesis, bioluminescence, chromosomal structural organization, ion channel transport across membranes, bacterial endosymbiosis, pheromones inducing sexuality during bloom decline and also serve as grazer deterrents. Allelopathy is mainly used by the algae to produce mono specific blooms by outcompeting other phytoplankton. It is closely associated with competition for resources, and limiting amounts of nutrients not only increase the production of allelochemicals but also accentuate their action.

The toxin production is mainly by polyketide pathways in which acetate units are added sequentially from acetyl CoA with a pathway regulated by polyketide synthase (Cembella, 1998). Algal toxins from different species vary in structure, atomic composition and functional activity. The factors stimulating toxin production in one algal species or group may have a different impact on another. Toxin production is stimulated by the presence of grazers and toxin content per cell changes with nutrient status, temperature and salinity (Bates, 1998).

Characteristics of Harmful Algae

The most important characteristic of the harmful algal species is the capacity to form blooms (Steidinger and Gracces, 2006). A bloom is a relative term where a species population increases over time and this increase leads to an observable or recordable effect. An algal bloom goes through sequential developmental phases such as initiation, growth, maintenance and dispersal/dissipation/termination. These different phases can act as "triggers" for the induction of transitions in life stages of a microalgal species and, there may be feedback mechanism such as cell

density and infochemicals. The physiochemical parameters that trigger the blooming of HABs include diffusion and advection of the population, salinity, turbulence, temperature, and nutrients. Life cycle of HAB species is particular in which phase alteration occurs between the diploid benthic and haploid planktonic forms. Resting cyst formation, phagotrophy and other resistant stages in life cycle are also observed along with polymorphic behaviour in life cycles. The harmful microalgae show a rapid intrinsic division rate and allelopathic defence against predation and competition.

Bacterial association of Harmful Algae

An ordered and structured bacterial community is associated with HABs, rather than a random assemblage of species recruited from the marine bacterial community. Bacterial communities are closely linked with both succession changes with phytoplankton assemblages and with algal physiological status. The algal-bacterial association is very specific, and the two-way interactions between phytoplankton and their attached microbial flora can influence the bacterial-algal succession and the cycling of organic matter in the ocean. Bacteria may be involved in regulating transitions between the stages of HAB events and play an important role in phytoplankton growth (Kodama *et al*., 2006).

The present scenario of Harmful Algal Blooms

The past five decades have witnessed a dramatic increase in the availability of nutrients on land, in the atmosphere, and in the oceans. This change has occurred largely due to the development of industrial fertilizers, changing practices in the raising of animals for consumption on land and in the sea and increased consumption of fossil fuels (Smayda, 2005). Over enrichment of coastal waters by nutrients is considered a major pollution

problem worldwide and one of the important factors contributing to global habitat change, including the geographic and temporal expansion of some harmful algal blooms (Anderson *et al*., 2002). Moreover, along with the increase in the frequency of HAB the geographic range of microalgal species is also expanding as a result of natural factors (climate change, catastrophic storm events, ocean currents, transport of spores via wind or bird feet etc) and human mediated vectors. Human mediated transport mainly includes the transportation of HAB species by ships ballast water (Hallegraeff and Bolch, 1992) and translocation of aquaculture products such as shell fish (Shumway *et al*., 2004).

Nowadays HABs have received growing and worldwide attention because of its high ecological implications. International Oceanographic Commission (IOC) has been giving top priority and coordinates several works related with HAB. Agencies like ICES (International Council for the Exploration of the Sea), SCOR (Scientific Committee on Ocean Research), EU (European Union) and APEC (Asia-Pacific Economic Cooperation) also focus on HABs and their impacts. With man's increasing use of the coastal strip, the impact of HAB has become even more prominent over the last thirty years (Hoagland and Scatasta, 2006). There is also evidence that in some places there has been an increase in harmful algal events. Study of harmful algal groups is very important as it helps in developing strategies for the prediction and control of harmful algal blooms.

The study organism- *Phaeocystis*

The harmful alga which is considered in this study is *Phaeocystis*, which is one of the widespread marine genera of phytoplankton, exhibiting

phase alteration between free living cells of 3-9µm in diameter and gelatinous colonies reaching several mm. The genus was erected by Lagerheim in 1893 to accommodate the colonial stage of an alga described originally as *Tetraspora pouchetii* by Hariot in Pouchet (1892). *Phaeocystis* is considered as a eurythermal and euryhaline alga and regularly form blooms in very contrasting nutrient rich areas of the world's ocean. This bloom forming alga is often recognised both as a nuisance alga and an ecologically important member of the phytoplankton. The uniqueness of the genus *Phaeocystis* rests not only in the fabulous biomass reached by its blooms but also mostly in its exceptional physiology and ecology. The polymorphic life cycle of the alga induces dramatic changes in the structure and functioning of the planktonic and benthic food-web as well as in the biogeochemistry of trace metals and sulphur. *Phaeocystis* is a major contributor to the global sulphur budget by releasing substantial quantities of dimethylsulfide propionate (DMSP) which is metabolized into dimethylsulfide (DMS) as the cells are grazed or infected and lysed by virus. This volatile sulphur compound is known to have an impact on the chemical quality of the atmosphere and on the global climate regulation as a contributor to cloud condensation nuclei.

Bloom of *Phaeocystis* was first documented more than a century ago (Gran, 1902). They form extensive blooms particularly in cold temperate, boreal and polar waters. In particular, the colony forms can produce massive bloom in spring and early summer in just a few days and these blooms of numerous, voluminous colonies have attracted the attention of scientists, fishermen and tourists. The blooms are often referred to as harmful blooms since they are avoided by fish (Chang, 1983) and appear detrimental to the growth and reproduction of shell fish and macrozooplankton or are

ichthytoxic. Massive areas of pollution are created when dissolved organic compounds released by *Phaeocystis* during declining bloom conditions, accumulate, foam and then wash onshore. *Phaeocystis* have negative effects on higher trophic levels in the marine ecosystem and consequently influence human activities such as fisheries and fish farming. Dense blooms have been responsible for causing net clogging, mortality in fish cultures and alteration of fish taste. *Phaeocystis* is also known to release many toxic compounds into the water column which appear to affect the growth and reproduction of other organisms. It appears to be a useful indicator species of long-term or chronic environmental changes such as climate and eutrophication.

Significance of the study

Considering the importance of diversity of microalgae in our ecosystem, and new invasion of many organisms, an attempt was made to monitor the Cochin estuary along the southwest coast of India for the qualitative distribution of phytoplankton and to study the growth kinetics and allelopathic effect of the *Phaeocystis* sp. isolated from the Cochin estuary. *Phaeocystis* blooms are common only in high latitude environments and they rarely occur in low latitude environments such as the subtropics and tropics. As *Phaeocystis* is grouped under harmful alga, in the present study it is taken as a model organism to study the factors causing the bloom formation in the ecosystem. The nutrient concentration of the water body along with other physiochemical parameters that includes temperature, salinity and pH play an important role in triggering the bloom of *Phaeocystis*. The *Phaeocystis* harbour specific bacterial flora associated with it and they exert an important role in the growth, haemolytic activity and the bloom phases of the alga. The harmful alga mainly depends on the

production of allelopathic compounds for the establishment of bloom in the marine environments. These physiological properties of the *Phaeocystis* were considered for the study, along with the role of nutrients in the allelopathic and haemolytic activity.

Objectives of the study:

1) To study the spatial and qualitative distribution of microalgae along the Cochin estuary, and to isolate an ecologically important microalga from the estuary.
2) To culture the *Phaeocystis* sp. isolated from Cochin estuary under laboratory conditions.
3) To study the growth characteristics and colony formation of *Phaeocystis* sp.
4) To study the haemolytic and allelopathic properties of *Phaeocystis* sp.
5) To study the bacterial association with *Phaeocystis* sp.

The thesis is presented in six chapters. The first chapter comprises of a general introduction including the importance of the work. The second chapter deals with the qualitative analysis of the phytoplankton along the selected stations in Cochin estuary and the development of clonal cultures of ecologically important microalgae. Among the different phytoplankton cultured, the *Phaeocystis* sp. was selected for the current study. The third chapter gives an account of the optimal culture conditions of *Phaeocystis* in laboratory and the effect of nutrients on the colony formation of *Phaeocystis* sp. The fourth chapter deals with the haemolytic and allelopathic property exhibited by *Phaeocystis* sp. The effect of nutrient concentration on the haemolysis of *Phaeocystis* was also studied. The effect of the culture filtrate

and cell extract of *Phaeocystis* sp. on the growth of other microalgae were also presented in this chapter. The fifth chapter projects the isolation of associated bacteria from the *Phaeocystis* sp. cultured in laboratory, their identification and the influence of associated bacterial flora on the growth and haemolysis of *Phaeocystis* sp. The major findings of the study were summarized in chapter 6.

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Chapter 2

ISOLATION OF *PHAEOCYSTIS* SPECIES FROM COCHIN ESTUARY

Contents

2.1 Introduction

The Cochin backwaters, is the longest estuarine system on the southwest coast of India, extending between $9^0$40'12" and $10^0$10'46"N and $76^0$09'52"and $76^0$23'57"E with its northern boundary at Azheekodu and southern boundary at Thannermukham bund. The total area of the backwater is about 157 sq. Km with depth ranging from 2m to 8m. The backwater stretches parallel to the coastline and is connected with the Arabian Sea at the Cochin harbour by a barmouth of 450m width and average depth of 8-10m. The estuary has all the characteristics of a tropical positive estuary and includes a system of interconnected lagoons, bays and swamps penetrating the mainland and enclosing many islands in between. The backwater system receives runoff during the southwest monsoon and also to some extent during the northeast monsoon season. The discharge of freshwater from the rivers, particularly during the southwest monsoon period reduces the salinity of backwater system considerably even in areas around the barmouth where salt water ingress occurs below 5m depth only.

Cochin is the major port on the southwest coast of India. As per the Environment Protection Act, 1985, the Cochin backwater has been classified under the "Ecologically Sensitive Zone".

The salinity gradient in the Cochin estuary supports diverse species of flora and fauna, according to their tolerance for saline environment. This tropical estuary with high productivity acts as a nursery ground for many species of marine and estuarine fin fishes, molluscs and crustaceans. The low lying swamps and tidal creeks dominated by sparse patches of mangroves provide shelter to juveniles of many important species. The areas of the backwater with fine sediments and rich organic matter support abundant and diverse benthic fauna. The changes in the hydrology of backwaters are controlled by the Arabian Sea and it plays an important role in regulating the migrant fauna of the estuary.

Several attempts have been made to study the qualitative and quantitative distribution of phytoplankton groups in Cochin estuary by various authors and the important groups identified include diatoms, dinoflagellates, chlorophytes and cyanobacteria (Subramanyan, 1946, 1958, 1959, Subramanyan *et al*., 1960 and 1965, Gopinathan, 1972). About 99% of the total phytoplankton collected from the Cochin backwaters were diatoms including *Chaetoceros, Coscinodiscus, Skeletonema, Pleurosigma and Nitzschia* and dinoflagellates of the genera *Peridinium, Gymnodinium and Ceratium* (Devassy and Bhattathiri, 1974; Joseph and Pillai, 1975; Gopinathan, 1984; Jayalakshmi *et al*., 1986; Jagadheeshan, 1986; Selvaraj *et al*., 2003; Qasim, 2004). Similar findings were also made by Desikachary (1988) from Indian Ocean, Joseph and Sreekumar (1993) from Cochin backwaters and Geetha and Kondalaroa (2004) from coastal waters of eastcoast of India. Systematic study of the microphytobenthos in Cochin

estuary was undertaken by Sivadasan and Joseph (2006) and 14 microalgae belonging to pennate diatoms were added as new records to the phytoplankton from the estuary.

Several toxic algal groups were recorded from the estuary, among the three taxonomic divisions, Cyanophyta, Bacillariophyta, and Dinophyta. Recently the pattern of harmful algal blooms (HAB) was changing worldwide along with the climate change, eutrophication, increased harvesting of fish and shellfish, altered freshwater runoff, redistribution and dispersal of species through ballast water. There occurs an increase in the global spreading of HAB species, novel occurrence of organisms, increase in bloom frequency and increase in the annual blooms along the marine ecosystem all over the world. In Indian coast, the frequency of HAB increased along with the blooming of new toxic microalgal species such as *Gymnodinium nagasakiense* in 1992 (Karunasagar and Karunasagar, 1992), *Phaeocystis globosa* in 1996 (Madhupratap *et al.*, 2000), *Cochlodinium polykreikoides* in 2004 (Ramaiah *et al*., 2005), *Chattonella marina* in 2011 (Padmakumar *et al*., 2011).

So far, there are no reports on the occurrence of *Phaeocystis* in the Cochin estuary and *Phaeocystis* appears to be a new introduction to the Arabian Sea, most probably through the introduction of ballast water. The presence of *Phaeocystis* in Indian waters is considered as an example of bioinvasion of new organism to our ecosystem (Madhupratap *et al.*, 2000). One of the important hypotheses for the causation of harmful algal blooms is the ballast water vectoring of HAB species which increases the geographical expansion of the species. The present study on the *Phaeocystis* sp. isolated from the Cochin estuary is quite relevant in this context.

2.2 Review of Literature

2.2.1 Distribution of *Phaeocystis*

The genus *Phaeocystis* has got a worldwide distribution and could acclimatise a wide range of temperatures (-2 to >20^{0}C) and occurs as different species in very contrasting marine systems. Cells in free-living forms are cosmopolitan in distribution and are an important component of the haptophycean assemblage, which dominates the oceanic nanoplankton in many areas. It contains the major problematic, bloom forming species distributed in varied geographical areas including southern and eastern parts of the North Sea Basin (Cadee and Hegeman, 1986; Weisse *et al*., 1986; Lancelot *et al*., 1987; Colijin *et al*., 1990; Lancelot, 1990), northern Norwegian fjords (Eilertsen *et al*., 1981) in the Fram Strait, Greenland Sea (Gunkel, 1988; Baumann, 1989; Gradinger and Baumann, 1991; Smith *et al*., 1991), Barents Sea (Wassmann *et al*., 1990,) and in the Antarctic ecosystems (Garrison *et al*., 1982, 1983, El-Sayed *et al*., 1983; Fryxell *et al*., 1984, 1985; Fryxell, 1989). As a general trend *Phaeocystis globosa* blooms in temperate and tropical waters while *Phaeocystis pouchetii* and *Phaeocystis antarctica* were better adapted to the cold temperature prevailing in Arctic and Antarctic waters (Garrison *et al*., 1982, 1983). It may also dominate phytoplankton assemblages of subtropical (Atkinson *et al*., 1978) and tropical marine ecosystems (Guillard and Hellebust, 1971; BjØrnland *et al*., 1988; Al-Hasan *et al*., 1990). Blooms of *Phaeocystis globosa* in the southeast coast of China were ascribed to globally abnormal climate and a very strong El Nino event (Chen *et al*., 2002).

A bloom of *Phaeocystis globosa* was reported from the central Arabian Sea in 1996 during the summer monsoon period (Madhupratap *et al*., 2000). Almost 95% of the phytoplankton population was composed of colonies of *Phaeocystis globosa*.

2.2.2 Taxonomy of *Phaeocystis*

The genus *Phaeocystis* was first described by Lagerheim in 1893. The *Phaeocystis* species was described based on the phenotypic characters such as the morphometry of the colonial stage and physiological and biochemical properties. *Phaeocystis* belongs to the phylum Haptophyta and class Prymnesiophyceae. The haptophyceae comprises mainly unicellular or colony-forming algae and the cells are yellow or yellow to brown, the chloroplast usually two in number and containing chlorophyll-a and two or three different chlorophyll-c (Kawachi *et al*., 1991). One of the main structural characteristics of the group is the haptonema, a filiform organelle which occurs together with the two flagella. The haptonema has not been found in any other group of organisms, and its length is a species characteristic. Some species lack haptonema or it is rudimentary. Haptonema can serve as an attachment organelle. It is usually stretched forward in swimming cells but long haptonema coil up on contact with other objects (Jeffrey *et al*., 1994). The cells of most haptophytes are covered with carbohydrate scales, the structure of which is species specific.

The class Prymnesiophyceae is further organized into subclass Prymnesiophycidae which includes 13 orders, Arkhangelskiales, Coccosphaerales, Discoasterales, Eiffellithales, Isochrysidales, Podorhabdales, Prinsiales, Prymnesiales, Rhabdosphaerales, Stephanolithiales, Syracosphaerales, Watznaueriales and Zygodiscales. Prymnesiophyceae includes about 762 species and subspecies. The order Prymnesiales includes 74 species and subspecies in three family Chrysotilaceae, Phaeocystaceae and Prymnesiaceae. Family Phaeocystaceae includes the genus *Phaeocystis* (Moestrup and Larsen, 1992).

Phylum	- Haptophyta
Class	- Prymnesiophyceae
Subclass	- Prymnesiophycidae
Order	- Prymnesiales
Family	- Phaeocystaceae
Genus	- Phaeocystis

Six species of *Phaeocystis* are now recognised based on small subunit (SSU) rDNA sequence analysis and morphological characterisation. These are *Phaeocystis antarctica* Karsten, *Phaeocystis globosa* Scherffel, *Phaeocystis pouchetii* (Hariot) Lagerheim, *Phaeocystis jahnii* Zingone, *Phaeocysits scorbiculata* Moestrup and *Phaeocystis cordata* Chretiennot-Dinet (Moestrup, 1979; Medlin *et al*., 1994; Zingone *et al*., 1999; Edvardsen *et al*., 2000; Lange *et al*., 2002). Colony forms were reported only for the first four species. It is now considered that probably more than six *Phaeocystis* species exist (Lange *et al*., 2002; Medlin and Zingone, 2007). But the species described were not considered as valid because of poor illustrations and unlikely features, including one chloroplast per cell and no haptonema and they include *Phaeocystis brucei* Mangin, *Phaeocystis amoeboidea* Buttner, *Phaeocystis sphaeroidea* Buttner, *Phaeocystis fuscescens* Braun and *Phaeocystis giraudyi*. (Medlin and Zingone, 2007) Out of this *Phaeocystis fuscescens* and *Phaeocystis giraudyi* were discarded from the list since they did not fit in the genus.

2.2.3 Morphological characteristics of *Phaeocystis*

Phaeocystis had a polymorphic lifecycle with both the colonial and flagellated cells (Kornmann, 1955). The colonial stage, with cells very loosely interconnected and enclosed in a thin colony skin (Hamm *et al*., 1999) was most easily recognized, although some species may form mucilaginous

colonies or did not seem to have a colonial stage. Thousands of cells could occur in a colony that might reach 2cm in diameter (Jahnke and Baumann, 1987; Verity *et al*., 1988; Davidson and Marchant, 1992; Rousseau *et al*., 1994; Whipple *et al*., 2005). Flagellated cells had two parietal chloroplast and two flagella, which might be equal or unequal in length and heterodynamic. A short haptonema was present between the two flagella, which might or might not have a swollen end. The flagellated cell might be naked or had two layers of different shaped organic scales. Some flagellated cells also produce groups of filaments, which were extruded from the cell and assume a characteristic pattern (Medlin and Zingone, 2007).

The colony cells of *Phaeocystis globosa* were diploid with a diameter of 4.5 – 8µm, evenly distributed in the colony beneath a thin skin and were weakly inter connected. Diameter of the colony ranges from 10µm to 8-9mm. The colonies were spherical but deviate into non spherical forms when growing into larger ones or when subjected to hydrodynamical stress. The colony cells had 2-4 parietal chloroplasts, and were deprived of body scales, haptonema and flagella. Non-motile free-living cells of colonial origin had also been reported in *Phaeocystis globosa* cultures. These cells were morphologically similar to colony cells and had same ploidy level. (Kornmann, 1955 ; Cariou *et al*., 1994 ; Vaulot *et al*., 1994 ; Rousseau *et al*., 1994 ; Hamm *et al*., 1999 ; Peperzak *et al*., 2000b). The flagellated cells had been reported as swarmmers (Scherffel, 1900), microzoospores, small and large zooids, microflagellates and mesoflagellates (Kornmann, 1955). They had a round shape, and were smaller than colonial cells, with a diameter of 3-5µm. These flagellates were capable of rapid vegetative reproduction and they possess two equal heterodynamic flagella and a short haptonema. They had an anterior

depression and two golden brown plastids. The cell body was covered by two types of organic scales showing a pattern of radiating ridges, visible on both sides (Parke *et al*., 1971). These flagellates were observed in senescent cultures, swimming inside spherical colonies of various sizes and were associated with colony disappearance (Kornmann, 1955). Diploid flagellates were observed with the same size range as colonial cells, 4.5- 8µm in diameter and containing two flagella and one haptonema but lacking scales and filaments. These flagellates had been reported as an asexual swarmmer and typically appear in culture when colonial cells were released mechanically from the colony and were able to form new colonies within a day after adhesion to a surface (Kornmann, 1955; Kayser, 1970; Rousseau *et al*., 1994).

In *Phaeocystis pouchetii*, the colony cells were diploid, in a size range of 5-7µm in diameter and had an anterior longitudinal groove and are deprived of filamentous appendages and scale coverings (Jakobsen and Tang, 2002). Actively growing colonial cells were distributed in groups on lobes of cloud like colonies (Jahnke and Baumann, 1987; Baumann *et al*., 1994; Rousseau *et al*., 1994). The colonies were characterised by delicate mucus which disrupts easily compared to the solid mucilage of *Phaeocystis globosa* (van Rijssel *et al*., 1997; Jakobsen and Tang, 2002; Wassmann *et al*., 2005). Non motile free living cells were morphologically similar to colonial cells, and could be found together with the colony stage during the colony disruption. Diploid flagellates were observed in culture which was round and had an average diameter of 5µm with two golden brown parietal chloroplasts, two heterodynamic equally long flagella (11µm), and a short non-coiling haptonema. The cell body was covered by scales with radiating ridges visible on both surfaces (Jacobsen, 2002). The cells originate from

the colony and were found to be precursors for the colony development. Wassmann *et al*. (2005) observed two types of flagellates based on size criteria i.e. large 6µm and heart-shaped 3-4µm. The large flagellates co-occurred with colonies and were abundant, sometimes being dominant over colonial cells in terms of cell density and small flagellates were found free living or within the decaying colonies before their disappearance.

The colony cells of *Phaeocystis antarctica* were found to be similar to *Phaeocystis globosa*. The cells were evenly distributed along the periphery of colonies characterized by solid mucus (Davidson, 1985; Vaulot *et al*., 1994; Mathot *et al*., 2000). Two types of flagellates were observed, one with scales and produce filaments and stars and the other is devoid of scales, filaments and stars. The scale bearing flagella with size range of 3.5µm to 7µm had an anterior depression, two chloroplast with a large central pyrenoid, and bears two flagella and a haptonema (Davidson, 1985). The body cell was covered by two types of scales with a pattern of radiating ridges visible on both sides. In the natural environments, such scale-bearing flagellates increase in number at the beginning of the colonial bloom, and decline during the bloom and were present in large number after the bloom (Davidson and Marchant, 1992). The second type of *Phaeocystis antarctica* flagellate had the same size of the colonial cell, 6.5µm to 7.5µm and bear two flagella and a haptonema but lack scales, filaments and stars. These flagellates were formed inside and released from the spherical or elongated colonies and they got attached to the spines and surface of large diatoms where it subsequently formed new colonies (Fryxell, 1989; Garrison and Thomsen, 1993; Marchant and Thomsen, 1994).

Only two cell types had been described for *Phaeocystis jahnii*-colonial cells and flagellates (Zingone *et al*., 1999). The colony cells had a

size range of 6-8.5µm in diameter and irregularly distributed in loose, irregular colonies. Non-motile free-living cells of the same size had also been reported. The flagellate cells were round, 3.5 – 5µm in diameter and had two or four golden brown parietal chloroplasts. It bears two unequally long flagella and a non-coiling haptonema (Zingone *et al*., 1999). *Phaeocystis cordata* and *Phaeocystis scrobiculata* had only been described as flagellated cell. The size range of *Phaeocystis cordata* is 3-3.5µm long and 3-4µm wide. They had two unequal flagella and a non-coiling haptonema. These cells produce filaments that form pentagonal figures (Zingone *et al*., 1999). The cells of *Phaeocystis scorbiculata* had a size of 8µm in length, bear two equal flagella and a non coiling haptonema. It was covered by a periplast and produce filaments longer than 50µm which form a nine ray figure rather than the five ray star observed in other *Phaeocystis* sp. (Moestrup, 1979; Hallegraeff, 1983).

2.2.4 Life cycle of *Phaeocystis*

The presence of different morphotypes supports the existence of a haploid/diploid life cycle in *Phaeocystis* especially in colony forming ones. In such a life cycle, both the haploid and diploid stages were related by sexual processes, meiosis and syngamy and both were capable of mitotic division (Valero *et al*., 1992; Houdan *et al*., 2004). The haploid flagellates occur in water between two blooms of diploid colonial cells. Two haploid flagellates of different mating types undergo syngamy at the time of colony bloom initiation (Parke *et al*., 1971; Peperzak *et al*., 2000a). Filament characteristics of haploid flagellates could possibly play a role in mating. At the end of the bloom, the colony cells undergo meiosis to produce haploid flagellates in the water column. The vegetative reproduction of the diploid stage occurs through two distinct pathways involving colonial cells

and diploid flagellates. The first pathway consists of the mitotic division of colonial cells within the colony for the colony growth, maintaining the bloom (Kornmann, 1955; Rousseau *et al*., 1994; Veldhuis *et al.*, 2005). The second pathway involves the transition through short lived diploid flagellates that were released from the colonies and were able to reinitialize the colonial stage within a day. Diploid flagellates, therefore co-occur with the colonial stage, and propagate the colonial cells.

Several factors had been hypothesized to play a role in transitions between the morphotypes which include nutrient depletion, light intensity chemical substance produced by diatoms especially *Chaetoceros* sp. and turbulence (Kornmann, 1955; Veldhuis and Admiraal, 1987; Cariou *et al*., 1994; Schapira *et al*., 2006).

2.2.5 Bioinvasion

The first biological study that suspected shipping as a vector of non-native species introductions was published in the early 1900s where Ostenfeld (1908) reported Asian phytoplankton species *Odontella (Biddulphia) sinensis*, after mass occurrence in the North Sea in 1903. A summary of European shipping studies revealed that more than 1000 species were transported in the ballast water of ships. A Japanese dinoflagellate was released to Australian waters, which led to the closure of shellfish beds along the Australian coast (Carlton and Butman, 1995). The toxic blooms of *Gyrodinium* sp. in 1998, along the coast of China, and the bloom of *Alexandrium catenella* along the coast of South of Chile in 2002, were considered as a result of bioinvasion (Rodriguez and Arancibia, 2002). According to Bhat and Matondkar (2004), the possible cause for the increase in HABs around Indian waters was due to the transport of exotic

species through ballast water from ships. The invasion could cause profound changes in biodiversity. On a typical day, more than 3000 species of marine animals and plants are in silent motion around the world, travelling in ballast water in ships. The first South Asia regional meeting on Ballast Water Control and Management held in Goa, 2004, puts this strongly as "Alien invaders are ordinary marine organisms, often harmless in their home environments, but assuming the role of natural terrorists when they are transported to another ecosystem". India is one of the active pilot countries among the six identified by IMO (International Maritime Organization) since the year 2000, for Global Ballast Water Management Programme. Under this, the Indian ports were monitored for various invading species, including HAB forming species (Anil and Venkat, 2004).

According to Madhupratap *et al*. (2000) who first reported the bloom of *Phaeocystis globosa* in the central Arabian Sea, the *Phaeocystis* appears to be a new introduction to the Arabian Sea through the ballast tanks and could be expected to bloom in coastal waters that lead to the formation of foams on the beaches or affect food chains and fisheries in the future. The brackish seas of the Europe (Baltic Sea, Black Sea, Sea of Azov, and Caspian Sea) were subjected to intense invasion of non-indigenous species that exerted a significant impact upon community structure and functions, by modifying food chain resources, with direct and indirect effect on different human uses of the Sea (Paavola *et al*., 2005).

Taxa transported through ballast water were found to be ranging from unicellular algae to fish (Gollasch *et al*., 2002; David *et al*., 2007). Recently the phytoplankton species composition and abundance in ballast waters of ships in China was studied by Chen *et al*, (2009), who identified 239 species belonging to seven phyla. Yasakova (2010) reported the bioinvasion

of *Phaeocystis pouchetii* along with diatoms *Asterionellopsis glacialis* (Castor) Round, *Lioloma pacificum* (Capp) Hasle and dinoflagellates *Dinophysis odiosa* (Pavillard) Tai & Scogsberg, *Alexandrium ostenfeldii* (Pauls) Balech et Tangen, *Oxytoxum variabile* Schill, and *Gymnodinium stellatum* Hulburt through the ballast water of commercial ships during the period of 1998-2009 on the north eastern part of Black Sea. Development of new ballast water treatment technologies for reducing the number of organism are progressing, worldwide (Liebich *et al*., 2010)

2.3 Materials and Methods

2.3.1 Sampling of phytoplankton

Phytoplankton samples were collected from 10 stations in the Cochin estuary for the isolation and culturing of harmful algae. The details of the sampling sites are presented in the Table 2.1 and Figure 2.1. The stations were selected based on the ecological importance.

Table 2.1 Details of the sampling sites

Sl No	Stations	Position		Description
		Latitude	Longitude	
1	Eloor	$10^0$5’23”N	$76^0$16’49”E	Industrial belt
2	Varapuzha	$10^0$4’30”N	$76^0$16’48”E	Industrial belt
3	Vaduthala	$10^0$12’13”N	$76^0$15’50”E	Disposal of domestic waste
4	Bolgatty	$9^0$58’52”N	$76^0$15’50”E	Inland navigations and other tourism operations
5	Barmouth	$9^0$58’26”N	$76^0$14’39”E	Cochin Harbour entrance
6	Marine Science Jetty	$9^0$57’37”N	$6^0$16’54”E	Near to land area and site of domestic pollution
7	Fishing harbour	$9^0$56’47”N	$6^0$15’52”E	The fishing and processing unit operations
8	Thevara	$9^0$55’35”N	$76^0$17’53”E	Sewage outfall
9	Edakochi	$9^0$54’33”N	$76^0$18’35”E	Domestic sewage outfall
10	Puduvaippu	$9^0$59’26.1”N	$76^0$4’8.4”E	Mangrove area

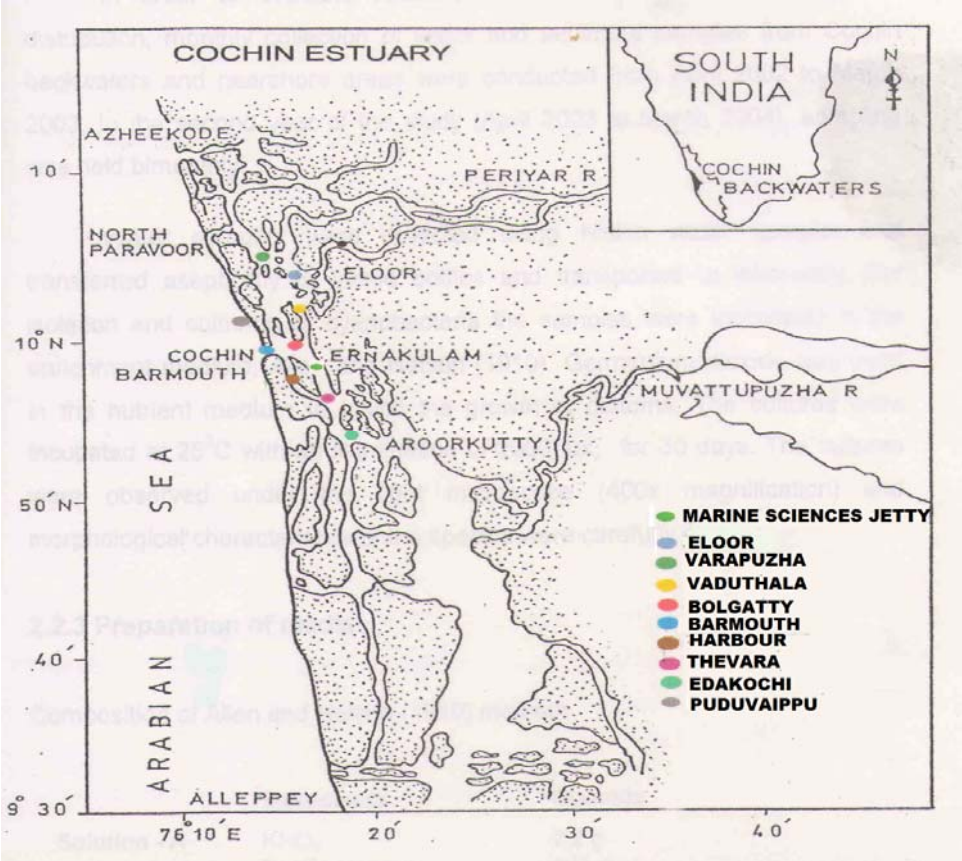

Figure 2.1 Map showing sampling sites in the Cochin estuary

Out of the ten stations Eloor, Varapuzha and Vaduthala recorded salinity near zero and hence are considered as freshwater regions, where as Bolgatty, Fishing harbour, Barmouth, Thevara, Edakochi and Marine Science Jetty are considered as saline areas with salinity ranging from 17-28ppt. Puduvaippu is one of the mangrove areas in Cochin. Sampling was done only once during the post-monsoon season (October – November, 2006).

2.3.2 Estimation of Hydrographical parameters

During the sampling of phytoplankton, water samples were collected from the ten stations using a Niskin water sampler and the hydrographical

parameters such as temperature (Standard mercury thermometer), salinity (Salinometer DIGI-AUTO model 3G, Tsurumi Seiko, Japan), pH (portable pH meter Perkin Elmer, accuracy ±0.01), dissolved oxygen (Winkler method, Strickland and Parson, 1972), Chlorophyll -a (Strickland and Parson, 1972), turbidity (Secchi-disc of 20cm in diameter), nitrate (Zhang and Fischer, 2006), nitrite (Strickland and Parson, 1972), phosphate (Strickland and Parson, 1972) and silicate (Strickland and Parson, 1972) were measured.

2.3.3 Statistical analysis

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

2.3.4 Collection of Phytoplankton sample

Phytoplankton samples were collected from Cochin estuary using phytoplankton net. 20 litres of water was allowed to flow through the net and the phytoplankton cells remained in the net was backwashed in to a sterile bottle with sterile seawater. The sample collected was transported to the laboratory. The samples were divided into two sections and one portion was inoculated in to Walne's medium (Walne, 1970) and f/2 medium (Guillard, 1975) which served as enrichment media, and incubated at 20^0C with an illumination of 2000 lux for 15 days. The other portion of the sample was preserved in 3% neutralized formaldehyde immediately after collection.

2.3.5 Qualitative estimation of the phytoplankton sample

The qualitative estimation of the preserved phytoplankton groups collected from the ten stations were done using Nikon E200 light microscope at 400X magnification. The phytoplankton groups present in the samples were determined at generic level from the ten stations (Desikachary, 1988; Thomas, 1996, 1997; Yamaguchi and Gould, 2007).

2.3.6 Preparation of culture medium

Sterile seawater of 25 ppt salinity was used for the preparation of the medium.

Composition of Walne's medium	
Trace metal solution	
$ZnCl_2$	2.1g
$CoCl_2.6H_2O$	2g
$CuSO_4.5H_2O$	2g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.9g
Distilled water	100ml
Vitamin solution	
Vit B_{12}	10mg
Vit B_1	10mg
Biotin	200µg
Distilled water	100ml
Nutrient solution	
$FeCl_3.6H_2O$	1.3g
$MnCl_2.4H_2O$	0.36g
H_3BO_3	33.6g
EDTA (Disodium salt)	45g
$NaH_2PO_4.2H_2O$	20g
$NaNO_3$	100g
Trace metal solution	1ml
Distilled water	1000ml

Nutrient solution was autoclaved and vitamin solution was sterilized by membrane filtration. 1 ml of nutrient solution and 0.1 ml of vitamin solution were added to 1000ml of sterilized seawater of 25ppt salinity for the preparation of the medium.

Composition of F/2 medium		
Trace metal solution	g/L	Quantity used per litre
$FeCl_3$. $6H_2O$	-	3.15g
Na_2EDTA. $2H_2O$	-	4.36g
$MnCl_2$. $4H_2O$	180	1ml
$ZnSO_4$. $7H_2O$	22	1ml
$CoCl_2$. $6H_2O$	10	1ml
$CuSO_4$. $5H_2O$	9.8	1ml
Na_2MoO_4. $2H_2O$	6.3	1ml
Vitamin solution	g/L	Quantity used per litre
Thiamine		200mg
Biotin	1	1ml
Cyanocobalamin	1	1ml
Final medium	g/L	Quantity used per litre
$NaNO_3$	75	1ml
NaH_2PO_4. H_2O	5	1ml
Na_2SiO_3. $9H_2O$	30	1ml
Trace metal solution		1ml
Vitamin solution		0.5ml

2.3.7 Preparation of clonal culture of phytoplankton

The qualitative analysis of the phytoplankton groups grown in the growth media from the ten stations was done using a phase contrast microscope (Nikon, Eclipse E200). Each phytoplankton groups that could be able to grow under laboratory conditions were isolated from the mixed culture of microalgae and were grown into single clonal culture for further study.

Mixed algal culture was made to single clonal culture by the following methods.

2.3.7.1 Single cell isolation by micropipette

A Pasteur pipette was heated in the flame, the tip of it was extended using forceps and the tip was broken in order to get a very small pore size and this was used for single cell isolation. Single algal cell was drawn out from a drop of algal culture under microscope using this micropipette and were released into sterile droplet of culture medium placed on a cavity slide. The process was repeated until a single cell, free of all other organisms could be confidently placed into sterile culture medium.

2.3.7.2 Isolation with the use of agar media

Agar plates were prepared using f/2 medium with 1.5% agar. A loop full of algal culture was placed on the agar and it was streaked over it using a nichrome wire loop. Then the plates were incubated at 20^0C in light for about 15 days. Individual colonies produced on the agar medium were picked up by a sterile loop and inoculated into sterile medium.

2.3.7.3 Dilution Techniques

Dilution blanks were prepared by adding 9ml of medium into 20 ml test tubes and sterilising the medium by autoclaving. I ml of mixed algal culture was added to the first test tube. Then serial dilutions were performed using the remaining test tubes, by transferring 1 ml from each tube, until a single cell was obtained.

Single cell culture of different microalgal groups was prepared by the above methods and out of which *Phaeocystis* sp. was selected for the study.

2.3.8 Identification of *Phaeocystis* species

Identification of the genus *Phaeocystis* was done based on the morphology, life cycle and culture characteristic of the alga. (Moestrup and Larsen, 1992; Baumann *et al*., 1994; Rousseau *et al*., 1994; Zingone *et al*., 1999; Jacobsen, 2000; Whipple *et al*., 2005; Barsanti and Gualtieri, 2006; Brodie and Lewis, 2007; Medlin and Zingone, 2007; Rousseau *et al*., 2007; Lee, 2008). The algal cells were observed under the compound microscope (Nikon E200) at 1000X magnification. Cell morphology, life cycle of the alga and culture characteristic were studied using phase contrast microscope at 400X magnification. Scanning electron microscopic images were taken to study the cell morphology in detail.

2.3.9 Sample preparation for Scanning electron microscopy (SEM)

Phaeocystis sp. cells growing exponentially in the Walne's medium were used for the SEM. Algal samples were centrifuged to separate algal cells from the culture filtrate. The cells were washed with filtered seawater for three times and were fixed with 2.5% glutraldehyde in distilled water and kept for 24 hours at 4^0C. The cells were again washed with distilled water and centrifuged for three times to remove the glutraldehyde completely. After final washing with distilled water, the samples were centrifuged to separate cells and the cells were dehydrated using alcohol. A series of alcohol treatments were given using 10% to 100% ethanol and after each alcohol treatment, the samples were centrifuged to separate the cells and the cells were finally placed in 100% ethanol and then dried overnight in a vacuum desiccator. The prepared samples were used for SEM and the images were taken at Sophisticated Test and Instrumentation Centre (STIC), Cochin University of Science and Technology.

2.4 Results

2.4.1 Hydrographical parameters

The various hydrographical parameters of the stations studied in the Cochin estuary were presented in Table 2.2

2.4.1.1 Temperature

The temperature observed in the various study areas was within the range of 27^0C (Station 10) to 30^0C (Station 6). One factor ANOVA and further comparison by Duncan's analysis revealed that station 10 significantly varied from the other stations and station 5 didn't show significant variation from station 2, but varied significantly from the remaining stations. The remaining stations didn't show any significant difference between each other.

2.4.1.2 Salinity

The salinity of various stations was in the range of 1ppt to 27ppt. Salinity was very low, almost reaching to fresh water condition at station 1 and station 2. All other stations were characterised by high salinity. Maximum salinity was observed in station 5. Station 6 and station 7 also showed high salinity of 25 and 26ppt. Salinity varied significantly from station to station except between station 1 and 2, and stations 4 and 9. There was a significant increase in salinity in the order, station (1, 2) < 3 < (4, 9) < 8 < 10 < 6 < 7 < 5.

2.4.1.3 pH

The average pH values obtained from various study areas were in the range of 6.5 to 7.9. Fresh water areas (station 1 and 2) showed slightly acidic pH, whereas other stations (stations 4, 5, 6, 7, 8, 9, 10) recorded slightly alkaline pH. The maximum value of surface pH recorded during the study was 7.9 in station 6. A neutral pH was observed in station 3.

Statistical analysis showed that station 1, 2, and 3 did not vary significantly in pH value, but these stations showed significant variation from all other stations.

2.4.1.4 Dissolved oxygen

The average DO value in the stations was in the range of 1.6 to 4.6ml/L. Dissolved oxygen always showed a lower value (1.6ml/L) at station 1(Eloor), which is an industrial belt. The maximum value was recorded at station 2. There was not much variation in the level of dissolved oxygen at other stations, except station 1. Station 1 varied significantly from other stations in its DO value.

2.4.1.5 Euphotic depth

The depth of light penetration at different stations of Cochin estuary ranged between 0.7m to 1.1m. Maximum light penetration was at station 4 (Bolgatty) and minimum at station 6 (Marine science jetty). Statistical analysis showed a significant increase in euphotic depth in the order, stations (6, 8, 10, 7, 5) < (2, 1) < 3 < 9 < 4.

2.4.1.6 Chlorophyll a

The quantity of the photosynthetic pigment ranged between 3.95µg/L and 71.9µg/L. Station 10 was strikingly different from other stations as it showed high chlorophyll-a content. Minimum chlorophyll-a value was observed at station 2. Statistical analysis by one way ANOVA showed that station 10 has significantly higher (p<0.05) productivity than the remaining stations. There was no significant variation between stations (2, 5, 4,), (5, 4, 1), (1, 3), (7, 6). Stations 8, 9,10 showed significant variation from all other stations.

Table 2.2 Hydrographical parameters of stations studied in Cochin estuary

Hydrographical parameters	Stations									
	1 Eloor	2 Varapuzha	3 Vaduthala	4 Bolgatty	5 Barmouth	6 Marine Sciences Jetty	7 Fishing Harbour	8 Thevara	9 Edakochi	10 Puduvaippu
Salinity (ppt)	1.5 ± 0.5^{a}	1.8 ± 0.26^{a}	7.5 ± 0.5^{b}	17.6 ± 0.5^{c}	27.9 ± 0.36^{h}	26.17 ± 0.76^{f}	26.6 ± 0.55^{g}	18.97 ± 0.87^{d}	17.78 ± 0.25^{c}	21.57 ± 0.4^{e}
Temperature (^{0}C	29.5 ± 0.47^{cd}	28.9 ± 0.3^{bc}	29.5 ± 0.5^{cd}	29.5 ± 0.4^{cd}	28.7 ± 0.3^{b}	29.7 ± 0.6^{d}	29.8 ± 0.2^{d}	29.87 ± 0.15^{d}	29.6 ± 0.5^{cd}	27.7 ± 0.6^{a}
pH	6.6 ± 0.4^{a}	6.5 ± 0.1^{a}	7.09 ± 0.1^{ab}	7.9 ± 0.3^{c}	7.7 ± 0.7^{bc}	8.0 ± 0.2^{c}	7.8 ± 0.2^{bc}	7.5 ± 0.2^{bc}	7.6 ± 0.3^{bc}	7.7 ± 0.6^{bc}
DO (ml/L)	1.63 ± 0.3^{a}	4.64 ± 0.54^{b}	4.2 ± 0.3^{b}	3.76 ± 0.6^{b}	3.37 ± 1.1^{b}	3.67 ± 0.76^{b}	3.23 ± 0.47^{b}	4.05 ± 0.75^{b}	4.09 ± 1.15^{b}	4.24 ± 0.9^{b}
Depth (m)	0.94 ± 0.15^{bcde}	0.93 ± 0.04^{bcde}	1.04 ± 0.06^{cde}	1.11 ± 0.11^{e}	0.92 ± 0.07^{abcd}	0.73 ± 0.08^{a}	0.86 ± 0.12^{abc}	0.77 ± 0.12^{ab}	1.06 ± 0.13^{de}	0.82 ± 0.08^{ab}
Chl-a (μg/L)	6.29 ± 0.86^{bc}	3.95 ± 0.9^{a}	7.72 ± 0.88^{cd}	5.55 ± 0.58^{ab}	4.4 ± 0.2^{ab}	10.4 ± 0.2^{e}	8.58 ± 0.56^{ef}	12.76 ± 0.38^{f}	15.52 ± 1.35^{g}	71.9 ± 2.7^{h}
Nitrite (μmol/L)	1.47 ± 0.06^{c}	0.58 ± 0.07^{ab}	0.85 ± 0.09^{ab}	0.74 ± 0.11^{ab}	0.54 ± 0.05^{b}	0.92 ± 0.32^{b}	0.53 ± 0.17^{a}	0.73 ± 0.28^{ab}	0.59 ± 0.22^{ab}	0.64 ± 0.30^{ab}
Nitrate (μmol/L)	43.2 ± 5.94^{e}	14.8 ± 0.91^{d}	8.8 ± 0.60^{c}	4.83 ± 0.65^{b}	5.49 ± 0.36^{b}	9.4 ± 0.62^{c}	3.78 ± 0.45^{ab}	3.58 ± 0.25^{ab}	4.2 ± 0.30^{b}	0.58 ± 0.27^{a}
Phosphate(μmol/L)	3.29 ± 0.56^{cd}	4.4 ± 1.20^{d}	2.39 ± 1.15^{abc}	1.66 ± 0.77^{abc}	0.85 ± 0.35^{a}	2.83 ± 1.25^{bcd}	1.28 ± 0.72^{ab}	1.89 ± 0.7^{abc}	1.58 ± 1.08^{ab}	9.5 ± 0.6^{e}
Silicate (μmol/L)	28.05 ± 15.5^{a}	22.3 ± 15.4^{a}	33.92 ± 21.5^{a}	20.62 ± 10.1^{a}	18.64 ± 5.1^{a}	14.4 ± 5.5^{a}	30.4 ± 11^{a}	$34.1 \pm 11a$	34.4 ± 10.5^{a}	26.07 ± 4.0^{a}

2.4.1.7 Nitrite

Nitrite originates in water by the reduction of nitrates and by the conversion of ammonia into nitrites. Nitrite concentration was maximum at station 1 (1.4µmol/L) and minimum at station 5 and 7 (0.53µmol/L). Statistical analysis showed that nitrite concentration of station 1 significantly varied from other stations.

2.4.1.8 Nitrate

The nitrate value ranged between 0.57 to 43.2µmol/L with maximum value at station 1 (Eloor) and minimum at station 10 (Puduvaippu). There was a gradual decrease in the nitrate value from station 1 towards station 10, that is from the northern part of estuary towards the south, except that there was a slight increase at station 6, Marine Science Jetty. The nitrate concentration in different stations was in the order (10, 8, 7) < (9, 4, 5) < (3, 6) < 2 < 1.

2.4.1.9 Phosphate

Inorganic phosphate content in surface waters varied from the minimum of 0.85µmol/L (station 5) to the maximum of 9.5µmol/L (station 10). The highest value of phosphate was observed from the mangrove region (station 10). Statistical analysis revealed that station 5 significantly varied from stations 6, 1, 2 and 10.

2.4.1.9 Silicate

Silicate content in surface water varied from a maximum value of 34.1µmol/L at station 8 and minimum value of 14.3µmol/L at station 6. One way ANOVA and subsequent analysis revealed that there was no significant variation in silicate content between the stations

2.4.2 Distribution of phytoplankton

The qualitative distribution and spatial variation of phytoplankton along the Cochin estuary was studied. During the study phytoplankton groups belonging to 67 genera were recorded from the ten stations. Phytoplankton groups identified mainly belong to Bacillariophyceae (32 genera), Chlorophyceae (17 genera), Dinophyceae (9genera), Cyanophyceae (8 genera) and Xanthophyceae (1 genus). Unidentified species were also recorded from many stations. List of phytoplankton identified from each station is given in Tables 2.3a to 2.3 j.

Table 2.3a Distribution of phytoplankton at station 1

Station 1 (Eloor)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Caloneis sp.	*Closteriopsis* sp.		*Protoperidinium* sp.
Coscinodiscus spp.	*Pediastrum* sp.		
Fragilaria sp.	*Tetraedon* sp.		
Leptocylindrus sp.	*Ulothrix* sp.		
Gyrosigma sp.			
Navicula spp.			
Skeletonema spp.			
Thalassiosira sp.			

Table 2.3b Distribution of phytoplankton at station 2

Station 2 (Varapuzha)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Amphora sp.	*Ankistrodesmus* sp.	*Anabaena* sp.	*Protoperidinium* sp.
Asterionella sp.	*Pediastrum* sp.	*Lyngbya* sp.	
Biddulphia sp.	*Planctonema* sp.	*Oscillatoria* sp.	
Caloneis sp.	*scenedesmus* sp.		
Chaetoceros sp.	*Staurastrum* sp.		
Coscinodiscus spp.	*Tetraedon* sp.		
Cyclotella sp.			
Fragilaria sp.			
Navicula spp.			
Pinnularia sp.			
Pleurosigma sp.			
Skeletonema sp.			
Thalassiosira sp.			
Triceratium sp.			
Unidentified spp.			

Table 2.3c Distribution of phytoplankton at station 3

Station 3 (Vaduthala)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Amphora sp.	*Cosmarium* sp.	*Anabaena* sp.	*Protoperidinium* sp.
Campylodiscus sp.	*Oedocladium* sp.	*Aphanizomenon* sp.	
Chaetoceros sp.	*Planctonema* sp.	*Oscillatoria* sp.	
Coscinodiscus spp.	*Tetraedon* sp.		
Fragilaria sp.	*Ulothrix* sp.		
Leptocylindrus sp.			
Navicula spp.			
Pinnularia sp.			
Pleurosigma sp.			
Skeletonema sp.			
Thalassiosira sp.			
Thalassiothrix sp.			
Triceratium sp.			

Table 2.3d Distribution of phytoplankton at station 4

Station 4 (Bolgatty)				
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**	**Xanthophyceae**
Amphora sp.	*Characium* sp.	*Anabaena* sp.	*Diplosalis* sp.	*Botrydium* sp.
Asterionella sp.	*Cosmarium* sp.	*Oscillatoria* sp.		
Biddulphia sp.	*Planctonema* sp.			
Coscinodiscus spp.	*Ulothrix* sp.			
Gyrosigma sp.				
Navicula spp.				
Pleurosigma sp.				
Skeletonema sp.				
Thalassiosira sp.				
Unidentified spp.				

Table 2.3e Distribution of phytoplankton at station 5

Station 5 (Barmouth)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Amphora sp.	*Ankistrodesmus* sp.	*Lyngbya* sp.	*Ceratium* sp.
Biddulphia sp.	*Tetraedon* sp.	*Oscillatoria* sp.	*Protoperidinium* sp.
Coscinodiscus spp.		*Phormidium* sp.	
Chaetoceros sp.			
Cylindrotheca sp.			
Gyrosigma sp.			
Navicula spp.			
Pleurosigma sp.			
Podocera sp.			
Skeletonema sp.			
Thalassionema sp.			
Thalassiosira sp.			
Unidentified spp.			

Table 2.3f Distribution of phytoplankton at station 6

Station 6 (Marine Sciences Jetty)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Amphora sp.		*Anabaena* sp.	*Ceratium* sp.
Bacteriastrum sp.		*Oscillatoria* sp.	*Dinophysis* sp.
Biddulphia sp.			*Gonyaulax* sp.
Chaetoceros sp.			*Gymnodinium* sp.
Coscinodiscus spp.			
Gyrosigma sp.			
Navicula spp.			
Pleurosigma sp.			
Skeletonema sp.			
Thalassionema sp.			
Unidentified sp.			

Table 2.3g Distribution of phytoplankton at station 7

Station 7 (Fishing Harbour)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Amphora sp.	*Cosmarium* sp.	*Aphanocapsa* sp.	*Alexandrium* sp.
Asterionella sp.	*Pediastrum* sp.	*Gleotheca* sp.	*Ceratium* sp.
Biddulphia sp.	*Staurastrum* sp.	*Oscillatoria* sp.	*Protoperidinium* sp.
Chaetoceros sp.		*Phormidium* sp.	
Coscinodiscus spp.			
Diploneis sp.			
Gyrosigma sp.			
Leptocylindrus sp.			
Navicula spp.			
Nitzschia sp.			
Skeletonema sp.			
Thalassionema sp.			
Thalassiosira sp.			

Table 2.3h Distribution of phytoplankton at station 8

Station 8 (Thevara)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Amphora sp.	*Ankistrodesmus* sp.	*Anabaena* sp.	*Ceratium* sp.
Asterionella sp.	*Cosmarium* sp.	*Oscillatoria* sp.	*Noctiluca* sp.
Biddulphia sp.	*Planctonema* sp.		*Protoperidinium* sp.
Chaetoceros sp.			
Coscinodiscus spp.			
Diploneis sp.			
Gyrosigma sp.			
Navicula spp.			
Nitzschia sp.			
Pleurosigma sp.			
Skeletonema sp.			

Table 2.3i Distribution of phytoplankton at station 9

Station No.9 (Edakochi)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Amphora sp.	*Ankistrodesmus* sp.	*Nostoc* sp.	
Biddulphia sp.	*Chlamydomonas* sp.	*Oscillatoria* sp.	
Caloneis sp.	*Closterium* sp.		
Campylodiscus sp.	*Cosmarium* sp.		
Chaetoceros sp.	*Genecularia* sp.		
Coscinodiscus spp.	*Pediastrum* sp.		
Cymbella sp.	*Planctonema* sp.		
Dictylum sp.	*Tetrastrum* sp.		
Eucampia sp.	*Ulothrix* sp.		
Fragilaria sp.			
Gyrosigma sp.			
Melosira sp.			
Nitzschia sp.			
Pleurosigma sp.			
Skeletonema sp.			
Thalassionema sp.			
Thalassiothrix sp.			
Unidentified spp.			

Table 2.3j Distribution of phytoplankton at station 10

Station 10 (Puduvaippu)			
Bacillariophyceae	**Chlorophyceae**	**Cyanophyceae**	**Dinophyceae**
Asterionella sp.	*Ankistrodesmus* sp.	*Lyngbya* sp.	*Ceratium* sp.
Bacteriastrum sp.	*Closterium* sp.	*Oscillatoria* sp.	*Dinophysis* sp.
Biddulphia sp.	*Genecularia* sp.		*Protoperidinium* sp.
Chaetoceros sp.	*Pediastrum* sp.		
Coscinodiscus spp.	*Planctonema* sp.		
Cyclotella sp.	*Tetraedon* sp.		
Navicula spp.	*Tetrastrum* sp.		
Podocera sp.			
Rhizosolenia sp.			
Skeletonema sp.			
Thalassionema sp.			
Thalassiosira sp.			
Unidentified spp.			

2.4.3 Isolation and purification of phytoplankton

Initial inoculation of water sample from the ten stations into two media, Walne's and f/2 gave a mixed culture of microalgae. By 15th day of inoculation, the media turned in to light brown colour indicating the growth of phytoplankton. Several species which could not be identified in the preserved samples also have grown in the mixed culture along with the identified algae. From the mixed culture of microalgae, single cell cultures of various phytoplankton groups were prepared under laboratory conditions. Table 2.4 shows the list of phytoplankton groups that have grown under laboratory condition in the growth media provided.

Table 2.4 List of clonal culture of phytoplankton grown under laboratory conditions

Bacillariophyceae	Dinophyceae	Haptophyceae
Asterionella sp.	*Ceratium* sp.	*Phaeocystis* sp.
Bacteriastrum sp.	*Gymnodinium* sp.	
Biddulphia sp.	*Noctiluca* sp.	
Chaetoceros sp.	*Protoperidinium* sp.	
Coscinodiscus sp.		
Gyrosigma sp.		
Navicula sp.		
Pleurosigma sp.		
Rhizosolenia sp.		
Skeletonema sp.		

During the analysis of preserved samples, *Phaeocystis* sp. could not be observed. It might be due to changes in the morphology of the organism during preservation. Many of the unialgal cultures could not be maintained under laboratory conditions for a long period as they perished after a few generations. Out of the successful cultures, *Phaeocystis* sp. was selected for the study considering its ecological importance in the natural ecosystems. The *Phaeocystis* sp. culture was obtained from samples collected from station 5 (Barmouth) and station 6 (Marine Sciences Jetty).

2.4.4 Identification of *Phaeocystis* species

Microscopic examination of the organism showed distinctive characters of the genus *Phaeocystis.* Images of *Phaeocystis* sp. single cells and colony cells are shown in PLATE 2 to PLATE 8.

Characters observed in the microalga

1) Cell polymorphism was observed in the algal culture with three types of cells observed in the microscopic field. Solitary non-motile cells with a spherical shape and an average size of 4-8µm in diameter, swimming cells with the cell size smaller than the non-motile cells and with a slight invagination on one side of the cell (heart shaped) for the insertion of two flagella and the colonies as loose aggregates of non-motile cells with no external layer or specific shape. Mucilage content in the colonies was very low. The colonies were cloud shaped (without a regular shape) with cells in packets of four or five. The size of colony increased along with the culture period, that is, from 12µm to 80µm in diameter.

2) Sequential development of colony from free-living cell dividing into two and then getting arranged in packets of four cells were observed. Several cell packets were then arranged together to form large cloud shaped colonies with lobes of four cells. The colonies underwent disruption in the later culture periods and were released into single solitary cells.

3) The alga showed a complex life cycle characterised by the alternance between different free-living cells and mucilaginous colonies of non-motile coccoid cells. The observations of the algal culture from the initial phase showed that the growth of the algae in the culture started as single non motile cell in the lag phase, then the cells underwent division and colonies were formed. The colonies were shown to vary widely in shape and size from the logarithmic growth phase towards the stationary

phase. The colony size increased along the logarithmic phase of the growth period of alga. Towards the stationary phase, the colonies were found disintegrating and single non-motile cells were seen in the culture. At this stage, the number of swimming cells in the culture increased and could be seen as motile flagellated cells moving in the microscopic field. These alternations of cells were observed in all cultures in the laboratory.

4) The cells had two golden brown parietal chloroplast which gave them golden brown colour with a small bright orange body in the space between the chloroplast. Body scales were observed in SEM images. Haptonema was present in the flagellated cells of the alga where it was absent in the non motile solitary cells and colony cells.

5) The live culture of the alga showed a brown colour with definite growth characteristics with an initial lag phase and a logarithmic growth phase, and finally stationary phase and decline phase which could be clearly observed by the intensity of the colour of the algal culture.

PLATE 1 *Phaeocystis* sp. culture in the environmental chamber

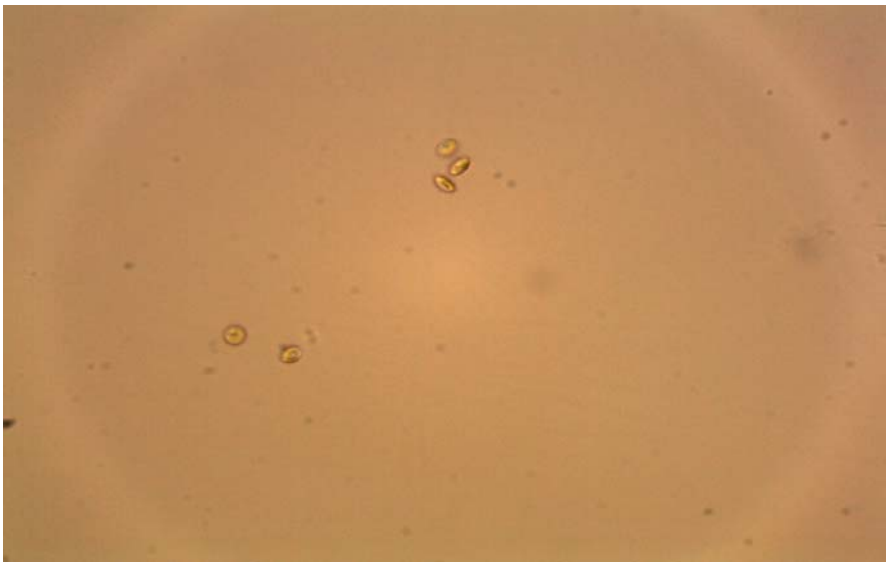

PLATE 2 Solitary cells of *Phaeocystis* sp. and initial stage of colony formation.

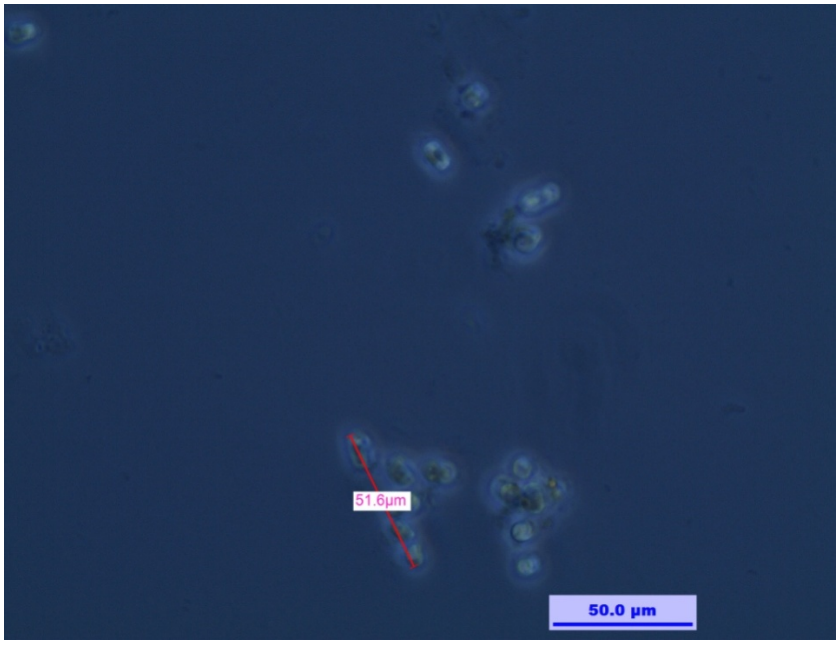

PLATE 3 Cloud shaped colonies of *Phaeocystis* sp.

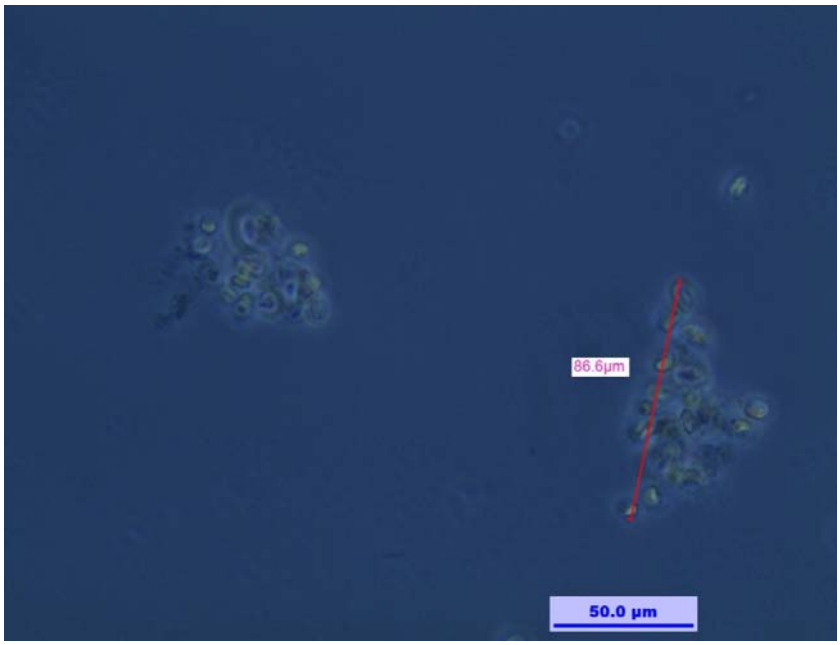

PLATE 4 Colonies of *Phaeocystis* sp. increasing in size

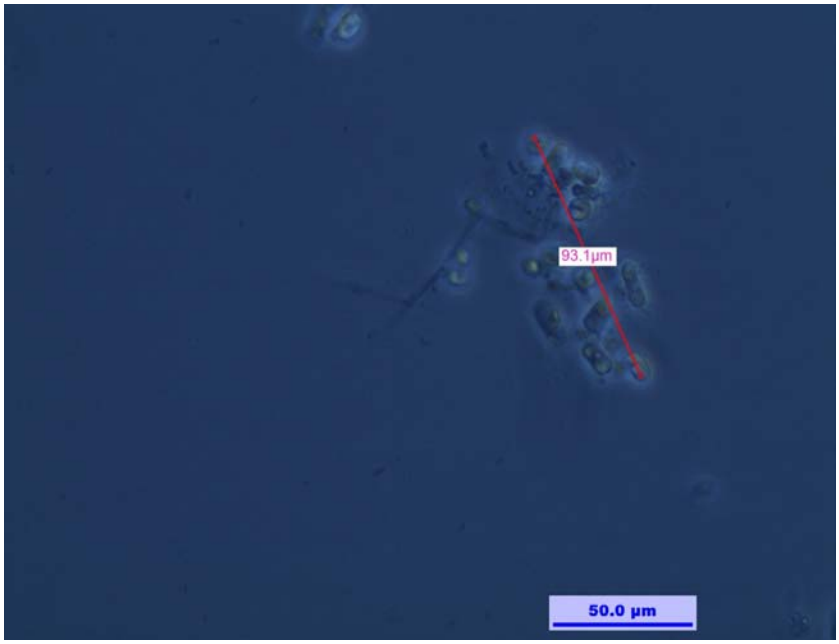

PLATE 5 Disintegration of *Phaeocystis* sp. colonies at the end of the logarithmic phase

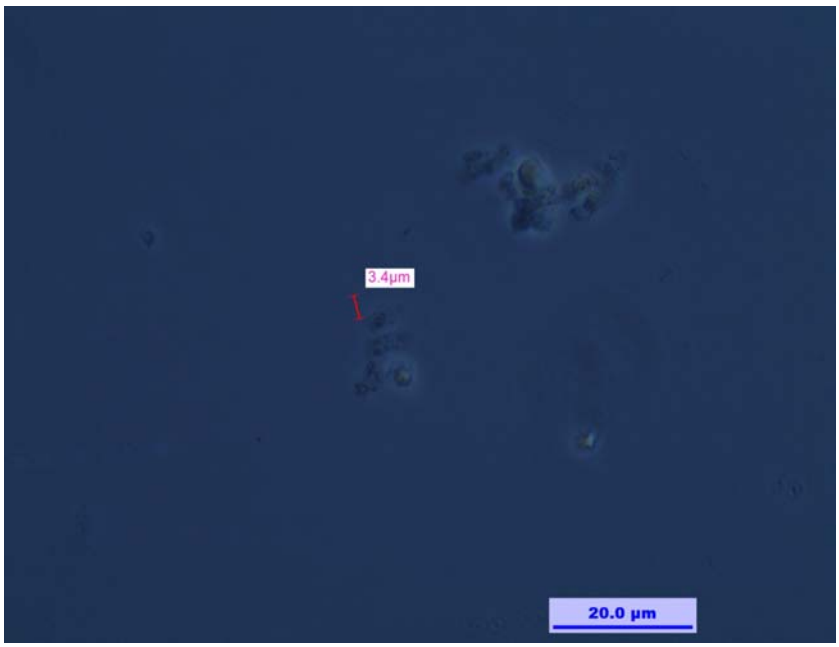

PLATE 6 Disintegrated colonies of *Phaeocystis* sp. and solitary cells released from the colonies

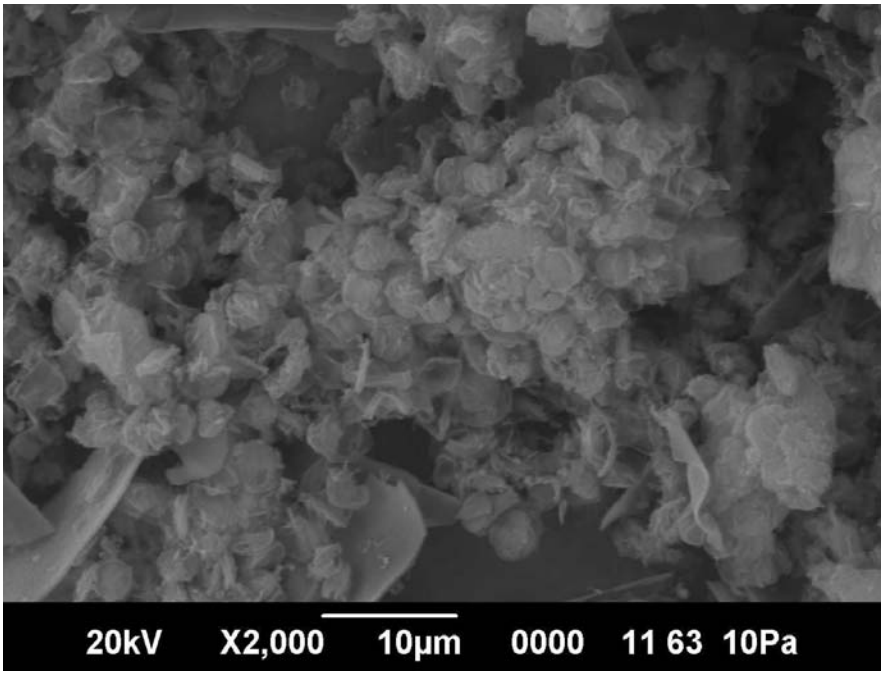

PLATE 7 SEM image of *Phaeocystis* sp. colonies

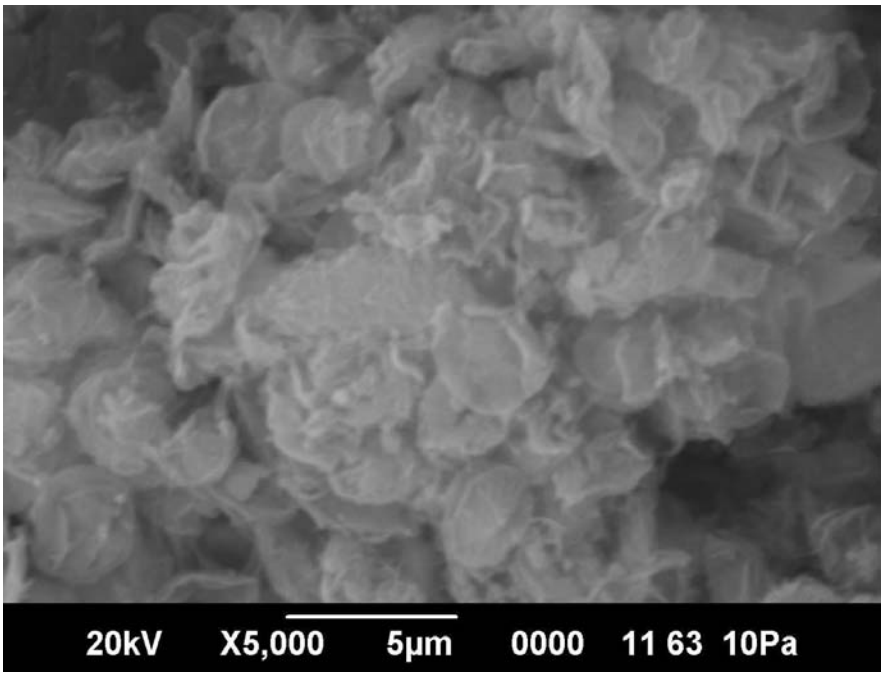

PLATE 8 SEM image of *Phaeocystis* sp. colonies

2.5 Discussion

In the present study, phytoplankton sampling was done with the main objective of isolation of an ecologically important alga from Cochin backwaters- one of the important tropical estuaries. The culturing of the phytoplankton sample collected from various stations in the estuary resulted in the growth of many important algal groups in the mixed culture and out of it the clonal culture of the *Phaeocystis* sp. was specially developed to study its growth characteristics, condition leading to bloom formation and its toxicity if any.

Several studies have been conducted in the Cochin estuary on the distribution of phytoplankton (Subramanyan 1946, 1958, 1959; Subramanyan *et al*., 1960 and 1965; Gopinathan, 1972; Devassy and Bhattathiri, 1974; Joseph and Pillai, 1975; Gopinathan, 1984; Jayalakshmi *et al*., 1986; Jagadheeshan, 1986; Selvaraj *et al*., 2003; Qasim 2004). The observations on the distribution of phytoplankton at various stations of Cochin estuary in the present study were in agreement with the earlier works, and major groups identified include diatoms, dinophytes, cyanophytes and chlorophytes. *Skeletonema* and *Navicula* were found to be dominant in the majority of stations studied. *Phaeocystis* was not isolated and reported in any of the previous phytoplankton studies in the Cochin estuary. Information regarding the geographical distribution of the *Phaeocystis* was mainly based on the presence of colonial stages. The probability of recognizing the flagellate stages in fixed samples is quite low because of the poor preservation. Furthermore, the colonies of *Phaeocystis* sp. were easily disrupted on preservation of algal samples. Observing the flagellated cells or the non motile solitary cells of the *Phaeocystis* in the phytoplankton samples is very difficult. In the present study also,

Phaeocystis was not observed in the preserved samples probably due to disruption of cells. *Phaeocystis* was observed only when the algal cultures were grown in the laboratory. Among the stations studied, two stations, Marine Science Jetty and Barmouth showed the presence of *Phaeocystis.*

The hydrographical conditions of the stations showed an average temperature of 30^0C, salinity 26ppt and slightly alkaline pH. Nutrients were present in the stations in the range of nitrite 0.73 to 0.92µm/L, nitrate 5.4 to 9.4µm/L, phosphate 1.2 to 4.1µm/L, and silicate 14 to 18µm/L. The concentration of nutrients in the estuary indicated large waste water inputs from industrial units and agricultural runoffs. High values of nutrients in the stations indicate the sign of eutrophication in the estuary.

The taxonomy of Prymnesiophyte is based mainly on the morphological characteristics. The dominant life cycle stage, cell size, length of flagella and haptonemal behaviour are considered as the key features for its identification. Different classification schemes have been proposed in the literature (Chretiennot-Dinot, 1999; Jordan and Gree, 1994). *Phaeocystis*, with its unusual and complex life cycle is unique within the Prymnesiophyceae and warrants the creation of the order Phaeocystales (Edvarsen *et al*., 1999). The retention of the family Phaeocystaceae was recognized in all earlier systematic schemes (Jordan *et al*., 1995).

The size of the flagellated and colonial cell types observed in the study is within the range of cell size reported for the genus *Phaeocystis* (Kornmann, 1955; Baumann *et al*., 1994; Medlin *et al*., 1994; Peperzak *et al*., 2000b).

Out of the species in the genus *Phaeocystis*, only 4 species were observed to form colonies, *Phaeocystis pouchetii*, *Phaeocystis globosa*, *Phaeocystis antarctica* and *Phaeocystis jahnii* (Baumann *et al*., 1994; Medlin and Zingone, 2007). The organism isolated in the study was observed to form colonies under laboratory condition with a size range of 10µm to 90µm. The colony size of the natural blooms of *Phaeocystis* reach up to 2mm in size. The complex life cycle, i.e. alternation of free-living cells with non-motile colonies observed in the present study was in agreement with the life cycle of the genus *Phaeocystis* as reported by many workers (Kornmann, 1955; Baumann *et al*., 1994; Medlin *et al*., 1994; Peperzak *et al*., 2000b; Medlin and Zingone, 2007). The *Phaeocystis* culture exhibited three stages in its life cycle. Non-motile single cells were observed in the earlier stages of growth. These cells underwent division to produce a chain of cells and then got arranged in packet of four cells irregularly without a definite colony envelope. According to Baumann *et al*. (1994) the diploid non-motile cells act as a stage of vegetative growth of the alga where it divides and form colonies. Later these colonies grew in size by cell division. The colony shape of the alga isolated in the present study varied from the colonies of *Phaeocystis globosa*, *Phaeocystis antarctica* (spherical colonies) and *Phaeocystis pouchetii* (cloud shaped colonies with defined colony wall).

The colonies formed by the alga, showed close similarity to the colonies of *Phaeocystis jahnii* , where the colonies were found as lacking a definite shape and a regular arrangement of cells as well as a visible external envelope (Zingone *et al*., 1999). In the SEM images, the colony cells were found to have an external scale arrangement, as in the case of

Phaeocystis jahnii, which have scale investments in the colonial cells (Zingone *et al*., 1999).

Studies on *Phaeocystis* has revealed that the colony formation occurs from cells which are produced by the syngamy of different mating types of haploid microzoospores which occur as flagellated cells released from the colony during the colony senescence (Rousseau *et al*., 2007). According to the study of Vaulot *et al*. (1994) the colonies were produced solely due to the presence of different mating zoospores in the culture itself. Here in the present study also the flagellated cells were found during the colony senescence which was smaller than the colony cells and non motile single cells.

Another common feature of the flagellated cells of the *Phaeocystis* species is the formation of filaments extruded by the cells in a characteristic pattern of five or nine rayed stars. These filaments mainly function as anchors for the attachment to the *Chaetoceros* setae or other solid structure giving stability for the formation of colonies and also as a defence mechanism against predations (Chretiennot-Dinet, 1999). Such filaments were not observed in the present algal culture. The filaments were not observed in the laboratory culture of *Phaeocystis jahnii* also (Zingone *et al*., 1999). Since the alga was cultured under laboratory as single species, in the absence of predation, the filaments may get degenerated leading to their complete absence in cultures without grazing pressure.

The importance of *Phaeocystis* increases as an example for bioinvasion, and the bioinvasion of *Phaeocystis* was reported in various ecosystems around the world (Yasakova, 2010). Blooms of a novel organism *Cochlodinium polykreikoides* reported along the Kerala coast in

September, 2004 was also considered as an example of bioinvasion (Ramaiah *et al.,* 2005). Madhupratap *et al.* (2000) who first reported *Phaeocystis* blooms from the Arabian Sea considered *Phaeocystis* as a bioinvasive species. The genus was introduced into the Arabian Sea through ballast water and the organism got adapted to the new environment. The frequency of harmful algal blooms is increasing worldwide along with novel occurrence of many algal species indicating the global spreading of the HAB species. These include diatoms, dinoflagellates, cyanobacteria, and prymnesiophytes including *Phaeocystis* (Smayda, 1997). The ecological importance of the bioinvasion of *Phaeocystis* to our ecosystem has to be studied in detail in the light of the global spreading of HAB species.

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Chapter 3

CULTURE CHARACTERISTICS OF *PHAEOCYSTIS* SPECIES

Contents

3.1 Introduction

Phaeocystis blooms were reported from a wide variety of ecosystems. Situations causing the bloom are characterised by high nutrient concentrations, high mixing, stratification, mesotrophy, and grazing. The widespread *Phaeocystis* blooms in polar, sub polar and temperate waters and the factors determining the fate of massive accumulation of suspended biomass during these blooms are important for understanding the ecology of this genus as well as the carbon and biogeochemical element cycles of ecosystems dominated by it.

Algal culture is an artificial environment in which the algae grow and the culture conditions should resemble the alga's natural environment as far as possible. After isolation from the natural environment, algal strains are maintained under artificial conditions of media, light, and temperature (Richmond, 2004). A culture has three distinct components: a culture medium contained in a suitable vessel, the algal cells growing in the medium and air to allow exchange of carbon dioxide between medium and atmosphere. For the culturing of autotrophic alga, all that is needed for growth is light, CO_2, water, nutrients, and trace elements. By means of

photosynthesis the alga will be able to synthesize all the biochemical compounds necessary for growth. Only a minority of algae is, however, entirely autotrophic; many are unable to synthesize certain biochemical compounds (certain vitamins) and will require these to be present in the medium. The most important parameters regulating algal growth are nutrient quantity and quality, light, pH, turbulence, salinity, and temperature. The most optimal parameters as well as the tolerated ranges are species specific and the various factors may be interdependent and a parameter that is optimal for one set of conditions is not necessarily optimal for another (Barsanti and Gualtieri, 2006).

The temperature at which cultures are maintained should ideally be as close as possible to the temperature at which the organisms grow in the natural environment. Light is the source of energy which drives photosynthetic reactions in algae. Light intensity plays an important role, and it varies with the culture depth and the density of the algal culture. The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2–8.7, though there are species that dwell in more acidic/basic environments. Complete culture may collapse due to the disruption of many cellular processes that can result from a failure to maintain an acceptable pH. Mixing is necessary to prevent sedimentation of the algae, to ensure all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification and to improve gas exchange between the culture medium and the air. Culture vessels using for the algal culture should be non-toxic (chemically inert), reasonably transparent to light, easily cleaned and sterilized, and provide a large surface to volume ratio. Recommended materials for culture vessels and media preparation include teflon, polycarbonate, polystyrene, and borosilicate glass (Andersen, 2005).

Another important factor is the culture media which provide the necessary nutrients for the growth of algae. Media can be classified as being defined or undefined. Defined media, which are often essential for nutritional studies, have constituents that are all known and can be assigned a chemical formula. Undefined media, on the other hand, contain one or more natural or complex ingredients, for example, liver extract or seawater, the composition of which is unknown and may vary. Natural seawater is mainly used for the culture of marine and estuarine algae. The quality of coastal water may be improved by ageing for a few months at 4^0C (allowing bacterial degradation), and by filtering through acid-washed charcoal (which absorbs toxic organic compounds).

Nitrate is often the nutrient that first limits primary production in the marine and freshwater habitats and the source of nitrogen is very important in the culture media. Most of the algae can utilize either ammonium salts, or nitrates when these are supplied in suitable concentrations in the media. Low concentration of nitrogen leads to the decrease of chlorophyll in the cell and subsequently the rate of photosynthesis will also get decreased. Phosphorous is another major element required for the normal growth of algae and it is assimilated mainly in the form of inorganic orthophosphate. Compounds containing phosphorus play an important role in metabolism, particularly energy transformation reactions. The pH of the medium alters the rate of phosphate uptake either by a direct effect on the permeability of cell membrane or by changing the ionic form of the phosphate. The trace metals that are essential for microalgal growth are incorporated into essential organic molecules, particularly a variety of coenzyme factors that enter into photosynthetic reactions. Of these metals, the concentrations of Fe, Mn, Zn, Cu and Co (and sometimes Mo and Se) in natural waters may

be limiting to algal growth. Both N and P should be co-managed in the development of strategies to minimize HAB. Other nutrients such as silicate and iron also can significantly influence the outcome of species dominance and the structure and abundance of phytoplankton communities under cultural eutrophication. Iron is the constituent of many enzymes and of cytochromes and certain other porphyrins. Among all the known vitamins and growth factors, only vitamin B_{12}, thiamine and biotin have been found to be of importance for algae. The assimilation of nutrients by phytoplankton depends on environmental factors such as light, temperature and water column stability.

When algae are grown in limited culture media, the algae pass though four stages of growth. The lag phase, where the growth rate is zero and the growth is attributed to the physiological adaptation of the cell metabolism to growth, such as the increase of the levels of enzymes and metabolites involved in cell division and carbon fixation. During exponential phase or steady state of growth, all the cell constituents are synthesised in constant proportion and the cell number increases at logarithmic rate. After a short logarithmic growth phase, the cells enter into stationary phase, where the limiting factor and the growth rate are balanced, which results in a relatively constant cell density. During the decline phase, water quality deteriorates and nutrients are depleted to a level incapable of sustaining growth. Cell density decreases rapidly and the culture eventually collapse. (Andersen, 2005).

The blooming success of *Phaeocystis* is due to the ability to form large gelatinous colonies. The colony matrix which acts as energy and nutrient reservoir gives a competitive advantage to *Phaeocystis* when resources are scarce or highly fluctuating. On the other hand, the ability of

Phaeocystis to resist infection, bacterial colonisation, the increased viscosity of seawater and the resistance of *Phaeocystis* colonies to mesozooplankton grazing further adds to the blooming success of the genus. The formation of colonies from solitary cells and the subsequent emigration of cells from the colonies have been observed in culture, but the induction and regulation of these processes are poorly understood.

With a view to understanding the conditions which results in the blooming of this alga, the culture parameters of *Phaeocystis* sp. such as salinity, pH, temperature and nutrients were studied under laboratory conditions. The effect of nitrate, phosphate and iron concentration on the colony formation and in the life cycle of *Phaeocystis* sp. was also investigated.

3.2 Review of Literature

3.2.1 Physiochemical factors influencing the growth and physiology of microalgae

Growth of microalgae is affected by various factors such as nutrients, temperature, salinity, pH etc. In estuarine systems the algal growth was primarily limited by nitrogen while phosphate limitation occurred only when light and temperature were likely to constrain the algal growth (Rosenberg and Ramus, 1982). Fabregas *et al.* (1985) observed a decrease in protein content with increase in salinity in many microalgal species. The effect of temperature on the growth rate of microalgae has been observed in many species. Lower microalgae growth rate could be a result of the increase in respiration due to rise in temperature above the specie's optimum level (Fogg and Thake, 1987). The extracellular releases of the phytoplankton were influenced by the nutrient status of the water and also the growth phases of the algae (Williams, 1990). Renaudl *et al*. (1994)

observed that maximum lipid content coincides with optimal range in growth temperature in many species and lipid content is lower at temperatures below and above this range. In the fast growing opportunistic algae with simple morphology and without tissue differentiation, all cells must have a complete physiological apparatus to support resource acquisition, photosynthesis and growth, and therefore, should contain high levels of N and P rich organic compounds. So the nutrient availability and the growth of fast growing algae were more related to nutrient limitation than the slow growing benthic algae (Pedersen and Borum, 1996). At temperatures of 20^0C and 25^0C, lipids and carbohydrates were higher than at 30^0C. Protein was not significantly affected by the temperature, but a tendency for lower values was observed at 25^0C (Araujo and Garcia, 2005). Many phytoplankton cells were known to release elevated amounts of organic compounds under nutrient limitation. The stress conditions imposed by the shifted nutrient supply ratios can stimulate the production of allelochemicals that inhibit potential competitors (Graneli *et al*., 2008).

The concentrations of the macronutrients, nitrate and phosphate largely control biomass development of *Phaeocystis* in temperate waters (Lancelot *et al*., 1987; Verity *et al*., 1988; Veldhuis *et al*., 1991). Riegman (1995), showed that in mixed phytoplankton assemblages in the laboratory, *Phaeocystis pouchetii* became dominant only when N:P ratios were 7.5 or lower, and at a N:P ratio 1.5, there was almost complete dominance of *Phaeocystis pouchetii*. The genus *Phaeocystis* occurs under a wide range of light intensities. In temperate regions, the ability of *Phaeocystis* to grow at low light intensity allows bloom development early in the season (Peperzak *et al*., 1998). *Phaeocystis* can utilize both organic phosphate and inorganic phosphate and can be competitive at high nitrate levels (Lancelot *et al*., 1998).

The molar ratio of the N/P of *Phaeocystis globosa* was less than the other algae, showing that the *Phaeocystis* converts nitrogen more efficiently into new biomass than phosphorus (van Boekel and Veldhuis, 1990; Hecky and Kilham, 1988). The cellular content of chlorophyll a and accessory light harvesting pigments of the *Phaeocystis* sp. isolated from Southern Ocean increased under low light intensities, whereas, the iron limitation resulted in a decrease of all light harvesting pigments. The high biomass of *Phaeocystis* cells may also be found under the sea ice, where light intensities were often <5µmol quanta m^{-2} s^{-1}. Massive colony blooms of *Phaeocystis* in Antarctic waters early in spring were terminated once nutrient became depleted (van Hilst and Smith, 2002),

In the ocean, algal growth was partly controlled by iron when this micronutrient is available in limiting concentrations, but variable light climate also plays an important role. A complex interaction between iron and light limitation has been described by many authors (Stefels and van Leeuwe, 1998; van Leeuwe and Stefels, 1998; Arringo and Tagliabue, 2005). Ratios of the pigments 19'-hexanoyloxyfucoxanthin: chlorophyll-a increased while ratios of fucoxanthin:chlorophyll-a decreased under increasing iron limited conditions. At low light intensities, the iron demand of cells increased in relation with enlargement of the photosynthetic apparatus (Raven, 1990; de Baar and Boyd, 2000). Bacterial degradation of phytoplankton in high nutrient low chlorophyll regions could be limited by iron availability (Arrieta *et al*., 2004). Under iron limitation, *Phaeocystis* cells showed characteristics of high light acclimation by the rapid conversion of the light harvesting pigment, diadinoxanthin into photo protective pigment, diatoxanthin. Thus photo acclimation can be successful under conditions of iron limitation. DiTullio *et al.* (2007) studied the

pigment composition of freshly isolated *Phaeocystis antarctica* under iron limitation. Sedwick *et al.* (2007) studied the iron requirements of *Phaeocystis antarctica* and recorded a relatively high iron requirement at low light intensity levels. Iron supplies either directly stimulate the microbial activity, or do so indirectly by enhancing DOC production of phytoplankton upon their release from iron limitation (Becquevort *et al*., 2007). Iron addition influenced the relative abundance of colonies versus single cells of *Phaeocystis* and caused a decrease in the C/N ratio of *Phaeocystis*.

3.2.2 Release of extracellular products by microalgae

Different substances such as polysaccharides, proteins, amino acids, nucleic acids, lipids vitamins, toxins, and other small molecules are released by the microalgae in to the growth media (Droop, 1968; Hellebust, 1974; Brochmann *et al*., 1979; Hoagland *et al*., 1993).

The extracellular release of organic matter by marine phytoplankton was a normal physiological process which was closely related to the rate of photosynthesis and constitutes upto 5µg C L^{-1} h^{-1} of the total primary production in coastal waters (Mague *et al*., 1980). The biochemical composition of microalgae could change with their growth rates, environmental conditions and the phase of their life cycle. Carbohydrates constitute the main part of the extracellular exudates. These exudates were primarily composed of polysaccharide chains of high molecular weight; the specific composition of which depends on the algal species (Myklestad, 1974; Haug *et al*., 1973; Paulsen and Myklestad, 1978; Monti *et al*., 1995). Lipids and carbohydrates are considered as cellular fuel, besides their important function as structural constituents of membranes

(Thompson *et al*., 1992). Hence, their decrease can negatively affect growth and metabolism of cells.

Phaeocystis produces copious amounts of acrylic acid (Guillard and Hellebust, 1971), a substance with known antibiotic properties (Sieburth, 1960). *Phaeocystis* also secretes large amounts of dimethylsulfide, a by-product of the reaction which forms acrylic acid (Barnard *et al*., 1984). The gelatinous matrix of *Phaeocystis* colonies was composed primarily of medium to high molecular weight polysaccharides (Guillard and Hellebust, 1971; Veldhuis and Admirral, 1985) a good substrate for bacterial metabolism. The combination of sterols and fatty acids could be used as a biomarker of *Phaeocystis*, and a set of fatty acids, sterols and pigments was diagnostic tool for the class of algae to which *Phaeocystis* belongs (Prymnesiophyceae). The fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential for the growth of multicellular animals, occur in only trace amounts in *Phaeocystis*, whereas, they are very common in other phytoplankton. The lipid composition of *Phaeocystis* was similar to that of other algae with 24- methylcholesta-5, 22E- dien- 3β- ol as dominant sterol. Due to the mucilaginous matrix, the average C/Chl a and C/N ratio values for colonies were higher than for flagellate cells and vary between 55 and 245 (w/w) and between 7 and 31 (w/w), respectively (Nichols *et al*., 1991).

Mostly based upon the fatty acids and lipid contents, solitary cells were more nutritious than colonies. *Phaeocystis* was well known for producing extravagant amounts of particulate and dissolved organic carbon, especially during the colonial stage of its life cycle. Alderkamp *et al.* (2006) reviewed the characteristic and dynamics of the organic matter produced by *Phaeocystis*.

3.2.3 Colony formation

Among the six recognised species of *Phaeocystis*, only four species are widely known to form colonies- *Phaeocystis pouchetii*, *Phaeocystis globosa*, *Phaeocystis antarctica* and *Phaeocystis jahnii*. Guillard and Hellebust (1971) cultured *Phaeocystis globosa* colonies at 20^0C and reported the growth of culture at temperature of even 27^0C. Cells within the colonies were diploid and lack flagella and were mostly immobilized within the colony skin, and thus could not directly participate in the sexual reproduction (Parke *et al*., 1971; Verity *et al*., 1988; Marchant and Thomsen, 1994).

Lancelot and Mathot (1985) reported that *Phaeocystis pouchetii* could utilize the carbon in the colony mucilage as an additional carbon source, and thus provide a mechanism for enhanced iron uptake by colonial cells, providing them a competitive advantage over solitary cells. The *Phaeocystis* colonies were capable of storing carbon, phosphorus and nitrogen in the light for subsequent assimilation in the dark (Lancelot and Mathot, 1985; Verity *et al*., 1991; Veldhuis *et al*., 1991). The carbon content relative to nitrogen increased with the size and age of the colony and also at low ambient inorganic nitrogen (Lancelot *et al*., 1991). In the North Sea, when phosphate was reduced to limiting concentrations, flagellated cells of *Phaeocystis pouchetii* appeared to be favoured than the colony cells (Riegman *et al*., 1992). It has been suggested (Kayser, 1970; Lancelot *et al*., 1991) that a solid substrate was necessary for the initial transition from single cells to the colonial morphology. Calcium stabilizes the colony structure; in the absence of Ca^{2+} colonies disintegrate (van Boekel, 1992). The extracellular colony matrix was formed by gelatinization of carboxylated chains, promoted by calcium and magnesium bridges (van Boekel, 1992).

Vaulot *et al.* (1994) observed that the strain of *Phaeocystis globosa* could form colonies in their culture at 22^0C. Riegman and van Boekel (1996) confirmed that colonial cells of *Phaeocystis globosa* were more effective competitors for nitrate and were favoured at irradiances above 50µmol photons $m^{-2}s^{-1}$. *Phaeocystis* was cosmopolitan in occurrence and regularly and regionally sequesters huge amounts of resources; generally in colder waters in the form of colonies (Lancelot *et al*., 1998; Zingone *et al*., 1999). The colony membrane might be largely responsible for minimising a variety of processes including grazing, cell lysis by viruses and sedimentation (Hamm *et al.,* 1999). *Phaeocystis* colonies had high C:N and C:P ratios, therefore they could remove more dissolved inorganic carbon per unit of nutrients assimilated and drive the biological pump to remove atmospheric CO_2 more efficiently than other phytoplankton (Verity *et al*., 1988; Arringo *et al*., 1999). The *Phaeocystis* colonies escape significant grazing by protozooplankton but were exposed to larger metazooplankton (Hamm, 2000; Verity, 2000).

Despite the fact that cells within the colonies must divert apparently significant portions of their photosynthate to the gelatinous matrix, and that the colony skin would appear to be a barrier to nutrient uptake, growth rates of the colony cells and flagellated cells did not differ substantially (Jacobsen, 2000). Growth rates of colony cells can even exceed those of solitary cells. Numerous studies had reported that the *Phaeocystis* colonies might not be eaten in proportion to their abundance due to either size mismatches with grazers, or due to production of some chemical product which renders them relatively less palatable (Verity, 2000). A variety of potentially important factors influencing the initiation of colonies from

solitary cells had been proposed, including light, temperature and nutrients (Verity *et al*., 1991; Vaulot *et al*., 1994; Peperzak *et al*., 2000b).

Low light and temperature favour colony formations, whereas high light and high temperatures favour solitary cells of *Phaeocystis poucehtii* (Verity *et al.,* 1991; Jacobsen, 2000). Schoemann *et al.* (2001) suggested that iron might complex with the colonial sheath of *Phaeocystis pouchetii*, thus making the absorbed iron more available to the colonial cells embedded in the mucilage. Colony formation in *Phaeocystis globosa* might even be enhanced by microzooplankton grazing on solitary cells (Jakobsen and Tang, 2002). It had been suggested that inorganic nutrients and grazing influence the ratio of solitary cells and colony cells of the *Phaeocystis.* A recent laboratory study of *Phaeocystis globosa* found that colony formation and survival were enhanced under conditions of enhanced mcirozooplankton grazing (Jakobsen and Tang, 2002), confirming the role of grazing. The iron limitation increased the ratio of solitary cells over the colony cells thus resulting in decrease in colonial cell abundance and a negative net growth rate for colonies (Sunda and Huntsman, 1997; Wassmann *et al*., 1990). The percentage of solitary cells relative to total cell (colonial + solitary) was high in the spring but decreased to minimum during late spring, specifically nearly 98% of the *Phaeocystis antarctica* cells were in colonies in late spring, coinciding with the seasonal chlorophyll maximum (Smith *et al*., 2003).

Another aspect of *Phaeocystis* colonies underappreciated was that they provide sites for temporary settlement of other plankton. Sazhin *et al.* (2007) enumerated the consortia of organisms associated with colonies during blooms of *Phaeocystis globosa* in the eastern English Channel and *phaeocysits pouchetii* in mesocosm in western Norway. In both

environments, mass development of the small needle shaped diatom, *Pseudonitzchia* species, occurred on *Phaeocystis c*olonies at the end of the bloom. The author proposed that the diatom used the organic substance of the colonial matrix for growth. There was an uncertainty about to what extent the *Phaeocystis* colonies form a good food for other zooplankton (Weisse *et al*., 1994; Schoemann *et al*., 2005; Nejstgaard *et al*., 2007). A variety of filtering or suspension feeding organisms including zooplankton and various bivalves decreased or stopped their feeding when *Phaeocystis* colonies were present. Active rejection of colonies had also been observed, (Kammermans, 1994; Weisse *et al*., 1994; Smaal and Twisk, 1997; Nejstgaard *et al*., 2007), and mechanical disturbance of the gills of bivalves and the mouth parts of zooplankton caused by colony mucus had been hypothesized.

Seuront *et al.* (2007) suggested that the biologically-induced increase in seawater viscosity might be a competitive advantage to *Phaeocystis* as a potential anti-predator strategy. The anti-predatory effect of *Phaeocystis* colonies was studied by many authors (van Rijssel *et al*., 1997; Hamm *et al*., 1999; Wolfe, 2000; Strom *et al.,* 2003; Pohnert, 2004; Yoshida *et al*., 2004; Stelfox-widdicombee *et al*., 2004; Wooten and Roberts, 2006; Nejstgaard *et al*., 2007).

3.2.4 Blooms of *Phaeocystis*

Numerous environmental factors had been invoked as bloom triggers, including temperature, edaphic factors (Jones and Haq, 1963), decreased concentrations of silicate and phosphate (Jones and Spencer, 1970) and trace metals (Morris, 1971).

Enormous strands of colonial *Phaeocystis* exceeding 10^8 colonies m^{-3} occured in both coastal and oceanic waters (Kashkin, 1963). In polar region, epidemics of *Phaeocystis* were a classical bloom phenomenon (Smayda, 1958; El-Sayed *et al*., 1983; Palmisano *et al*., 1986). Blooms of *Phaeocystis* play a dominating role for the carbon flux dynamics in the fjords and coastal environments of northern Norway. *Phaeocystis* blooms usually take place between mid April and late May and represent a prominent, recurrent phenomenon in the north Norwegian coastal zone (Eilertsen *et al*., 1981). *Phaeocystis* accounted for as much as 80% of the phytoplankton biomass during blooms in Norwegian waters and it exhibited a remarkable year-round occurrence in Balsfjord over an annual temperature range of 1-7^0C (Haug *et al*., 1973; Eilertsen *et al*., 1981). *Phaeocystis* was abundant along the Dutch, German and Belgian coasts, where there was the provocative historical implication that it had become a weed species in the progressively eutrophic Wadden Sea (Cadee and Hegemann, 1974, 1979, 1986; Lancelot and Mathot, 1987).

There are several reports of *Phaeocystis* blooms in Antarctica (Buck and Garrison 1983; El-Sayed *et al*., 1983; Sasaki and Watanabe, 1984; Garrison and Buck, 1985; Palmisano *et al*., 1986). Blooms of *Phaeocystis* colonies and their sedimentation were observed in the Bransfield Strait during ice-free conditions in November/December 1980 (Schnack *et al*., 1985; Bodungen *et al*., 1986). Blooms exceeding 10^7 colonies/m^3 were observed in Narrangansett Bay, the eastern Irish Sea, the Liverpool Bay and the North Sea (Jones and Haq, 1963; Jones and Spencer, 1970; Weisse *et al*., 1986; Verity *et al*., 1988). *Phaeocystis* blooms were predictable events in the Ross Sea and they start before the major diatomaceous blooms (Ainley and Jacobs, 1981; El-Sayed *et al*., 1983; Palmisano *et al*., 1986;

SooHoo *et al*., 1987). *Phaeocystis* was prominent in the marginal ice edge zone as well as in the open Barents Sea where it blooms following a diatom bloom in late spring or in concert with the diatoms (Rey and Loeng, 1985; Skjoldal and Rey, 1989).

Annual massive blooms of *Phaeocystis* colonies were observed mainly from mid April to mid May in the southern North Sea, generally following a diatom spring bloom (Cadee and Hageman, 1986; Lancelot, 1990; Reid *et al*., 1990; Fernandez *et al*., 1992). Blooms of an unidentified *Phaeocystis* sp. were reported for the first time in Kuwaiti coastal waters at temperatures of 20^0C (Al-Hasan *et al*., 1990). *Phaeocystis* colonies may dominate the entire spring bloom in the Barents Sea and mass sedimentation of *Phaeocystis* was recorded from the Barents Sea in May/June 1987 (Wassmann *et al*., 1990). In the North Sea, two *Phaeocystis* species were recognized, a northern coldwater species *Phaeocystis pouchetii,* and a southern North Sea species, *Phaeocystis globosa*, that thrives at somewhat higher temperature (Cadee, 1991). Blooms of *Phaeocystis* colonies were regularly observed in the Greenland Sea (Gradinger, 1986; Baumann, 1990; Smith *et al*., 1991).

Dense layers of healthy, non-aggregated *Phaeocystis* colonies were present in the turbulent, nephleoid layer in the central North Sea off Helgoland (Riebesell, 1993). The *Phaeocystis* bloom termination was influenced by the sedimentation process. Factors influencing the fate of senescent *Phaeocystis* blooms were, probably, water depth, turbulent energy supply, aggregate formation, release of flagellated cells from colonies, microbial degradation, zooplankton grazing as well as lysis of colonies and cells (Wassmann, 1994). *Phaeocystis* sp. colonies were also reported in the open Arabian Sea at 22-28^0C during the upwelling driven by

the strong southeast monsoon (Garrison *et al*., 1998). Massive blooms of *Phaeocystis antarctica* occurred in the southern Ross Sea, and they had been reported to produce some of the highest concentrations of DMS observed in the ocean (DiTullio and Smith, 1995; Kettle *et al*., 1999).

Members of the genus *Phaeocystis* occurred throughout the world's ocean, often forming large blooms in a variety of diverse locations, such as the Ross Sea, North Sea, Greenland Sea, Bering Sea shelf break, the Arabian Sea and the Barents Sea (Lancelot *et al*., 1998). A bloom of *Phaeocystis globosa* was first reported from the central Arabian Sea in 1996 during the summer monsoon period (Madhupratap *et al*., 2000). Almost 95% of the phytoplankton population was composed of colonies of *Phaeocystis globosa*. In 1997, and again in 1999, massive blooms of *Phaeocystis globosa* colonies occurred in coastal water of the South China Sea at temperature of 17-30^{0}C (Huang *et al*., 1999; Chen *et al*., 2002).

Phaeocystis blooms compared to diatoms in the Belgian coastal zone was related to the combined effects of riverine nutrient load and the north Atlantic oscillation (Breton *et al*., 2006). *Phaeocystis globosa* bloom was also observed in the Eastern English Channel (Seuront *et al*., 2006). Extensive data set on *Phaeocystis* that was provided by the continuous plankton recorder (CPR), from the North Atlantic since 1948, showed that *Phaeocystis* abundance had mainly been restricted to the neritic regions and limited to spring time, with the south eastern North Sea as a hotspot. Dissolved iron availability plays a primary role in regulating blooms of colonial *Phaeocystis antarctica* in the southern Ross Sea during summer (Verity *et al*., 2007).

3.3 Materials and Methods

3.3.1 Growth characteristics

The growth characteristics of *Phaeocystis* were studied by culturing in different salinity, pH, temperature and nutrient level. The growth was calculated by the estimation of Chlorophyll-a as per the method of Strickland and Parson (1972).

3.3.1.1 Estimation of Chlorophyll-a

For the estimation of Chlorophyll-a, the algal culture was filtered through 1µm filter paper under moderate vacuum, the filter paper was dried until constant weight is obtained and weighed out. The dried filter paper was transferred to clean stoppered test tube and 10ml of 90% acetone was added. The test tubes were kept at 4^0C for 24 hours in order to facilitate complete extraction. The chlorophyll-a-acetone solution was centrifuged for 10 minutes at 5000 rpm and the absorbance of the clear solution was measured at 630, 645, 665 and 750nm. All the absorbance values were subtracted from the absorbance of 750, thus minimising the error in the chlorophyll-a measurement. The Chlorophyll-a value was expressed as µg/ml using the equation

$$Ca = 11.85\ E_{665} - 1.54\ E_{645} - 0.08\ E_{630} \quad \text{---------- (3.1)}$$

E is the absorbance of chlorophyll-a samples at respective wavelengths.

$$\text{Chlorophyll-a } (\mu g/ml) = (Ca \times v)/V \times 1$$

Where:

v = Volume of acetone

V = volume of water sample filtered (L)

1 = path length of cuvette (cm).

3.3.1.2 Effect of variation of salinity, pH and temperature on the growth of *Phaeocystis* species

The effect of salinity, pH and temperature on the growth of *Phaeocystis* sp. was studied to find out the optimum salinity, pH and temperature for the maximum growth. Experiments were carried out in Walne's medium. Effect of salinity was examined at 0, 10, 20, 30 and 40ppt. Effect of pH was studied at pH 6, 7, 8 and 9. The effect of temperature on the growth of *Phaeocystis* sp. was observed at five temperatures, 10^0C, 15^0C, 20^0C, 25^0C and 30^0C. Growth media prepared at different salinity, pH and temperature were inoculated with *Phaeocystis* sp. cells from the stock culture of alga maintained in the laboratory. Incubation was done for 24 days in an Environmental Chamber (Sanyo, Versatile Environmental Chamber) and growth was measured by the estimation of chlorophyll-a at 3 day intervals of the total growth period.

3.3.1.3 Effect of growth media on the growth of *Phaeocystis* species

The effect of various culture media on the growth of *Phaeocystis* sp. was studied. Walne's medium and different concentrations of f/2 medium were used for the study. All media used were prepared at salinity 30ppt, pH 8.00 and incubated at 20^0C, 12h:12h light:dark cycle in the environmental chamber for a period of 24 days.

Different combinations of f/2 medium were used as follows:

1) f/2-Si ---------- Na_2SiO_3. $9H_2O$ was omitted from the composition of f/2 medium
2) f/4 ---------- Half the concentration of f/2
3) f/20---------- One-tenth concentration of f/2
4) f/50---------- $1/25^{th}$ concentration of f/2.

The growth was measured at 3 day intervals by measuring the chlorophyll-a concentration.

3.3.1.4 Effect of nitrate and phosphate on the growth of *Phaeocystis* species

Concentrations of the macronutrients nitrate and phosphate were varied from a high to low level to study the effect of these nutrients on the growth of *Phaeocystis* sp. Six different media with varying concentrations of nitrate were prepared in Walne's medium of salinity 30ppt, initial pH of 8.00 to study the effect of nitrate on the growth of *Phaeocystis* sp.

1) Nitrate 2000µM and Phosphate 100µM
2) Nitrate 1500µM and Phosphate 100µM
3) Nitrate 1000µM and Phosphate 100µM
4) Nitrate 500µM and Phosphate 100µM
5) Nitrate 200µM and Phosphate 100µM
6) Nitrate 100µM and Phosphate 100µM

Another set of six different media with varying concentrations of phosphate were prepared in Walne's medium of salinity 30ppt, initial pH of 8.00 to study the effect of phosphate on the growth of *Phaeocystis* sp.

1) Phosphate 100µM and nitrate 2000µM
2) Phosphate 75µM and nitrate 2000µM
3) Phosphate 50µM and nitrate 2000µM
4) Phosphate 25µM and nitrate 2000µM
5) Phosphate 10µM and nitrate 2000µM
6) Phosphate 5µM and nitrate 2000µM

Another set of media was prepared with both nitrate and phosphate in low concentrations-nitrate 100µM and phosphate 5µM to study the effect of growth of *Phaeocystis* sp. in limiting conditions of both nitrate and phosphate. Incubation of all experimental flasks were done at a temperature of 20^0C, 12h:12h light: dark cycle in the environmental chamber for a period of 24 days and growth was measured by the estimation of Chlorophyll-a.

3.3.1.5 Specific growth rate

Growth rate of *Phaeocystis* sp. was calculated under different conditions of salinity, pH, temperature, media and nutrients and expressed as the specific growth rate (K'). The specific growth rate (K') was calculated from biomass increase per unit time as per Pirt (1975).

$$K'\left(day^{-1}\right) = \frac{\ln\left(N_1 / N_0\right)}{t_1 - t_0}$$

Where N_0 and N_1 are quantitative expression of the biomass of cells given in terms of chlorophyll-a concentration at the beginning (t_0) and at the end (t_1) of selected time interval during incubation.

3.3.1.6 Statistical analysis

The results of the study were analysed using two-way ANOVA by Duncan's multiple comparison of the means using SPSS (Statistical Package for Social Sciences) 10.0 for Windows. Significant differences were indicated at $p<0.05$.

3.3.2 Polymorphic behaviour of cells

The polymorphism exhibited by the cells of the *Phaeocystis* sp. during the growth period was determined by culturing the alga in Walne's

medium. Live cells were observed under phase contrast microscope (Nikon, Eclipse E200) and the number of colony cells and solitary cells were measured using a haemocytometer. The development of single cell to colony cells was noted in the culture at three days interval of the entire growth period of 24 days.

3.3.3 Biochemical composition

100 ml of Walne's medium of salinity 30ppt and pH 8.00 was inoculated with 3ml *Phaeocystis* sp. culture taken from exponential growth phase and incubated in the environmental chamber for 30 days. Sampling was done at 15^{th} day and 30^{th} day of growth in order to determine the biochemical composition of the alga in logarithmic and stationary growth phase. 100ml culture was filtered through 1µm pore size GF/F filter paper and the filter paper with cells was dried in an oven at 50^0C until constant weight is obtained. Dry weight of filter paper with cells was taken, and it was crushed and transferred to test tube. The weight of the filter paper was taken initially before filtration and the final weight of the alga was obtained by subtracting the weight of filter paper with alga from the initial weight of the filter paper. The dried samples were used for biochemical analysis.

3.3.3.1 Total Proteins

5ml 1N NaOH was added to the test-tubes containing *Phaeocystis* samples. The tubes were heated in a boiling water bath for 10 min. The samples were cooled and diluted to 10ml and centrifuged at 3000rpm and supernatant was taken for analysis. Total protein was analysed using Lowry's method (Lowry *et al*., 1951).

3.3.3.2 Total carbohydrates

1ml 80% sulphuric acid was added to samples kept in an ice bath and incubated for 20hrs at 20^0C and the mixture was diluted to 10ml. Total Carbohydrates was estimated using phenol sulphuric acid method (Dubois *et al*., 1956).

3.3.3.3 Total lipids

10ml of organic solvent (chloroform-methanol) was added to the sample and centrifuged at 2500 rpm for 5 min. Supernatant was taken and 0.9% NaCl solution was added. Allowed to stand overnight at 4^0C in an open condition without plugging. A biphasic layer was formed in which the lower phase contained lipids. Top layer was removed carefully and volume adjusted to 10ml by adding chloroform:methanol solution and used as test solution. Total lipid was estimated by Phosphovanillin method (Barnes and Black stock, 1973).

3.3.3.4 Extracellular proteins and carbohydrate

Walne's medium was inoculated with *Phaeocystis* sp. cells from exponential growth phase and cultured for 30 days. Sampling was done at 15^{th} day and 30^{th} day of growth. 100ml of *Phaeocystis* sp. culture was filtered through 1µm pore size filter paper to remove the cells and the filtrate was separated and lyophilized to reduce the volume to 10ml. Total protein was estimated by direct reading at 280nm and total carbohydrate was estimated by phenol sulphuric acid method (Dubois *et al*., 1956).

3.3.3.5 Statistical analysis

The results of the tests were analysed by one way ANOVA using Microsoft Excel and significant difference was calculated at $p<0.05$.

3.3.4 Effect of nutrients on the colony formation

The influence of two macronutrients, nitrate and phosphate and micronutrient, iron on the colony formation of *Phaeocystis* sp. was determined. The experiments were carried out in Walne's medium using artificial seawater at salinity 30ppt, and pH 8.00 and the media were inoculated with *Phaeocystis* sp. cells (10^7cells/ml) and incubated in the environmental chamber for a period of 24 days.

Composition of Artificial Seawater

Anhydrous salts	g/L
NaCl	24.54
Na_2SO_4	4.09
KCl	0.7
$NaHCO_3$	0.2
KBr	0.1
H_3BO_3	0.003
NaF	0.003
Hydrous salts	
$MgCl_2.6H_2O$	11.1
$CaCl_2.2H_2O$	1.54
$SrCl_2.6H_2O$	0.017

Dissolved anhydrous salts in 600ml distilled water and hydrous salts in 300ml distilled water. Combined the two solutions and the final volume was brought to 1000ml.

3.3.4.1 Effect of nitrate and phosphate on the colony formation of *Phaeocystis* species

To determine the effect of macronutrients nitrate and phosphate on the colony formation of *Phaeocystis* sp. four different growth media were prepared in Walne's medium using artificial seawater.

1) NP non limiting medium (Nitrate 2000µM and Phosphate 100µM)

2) NP limiting (Nitrate 100µM and Phosphate 5µM)

3) P limiting (Nitrate 2000µM and Phosphate 5µM)

4) N limiting (Nitrate 100µM and Phosphate 100µM)

Growth of *Phaeocystis* sp. in each medium was estimated by measuring the chlorophyll-a value at three days interval. At every 3rd day of growth period, the colony cells and the solitary cells were counted in the algal culture using haemocytometer under a Nikon E200 light microscope at 400X magnification.

3.3.4.1.1 Estimation of nitrate and phosphate concentrations in the culture media

Concentration of nitrate and phosphate in the various culture media were estimated at 4 days interval. Nitrate was estimated by the method of Zhang and Fischer (2006) by using resorcinol reagent. 5ml of algal culture was filtered through 1µm pore size GF/F filter paper to remove the cells, and 2% 0.6ml resorcinol was added to the filtrate. To the mixture, 5ml concentrated sulphuric acid was added, mixed well and allowed to stand for 30 minutes in dark. The reaction mixture was placed in a water bath at room temperature to reduce the heat produced during the reaction. When the temperature became normal, the volume was made up to 25 ml with distilled water and absorbance was read at 505 nm.

Phosphate was estimated by colorimetric method of Strickland and Parson (1972). 25ml of algal culture was taken and filtered through 1µm pore size GF/F filter paper to remove the cells and intact particles. 0.5ml of

ascorbic acid was added to the 25 ml algal filtrate, followed by 0.5ml of mixed reagent (ammonium molybdate+potassium antimony tartarate+sulphuric acid). A blank was prepared with distilled water. Phosphate ions in water react with an acidified molybdate reagent to yield molybdo phosphoric acid, which was reduced using ascorbic acid to a highly coloured blue compound. The absorbance was read at 880nm.

3.3.4.2 Effect of iron on the colony formation of *Phaeocystis* species

The concentration of iron in the Walne's medium was varied to values of 10µM, 5µM, 2.5µM and 0µM (without addition of iron). Growth was observed at 3 days interval by estimating the chlorophyll-a value. At every 3rd day of growth period, the colony cells and the solitary cells were counted in the algal culture using a haemocytometer under the Nikon E200 light microscope at 400X magnification.

3.3.4.3 Statistical analysis

The results of the study was analysed statistically by two-way ANOVA following Duncan's multiple comparison using SPSS 10.0 for Windows. Significant difference was calculated at $p<0.05$.

3.4 Results

3.4.1 Growth Characteristics

3.4.1.1 Effect of variation of salinity on the growth of *Phaeocystis* species

Salinity tolerance of *Phaeocystis* sp. was studied by growing the culture in Walne's medium having different salinity levels (0-40ppt) and measuring growth as chlorophyll-a content up to 24 days at three day intervals (Figure 3.2 and Table 3.1 of Appendix). No growth was observed at 0ppt salinity.

Phaeocystis showed wide salinity tolerance. The chlorophyll-a content increased remarkably by 9th day of growth at all salinities. Maximum growth was observed at 12th day. Highest chlorophyll-a content (3.6µg/ml) was obtained at 30ppt on 12th day of growth. The growth curve of *Phaeocystis* sp. at all salinities was found to be more or less similar. At 10ppt the highest chlorophyll-a concentration was 3.4µg/ml on 12th day. Highest chlorophyll-a concentration was 3.47 µg/ml at 20 ppt. At 40ppt, maximum chlorophyll-a concentration was 3.07µg/ml. Growth of *Phaeocystis* sp. in different salinity varied significantly (p<0.05) between 20ppt and 30ppt.

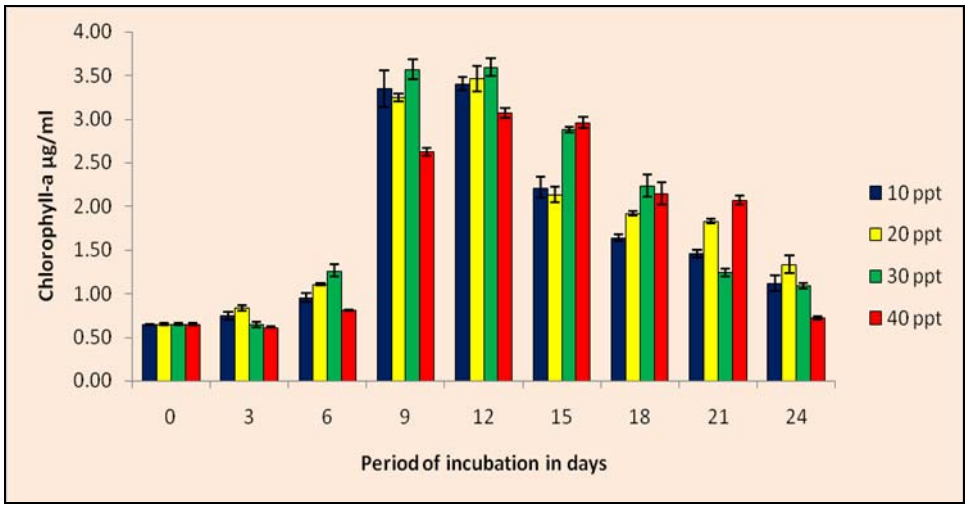

Figure 3.2 Effect of variation of salinity on the growth of *Phaeocystis* sp.

The specific growth rate (k') showed the same trend as the growth curve of *Phaeocystis* sp. in different salinity (Figure 3.3). Highest specific growth rate (0.189 day^{-1}) was obtained at 30ppt on 9th day. Highest specific growth rate obtained for 10ppt, 20ppt and 40ppt were 0.182 day^{-1}, 0.178 day^{-1} and 0.155 day^{-1} respectively (Table 3.1 of Appendix).

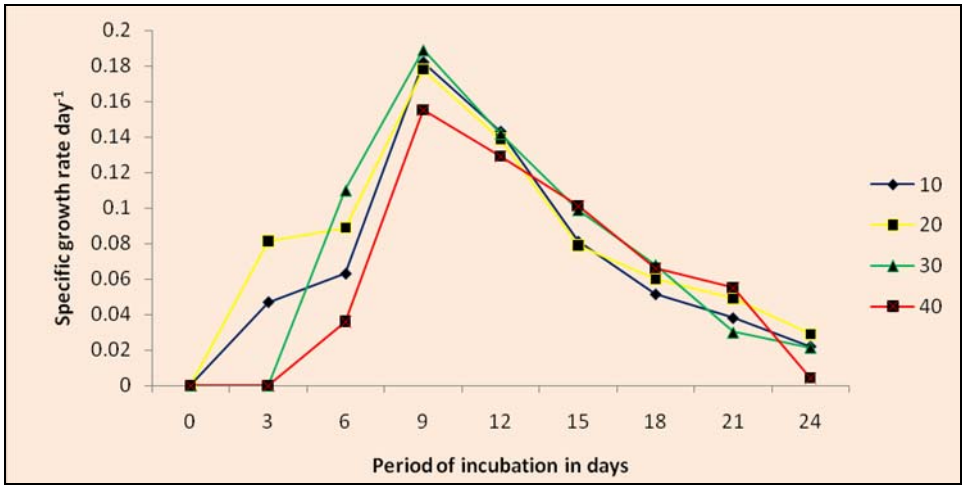

Figure 3.3 Specific growth rate of *Phaeocystis* sp. at different salinity

3.4.1.2 Effect of variation of pH on the growth of *Phaeocystis* species

The effect of pH on the growth of *Phaeocystis* was studied for 24 days by inoculating the cultures in Walne's medium having varying pH (6-9) and it was found to grow maximally at pH 8.00 (Figure 3.4). Growth was very little at pH 6 and the cells entered in the decline phase on 12th day and there was no growth from 15th day onwards. Cells at pH 8 and 9 remained in exponential phase till 15th day whereas at pH 7 the culture started declining from 5th day onwards. Growth was found to be highest at pH 8 with the highest chlorophyll-a value 4.34µg/ml. (Table 3.2 of Appendix).

The ANOVA results showed that the growth of *Phaeocystis* sp. varied significantly (p<.05) at all pH values tested. The specific growth rate of *Phaeocystis* sp at pH 7, 8 and 9 showed the same pattern. Highest specific growth rate was obtained for pH 8 (Figure 3.5 and Table 3.2 of Appendix).

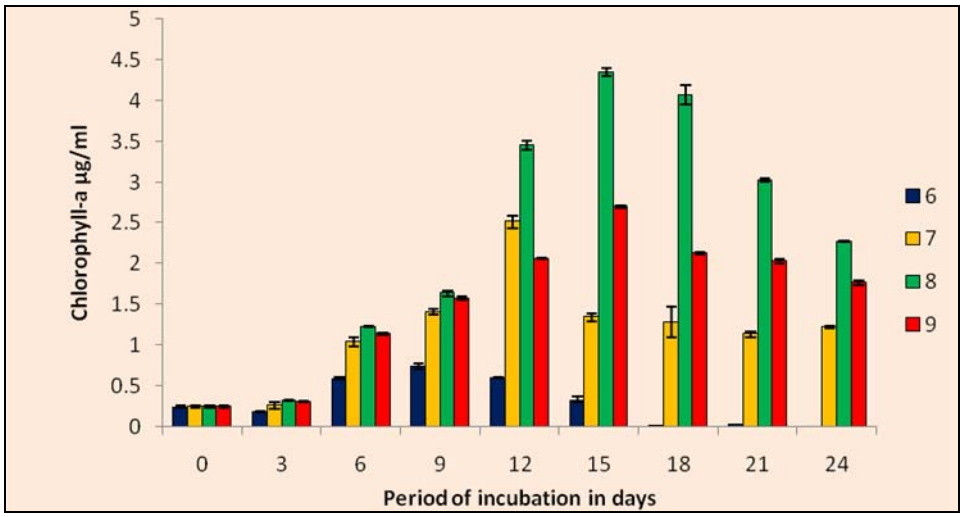

Figure 3.4 Effect of different pH on the growth of *Phaeocystis* sp.

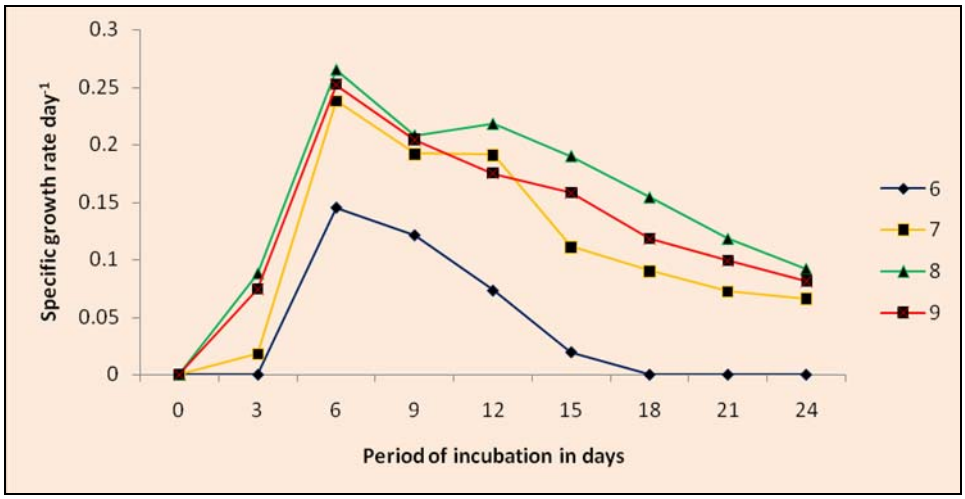

Figure 3.5 Specific growth rate of *Phaeocystis* sp. at different pH.

3.4.1.3 Effect of temperature on the growth of *Phaeocystis* species

Figure 3.8 shows the growth of *Phaeocystis* sp. in different temperatures 10^0C, 15^0C, 20^0C, 25^0C and 30^0C. *Phaeocystis* sp. showed maximum growth at 25^0C on 12^{th} day and at 20^0C on 15^{th} day. No growth or negligible growth was observed at 10^0C. At 15^0C, growth of *Phaeocystis*

seems to be almost steady from 3rd day to 24th day, with maximum growth on 18th day of experiment. At 25^{0}C, maximum growth was on 12th day, after that chlorophyll-a concentration decreased drastically from 15th day on wards. The turnover rate was high at 25^{0}C for *Phaeocystis* sp. Growth was observed at 30^{0}C till 6th day of growth, and then chlorophyll-a value decreased indicating that the cells have entered into the declining phase of growth (Figure 3.6 and Table 3.3 of Appendix).

Specific growth rate showed highest value at 25^{0}C (0.263day^{-1}). The maximum specific growth rate at 20^{0}C was 0.254 day^{-1} and at 15^{0}C was 0.135 day^{-1}. At 30^{0}C, maximum specific growth rate was 0.145 day^{-1} (Fig 3.7 and Table 3.3 of Appendix).

Statistical analysis showed that the growth at different temperatures 10^{0}C, 15^{0}C and 30^{0}C varied significantly from each other, but the growth at temperatures 20^{0}C and 25^{0}C did not show significant variation.

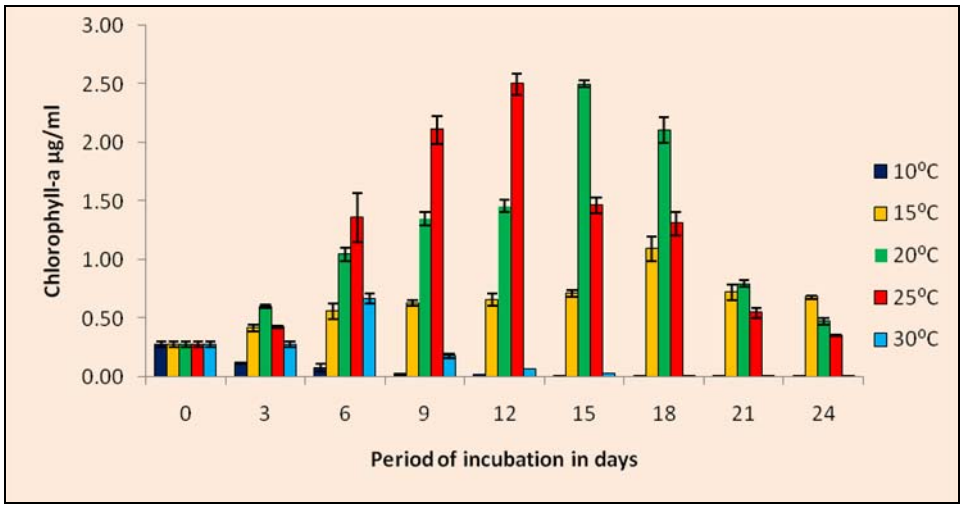

Figure 3.6 Effect of temperature on the growth of *Phaeocystis* sp.

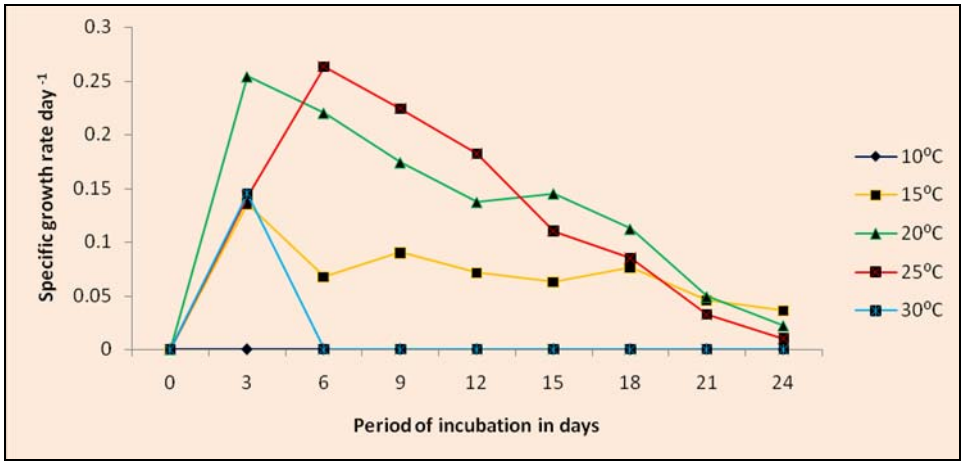

Figure 3.7 Specific growth rate of *Phaeocystis* sp. at different temperature

3.4.1.4 Effect of different growth media on the growth of *Phaeocystis* species

The ability of *Phaeocystis* sp. to grow in different media, Walne's, f/2, f/2-Si, f/4, f/20, and f/50 were tested. Figure 3.6 shows the growth profile of *Phaeocystis* sp. in different media. Cells exhibited maximum growth in Walne's medium with highest chlorophyll-a value 0.66µg/ml on 18th day of growth. In f/2 medium maximum growth was obtained at 15th day and cells remained in stationary phase from 18th to 21st day. Growth was very less in f/50 medium with a complete decline in growth by 9th day. In f/2-Si medium exponential phase extended from 12th day to 21st day (Table 3.4 of Appendix).

In f/4 medium growth started declining by 18th day and the exponential phase extended from 6th to 15th day. In f/20 medium maximum chlorophyll-a concentration was obtained on 6th day (0.23µg/ml) and growth declined afterwards. Specific growth rate showed the same pattern for Walne's medium, f/2 and f/2-si, with the maximum specific growth rate in the Walne's medium (0.178day^{-1}) (Figure 3.7 and Table 3.4 of Appendix).

Statistical analysis showed that the growth in different media varied significantly (p<.05), with Walne's medium supporting the maximum growth of *Phaeocystis* sp.

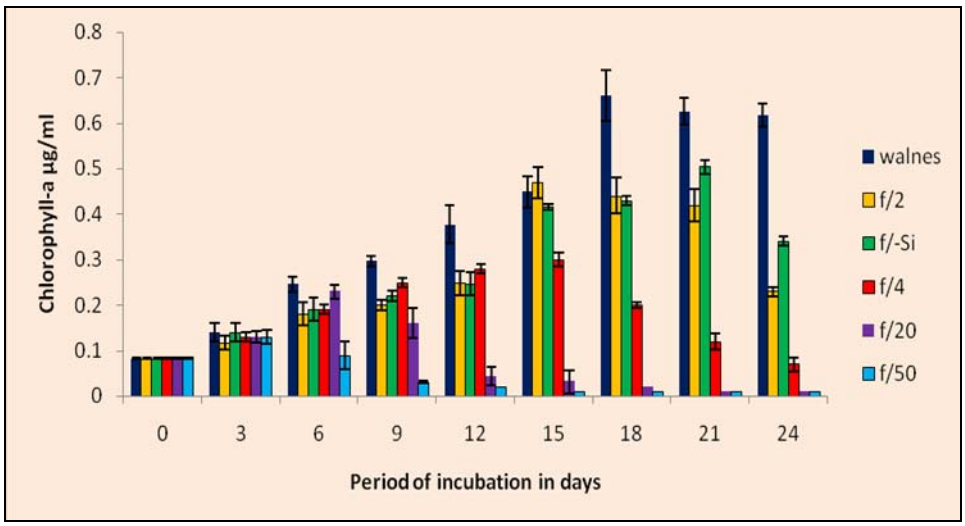

Figure 3.8 Effect of different growth media on the growth of *Phaeocystis* sp.

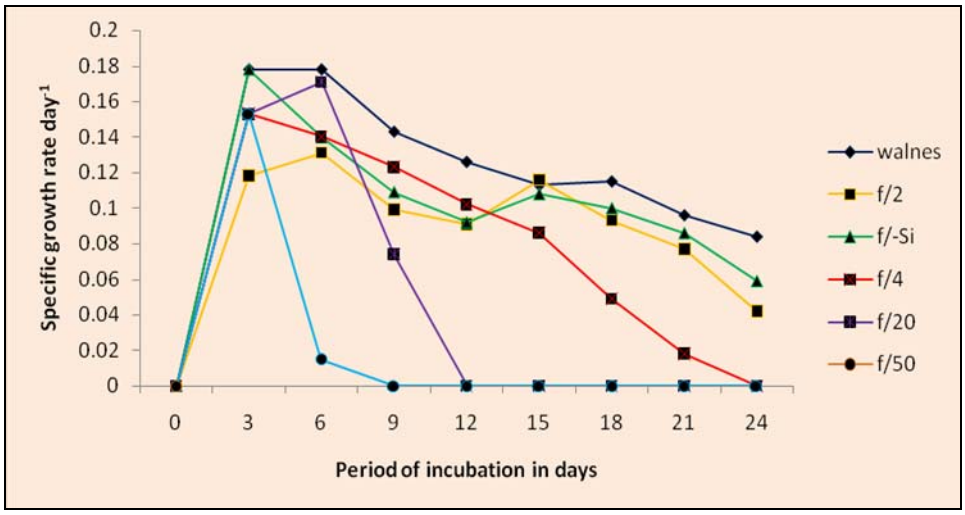

Figure 3.9 Specific growth rate of *Phaeocystis* sp. in different growth media

3.4.1.5 Effect of nitrate on the growth of *Phaeocystis* species

Figure 3.10 and Table 3.6 (Appendix) show the growth profile of *Phaeocystis* sp. in different media with varying concentration of nitrate, where the phosphate concentration was kept constant. Maximum chlorophyll-a value (1.6µg/ml) was obtained in the medium with the nitrate level 1500µM. Till 6^{th} day the growth was almost similar in all media. By 9^{th} day growth started decreasing in media containing low nitrate concentration when compared to media containing high nitrate concentration. In medium with nitrate level 1500µM, growth remained at high level till the end of the experiment.

Specific growth rate was maximum on the 6^{th} day of growth in all the nutrient media except in the media with nitrate concentration 500µM. Specific growth rate decreased considerably when the nitrate concentration was below 200µM. Maximum growth rate was obtained in the medium with the highest nitrate concentration of 2000µM (0.418 day^{-1}). (Figure 3.11 and Table 3.5 of Appendix).

Statistical analyses showed that the chlorophyll-a value varied significantly for the different media with varying concentrations of nitrate. However, there was no significant difference between media with low nitrate value of 200µM and 100µM. Media with nitrate levels 2000µM and 1000µM also did not vary significantly.

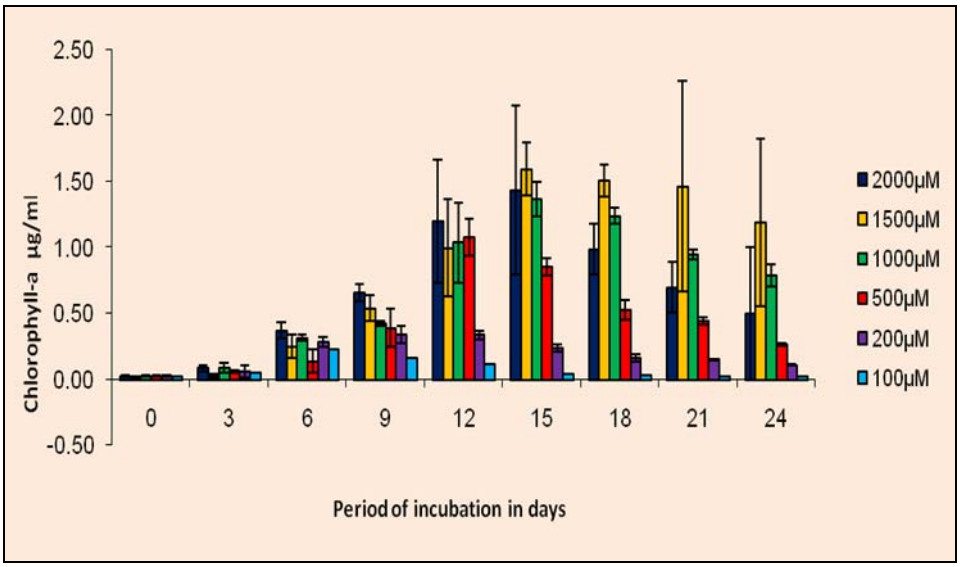

Figure 3.10 Effect of nitrate concentration on the growth of *Phaeocystis* sp.

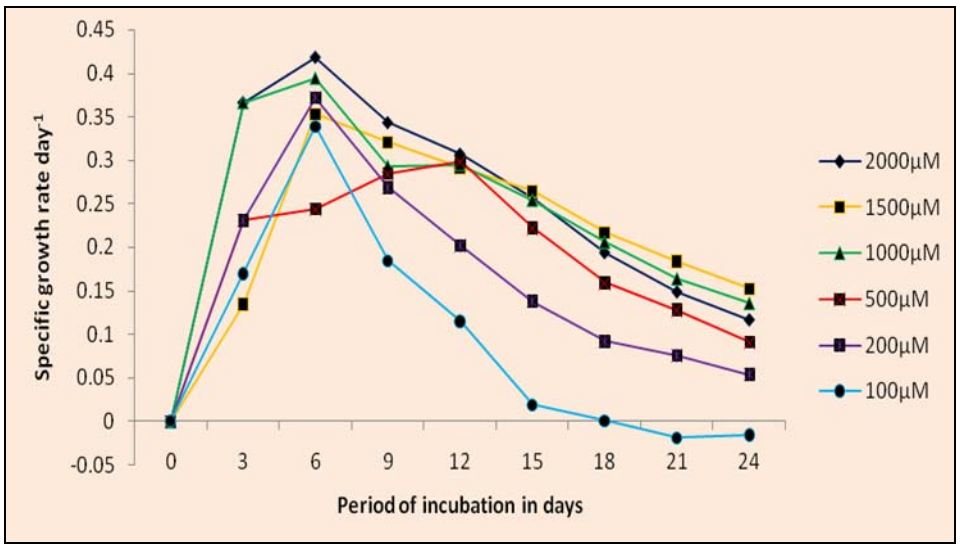

Figure 3.11 Specific growth rate of *Phaeocystis* sp. at different nitrate concentration

3.4.1.6 Effect of phosphate on the growth of *Phaeocystis* species

Figure 3.12 shows the growth profile of *Phaeocystis* sp. in media with varying concentrations of phosphate. *Phaeocystis* sp. showed maximum growth in the medium with highest phosphate level 100µM on 15th day of growth period with chlorophyll-a value 1.44µg/ml. By 12th day, growth started decreasing in media containing low phosphate levels (25µM, 10µM, 5µM), when compared to media containing higher phosphate levels. Minimum growth was observed in medium where the phosphate concentration was 5µM. Growth decreased significantly in media with low phosphate levels (Table 3.6 of Appendix).

Maximum specific growth rate was observed at 6th day of growth for all media and the highest value (0.418day^{-1}) was obtained for medium with highest concentrations of phosphate (100µM) (Figure 3.13 and Table 3.6 of Appendix).

Statistical analysis showed that significant variation in the growth of *Phaeocystis* sp. was observed only when the phosphate concentration becomes lower than 50µM. No significant variation was observed in growth in the media with phosphate concentrations of 100µM, 75µM and 50µM.

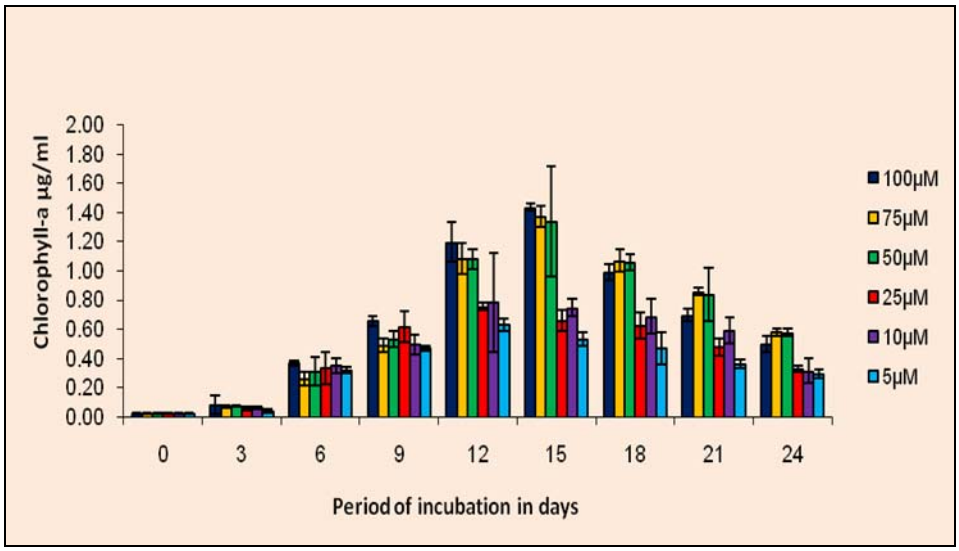

Figure 3.12 Effect of phosphate concentration on the growth of *Phaeocystis* sp.

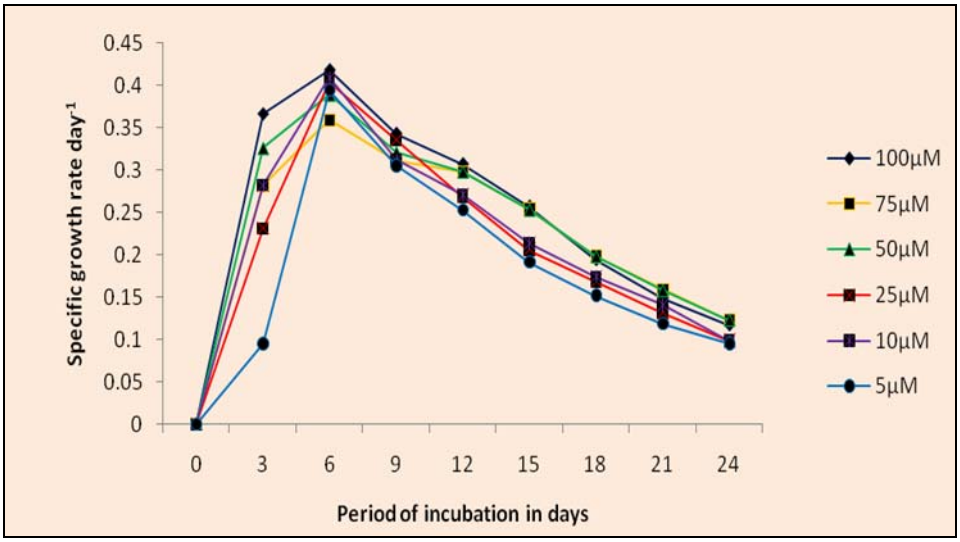

Figure 3.13 Specific growth rate of *Phaeocystis* sp. at different phosphate concentration

The growth of *Phaeocystis* sp. in media with different levels of nitrate and phosphate is shown in figure 3.14. Growth was highest in the medium with high nitrate and high phosphate levels. Growth was minimum in the medium containing low nitrate level. In the medium where phosphate level was high growth was low as nitrate level was minimum. In medium with low phosphate (5µm), growth was observed as the nitrate level was high in the medium. Growth was almost negligible in medium with both low nitrate and phosphate level. Maximum chlorophyll-a value (1.44µg/ml) was obtained in NP non limited media on 15th day of growth (Table 3.7 of Appendix)

Highest specific growth rate was obtained in NP non limiting medium (0.418 day^{-1}). For all nutrient media tested, maximum specific growth rate was observed on the 6th day of growth. In NP limiting medium, the specific growth rate was very low (Figure 3.15 and Table 3.7 of Appendix).

Statistical analysis showed no significant difference in growth between the NP limiting and N limiting medium, whereas both the media differed significantly from NP non limiting and P limiting media.

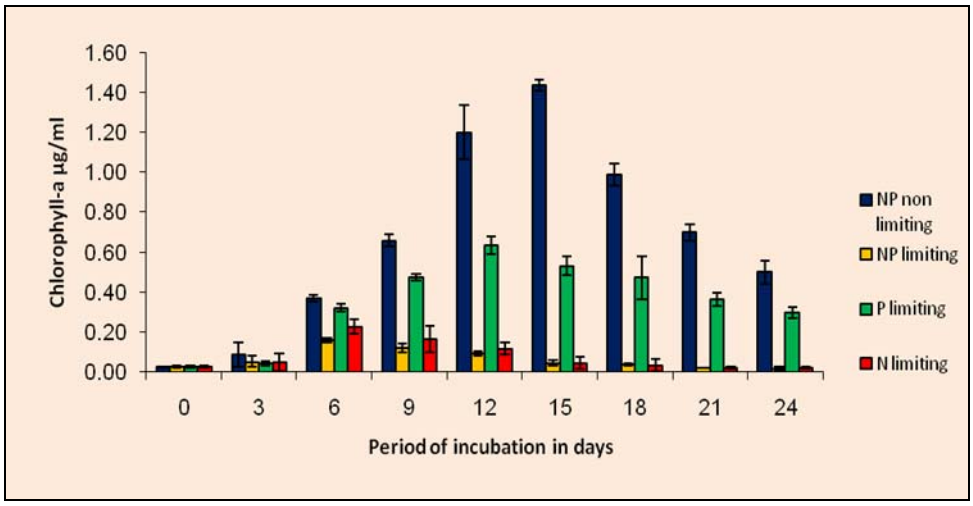

Figure 3.14 Growth of *Phaeocystis* sp. in different nutrient concentrations

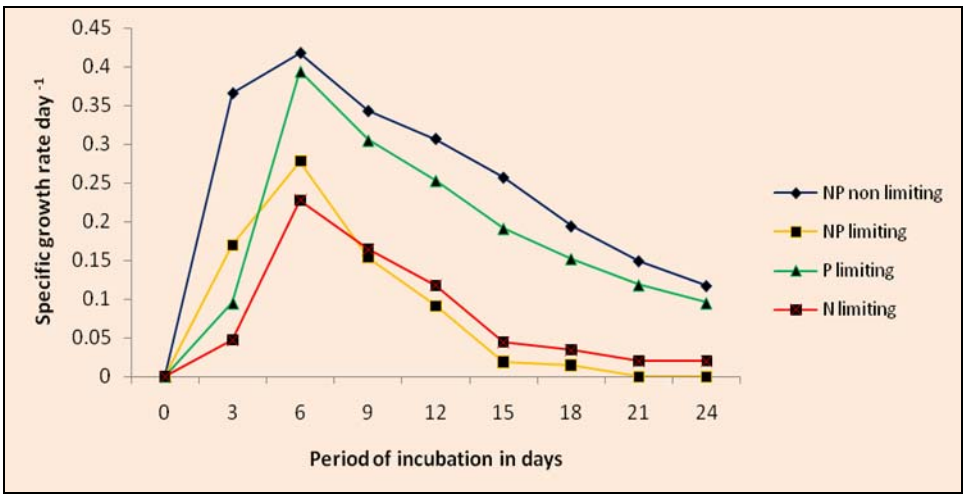

Figure 3.15 Specific growth rate of *Phaeocystis* sp. in different nutrient conditions

3.4.2 Polymorphic behaviour of cells

Cell morphology of *Phaeocystis* sp. was observed in the growth medium at 3 days interval (Plate 1 to plate 11). On the 3rd day of growth of algae, the cells were observed as single cells in the culture medium. Colonies were absent. Single flagellated motile cells were seen moving

randomly in very small number. Flagellated cells could not be photographed since they move randomly in live condition and under preservation the cells were ruptured. The size of the solitary cells was 3.9µm in diameter and the total number of solitary cells was 9×10^4 cells/ml. By the 6th day of growth, the solitary cells started dividing and got arranged in packets of four or five cells in number. Colonies started developing in the culture medium. Flagellated motile cells were present in the culture medium in few numbers. The cell number of solitary cells and colony cells were 10.5×10^5 and 2.9×10^5 cells/ml respectively. The size of solitary cells increased and ranged from 3.9µm- 7.5µm in diameter.

On the 9th day of growth, the number of colony cells started increasing in the culture medium (1.6×10^6 cells/ml) over the number of solitary cells (14.4×10^5 cells/ml). Average size of solitary cells was 8.1µm in diameter and colony size was 59.3µm. The flagellated motile cells were found to be absent.

By 12th day of growth of *Phaeocystis,* the size of the colonies started increasing to 78µm in size. Flagellated cells were found to be absent in the culture and the number of solitary cells and colony cells were 20.8×10^5 cells/ml and 2.5×10^6 cells/ml respectively. The average size of the solitary cells was found to be 8µm in diameter. The maximum number (8×10^6 cells/ml) of colony cells were obtained on the 15th day of growth and cells were mostly found in colonies. The diameter of colonies was in the range of 75.2 to 85.1µm. Motile cells reappeared in the growth medium in few numbers.

From 18th day of growth onwards the colonies started disintegrating in the culture medium and solitary cell number (3.52×10^6 cells/ml)

increased over the number of colony cells (1.6×10^6 cells/ml). Motile cells were also present in the growth medium. The size of the colony was 86.6µm in diameter. From 21st day the growth started declining along with the disintegration of colonies. The cells got elongated in size and the shape of the colonies got distorted. The number of solitary cells increased in culture medium than the colony cells. The size of the colonies started decreasing and was about 40.6µm in diameter. The size of the solitary cells also decreased from 7.2µm to 3.2µm in diameter. Numerous flagellated cells were seen moving vigorously in the culture medium. On 24th day of growth the size of the colony was in the range of 19.5µm to 33.3µm and the number of colony cells were found to be 1×10^5 cells/ml, whereas, the number of solitary cells was about 2.4×10^6 cells/ml. (Figure 3.16).

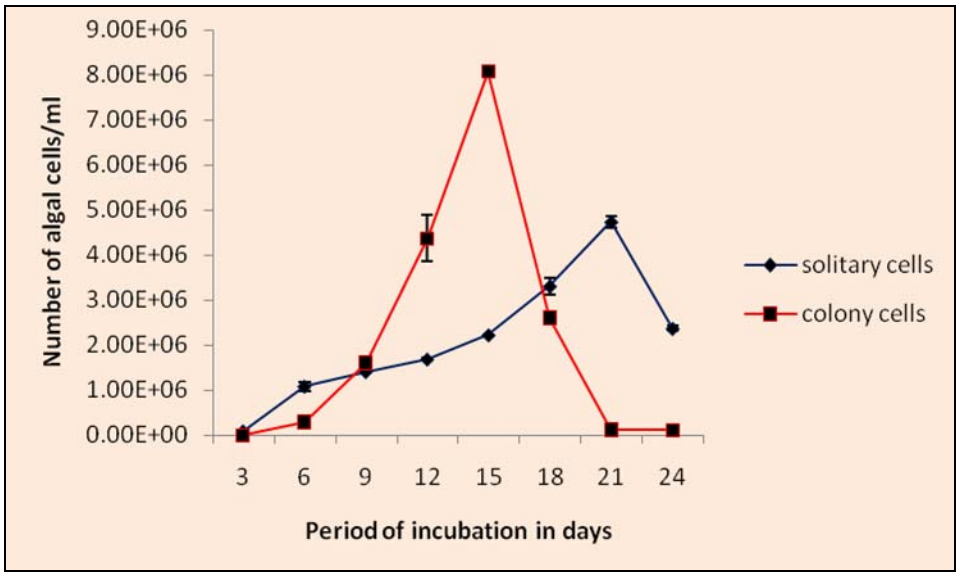

Figure 3.16 Number of colony cells and solitary cells of *Phaeocystis* sp. in Walne's medium.

Plate 1 *Phaeocystis* sp. on the 3rd day of growth

Plate 2 *Phaeocystis* sp. on the 6th day of growth

Plate 3 *Phaeocystis* sp. on the 9th day of growth

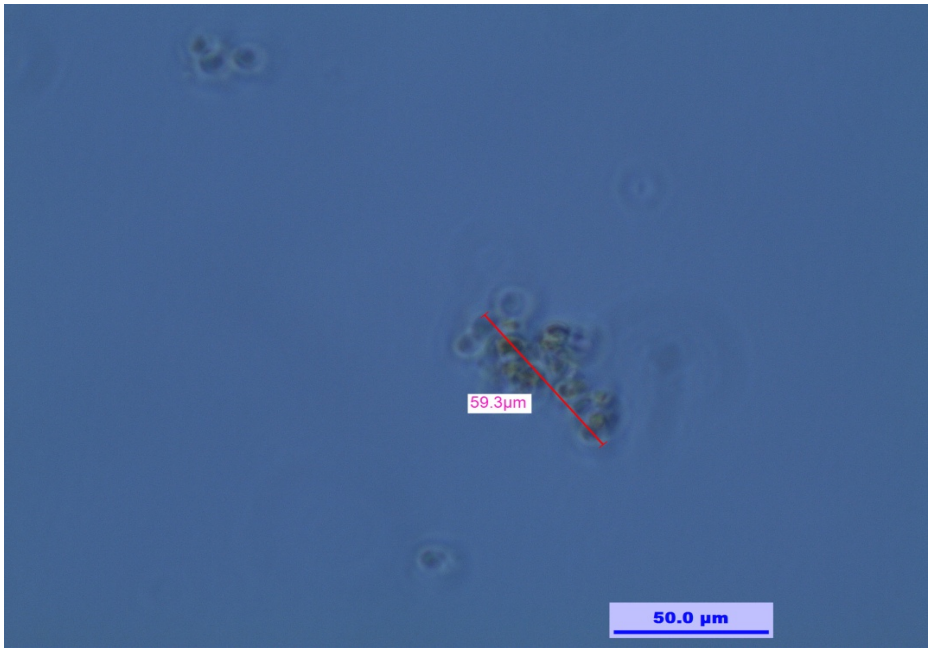

Plate 4 *Phaeocystis* sp. colony on the 9th day of growth

Plate 5 *Phaeocystis* sp. colony on the 12th day of growth (78µm in diameter)

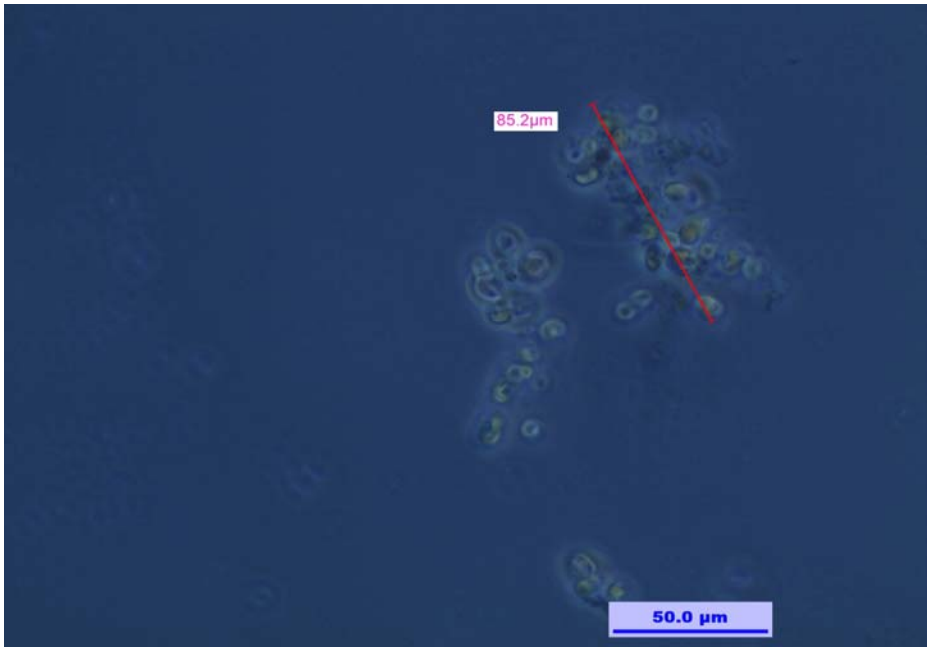

Plate 6 *Phaeocystis* sp. colony on the 12th day of growth (85.2µm in diameter)

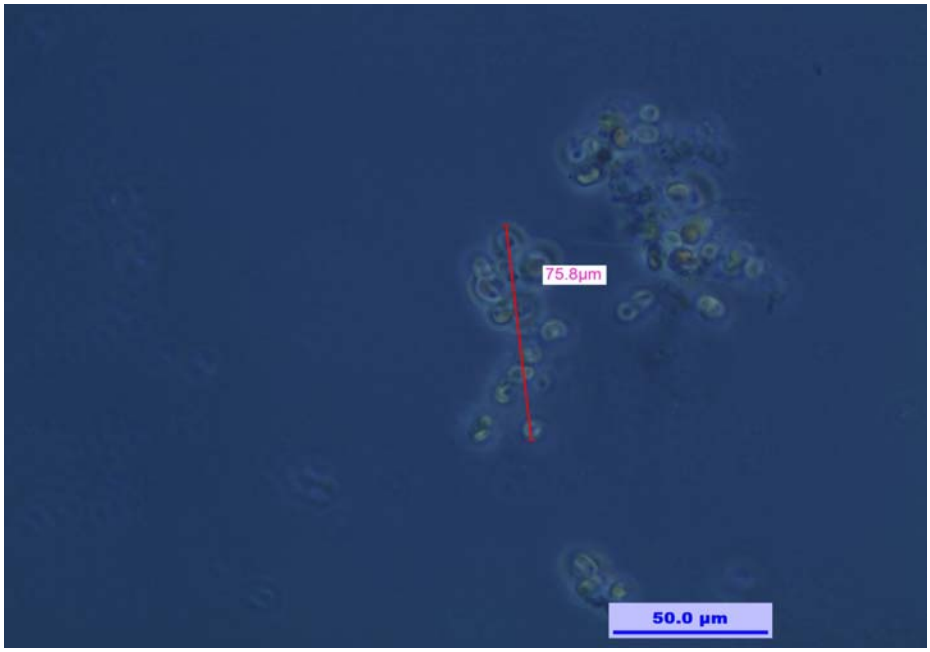

Plate 7 *Phaeocystis* sp. colony on the 15th day of growth

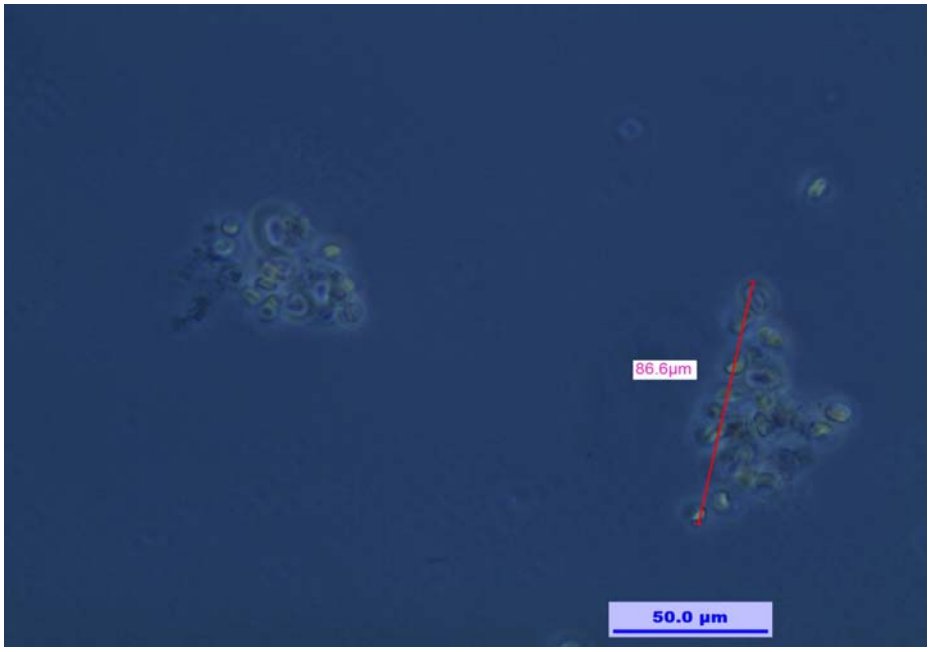

Plate 8 *Phaeocystis* sp. colony on the 18th day of growth

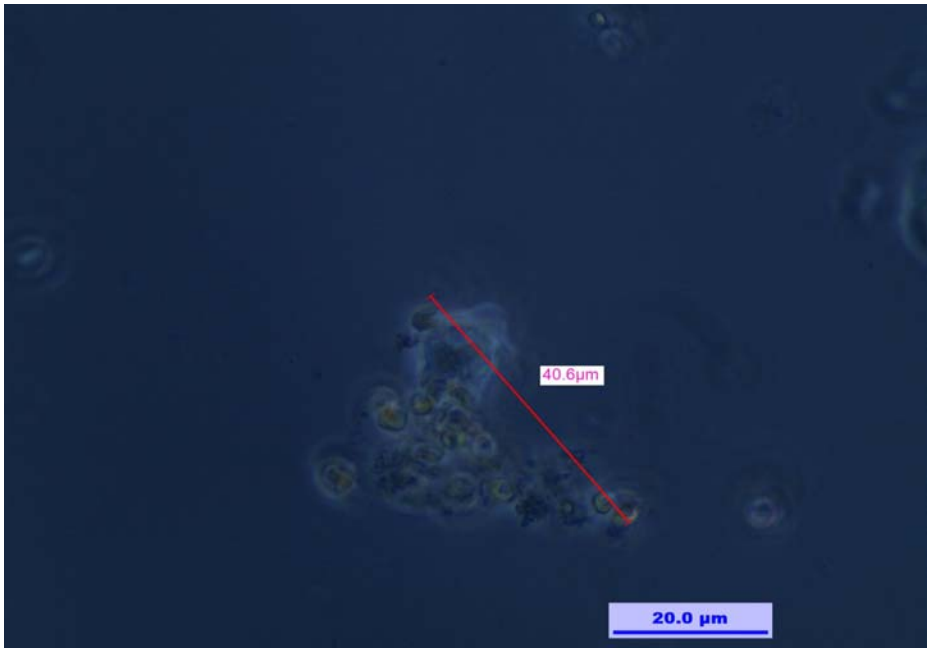

Plate 9 *Phaeocystis* sp. colony on the 21st day of growth

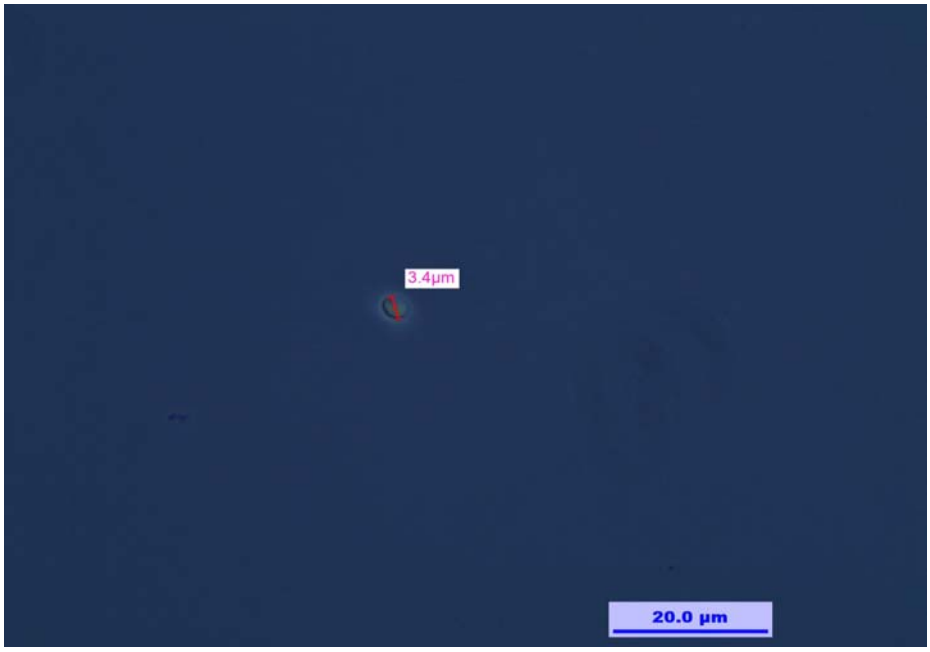

Plate 10 *Phaeocystis* sp. cell on the 24th day of growth

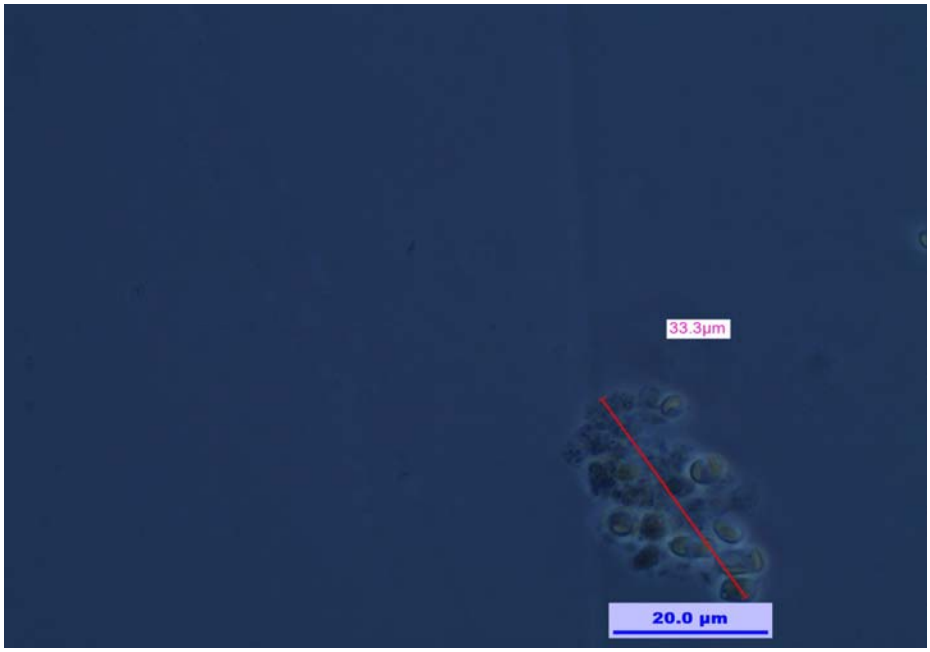

Plate 11 *Phaeocystis* sp. colony on the 24th day of growth

3.4.3 Biochemical composition

Total carbohydrate, protein and lipid content of *Phaeocystis* sp. were estimated using the standard methods.

Figure 3.17 to 3.19 show the total carbohydrates, total protein and total lipid concentration in logarithmic and stationary phases of growth of *Phaeocystis*. Carbohydrate content was higher in stationary phase (34.7mg/g) when compared to the logarithmic phase (24.6mg/g). Total protein was high in logarithmic phase (40.93mg/g) than the stationary phase (25.1mg/g). Total lipids seem to be almost same in both the logarithmic phase (12.2mg/g) and the stationary phase (11.6mg/g).

Statistical analysis showed that the total protein and carbohydrate content of the *Phaeocystis* sp. varied significantly between the logarithmic phase and stationary phase, but there was no significant difference in the

total lipid content at logarithmic and stationary growth phases. (Tables 3.8 to 3.10 of Appendix)

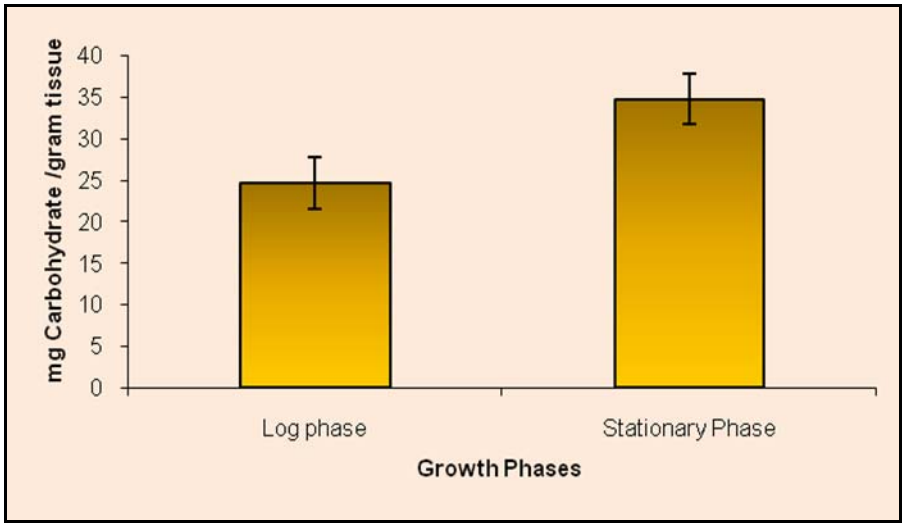

Figure 3.17 Total carbohydrates content of *Phaeocystis* sp. at different growth phases

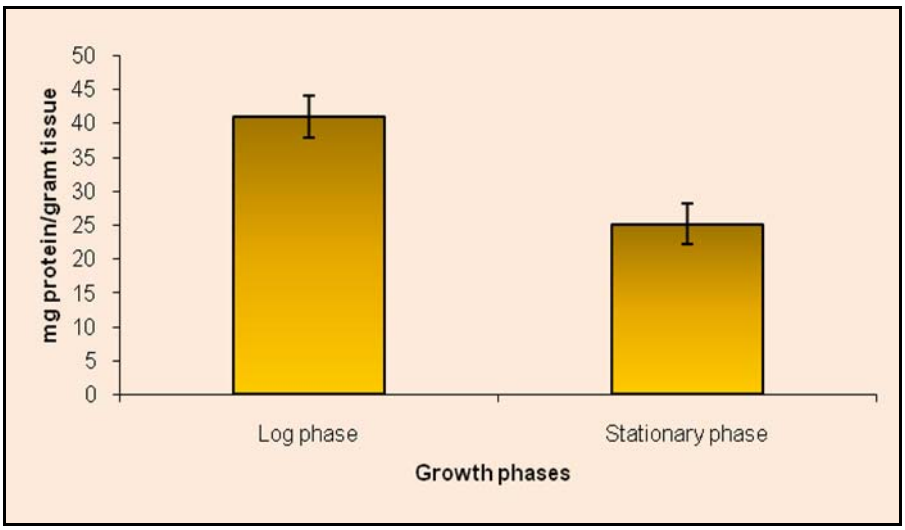

Figure 3.18 Total proteins content of *Phaeocystis* sp. at different growth phases

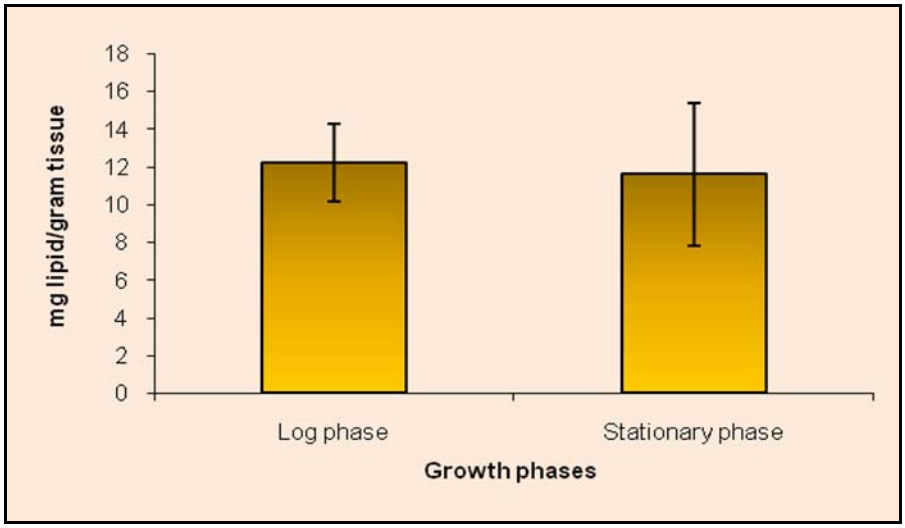

Figure 3.19 Total lipids content of *Phaeocystis* sp. at different growth phase

3.4.4 Extracellular release of biomolecules by *Phaeocystis* species

The extracellular proteins and carbohydrates released by *Phaeocystis* at logarithmic phase and stationary phase were estimated (Figure 3.20).

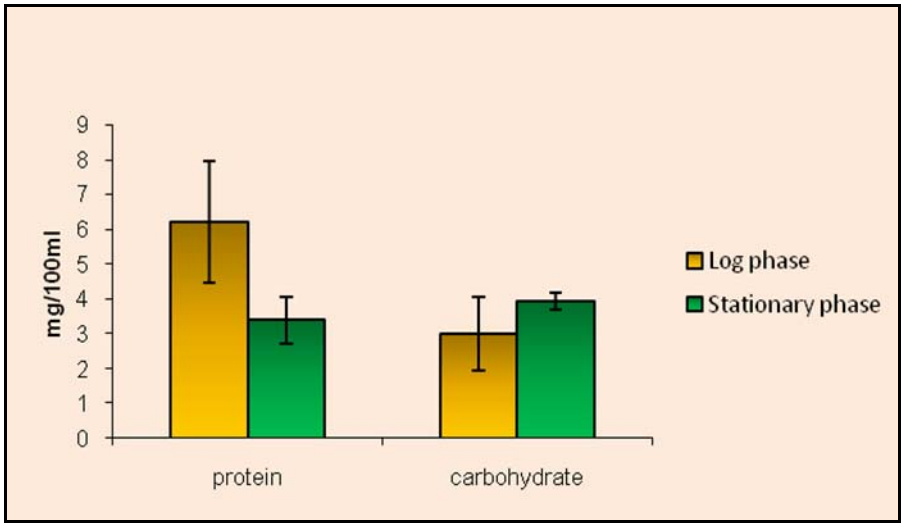

Figure 3.20 Extracellular releases of proteins and carbohydrates by *Phaeocystis* sp. at different growth phases

The quantity of proteins released from the cells was more in logarithmic phase (6.2 mg/100ml) than in stationary phase (3.4 mg/100ml), whereas, in the case of carbohydrates more quantity was released from the cells during stationary phase (3.94 mg/100ml) than in the logarithmic phase (3.10mg/100ml) (Tables 3.11 and 3.12 of Appendix).

3.4.5 Effect of nitrate and phosphate on the colony formation

The influence of macronutrients nitrate and phosphate on the colony formation of *Phaeocystis* was studied using Walne's medium prepared with artificial seawater in which the concentrations of the nitrate and phosphate were varied.

In NP non limiting medium, the colony cells were more abundant than the solitary cells. In this medium maximum colonial cells were found by the 15th day of growth period and after the logarithmic growth phase, the number of colony cells decreased, while the solitary cells increased. By 24^{th} day, the solitary cells were abundant in the nutrient non limiting medium. In nutrient limiting medium, where both the nitrate and phosphate were in low concentrations, the number of colony cells was very low and the algal culture was mainly dominated by the solitary cells. In N limiting medium and P limiting medium the number of solitary cells were higher than the number of colony cells (Figure 3.21 to 3.24 and Table 3.15 of Appendix).

Table 3.14 shows the nutrient concentrations of each media studied till the end of the growth experiment. Nitrate and phosphate were in surplus amount in the case of nutrient non limiting medium throughout the experiment. Both phosphate and nitrate were found to be very low in nutrient limiting medium by the end of the experiment. In P limiting

medium sufficient nitrate was present in the medium till 24th day but phosphate was very low. In N limiting medium, nitrate content of the medium was very low with high amount of phosphate.

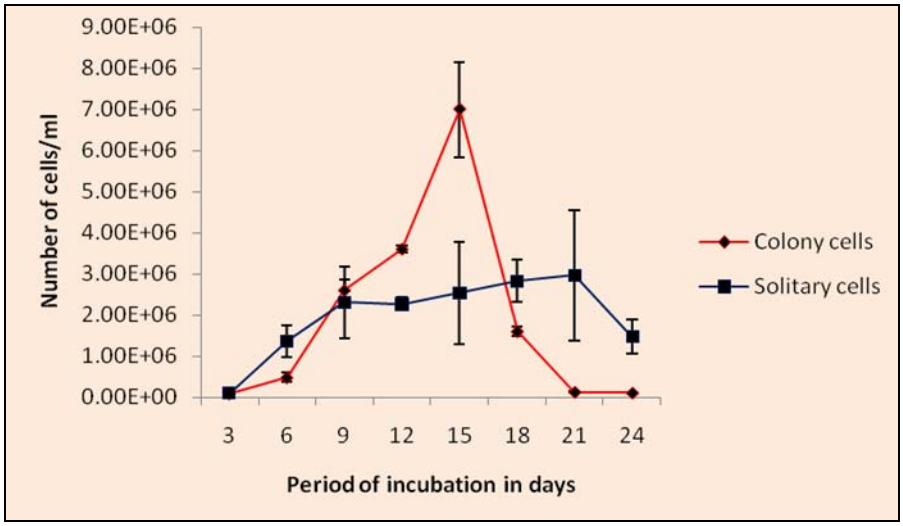

Figure 3.21 Number of colony cells and solitary cells in NP non limiting medium

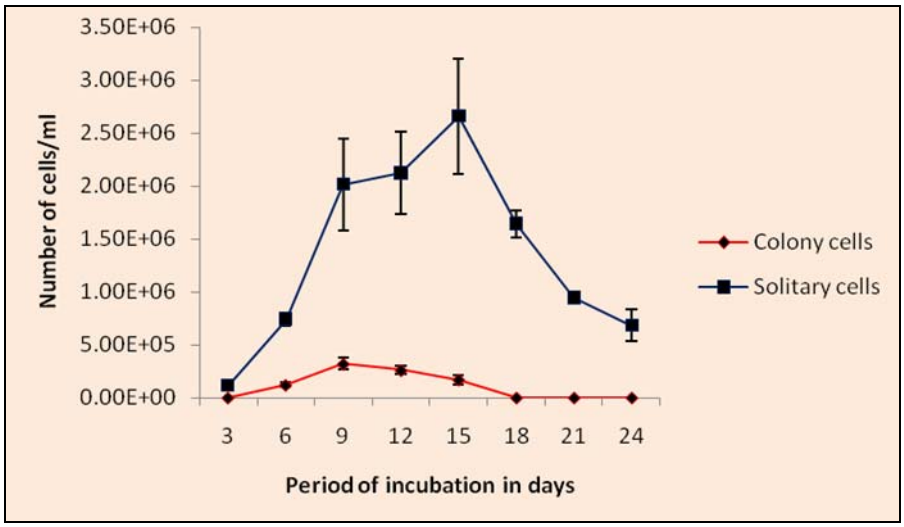

Figure 3.22 Number of colony cells and solitary cells in NP limiting medium

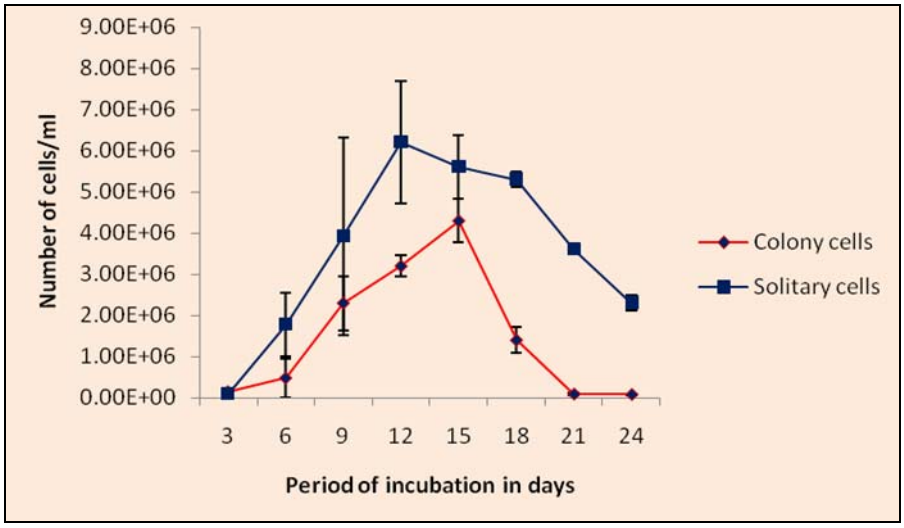

Figure 3.23 Number of colony cells and solitary cells in P limiting medium

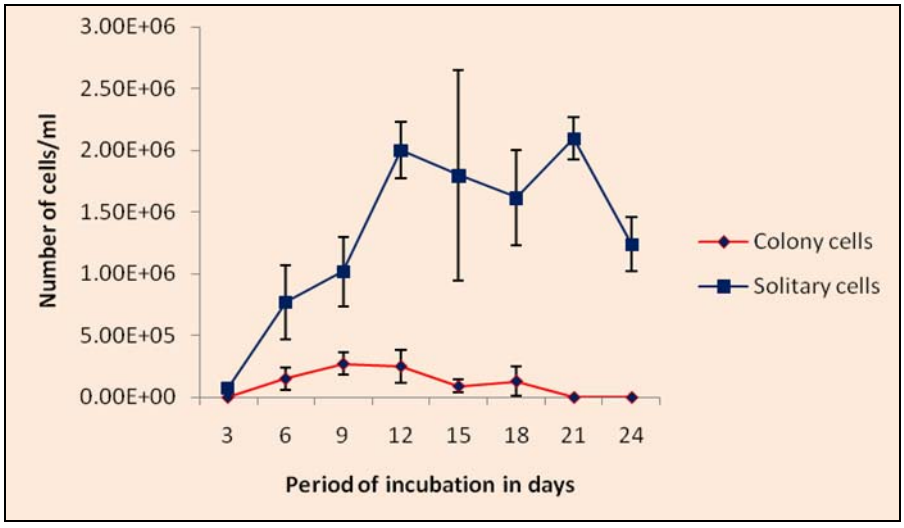

Figure 3.24 Number of colony cells and solitary cells in N limiting medium

Table 3.13 Concentration of nitrate and phosphate indifferent nutrient media during algal growth

Time period		4th day	8th day	12th day	16th day	20th day	25th day
NP non-limiting	Nitrate (µM)	1843 ± 40	1585±30.4	1233±57.8	1042±79.1	605.3±22.4	271.3±25.8
	Phosphate (µM)	34±4	18.09±6.02	6.6±3.05	1.58±0.155	0.82±0.46	0.55±0.34
NP limiting	Nitrate (µM)	92.3±2.5	80.3±5.5	52±10.4	12.4±2.5	4.5±0.5	0.70±0.2
	Phosphate (µM)	4.3±0.2	1.2±0.6	0.69±0.1	0.66±0.05	0.65±0.05	0.57±0.15
P limiting	Nitrate (µM)	1843±106	1486±80.3	1152±131	838±53.9	716±28.8	733±28.9
	Phosphate (µM)	2.6±0.55	1.6±0.2	0.42±0.017	0.34±0.017	0.32±0	0.25±0.05
N limiting	Nitrate (µM)	48.3±2.08	18±1.7	8.2±1.5	4.2±0.5	0.7±0.05	0.73±0.05
	Phosphate (µM)	58±2.5	27±1.5	14.6±0.58	12.6±0.5	11.6±0.55	10.03±1.05

3.4.6 Effect of iron on the growth and colony formation

The growth and the colony formation of *Phaeocystis* sp. were observed in Walne's medium with different concentrations of iron. The pattern of growth was almost similar in media with an iron concentration of 10µM and 5µM. Growth was seriously affected when the iron concentration was below 5µM. Growth was completely declined in medium with low or nil concentration of iron by the 18th day of growth. Maximum chlorophyll-a value (1.43µg/ml) was obtained in medium with high concentration of iron on the 15th day of growth period (Figure 3.25 and Table 3.14 of Appendix).

Statistical analysis revealed that growth of *Phaeocystis* sp. in different growth media with varying concentration iron showed significant variation (p<0.05).

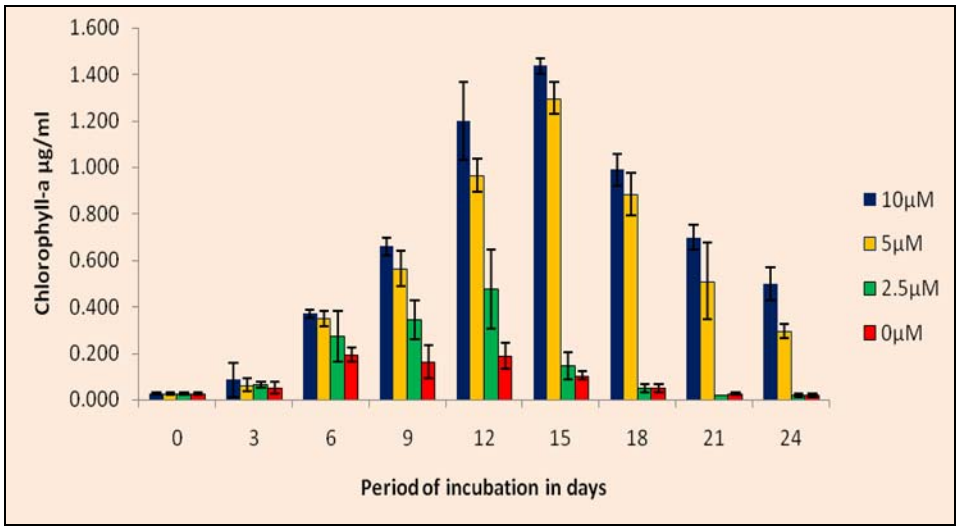

Figure 3.25 Effect of iron concentration on the growth of *Phaeocystis* sp.

The number of colony cells and solitary cells in the *Phaeocystis* sp. culture grown in different concentration of iron is shown in figures 3.26 to 3.29. Higher number of colony cells was observed only in culture with high concentration of iron (10µM). In media with iron concentration of 5µM the colony cells and solitary cells were present in almost same number with slight increase in the solitary cells though out the growth period. No colony formation occurred in medium with low concentration of iron and also in media without addition of iron (Table 3.16 of Appendix)

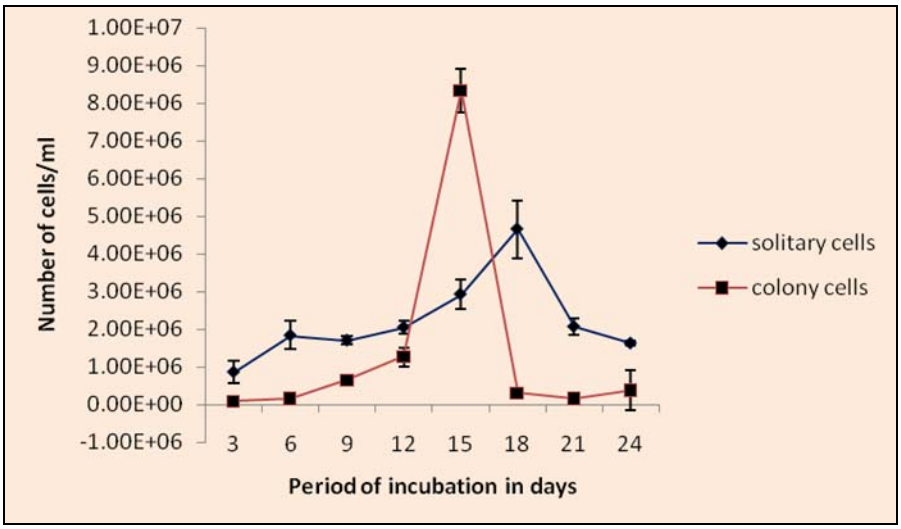

Figure 3.26 Number of colony and solitary cells in the medium with an iron concentration of 10µM

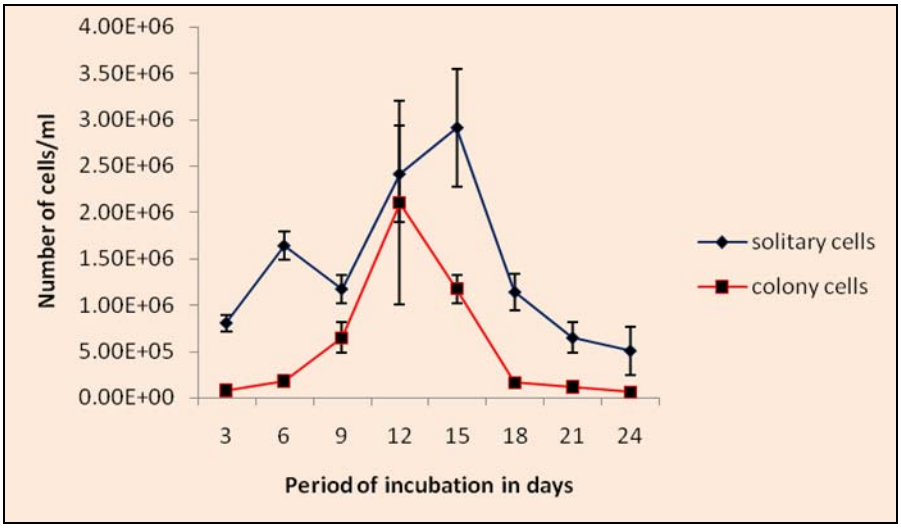

Figure 3.27 Number of colony and solitary cells in the medium with an iron concentration of 5µM

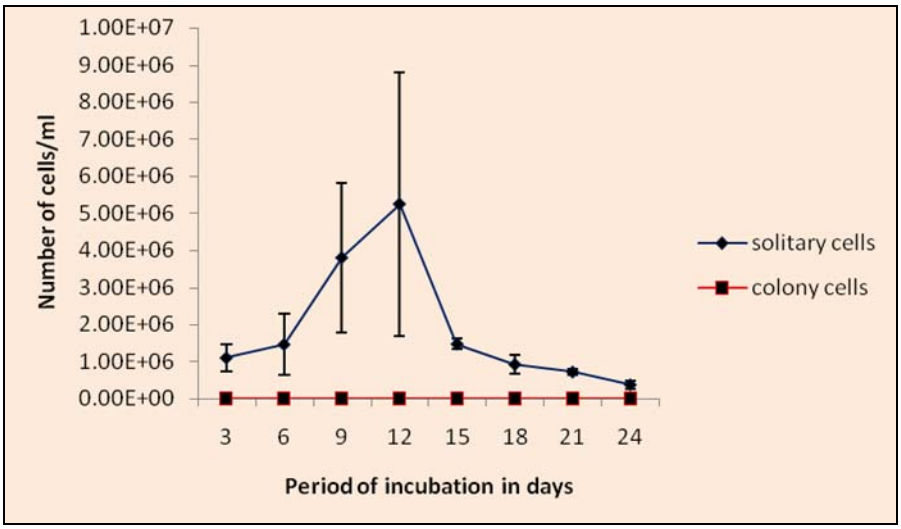

Figure 3.28 Number of colony and solitary cells in the medium with an iron concentration of 2.5µM

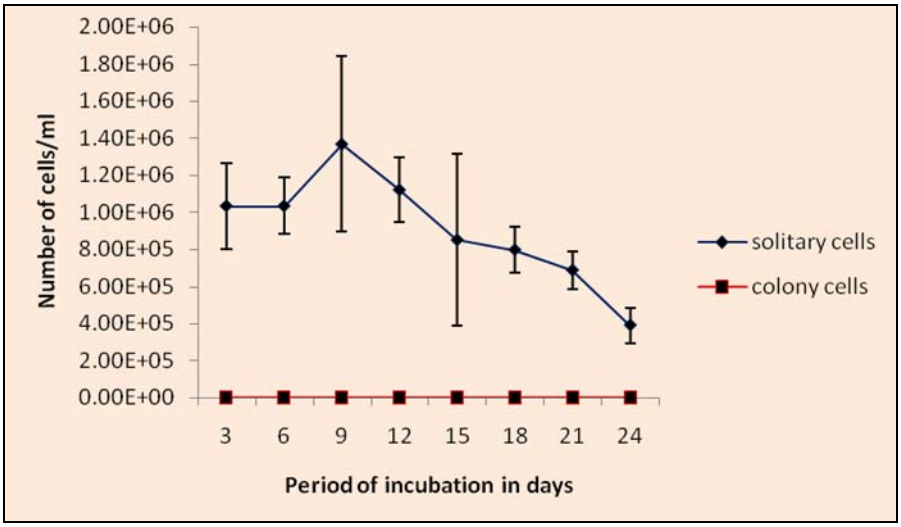

Figure 3.29 Number of colony cells and solitary cells in the iron deficient medium

3.5 Discussion

3.5.1 Effect of salinity, pH and temperature on the growth of *Phaeocystis* species

The Cochin estuary was found to experience wide fluctuations in the hydrographical parameters such as salinity, temperature, pH, and nutrients concentration. The salinity of the estuary varied between 2-18ppt during monsoon, 23-30ppt during post monsoon and 25-36 during pre-monsoon period and pH ranged from 6.7 to 8. The average atmospheric temperature ranged from 23.8^0C to 34.52^0C with minimum and maximum values in January and May. Sea surface temperature varied from 23.3^0C to 31^0C. Humidity value ranged from 77.09% to 93.66% and the region is subjected to semidiurnal tidal influence with a variation of about 1m (Critical Habitat Information System for Cochin Backwaters-Kerala, 2002). The growth of *Phaeocystis* sp. isolated from the Cochin estuary was tested in varying salinity, temperature, and pH and the organism was found to be successfully growing over a wide range of these factors. The species was found to be growing at salinity 10 to 40ppt and pH 7 to 9. But maximum chlorophyll-a value was obtained at salinity 30ppt and optimum growth was observed at pH 8.

The growth of *Phaeocystis* sp. was tested at different temperatures ranging from 10 to 30^0C and the strain was found to be able to grow at temperature ranging from 15 to 30^0C with maximum growth at 20 and 25^0C. But the turnover rate of the alga varied with different temperature and the cells entered the different growth phases (lag phase, log phase and stationary phase) at different rates. The lag phase was considerably prolonged when the incubation temperature was 15^0C. At 25^0C, maximum growth was obtained on 12^{th} day. As temperature increases, there occurs

shortening of the generation time and the cell metabolism increases causing the cells to enter into different growth phases with minimum time period (Jahnke, 1989). The optimum temperature varied for different species of *Phaeocystis*, with 15^0C for *Phaeocystis globosa* (Jahnke, 1989), whereas the *Phaeocystis pouchetii* could grow even at temperature 1.5^0C (Verity *et al*., 1988). Many scientists could successfully culture *Phaeocystis* species at temperatures ranging from 20 to 27^0C (Guillard and Hellebust, 1971; Vaulot *et al*., 1994). The results of the present study show that *Phaeocystis* sp. could well establish in tropical waters and could form blooms. *Phaeocystis* was considered to be a blooming alga only in the temperate and polar waters earlier, but the blooms in warm waters showed that the genus was well adapted to the conditions of the tropical waters.

3.5.2 Effect of nutrients on the growth

The growth of *Phaeocystis* sp. in different growth media was tested in the laboratory and Walne's medium was found to be supporting the maximum growth of *Phaeocystis*. Good growth was also observed in f/2 medium and f/2-Si medium showing that silicate is not important for the growth of *Phaeocystis* unlike the diatoms. But the growth was decreased in f/4, f/20 and minimum growth was observed in f/50 medium, showing that as the dilution of the media components increased, the growth rate decreased and growth completely diminished in minimum concentrations of nutrients. In the Walne's medium, the concentrations of the major nutrients, nitrate and phosphate was varied from high value to low value and tested the growth in each concentrations. The growth was more influenced by the nitrate concentration than the phosphate concentration of the growth medium.

Growth was very low in media where both nitrate and phosphate were limiting, whereas growth was still observed in media where only phosphate was limiting. The media with surplus concentration of phosphate and low nitrate concentration also did not support the growth of *Phaeocystis* sp. Most of the marine and estuarine algae are usually limited by nitrate than the phosphate concentration and *Phaeocystis* is a good competitor for nitrogen and a poor competitor for inorganic phosphate under light saturated conditions (Riegman *et al*., 1992). In general, the competitive ability of algae is determined by a combination of uptake characteristics and the efficiency with which nutrients are assimilated into new cell material. In nitrate controlled cultures, colony formation is stimulated by nitrate. Calculation of the N/P ratio of *Phaeocystis globosa* based on net uptake during growth in batch cultures under non limiting conditions yielded a molar ratio of 9.8 (van Boekel and Veldhuis, 1990). This means in comparison with other algae, which show an average ratio of 11.1 (Hecky and Kilham, 1988), *Phaeocystis globosa* converts nitrogen more efficiently into new biomass than phosphorous. The physiological mechanism of this phenomenon is unknown, but when the *Phaeocystis* is distributed in the controlled environments, the competition for nutrients occur only if nitrate is the major controlling factor (Riegman *et al*., 1992). Field observations along the continental coast of the North Sea (Cadee and Hegeman, 1986), the Barents Sea (Sakshaug and Slagstad, 1992), and in the Green land Sea (Smith *et al*., 1991) are examples for the *Phaeocystis globosa* blooming in environments where nitrate is available. In the present study also, *Phaeocystis* grew maximally when there is high quantity of nitrogen in the medium which confirms that nitrogen is very important in algal blooming.

3.5.3 Cell polymorphism

The growth characteristic of *Phaeocystis* sp. was studied thorough out the growth period of 24days and the polymorphic behaviour of the *Phaeocystis* cells during the entire growth period was tested. The heteromorphic lifecycle was observed in the laboratory that involves a diploid stage of one morphology which undergoes meiosis to form haploid cells of a different morphology, which then transform into gametes that fuse and reform the diploid stage, thereby restoring the first morphology (Billard, 1994).Three types of cells were found to occur in the culture from the lag phase towards the end of the culture. Flagellated single cells were seen moving randomly in the culture in the initial growth period in very little number along with the non-motile solitary cells. By the 6^{th} day of growth, the colonies started developing in the culture with the aggregation of four or five cells along with the single solitary cells. The size of the colonies was found to be gradually increasing towards the exponential growth phase and number of colony cells increased over the solitary cells. The flagellated cells were found to be less or absent towards the logarithmic phase with reappearance towards the end of the growth period.

The colonies observed in the present study showed similarity to *Phaeocystis jahnii*. The functional difference of the colonies of different *Phaeocystis* sp., varies in antigrazing property, in which *Phaeocystis pouchetii* and *Phaeocystis antarctica* have analogous structures of large diameter and in the case of the well organised colonies of *Phaeocystis globosa*, the colony skin give a mechanical protection of cells (Jacobsen, 2000). In the present study the colonies of the *Phaeocystis* species were found to be amorphous and less organised compared to other *Phaeocystis* species, and the cells were found distributed throughout the colonies. The

colonies have an irregular and asymmetric shape showing similarity to the colonies of *Phaeocystis jahnii*, (Medlin and Zingone, 2007) and colonies of cyanophyta, *Microcystis* and *Aphanizomenon* (Hamm, 2000). The colony skin has less effective mechanical defence than the highly specialized colonies of other *Phaeocystis* species.

The solitary cells (either motile or non-motile) were found to be present in the culture though out the growth period, and two main advantages were proposed for the retention of solitary cells. The most important was that the haploid (motile) solitary cells stage is required for the sexual reproduction within the genus (Cariou *et al*., 1994; Rousseau *et al*., 1994; Vaulot *et al*., 1994; Peperzak *et al*., 2000b). Cells within colonies are diploid, lack flagella and are immobilized and thus cannot take part in sexual reproduction. Secondly the solitary cells (mainly non motile) were needed for the development of new colonies where they stick to a surface and then divide to form new colonies. However other evidence for mixed culture of solitary cells and colonies implied that colonies might also able to utilize organic phosphates (Veldhuis *et al*., 1991). During the end of the culture, colonies started disintegrating with the release of flagellated solitary cells to the medium and the biomass of non motile solitary cells also increased that was released from the colonies by its disintegration. The release of the single celled swarmmers had been documented in culture (Kayser, 1970; Verity *et al*., 1988) and in other field studies (Jones and Haq, 1963; Parke *et al*., 1971).

3.5.4 Biochemical composition

The biochemical composition of the *Phaeocystis* sp. was found to be differing with the growth phase of the cells and it was found that total

protein content were more during the logarithmic phase compared to stationary phase. Total carbohydrates were more during the stationary phase of the algae compared to the logarithmic phase. The carbohydrates and lipids mainly form the structural components of the cells and carbohydrates form the main component of the gelatinous matrix of the colonies (Guillard and Hellebust, 1971; Veldhuis and Admirral, 1985) where, the proteins determine the physiological properties of the algae. When nutrients become limiting and *Phaeocystis* blooms reach a stationary growth phase, excess energy was stored as carbohydrates that leads to an increase in C/N and C/P ratios of *Phaeocystis* organic material. At the end of the bloom, deterioration of colonies occur due to various processes like autolysis of cells, grazing of colonies and cells, and lysis of cells that were virally infected causing the release of dissolved organic matter into the surrounding water column that was rich in glucan and mucopolysacharides. This organic matter is potentially readily degradable by heterotrophic bacteria surrounding the algal cell (Brusaard *et al*., 2005; Ruardy *et al*., 2005). The *Phaeocystis* cells also release large amount of organic material into the outside environment especially during its colonial stage (Alderkamp *et al.,* 2006). In the present study also there observed secretion of organic matter mainly proteins and carbohydrates by the cells into the culture medium. This secretion is more during the logarithmic phase than the stationary phase especially the proteins. These released organic materials form good substrates for the bacterial population and they mainly depend on this release. . The production of dissolved organic matter and subsequent formation of transparent exopolymers may influence the microbial population dynamics directly through bacterial colonization and indirectly though scavenging of predators and viruses (Brussaard *et al*., 2005). The relationship between *Phaeocystis* photosynthesis, DOC release

and microbial dynamics is thus complex and likely mediated by nutrient availability (Thingstad and Billen, 1994).

3.5.5 Effect of nutrients on the colony formation

The colony forms mainly dominate the *Phaeocystis* blooms in natural waters and sustained by the nutrients of natural or anthropogenic origin. The recent appearance of *Phaeocystis* colonies in the Arabian Gulf due to the nutrient enrichment by industries and sewages and the long term increase of *Phaeocystis* bloom occurrences in the eutrophicated temperate waters gave support for the good adaptability of colony forms to grow in enriched coastal waters. This makes *Phaeocystis* a useful indicator of long-term or chronic environmental changes in the respective areas (Lancelot *et al*., 1994). It is interesting to consider that the natural blooms of *Phaeocystis* colonies in subtropical waters described were reported mainly from the coastal and upwelling locations, giving additional evidence for the potential stimulatory effect of elevated nutrients either on rates of colony developments or accumulation of colonies. A range of nutrient and light conditions that occurs in subtropical and tropical waters supports the range of growth conditions for the colony developments. Stress, mainly nutrient deprivation, apparently can influence colony formations from solitary cells and vice versa (Cadee, 1996; Verity *et al*., 1988).

The influence of nutrients mainly nitrate, phosphate and iron in the growth media on the colony formation of *Phaeocystis* was tested. The number of colony cells was found to be more than the solitary cells in the nutrient non limitng culture. But when the phosphate alone was limitng in the culture medium, the number of solitary cells were higher compared to colony cells. At the same time the total chlorophyll-a value of the

Phaeocystis sp. culture was not affected much by the phosphate limitation. Under phosphate limitation, solitary cells are able to assimilate phosphate more effectively than the colonial cells (Riegman *et al*., 1992). The specific affinity of phosphate uptake of P limitng single cells is about ten times higher than the affinity of colonial cells (Veldhuis *et al*., 1991). When a batch culture of *Phaeocystis* grows into a stationary phase induced by P depletion, the percentage of colonial cells decreases (Veldhuis *et al*., 1991), indicating that flagellate cells are more capable of continued cell division at low phosphate concentrations than the colonial cells. It was suggested that the lower rate of division of colonial cells is due to diffusion limitation by the colonial mucus. The diffusion limitation of phosphate uptake by algae has been reported for the freshwater cyanobacteria *Oscillatoria agardhii* (Riegman and Mur, 1984) and *Synechococcus leopoliensis* (Mierle, 1985). It is also possible that the higher affinity for phosphate of single flagellate cells than of colonial cells is a consequences of physiological differences which produce another type of phosphate uptake carriers in single cells that is absent in the colonial cells (Riegman and van Boekel, 1996).

When the nutrients, both nitrate and phosphate were in limiting concentrations, the colony cells were found to be low or absent in the culture medium than the solitary cells. The nutrient deprivation may leads to the deterioration of colonies and the release of solitary cells from the colonies. In nitrate limitng cultures also, there observed the reduction of colonies in the culture even though phosphate was present in adequate amount. The chronic nutrient deprivation gradually leads to ghost colonies, suggesting that nutrient stress induce their life cycle event. Development of solitary cells and swarmmers may be the direct result of nutrient effects on the metabolism of colony cells, or an indirect effect of chemical inducers

released extracellularly by the stressed colony cells (Verity *et al*., 1988). The disintegration of large colonies and the concurrent release of solitary cells of small size (3-8µm), significantly alter the community composition and feeding behaviour of herbivores, microzooplankton where they form a good source of food. Moreover, bacterial population is enhanced following the collapse of colony bloom. Thus release of swarmmers alters the ecological efficiency of the plankton food webs.

Another difference in uptake characteristics is that, in contrast to flagellate cells, colonial cells are able to maintain their inorganic phosphate uptake rate in the dark (Veldhuis *et al*., 1991). So to some extent their poor uptake characteristics are compensated by prolongation of the uptake period. There is no evidence that either nitrogen or phosphorus is stored extracellularly in the colony matrix. Blooms of *Phaeocystis* sp. are usually restricted to areas with non limiting conditions of phosphate and nitrate.

The micronutrient iron has been shown to limit phytoplankton photosynthesis and growth (Martin *et al*., 1990; Sedwick and DiTullio, 1997; Olsen *et al*., 2000). An iron limitation in the Ross Sea during the summer is suggested as the reason for an increase in the flagellated and solitary cells in the summer (Smith *et al*., 2003). In the present study, iron limitations lead to complete absence of the colony in the culture along with the decrease in the total growth of the *Phaeocystis*. When the iron was in moderate concentration, the colony cells were produced in the culture during the early growth period, but along with the decrease in the iron concentration in the culture both the chlorophyll concentration and the colony formation was seriously affected. When the colony abundance decreased, the mean irradiance available to the remaining cells would increase and the iron demand per cells would decrease due to the

synergistic effect between irradiance and iron uptake (Sunda and Huntsman, 1997). Thus the iron limitation could result in enhanced colony degradation and liberation of flagellated cells (Wassmann *et al*., 1990), thereby directly producing an increase in the total abundance of solitary cells. Dissolved iron availability plays a primary role in regulating blooms of colonial *Phaeocystis antarctica* in the southern Ross Sea during summer (Verity *et al*., 2007).

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Chapter 4

ALLELOPATHIC EFFECTS OF *PHAEOCYSTIS* SPECIES

Contents

4.1 Introduction

Phytoplankton have been shown to compete for light and nutrients through different physiological and biochemical adaptations, such as, variation in surface area to volume ratio, production of specific enzymes, different nutrient requirements, luxury uptake, pigment composition, photosynthetic capacity, mixotrophy and vertical migration. Algal proliferations with respect to competition are part of plankton dynamics in aquatic environments. The negative influence of microalgae in terms of allelopathy and production of toxic compounds is also an adaptation by which some phytoplankton species could achieve a competitive advantage over other species, and hence it should be included as a relevant factor in competition.

Allelopathy refers to any process involving secondary metabolites produced by plants, algae, bacteria, and fungi that influence the growth and development of agricultural and biological systems (International Allelopathy Society, 1996). This includes both stimulatory and inhibitory effects, and both direct and indirect biochemical interactions. In fact, allelopathy is part of a

whole range of chemical communication mechanisms between organisms in a community. Allelopathy was considered as one of the key factors that promote the dominance of marine and freshwater harmful algal bloom forming species over other phytoplankton species. Harmful algal blooms are a serious ecological and socioeconomic problem facing the planet today. Majority of these HAB species are known to produce secondary metabolites, which can be toxic to other microorganisms, zooplankton, fish, shellfish, livestock and other large animals. The toxin production by HAB species through the release of degradation products may function in grazing deterrence, or as pheromones or allelochemicals (Turner and Tester, 1997; Wyatt and Jenkinson, 1997; Wolfe, 2000; Schmidt and Hansen, 2001)

In a complex community with a mixture of different species, the scope of the allelopathic compounds differ, some target organism may become adapted to an allelopathic compound, some will remain sensitive, and growth of some species are promoted by the release of compounds from the specific organism. The importance of allelopathy is enhanced in cases of abiotic stress (eg. nutrient availability, light), invasion of exotic organism, and synthesis of new molecules by the producer, delayed adaptation of the target organism and continuous release and limited degradation of allelochemical which lead to their accumulation in the environment (Reigosa *et al.*, 1999). In the ecological point of view, allelopathy is important in algal successions, explaining community structure, and the induction and termination of blooms. The microalgal allelochemicals include cyclic peptides, algaloids, organic acids, and long chain polyunsaturated fatty acids (Legrand *et al.*, 2003). The allelochemicals mainly inhibit photosynthesis, protein activity of the target

species, modify or activate its physiological functions, damage cell membranes, kill the competitor or exclude it from the donor vicinity.

Nutrient limitation enhances the toxin content and the release of toxin to extracellular medium in some marine HAB groups, such as haptophytes, dinoflagellates, diatoms and cyanobacteria. Both, the biotic and abiotic factors influence the production and accumulation of the toxic principle of the microalgae. The production of the allelopathic compound changes with the concentration and the age of the algal culture, pH, temperature and light. Studies show that the allelopathic effect of non toxigenic species on HAB species can be used as an agent for the biological control of harmful algal blooms in the aquatic systems.

The microalgae are known to produce a wide range of secondary metabolites with various biological actions. In the present study the action of the secondary metabolites produced by *Phaeocystis* sp. were tested in terms of allelopathic property on other microalgae and the haemolytic activity on human red blood cells.

4.2 Review of Literature

Phaeocystis regularly dominates the vernal blooms in coastal regions all over the world, especially in temperate and higher-latitude waters (Lancelot *et al*., 1987). It belongs to the phylum Haptophyta which includes organisms that are associated with toxin production. Substances with cytotoxic, icthyotoxic and haemolytic properties have been identified from several species of Prymnesiophyceae, a class of Haptophyta (Yasumoto *et al*., 1990; Aure and Rey, 1992; Stabell *et al*., 1993). The harmful effects of *Phaeocystis* were tested by various authors in terms of its haemolytic property, allelopathic effect on other phytoplankton groups and also

towards organisms of higher taxa (Stabell *et al*., 1999; Hansen *et al*., 2003; Rijssel *et al*., 2007; Hansen and Eilertsen, 2007).

4.2.1 Allelopathy in phytoplankton

Allelopathy has been observed in diverse ecological systems and also been shown to occur among various classes of marine algal species including cyanophyceae, bacillariophyceae, dinophyceae, haptophyceae, and raphidophyceae in both invitro and insitu.

Member of the class cyanophyceae show allelopathic action on other microalgal groups including chlorophytes, cryptophytes, dinoflagellates, chrysophytes, cyanophytes and diatoms. The cell free extract of *Oscillatoria elegans* and *Oscillatoria rubescens* were found to inhibit the growth of diatoms and chlorophytes (Keating 1977, 1978). Live cells and cell free filtrate of *Nostoc spumigena* caused growth inhibition and decrease of cellular chl-a and CO_2 uptake of diatoms (Flores and Wolk, 1986). The allelopathic compounds- microcystin, polyunsaturated fatty acids, and Kasumigamide (linear tetra peptide) were isolated from *Microcystis aeruginosa* by many workers (Ikawa *et al*., 1996; Sedmak & Kosi, 1998; Ishida & Murakami, 2000). A cyclic peptide isolated from the *Lyngbya* sp. was shown to inhibit growth and hormogonia development in other cyanobacteria and chlorophytes (Berry *et al*., 2004). The methanol extract of *Phormidium* sp. was found to be inhibiting growth and causing morphological and ultra structural alterations on other cyanobacterial species (Valdor and Aboal, 2007).

Chattonella antique and *Heterosigma akashiwo*, which belongs to raphidophyceae cause both growth inhibition and growth stimulation of phytoplankton (Matsuyama *et al*., 2000). Among the class bacillariophyceae

Pseudo-nitzschia pungens, Rhizosolenia alata and *Skeletonema costatum* were found to cause growth inhibition of other microalgal species (Legrand *et al*., 2003; Yamasaki *et al*., 2007).

Allelopathy was observed in the dinoflagellates - *Alexandrium catenella, Alexandrium minutum, Alexandrium tamarense* (Arzul *et al*., 1999; Wang *et al*., 2006), *Ceratium* sp., *Coolia monotis, Gambierdiscus toxicus, Prorocentrum lima* (Sugg and van Dolah, 1999; Legrand *et al*., 2003), *Gymnodinium breve* (Kubanek *et al.,* 2005), *Gymnodinium mikimotoi* (Fistarol *et al*., 2004) and *Peridinium aciculiferum* (Rengefors and Legrand, 2001). The chemical natures of the allelochemicals produced by these organisms were unknown and they were found to cause death, growth inhibition, growth stimulation, promotes cyst formation and immobilisation on other microalgal species.

A non protein light molecular weight compound produced by *Pandorina morum*, a chlorophyte cause photosynthesis inhibition and death (Harris and Cladwell, 1974). *Cosmarium vexatum*, and *Scenedesmus reinhardtii* were known to cause growth inhibition of microalgae (Wolfe and Rice, 1979).

The allelopathic effect of macroalgal species on microalgal community was demonstrated by various authors. The culture filtrate of *Ulva lactuca* inhibited the growth of three species of red tide microalgae- *Heterosigma akashiwo, Alexandrium tamarense* and *Skeletonema costatum* under laboratory conditions (Nan *et al*., 2008). The fresh tissue, culture filtrate and organic solvent extracts of *Ulva linza* (chlorophyta), *Corallina pilulifera* (rhodophyta) and *Sargassum thunbergii* (phaeophyta) inhibited the growth of *Prorocentrum donghaiense* in mesocosms experiments

(Wang *et al*., 2007). The growth of red tide microalgae *Skeletonema costatum* was inhibited by the crude extracts of five macroalgal species including *Porphyra tenera, Laminaria japonica, Ulva pertusa, Enteromorpha clathrata* and *Undaria pinnatifida* (Zhen *et al*., 2008). Xu *et al.* (2005) studied the allelopathic effects of *Enteromorpha linza* on *Heterosigma akashiwo*. The chlorophytes *Ulva pertusa* and *Ulva linza* were found to cause growth inhibition of *Prorocentrum micans* (Dinophyta) (Jin *et al*., 2005).

Allelopathy could affect phytoplankton population dynamics along with light, nutrient, grazing, or hydrodynamics (Pratt, 1966; Keating, 1977; Rojo *et al.*, 2000). Allelopathy acts as a key factor in promoting the dominance of marine harmful algal bloom forming species by the production of secondary metabolites by the HAB species which can be toxic to other microorganisms, zooplankton grazers, fish, shellfish, and livestock and other large animals (Carmichael, 1992; Anderson and Garrison, 1997; Wolfe, 2000).

4.2.2 Allelopathic property of *Phaeocystis*

Phaeocystis had been suspected for a long time of having a negative effect on co-occurring organisms. Schools of herrings seemed to avoid *Phaeocystis* blooms (Savage, 1930). Penguins died after consumption of krill that fed on *Phaeocystis antarctica* (Sieburth, 1960, 1961). Negative effects of *Phaeocystis* towards bryozoan, *Electra pilosa* were recorded by Jebrem (1980). *Phaeocystis* exerts its harmful effect mainly by clogging of fishing nets and accumulation of foam on beaches (Lancelot *et al*., 1987). *Phaeocystis pouchetii* was listed as a nuisance species by the Intergovernmental Oceanographic Commission (IOC) (Moestrup and Thomsen, 1995).

Mass mortality of caged fish occurred during a *Phaeocystis globosa* bloom in China Sea (Huang *et al*., 1999). The organic extracts from *Phaeocystis pouchetii* inhibited cell divisions in newly fertilised eggs of sea urchin, *Sphaerechinus granularis* in a dose dependent manner. It was found that *Phaeocystis pouchetii* release some compound to its surroundings that was toxic to cod larvae (Aanesen *et al*., 1998). The lethality of cod larvae exposed to cultures of *Phaeocystis pouchetii* increased with irradiance.

The allelopathy results from the increase in cell concentration during colony formation of *Phaeocystis* which was inturn depends on the competition for nutrients. *Phaeocystis pouchetii* showed anti mitotic activity towards sea urchin embryos along the coast of Northern Norway (Hansen *et al*., 2003). The allelopathic effect of *Phaeocystis pouchetii* towards diatom growth was studied by Hegarty and Villaereal (1998) and Hansen and Eilertsen (2007). Negative effects of *Phaeocystis* was studied by Jiesheng *et al*. (2010) who found that, the organic cell extract and culture filtrate of *Phaeocystis globosa* inhibited the growth of *Prorocentrum donghaiense*, *Chattonella marina* and *Chattonella ovata* under laboratory conditions, whereas the extracts were mildly toxic to *Artemia salina* causing only 10% mortality.

4.2.3 Haemolytic property

The class Haptophyceae contains several species associated with toxin production; *Chrysochromulina polylepis* produced and excreted glycolipids with haemolytic property (Yasumoto *et al*., 1990). Several microalgal species were known to produce haemolytic compounds capable of lysing vertebrate RBC. Haemolytic compounds were identified in various microalgal groups including *Amphidinium carterae* (Nayak *et al*., 1997), *Chrysochromulina*

polylepis (Johansson and Graneli, 1999), *Pfiesteria piscicida* (Marshall *et al*., 2000), *Alexandrium tamarense* (Eschbach *et al.*, 2001), *Prymnesium parvum* (Yariv and Hestrin, 1961; Shilo, 1971; Uronen *et al*., 2005), *Karenia brevis* (Neely and Campell, 2005) and *Fibrocapsa japonica* (de Boer *et al*., 2009).

The haemolytic toxic principle in *Phaeocystis* was different from that described for other prymnesiophytes and the proposed toxins released by *Phaeocystis* appear to be compounds that hold anaesthetic properties, possibly expressing toxic effects when presented in surplus dose (Stabell *et al.*, 1999). The cytotoxic, closely related compounds, 2-trans-4-cis-7-cis-decatrienal and 2-trans-4-trans-7-cis-decatrienal were identified in extracts of *Phaeocystis* culture fluid which reduced the growth of yeast cells (Hensen *et al.*, 2004). Haemolytic property in the extract of *Phaeocystis pouchetii* was characterised and it was shown that the toxin make pores in the surface of red blood cells and the toxin has no specific membrane receptor in the red blood cell membrane (Peng *et al*., 2005).

Haemolytic activity of live *Phaeocystis* cells was studied during mesocosm blooms by Rijssel *et al.* (2007). Samples containing live *Phaeocystis pouchetii* cells showed higher haemolytic activity, whereas, culture filtrate and cell extracts were less haemolytic or without effect. Haemolytic activity of *Phaeocystis* cells was further enhanced by increased temperature and light. Both the isolated toxin and supernatant of the *Phaeocystis globosa* cultures inhibited cultures of other microalgae (Jiesheng *et al*., 2010).

4.2.4 Haemolytic compounds

Up to now, three toxic components that could be involved in chemical deterrence had been identified in *Phaeocystis* species, acrylate, a

polyunsaturated aldehyde, and a haemolytic glycolipid. Acrylate was produced by *Phaeocystis* (Guillard and Hellebust, 1971) upon enzymatic cleavage of dimethyl-sulphonio-propionate and accumulates in mM concentrations in the colonial mucous layer (Noordkamp *et al*., 1998). During growth of the alga, however, acrylate was unlikely to cause harmful effects on nearby living cells because it was not excreted from the colonies. Additionally the concentration of acrylate present in the water column was not expected to exceed a concentration of above 4µM, which was much lower than the mM range of LC_{50} values for acrylate reported for marine organisms (Wolfe *et al*., 1997; Noordkamp, 2000; Sverdrup *et al*., 2001). But the acrylate could have a negative effect when *Phaeocystis* cells accumulate in the guts of grazers and also in acidic environment, where the acrylate would be in the protonated toxic form (below pH 4.25).

Polyunsaturated aldehyde isolated from *Phaeocystis pouchetii* (Hensen *et al*., 2004), form a line of defence. Membrane lipids are converted to mildly toxic polyunsaturated fatty acids, by a grazing activated enzymatic conversion. In the presence of reactive oxygen species PUFAs may be converted in to highly toxic polyunsaturated aldehydes (PUAs). PUAs negatively affect copepod fecundity and egg hatching and induce apoptosis in sea urchin embryos and cytotoxicity in human cell lines (Pohnert and Boland, 2002). Extracted culture fluid of *Phaeocystis pouchetii* containing PUAs and densities of 10^6 cells/ml, completely blocked DNA replication in sea urchin embryos (Hansen *et al*., 2003; Hensen *et al.*, 2004).

A haemolytic glycolipid isolated and characterised with a digalactose and a PUFA (heptadecadienoyl) group was found to be responsible for fish

mortality in coastal waters of southeast China (Huang *et al*., 1999) by induction of pores in the cell membrane of target cells (Peng *et al*., 2005).

4.2.5 Effect of nutrients on allelopathy and haemolytic property

Shilo (1967) first showed a correlation between the nutrient limitation and enhanced toxicity in *Prymnesium parvum*. This was also later confirmed in *Chrysochromulina polylepis* (Edverdsen *et al*., 1990). Phytoplankton was regularly subjected to environmental conditions that were less than optimal for them and respond to changes in the availability of nutrients by adapting their chemical composition to maintain growth (Laws and Bannister, 1980). A common feature of nutrient limitations was a decrease in the cellular concentration of the limiting nutrient which affects the physiological status of the algae (Campbell *et al*., 1984; Sakshaung and Olsen, 1986). Although, the optimal ratios may vary substantially between different species, both allelopathy and haemolytic property was closely associated with nutrient supplies in the aquatic environment (Einhelling, 1995). Nutrient limiting conditions caused algal cells to release organic compounds in elevated amounts into the water which caused deleterious effects on other organisms (Myklestad, 1977, 1995; von Elert and Juttner, 1997). Blooms of *Phaeocystis globosa* along the coastal waters of southeast China exhibited enhanced haemolytic activity in N-deficient medium (Liu *et al*., 2006).

In the present study the allelopathic effect of *Phaeocystis* sp. on the phytoplankton, *Chlorella marina, Chaetoceros calcitrans,* and *Isochrysis galbana* were studied under laboratory conditions. The haemolytic property was studied using human RBC. The effect of nutrients on the allelopathy and haemolytic property was also investigated.

4.3 Materials and Methods

4.3.1 Effect of *Phaeocystis* species cell extract on phytoplankton

4.3.1.1 Algae and experimental conditions

The *Phaeocystis* sp. was grown in artificial sea water supplemented with Walne's medium with an initial pH of 8.00 and salinity 30ppt. The incubated flasks were shaken manually, twice daily at set times. The microalga was cultivated to the exponential growth phase for use. The initial cell density of *Phaeocystis* sp. was set at 1×10^4 cell/ml.

The test microalgae *Chlorella marina, Chaetoceros calcitrans* and *Isochrysis galbana* were obtained from the algal collection in the Department of Marine Biology, Microbiology and Biochemistry, CUSAT and were maintained in the environmental chamber under the same culture conditions as that of *Phaeocystis* sp.

Artificial sea water was used for medium preparation to avoid the presence of biomolecules that were not produced by *Phaeocystis.* Artificial sea water was autoclaved, cooled and added the nutrients and vitamins (filter sterilized) according to the composition of Walne's medium. Culture temperature was maintained at 20^0C on a 12h:12h light:dark cycle with an illumination of 2000lux. All the microalgae, *Phaeocystis* sp, *Chlorella marina, Chaetoceros calcitrans and Isocrysis galbana* were grown as batch cultures in conical flasks containing 300ml of Walne's medium. Culture media were inoculated with microalgal cells at an initial cell density of 1×10^4 cell/ml and grown for 15 days. The experiments were carried out in triplicates.

4.3.1.2 Preparation of cell extract of *Phaeocystis* species

Phaeocystis cells were harvested at logarithmic growth phase (15^{th} day of experiment) by filtering 100 ml of culture using 1µm pore size GF/F filter

paper to separate the cells completely from the culture filtrate. The algal cells were re-suspended in a mixture of methanol:chloroform:water (13:7:5 v/v), and were vigorously sonicated using an ultrasonicater at 70 amplitude for 1 minute in ice to reduce the temperature rise. It was then centrifuged to remove the cell debris and the supernatant was evaporated to dryness in a vacuum desiccator and subsequently re-dissolved in 70% methanol.

4.3.1.3 Evaluation of the allelopathic activity of cell extract of *Phaeocystis* species

The allelopathic activities of cell extract on microalgae were tested following the method of Jiesheng *et al.* (2010). The microalgae *Chlorella marina* (chlorophyte), *Chaetoceros calcitrans* (diatom) and *Isochrysis galbana* (haptophyte) were used as test organisms. 1ml of the crude extract was added to 100ml sterile conical flasks. 1ml methanol was used as control. The flasks were kept open for the evaporation of the solvent. 30ml of the test algal cultures in the logarithmic growth phase were added to the flasks separately and shaken well. 70 ml of sterile Walne's medium in artificial sea water was added to obtain a final volume of 100ml. The chlorophyll-a concentration in the test and control flasks were estimated using the method of Strickland and Parson (1972) at 0hr, 24hr, 48hr, and 72 hr.

4.3.1.4 Statistical analysis

The results of the study were statistically analysed by one- way ANOVA using Microsoft Excel.

4.3.2 Effect of *Phaeocystis* species culture filtrates on phytoplankton

The allelopathic effect of culture filtrate of *Phaeocystis* sp. was also tested on the microalgae, *Chlorella marina*, *Chaetoceros calcitrans* and *Isochrysis galbana*.

4.3.2.1. Experimental set up

0.5 ml of microalgae *Chlorella marina*, *Chaetoceros calcitrans* and *Isochrysis galbana* from the stock cultures were inoculated into 150 ml of sterile Walne's medium prepared in artificial seawater of pH 8.00 and salinity 30ppt. The cultures were incubated in the environmental chamber as described in section 4.3.1.1.

Different nutrient conditions were used to study the effect of nutrient concentration on the release of allelopathic compounds from *Phaeocystis* sp. into the medium. Four different nutrient media (Table 4.1) were prepared in artificial seawater of salinity 30ppt and pH 8.00 for culturing *Phaeocystis* for the experiment.

Table 4.1 Concentration of nitrate and phosphate in the media

Nutrient conditions	Concentrations of Nitrate and Phosphate	
Nutrient sufficient medium (NP non limiting)	NO_3	2000µM
	PO_4^{3-}	100µM
Nutrient deficient medium (NP limiting)	NO_3	100µM
	PO_4^{3-}	5µM
Phosphate deficient medium (P limiting)	NO_3	2000µM
	PO_4^{3-}	5µM
Nitrate deficient medium (N limiting)	NO_3	100µM
	PO_4^{3-}	100µM

Trace metals, EDTA, iron and vitamins were added at levels corresponding to Walne's medium. *Phaeocystis* sp. cells were inoculated into the four nutrient media (100ml) to give the initial cell concentration of 1×10^4 cells/ml and incubated under optimal conditions.

4.3.2.2 Preparation of cell-free filtrates of *Phaeocystis* species

The cell-free filtrates were collected during the exponential phase for the nutrient sufficient medium (day 12) to make sure that the alga did not experience nutrient limitation and in early stationary phase for nutrient deficient media (day 18) to ensure the nutrient limitation. The cells in the culture media were removed by filtering under sterile condition in a laminar flow chamber, through 1µM pore size sterile GF/F filter paper and cell free culture filtrate was used for studying the allelopathic property.

4.3.2.3 Nutrient and chlorophyll-a estimation

The concentration of nitrate and phosphate was determined in the cell free filtrate of *Phaeocystis* prepared in order to find out the nutrient level after adding the culture filtrate into the cultures of test microalgae for the allelopathic experiment. Nitrate concentration was determined by spectrophotometric measurement at 505 nm using resorcinol as the reagent (Zhang and Fischer, 2006). Phosphate concentration was measured spectrophotometrically at 880nm using UV/Vis spectrophotometer following the method of Strickland and Parson (1972). Chlorophyll-a was estimated to understand the growth of *Phaeocystis* sp. in each nutrient conditions, since allelopathic compound released by *Phaeocystis* cell to the outside medium depends on the concentration of the algal cells. Chlorophyll-a was estimated following the method of Strickland and Parson (1972) as described in section 3.3.1

4.3.2.4 Estimation of allelopathic effect of culture filtrate of *Phaeocystis* species

The influence of the cell free filtrate from *Phaeocystis* sp. culture on *Chlorella marina, Chaetoceros calcitrans* and *Isochrysis galbana* were

evaluated using the method of Graneli and Johansson (2003). After 8 days of growth of the microalgae, 90ml from the 150ml algal culture were removed from each flask and replaced with equal volume of the cell- free filtrates of *Phaeocystis* sp. culture grown at four different nutrient conditions. As control, autoclaved seawater was used instead of filtrate. Nutrients (NO_3^- and PO_4^{3-}), trace metals, iron, EDTA and vitamins were added to all flasks in non-limiting (Walne's) concentrations to ensure that the growth of the different species was not limited by nutrients. Growth was calculated by estimating the chlorophyll-a concentration at 0hr, 24hr, 48hr, 72hr, and 96hrs.

4.3.2.5 Statistical analysis

To determine whether growth rate of the three microalgae tested varied significantly among different treatments, one-way ANOVA was done followed by Duncan's multiple comparison using SPSS 10.0 for Windows. Significant difference was calculated at $p<0.05$.

4.3.3 Artemia toxicity test

4.3.3.1 Hatching of *Artemia salina* cyst

Artemia salina, the brine shrimp, was used as a model organism to test the toxicity of *Phaeocystis* against animals. The toxicity test was done as per Venhaecke *et al.* (1981) and Graneli and Johansson (2003). The *Artemia* cysts were washed initially with sea water for four times and finally once with hypochlorate solution just to remove the thick outer cyst wall. It was then washed several times in sea water to remove the chlorine completely.

The cysts of *Artemia salina* were hatched, in autoclaved seawater with a salinity of 30ppt and pH 8.00. The cysts were kept under continuous

aeration at a temperature of 25^0C under continuous light. After 18-24hrs, hatched *Artemia* naupli (developmental stage 1) were transferred to fresh seawater (30ppt) and kept for an additional 24h. The transfer was made in order to be sure that all the naupli used for the test had reached the same developmental stages. The naupli were kept under the same conditions as the cysts. The toxicity of *Phaeocystis* cells and culture filtrate towards *Artemia salina* were examined using 48h old naupli. (Developmental stages 2 and 3).

4.3.3.2 Toxicity test on *Artemia salina*

Toxicity test was done using live *Phaeocystis* sp. cells and culture filtrate. Culture filtrate was prepared by filtering the *Phaeocystis* culture using 1µm pore-size filter paper to remove the cells completely and the supernatant was used for the test. The *Phaeocystis* cells were taken in two concentrations. Low concentration contained 6×10^6 cells/ml and high concentration contained 1.6×10^7 cells/ml. As a control, filtered autoclaved seawater was used. Ten ml of each cell suspension and culture filtrate were added to a 10ml well (six-well plate) and 10 naupli were transferred to each well. Six replicates were used for each test. One set of replicates (three in number) were kept in light and other set in dark conditions at a temperature of 25^0C, to verify whether there is any effect of light on the toxicity. Mortality of naupli was examined for three consecutive days under a stereomicroscope, and the naupli were considered dead if no movement of the appendages was observed within 10 seconds (Venhaecke *et al*., 1981).

4.3.4 Haemolytic property

The haemolytic property of *Phaeocystis* sp. was studied by growing the cells under different nutrient conditions to study the haemolytic property of the strain and the effect of culture conditions on haemolytic property.

4.3.4.1 Experimental set up

Walne's medium was prepared with filtered autoclaved artificial seawater with salinity of 30ppt, pH 8 and four nutrient conditions that vary in nitrate (N) and phosphate (P) were prepared. NP non-limited (NO_3 2000µM, PO_4^{3-} 100 µM), P limited (NO_3 2000µM, PO_4^{3-} 5µM), N limited (NO_3 100 µM, PO_4^{3-} 100µM) and NP limited (NO^{3-} 100 µM, PO_4^{3-} 5 µM). Trace metals, iron, EDTA and vitamins were added to all cultures at levels corresponding to Walne's medium (Walne, 1970). 1.2×10^6cells /ml of *Phaeocystis* were added to the culture medium and incubated in the Environmental Chamber.

Phaeocystis cell number were determined every day by light microscope (Nikon E200) at 400X magnification and growth was calculated at every 3 days in all the cultures by estimation of chlorophyll-a.

4.3.4.2 Nutrient Estimation

Sampling for nutrient analyses was done at 15th day when the cultures were in the logarithmic phase of growth. 20ml of the sample was filtered through 1 µm GF/F filter paper using a vacuum pump. The cell free supernatant was used for the analyses of phosphate and nitrate concentration in the medium. Nitrate and phosphate concentration were determined as mentioned in section 4.3.2.3.

4.3.4.3 Erythrocyte Lysis Assay

The haemolytic property of the strain was assessed as per the method of Rijssel *et al.* (2007). Sampling was done at logarithmic phase of growth when the cells were actively growing. Four types of samples were prepared, whole sample including cells (solitary and colony cells) and filtrate, filtrate alone (cell free culture medium), water extract of the cells and chloroform

methanol extract of the cells. For preparing the chloroform methanol extract 100 ml culture was centrifuged to separate cells and extracted with chloroform: methanol in the ratio 13:7 v/v. The extract was dried in a desiccator and redissolved in 70% methanol. For preparing the water extract, 100 ml algal sample was centrifuged, supernatant discarded and cell pellet was put in sterile sea water and disrupted by 3 sonic bursts (70 amplitude for 1 minute). Sonicated sample was centrifuged and supernatant was used for the assay.

Whole sample, filtrate and extracts were diluted 1:1 with blood cell suspension. For transferring the whole sample, inlet diameter of pipette was enlarged using scissors, making sure to include colonies while sampling. The blood cell suspension was prepared by adding 5 drops of fresh human blood to 30 ml lysis buffer (Eschbach *et al*., 2001), centrifuged (3000rpm, 1 minute) to separate the intact RBC from plasma. The supernatant was discarded and again added 30ml buffer. Repeatedly washed with buffer to remove the plasma completely and the collected RBC pellet was resuspended in 30ml buffer to get the final blood cell suspension for the assay. The mixture of blood cell suspension and algal sample was incubated in triplicate in test tubes (15ml) at 23^0C in light for 20 hours. Artificial seawater incubated with blood cell suspension was used as a control. For the chloroform: methanol extract, 70% methanol was used as control. After incubation, intact blood cells and *Phaeocystis* cells were removed by centrifugation (5min, 7000rpm) and the supernatant was transferred into a cuvette and measured at 414 nm to quantify the released haemoglobin. Sonified blood cell suspension diluted with artificial seawater was used as 100% lysis control. Haemolysis of *Phaeocystis* sp. was compared with a non toxic alga, *Chlorella marina* under the same conditions.

Haemolysis was also determined with *Phaeocystis* cells at various phases of growth i.e. lag phase, logarithmic phase and stationary phase of growth.

4.3.4.4 Statistical analysis

The results of the study were statistically analysed using two-way ANOVA followed by Duncan's multiple comparison using SPSS 10.0. Significant difference was calculated at $p<0.05$.

4.3.5 Dose- Response curve

Dose-response curve was made by diluting the whole culture sample of *Phaeocystis* in artificial sea water. Samples were taken when the cells were in the logarithmic phase of growth in Walne's medium. The sample was serially diluted with artificial seawater to get different dilutions of culture. 10 set of dilutions were made in artificial sea water. Erythrocyte lysis assay was done as described in the section 4.3.4.3 with varying cell concentrations.

4.4 Results

4.4.1 Allelopathic property

Allelopathic effects of *Phaeocystis* sp. cell extract and culture filtrate on the microalgae *Chlorella marina*, *Chaetoceros calcitrans* and *Isochrysis galbana* were studied.

4.4.1.1 Effect of *Phaeocystis* species on the growth of *Chlorella marina*

4.4.1.1.1 Effect of *Phaeocystis* species cell extract on the growth of *Chlorella marina*

Allelopathic effect of *Phaeocystis* sp. on *Chlorella marina* was tested by estimating chlorophyll-a concentration at 0hr, 24hr, 48hr and 72 hrs. (Figure 4.1 and Table 4.2 of Appendix). No difference in growth was

observed at 24 hr between the sample and control. Chlorophyll-a value was found slightly decreased in sample compared to control at 48 hr (0.041 µg/ml) and 72 hr (0.076 µg/ml) of growth, but the difference in growth was not statistically significant. The decrease of growth in sample showed that the cell extracts of *Phaeocystis* sp. had a negative influence on the growth of *Chlorella marina* (Table 4.3 and Table 4.4 of Appendix).

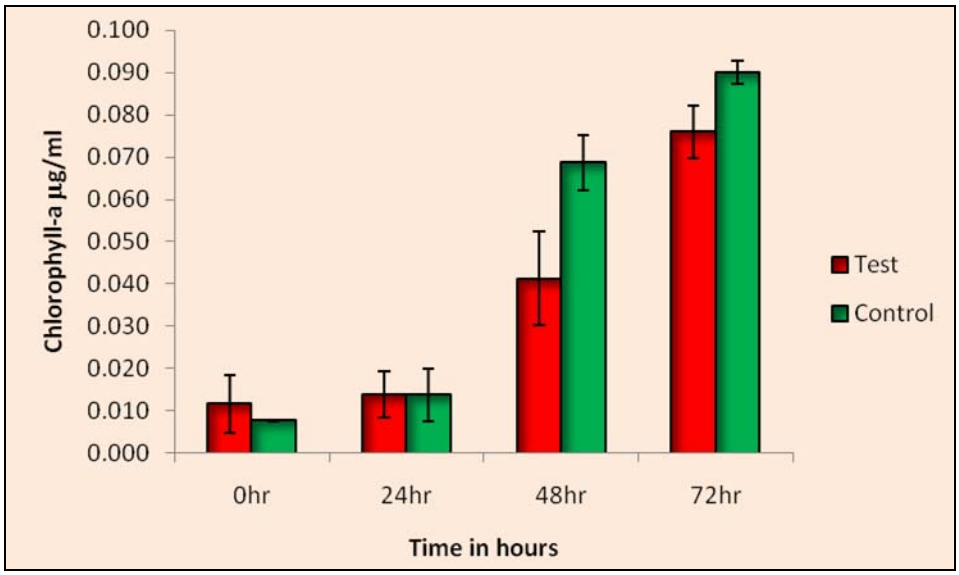

Figure 4.1 Effect of cell extract of *Phaeocystis* sp. on the growth of *Chlorella marina.*

4.4.1.1.2 Effect of *Phaeocystis* species culture filtrate on *Chlorella marina*

The allelopathic effect caused by the culture filtrate of *Phaeocystis* grown in different nutrient condition (nutrient sufficient and deficient) is shown in Figure 4.2. The culture filtrate of *Phaeocystis* showed an inhibitory action on the growth of *Chlorella marina*. The chlorophyll-a in the experimental cultures varied significantly from control at 24hr, 48 hr, and 72 hr and 96hrs (Table 4.5 of Appendix). By 96 hr, the difference in the chlorophyll-a value in the experiment conditions were found to be less compared to control, showing that the inhibitory effect caused by

Phaeocystis was transient or they had only an immediate effect. The difference in growth in nutrient deficient conditions varied significantly from nutrient sufficient conditions. The growth inhibition caused by the NP non limitng cultures was significantly less than the growth inhibition caused by nutrient deficient conditions at 24hr, 48hr and 72hr. The allelopathic effect caused by NP limiting, N limiting and P limiting culture filtrates didn't not vary significantly showing that, under nutrient limitation, the cells are in a stressed condition causing the release of allelopathic compounds from cell into the media. Estimation of nitrate and phosphate concentration in the culture filtrate from different nutrient conditions showed that nutrient level in NP limiting, N limiting and P limiting cultures were at the level of limiting and in NP non limiting culture nutrients were in nonlimiting concentration (Table 4.6 of Appendix). The growth of *Phaeocystis* was high in NP non limiting and P limiting cultures (Figure 4.3). The high growth of *Phaeocystis* sp. in nutrient non limiting cultures didn't cause an increase in the allelopathic effect of the respective culture showing that secretion of allelopathic compound was not proportional to the cell concentration, but was mainly due to nutrient depletion.

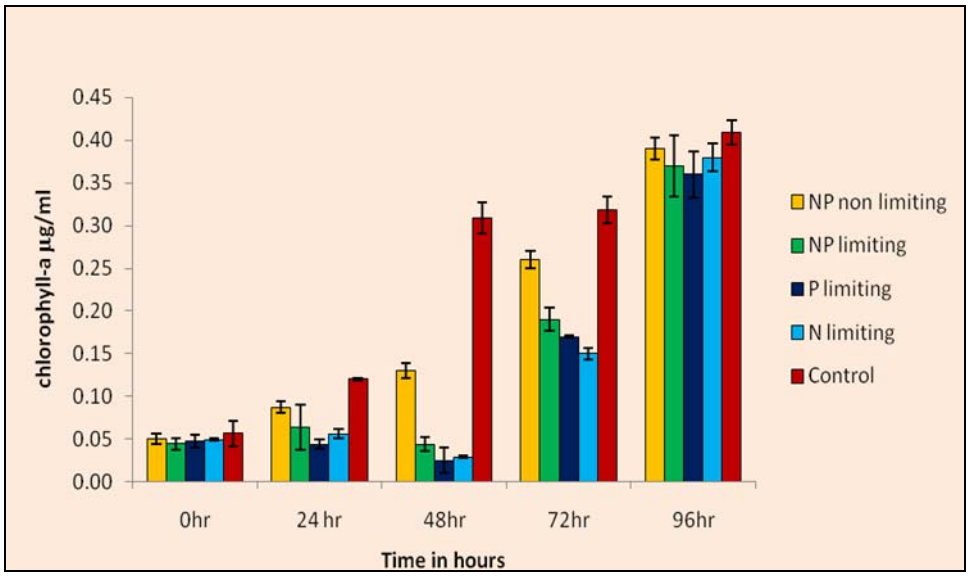

Figure 4.2 Effect of *Phaeocystis* sp. culture filtrate on the growth of *Chlorella marina*

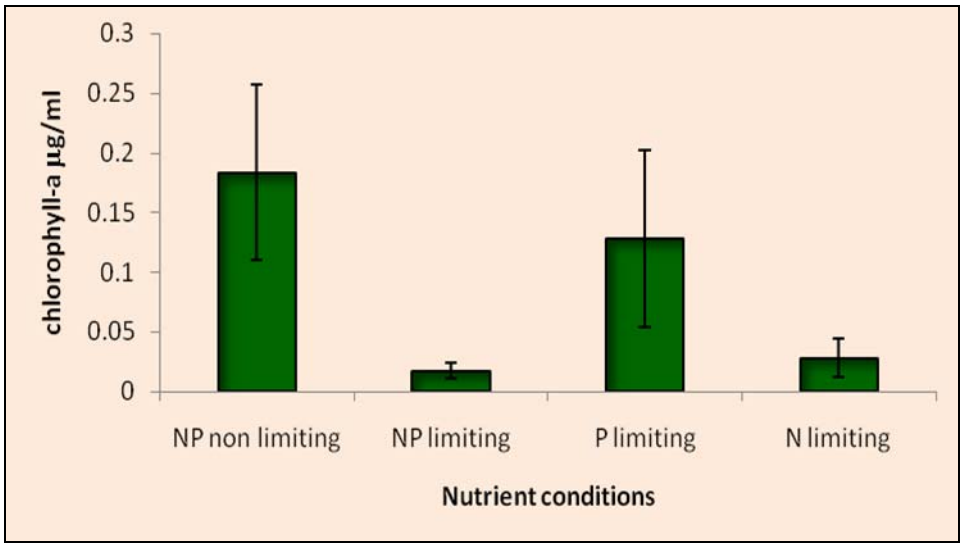

Figure 4.3 Growth of *Phaeocystis* sp. in different nutrient treatments

4.4.1.2 Effect of *Phaeocystis* species on *Chaetoceros calcitrans*

4.4.1.2.1 Effect of *Phaeocystis* species cell extract on *Chaetoceros calcitrans*

The cell extract of *Phaeocystis* sp. showed no significant effect on the growth of *Chaetoceros calcitrans*. There was a slight difference in chlorophyll-a value between the sample and control at 24hr (0.30 µg/ml) and 48 hr (0.49 µg/ml). However, from 72 hour onwards the growth difference between the sample and control was completely absent, showing that the cell extract of *Phaeocystis* sp. had no effect on the growth of the diatom *Chaetoceros calcitrans* (Figure 4.4 and Table 4.7 of Appendix).

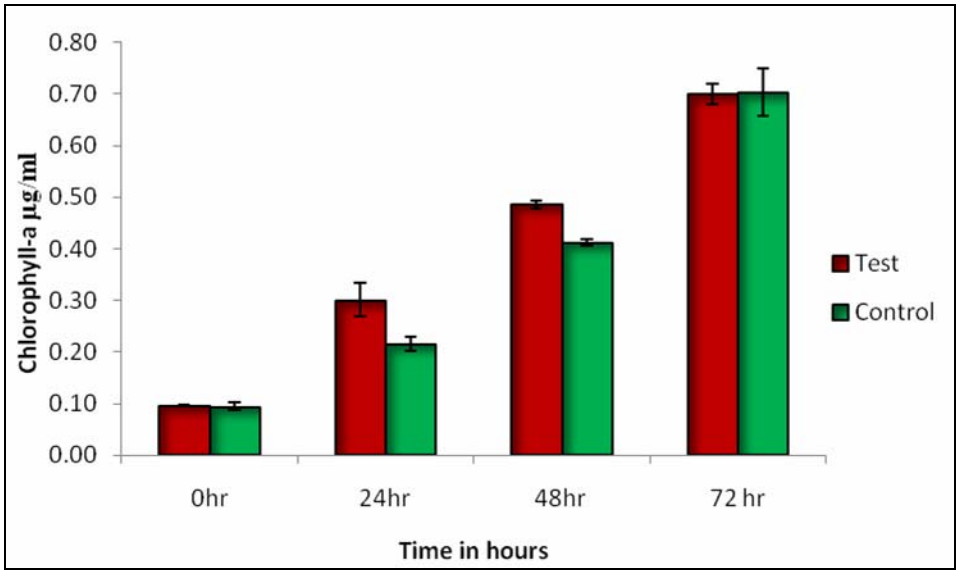

Figure 4.4 Effect of the cell extract of *Phaeocystis* sp. on the growth of *Chaetoceros calcitrans*

4.4.1.2.2 Effect of *Phaeocystis* species culture filtrate on *Chaetoceros calcitrans*

Phaeocystis sp. culture filtrate also had no effect on the growth of the diatom *Chaetoceros calcitrans* at 24hr, 48hr, 72hr and 96hr. (Figure 4.5 and Table 4.8 of Appendix). The extracellular compounds produced by the

Phaeocystis sp. under nutrient limitation and nutrient sufficient conditions could not elicit any response on *Chaetoceros calcitrans.* Though a slight difference in growth was observed, the difference was not statistically significant.

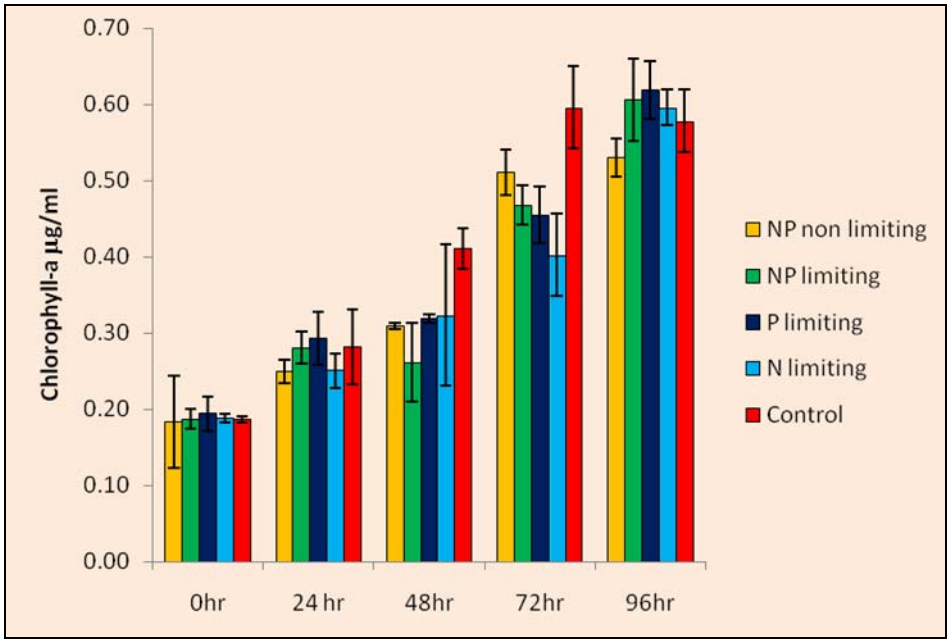

Figure 4.5 Effect of *Phaeocystis* sp. culture filtrate on the growth of *Chaetoceros calcitrans*

4.4.1.3 Effect of *Phaeocystis* species on *Isochrysis galbana*

4.4.1.3.1 Effect of *Phaeocystis* species cell extract on *Isochrysis galbana*

Phaeocystis sp. cell extract had a stimulatory effect on the growth of *Isochrysis galbana*. The difference in growth was observed at 24hr (0.46 µg/ml), 48hr (0.82 µg/ml and 72hr (1.25 µg/ml) of growth of *Isochrysis galbana*. (Figure 4.6 and Table 4.9 of Appendix).The growth difference between the test and control at 48hr and 72 hr were found to be statistically significant (p<0.05). (Table 4.10 and Table 4.11 of Appendix). The result showed that the extract of *Phaeocystis* sp. contain compounds that stimulate the growth of *Isochrysis galbana*.

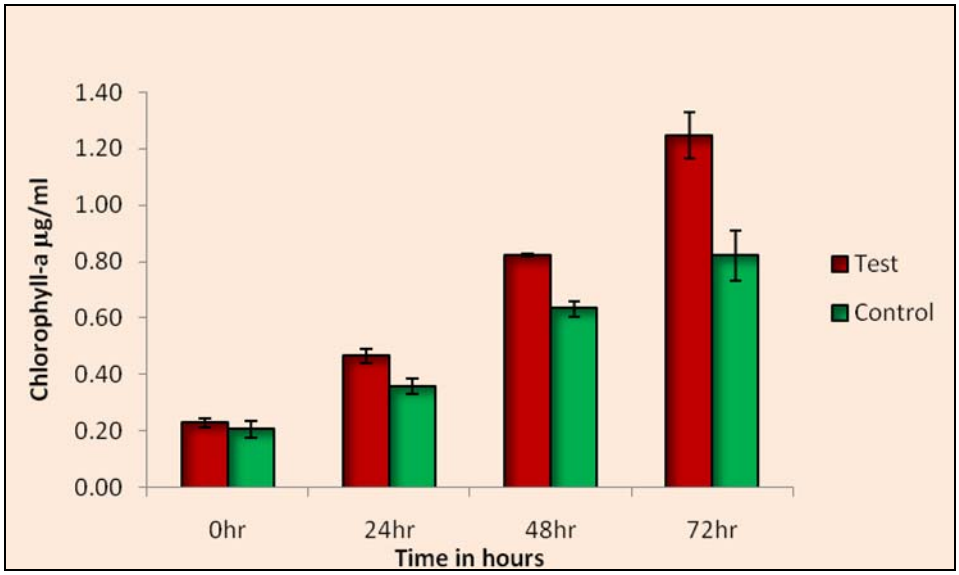

Figure 4.6 Effect of the cell extract of *Phaeocystis* sp. on the growth of *Isochrysis galbana*

4.4.1.3.2 Effect of *Phaeocystis* species culture filtrate on *Isochrysis galbana*

The addition of cell free culture filtrate of *Phaeocystis* from different nutrient conditions also showed a stimulatory effect on the growth of *Isochrysis galbana* (Figure 4.7 and Table 4.12 of Appendix). The growth stimulation was high for NP non limitng medium and P limiting medium that contain high chlorophyll-a (Figure 4.3) than the nutrient deficient cultures (NP limited and N limited). At 48 hr, significant variation was not observed between NP non limiting and P limiting cultures but they significantly varied from NP limiting culture, N limiting cultures and the control. At all hours tested, the growth of *Isochrysis galbana* in cultures added with filtrates from *Phaeocystis* culture showed significant variation from the control. The stimulatory effect of *Phaeocystis* sp. on *Isochrysis galbana* was increased with increase in the chlorophyll-a concentration of *Phaeocystis* culture. NP non limiting *Phaeocystis* culture and P limiting culture stimulated maximum growth of *Isochrysis galbana* compared to

other nutrient condition and control and the growth of *Isochrysis galbana* was lowest in the control.

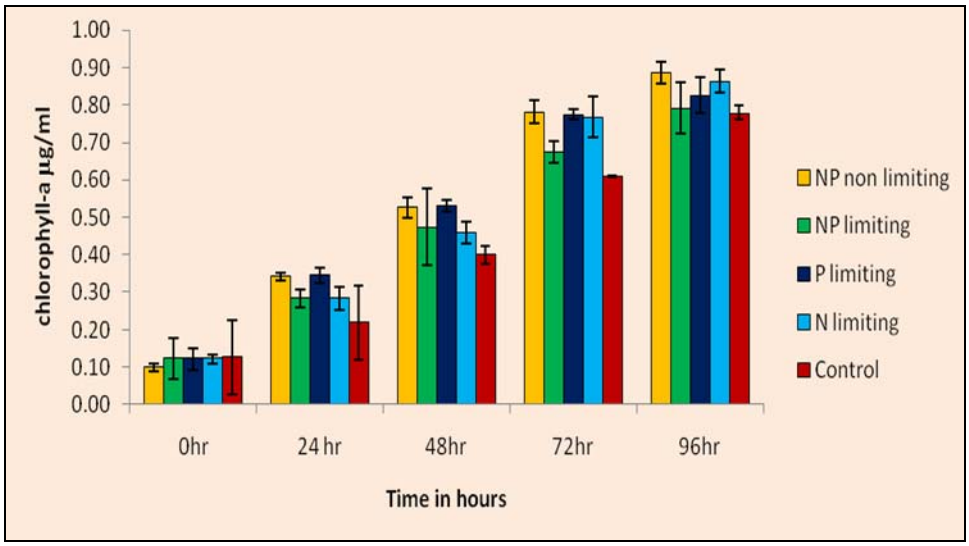

Figure 4.7 Effect of *Phaeocystis* sp. culture filtrate on the growth of *Isochrysis galbana*

4.4.1.2 *Artemia salina* toxicity test

The toxic effect of *Phaeocystis* sp. on the larvae of *Artemia salina* was tested using the live cells of *Phaeocystis* sp. and the culture filtrate. The effect of light on the toxicity was also tested by incubating *Artemia* larvae and the *Phaeocystis* sp. in light and dark condition. Incubation in light and dark condition didn't show any difference in the activity of *Phaeocystis* sp. on the larvae of *Artemia salina* (Table 4.13). *Artemia salina* was affected by the live cell suspension of *Phaeocystis* sp. causing mortality of 20% at a *Phaeocystis* cell concentration of 6×10^6 cell/ml and mortality of 30 % at a *Phaeocystis* cell concentration of 1.6×10^7 cells/ml. No mortality was observed in the control and culture filtrate-treated *Artemia salina*. The effect was only for a short duration, about 24 hours. At 24 hours, up to 30% mortality was observed. Motility of the remaining live

larvae was restricted, with a vibratory movement. By 48 hours, the larvae regained the healthy condition showing active swimming in water. No mortality was observed after 24 hours of exposure.

Table 4.13 Artemia toxicity test

		Time		
		24 hours	**48hours**	**72hours**
Dark	Control	10 alive, no mortality	10 alive, no mortality	10 alive, no mortality
	Culture filtrate-treated	10 alive no mortality	10 alive	10 alive, no mortality
	Phaeocystis cell-treated (6×10^6 cell/ml)	8 alive Motility restricted, with vibratory movement	8 alive. Larvae actively moving and seems to be in a healthy condition.	No more mortality. Organism in healthy condition
	Phaeocystis cell-treated (1.6×10^7 cells/ml)	7 alive Motility restricted, with vibratory movement	7 alive. Larvae actively moving and seems to be in a healthy condition.	No more mortality. Organism in healthy condition
Light	Control	10 alive, no mortality	10 alive	10 alive, no mortality
	Culture filtrate-treated	10 alive, no mortality	10 alive	10 alive, no mortality
	Phaeocystis cell-treated (6×10^6 cell/ml)	8 alive Motility restricted, with vibratory movement	8 alive. Larvae actively moving and seems to be in a healthy condition.	No more mortality. Organism in healthy condition
	Phaeocystis cell-treated (1.6×10^7 cells/ml)	7 alive Motility restricted, with vibratory movement	7 alive. Larvae actively moving and seems to be in a healthy condition.	No more mortality. Organism in healthy condition

4.4.2 Haemolytic property

4.4.2.1 Effect of different nutrient concentrations on the haemolytic property of *Phaeocystis* species

Haemolysis was shown by *Phaeocystis* sp. grown under different nutrient conditions (Figure 4.8 Table 4.14 of Appendix). The haemolytic activity varied significantly among whole culture sample and culture filtrate tested (ANOVA $p<0.05$), whereas the water extract and chloroform methanol extract didn't show any significant variation. Haemolytic activities of cell extracts were very negligible, even though the extracts were concentrated 100 times compared to whole culture sample and filtrate. The whole culture sample from NP non limited nutrient medium showed maximum haemolysis and the activity significantly varied from NP limited culture and N limited culture. In the case of culture filtrate, maximum haemolysis was caused by P limited culture and the activity showed significant variation from other nutrient treatments. No significant variation in haemolytic activity was observed between the NP non limited and NP limited cultures.

Chlorophyll-a concentration and cell density was high in nutrient balanced culture compared to nutrient limited culture (Figure 4.9). Haemolysis caused by the nutrient balanced culture was high, showing that the haemolysis was mainly caused by the intact cells and the activity is dependent on cell number.

In NP limited cultures, cell growth of *Phaeocystis* sp. was very low, and the haemolysis caused by the whole sample was less compared to culture filtrate. Under nutrient limitation, the haemolytic compound was secreted out of the *Phaeocystis* sp. cell into the culture filtrate, due to the physiological stress of the cell. Filtrates of phosphate limited cultures showed maximum haemolysis than nitrate limited cultures. Significant

variation was observed in the haemolysis caused by filtrates of N limited and P limited cultures ($p< 0.05$).

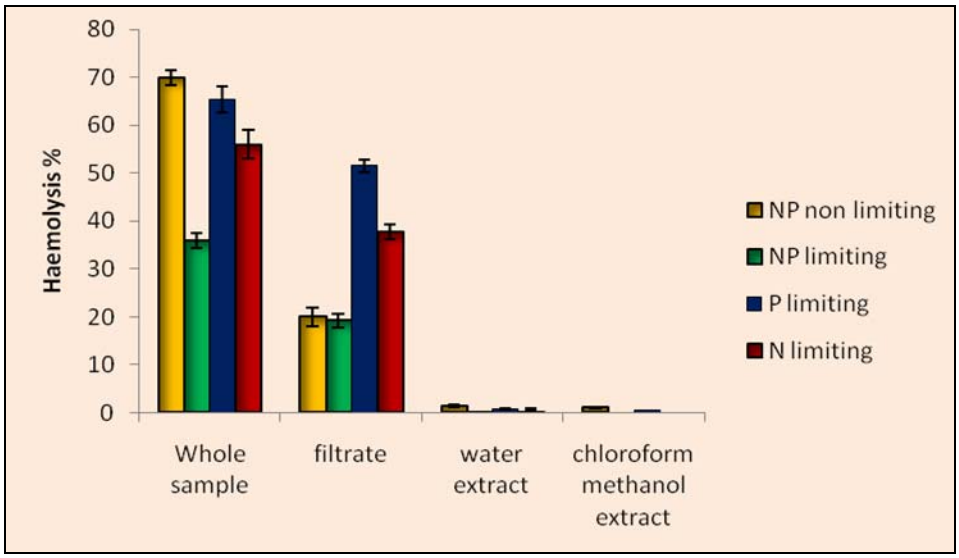

Figure 4.8 Effect of different nutrient conditions on the haemolytic property of *Phaeocystis* sp.

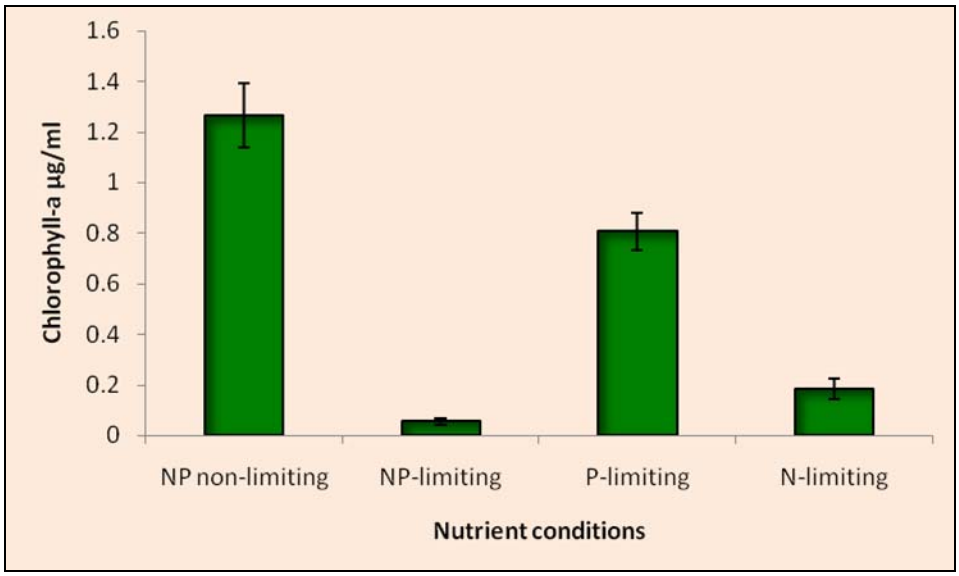

Figure 4.9 Chlorophyll-a concentration of *Phaeocystis* sp. culture in different nutrient treatments

Table 4.15 shows the concentrations of nitrate and phosphate in different nutrient conditions in which *Phaeocystis* sp. was cultured for the experiment. Nitrate and phosphate were in non limited concentrations in NP non limited cultures, where as in nutrient limited cultures nitrate and phosphate were in limiting concentrations.

Table 4.15 Concentrations of nitrate and phosphate in different nutrient treatments.

NP non limiting		NP limiting		P limiting		N limiting	
NO^{3-} (µM)	PO_4^{3-}(µM)	NO^{3-} (µM)	PO_4^{3-}(µM)	NO^{3-} (µM)	PO_4^{3-}(µM)	NO^{3-}(µM)	PO_4^{3-}(µM)
1233 ± 0.06	6.6± 0.11	51.6± 0.6.1	0.69± 0.5	1300±10.1	0.42±0.01	8.2±0.04	14.1± 5.1

4.4.2.2 Haemolysis during different growth phases of *Phaeocystis* species

Phaeocystis culture in different growth phases exhibited different rate of haemolysis that vary significantly (Figure 4.10 and Table 4.15 of Appendix).

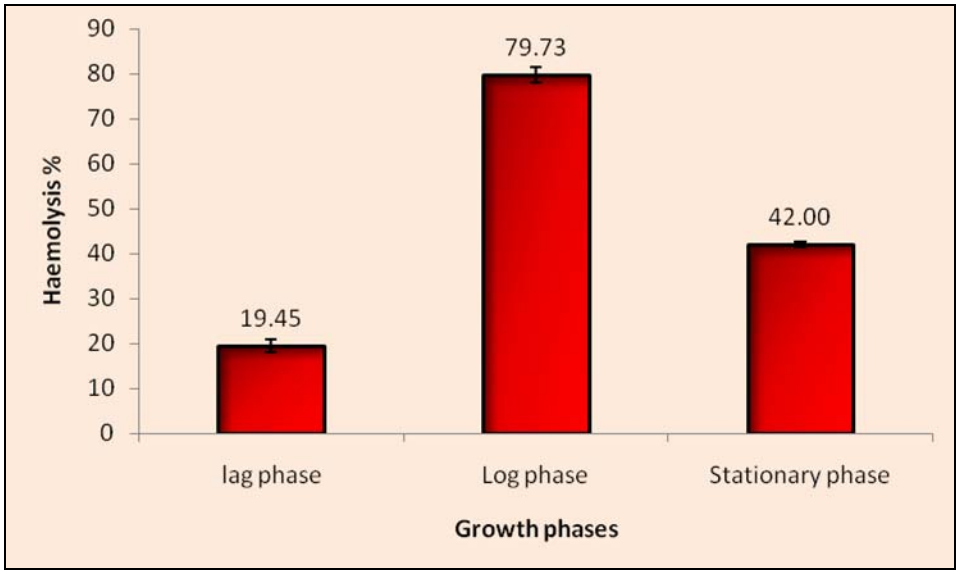

Figure 4.10 Haemolysis during different growth phases of *Phaeocystis* sp.

The haemolytic property was high during logarithmic phase where cells were actively growing (0.32µg/ml). In this phase the number of colony cells in the culture was higher compared to solitary cells. Flagellated cells were absent in the logarithmic growth phase. By stationary phase, under nutrient limitation, the colonies were disintegrated into single isolated cells, and at the same time colonies liberate flagellated cells into the culture medium. Haemolysis was low in stationary phase (42%) compared to logarithmic phase.

4.4.2.3 Dose- Response curve

Haemolysis exhibited by *Phaeocystis* sp. increased linearly with the corresponding increase in cell concentrations up to a particular level. 54.65% of haemolysis was caused by a cell number of 1.4 $\times 10^6$ cells/ml. Beyond the cell count of 2.1$\times 10^6$ cells/ml the haemolysis was in a steady state, without high rise in the haemolysis value (75% to 80%) (Figure 4.11 and Table 4.16 of Appendix).

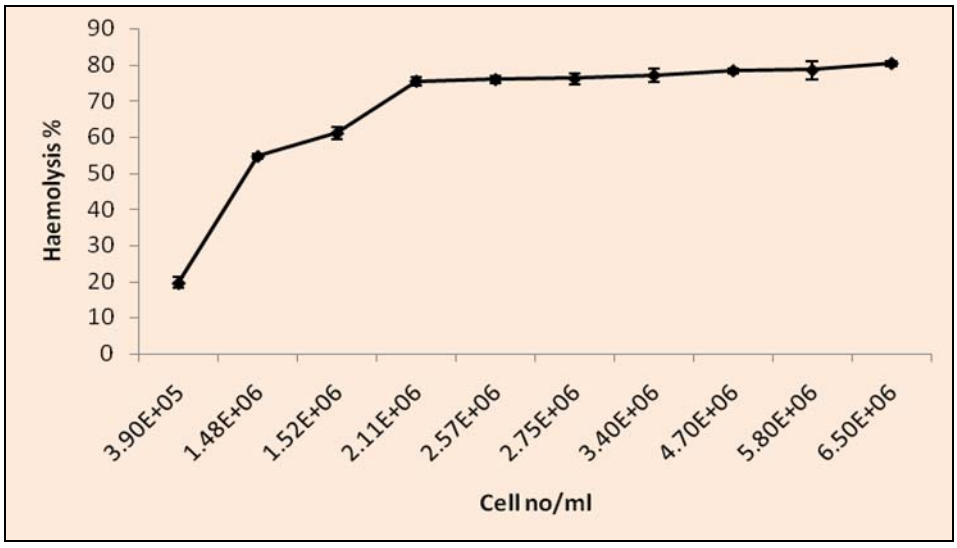

Figure 4.11 Effect of cell number on the haemolytic property of *Phaeocystis* sp.

4.5 Discussion

4.5.1 Allelopathic property

Phytoplankton species compete with each other for limiting resources, and consequently they have evolved different strategies to better exploit a resource. Phytoplankton may compete by directly interfering with each other, or through the release of chemical compounds. Any direct or indirect, harmful or beneficial effect caused by plants, protists, bacteria or viruses on another, through the production of chemical compounds that escape into the environments is called allelopathy (Rice, 1984). The effect of *Phaeocystis* sp. on the three phytoplankton *Chlorella marina, Chaetoceros calcitrans,* and *Isochrysis galbana* were studied. These three microalgal species are regular components of the phytoplankton flora in the Cochin estuary. Target phytoplankton was grown in a medium enriched with cell-free filtrate from the *Phaeocystis* sp. culture to study the effect of allelochemicals released by the *Phaeocystis* sp. into the medium. The effect of cell extracts of *Phaeocystis* sp. on the growth of the phytoplankton was also studied. *Phaeocystis* sp. produce toxic compounds that have different effects; some may be haemolytic, while other substances produced by these algae are allelopathic (Legrand *et al*., 2003). In the present study, the three microalgae responded differently, to the cell extract and culture filtrate of *Phaeocystis* sp.

4.5.1.1 Allelopathic effect of *Phaeocystis* species on *Chlorella marina*

The cell extract as well as the culture filtrate of *Phaeocystis* sp. produced a negative effect on the growth of *Chlorella marina*, which indicates that *Phaeocystis* sp. has a competitive advantage over *Chlorella marina*. This effect may be due to competition for available nutrients or allelopathy. Since, sufficient nutrients were added to the culture to prevent

nutrient limitation; the growth inhibition might be purely due to the presence of allelopathic compounds in the culture filtrate.

The allelopathic compound may bound to the *Phaeocystis* sp. cell, and under nutrient limitation, these are excreted outside and is harmful for other algae. In this study, *Chlorella marina* was initially subjected to severe cell loss when exposed to cell free filtrate of *Phaeocystis* sp. grown under nutrient deficient conditions. Although there was retardation of growth caused by nutrient balanced cultures, the allelopathic activity was more pronounced in nutrient deficient cultures. This suggests that toxic exudates from *Phaeocystis* sp. have an allelopathic influence on algal species and the availability of nutrients is an important factor regulating the production of allelopathic substances.

Several workers, (Pratt, 1966; Keating, 1977; Tillman and John, 2002) have reported the presence of toxic metabolites in culture filtrates of various algae, but it had rarely been related to the nutrient condition of the ambient water. Myklestad *et al.* (1995) reported that the growth of the diatom *Skeletonema costatum* was strongly inhibited when cultured in the presence of cell free filtrate of phosphorus deficient *Chrysochromulina polylepis* culture. This suggests that excretion of toxic metabolites might be a general mechanism among toxic haptophytes when exposed to nutrient-deficient conditions. In the literature, a close relationship between nutrient stress and enhanced allelopathic activity are well documented for several microorganisms other than phytoplankton (Vining, 1995). The organism growing without stress at their optimum rate, do not produce allelopathic substance to a large extend, but when growth rate declines due to nutrient limitation, the secondary metabolites become active. The consequence of this control system is that toxins are not produced when the organism is

able to compete effectively by virtue of its rapid growth, but toxin production is switched on as a defence system when the organism is no longer able to compete for the limiting nutrient. In the present study, the cell free filtrate from nitrate and phosphate deficient cultures inhibited the growth of *Chlorella marina*. This result supports the observation that allelochemicals are only produced/released under conditions of limited growth. By 96 hrs of growth, the culture of *Chlorella marina* exposed to culture filtrate, no longer exhibited cell loss. This suggests the allelopathic agent secreted by *Phaeocystis* is not highly stable. Studies have shown that toxins produced by the haptophytes are unstable and has high sensitivity towards pH, temperature and light (Shilo, 1967; Skulberg *et al*., 1993).

The cell free filtrates of *Prymnesium parvum* added to cultures of *Thalassiosira weissflogii, Rhodomonas baltica* and *Prorocentrum minimum* had significant negative effect on cell numbers, but within a few days the exposed species began to recover (Graneli and Johansson, 2003; Fistarol *et al*., 2005). Similar findings were observed for the dinoflagellate *Scrippsiella trochoidea* which recovered from the effects of the exudates of *Alexandrium ostenfeldii* excreted into the medium (Tillman *et al*., 2007). The study of Sole *et al.* (2005) showed that the allelopathic effect between *Chrysochromulina polylepis* and *Heterocapsa triquetra* was only important for high population densities of the toxic cells. The general pattern is that higher cell numbers of the donor species result in more magnified detrimental effects, and vice versa. Allelopathic effects could be important for inhibiting competition once a sufficiently high cell concentration had been reached. However, the general observation is that detrimental effects tend to appear at higher concentrations of the toxic algae around 10^6 cells/ml which suggests that, in general,

allelopathy among phytoplankton should not be considered as a critical factor in bloom initiation (Sole *et al*., 2005).

4.5.1.2 Allelopathic effect of *Phaeocystis* species on *Chaetoceros calcitrans*

The cell extract and culture filtrate of *Phaeocystis* sp. could not elicit any response in *Chaetoceros calcitrans.* The insensitivity of *Chaetoceros calcitrans* to excreted allelopathic compounds suggests its specific adaptations to defend itself. The adaptation of the different species of phytoplankton to allelopathic substances produced by *Phaeocystis* sp. may be a specific physiological characteristic to each receptor species (Arzul *et al*., 1999). In a similar study, Hansen and Eilertsen (2007), observed that the diatom *Skeletonema costatum* was not inhibited by the acrylic acid produced by *Phaeocystis pouchetii* at a concentration of 0.051 µg/ml, but the growth of *Skeletonema* was inhibited by acrylic acid at a higher concentration of 230µg/ml (Sverdrup *et al*., 2001). Normally occurring concentrations of acrylic acid in situ were therefore probably far too low to have significant effect on competing phytoplankton species. Graneli and Johansson (2003) also observed the insensitivity of *Prymnesium patelliferum* towards the excreted prymnesin. Windust *et al*. (1996) showed that micromolar concentrations of the dinoflagellate toxins okadaic acid and dinophysistoxin-1 effectively inhibited the growth of several microalgae, but did not affect the growth of the algae producing the toxin. These reports suggest that the allelochemicals produced by algae will not affect closely related species or species which can produce related allelochemicals. The haptophyte *Prymnesium patelliferum* produced similar prymnesin toxins as *Prymnesium parvum* and was not inhibited by cell free filtrates from *Prymnesium parvum* and instead, had shown a positive growth (Graneli and Johansson, 2003).

4.5.1.3 Allelopathic effect of *Phaeocystis* species on *Isochrysis galbana*

The stimulatory effect of *Phaeocystis* sp. culture filtrate and cell extract on the growth of *Isochrysis galbana* can be explained as the nutrifying effect of the extracellular products of the *Phaeocystis* sp. The stimulatory compounds were excreted during active growth and were less in the extracts. Though the cell extract of *Phaeocystis* sp. produced stimulatory effect on *Isochrysis galbana* the effect was very low compared to filtrate. Moreover, *Isochrysis galbana* and *Phaeocystis* sp. belong to the group haptophytes, so the extracellular compounds liberated to the medium by *Phaeocystis* may accelerate the growth of other haptophytes. The stimulatory effect was high in filtrates from nutrient sufficient cultures, where the cell growth was high and hence the production of extracellular products also would be high. The stimulatory effect of *Phaeocystis* sp. was directly proportional to the cell concentration. Similar result was obtained in the study of Gantar *et al.* (2008) where the filtrate of green algae *Chlamydomonas reinhardtii* showed stimulatory effect towards the cyanobacterium *Anabaena flosaquae*.

The mode of action of allelopathic substances include inhibition of growth (Keating 1978; Schlegal *et al*., 1999), inihibition of photosynthesis (Hagmann and Juttner, 1996) and inhibition of cellular motility (Kearns and Hunter, 2001). The study on allelopathic effect of *Phaeocystis* sp. on *Chlorella marina*, *Chaetoceros calcitrans* and *Isochrysis galbana* confirmed that the allelopathic substances are found in exudates, and under different nutrient conditions, the production of allelopathic substances changes. Under NP limiting conditions, the production of the allelopathic compounds is higher than when the cells are growing under either N or P deficient conditions. Phytoplankton species respond in different way to the allelochemicals

produced by *Phaeocystis* sp. with no effect on *Chaetoceros calcitrans*, inhibitory effect on *Chlorella marina* and stimulatory effect on *Isochrysis galbana.*

4.5.2 The effect of *Phaeocystis* species on *Artemia salina*

The allelopathic effect of the *Phaeocystis* sp. cell and culture filtrate on the brine shrimp *Artemia salina* was studied both in light and dark conditions and it was found that *Phaeocystis* sp. could not cause high mortality of the *Artemia salina*. The culture filtrate could not elicit any response, but the cells produced 20% to 30% mortality of *Artemia* suggesting that the *Phaeocystis* sp. culture was only mildly toxic towards the brine shrimp, indicating that the causative agents for allelopathy are not very potent toxins. These findings go in agreement with the studies of Jiesheng *et al.* (2010) on *Phaeocystis globosa* where the cell culture and cell-free filtrates were not toxic to *Artemia salina*. The cell extract containing the haemolytic substances induced only 10% mortality of *Artemia salina* after 24hour of culture. In the present study, light didn't seem to have any effect on the toxicity of *Phaeocystis* sp. towards *Artemia salina*. But the studies of Eilertsen and Raa (1995) and Aanesen *et al.* (1998) showed that the chemical compounds produced by *Phaeocystis* sp. were lethal to the cod larvae and the production of these toxins appeared to be dependent on the level of irradiance.

4.5.3 Haemolytic property

The haemolytic property of *Phaeocystis* sp. was studied using unprotected cell, human RBC and the effect of different levels of nitrate and phosphate in the medium on the haemolytic property was also studied.

4.5.3.1 Haemolytic property at different growth phases of *Phaeocystis* species

The haemolytic activity exhibited by *Phaeocystis* sp. during different phases of growth was studied. An important biotic factor that affects the haemolytic property is the growth phase and it varies in different microalgae. In this study exponentially growing populations showed the highest haemolytic activity followed by the cells in stationary phase. In a similar study, Shilo (1967) also observed that haemolytic activity of *Prymnesium parvum* was greatest in the exponential phase and continued to stationary phase before it eventually decreased.

However, haemolytic activity of *Chrysochromulina polylepis* was greatest during stationary phase and decreased after few days in stationary phase (Schmidt and Hansen, 2001). The haemolytic activity of *Peridinium aciculiferum* was also high in stationary growth phase (Rengefors and Legrand, 2001). The polymorphic behaviour of *Phaeocystis* sp. shows that during the exponential growth phase, the algal biomass was high with high density of non-motile cells (colony cells) in the culture. Since the haemolytic activity was high during the exponential growth phase, the non-motile cells present in the colonies may be responsible for the increased haemolytic activity. Rijssel *et al.* (2007) also observed high haemolytic activity when the algal culture was mainly dominated by the colony cells. During nutrient limitation, (stationary phase), colonies disintegrate to individual cells and the number of flagellated cells increase over the colony cells and, this results in the reduction of haemolysis. Under nutrient limitation the isolated cells secrete out the haemolytic compound to the surrounding medium.

4.5.3.2 Dose-response curve

The EC_{50} value of cell density of different microalgae was studied by various authors and was found to be as *Phaeocystis pouchetii* 1.86×10^7cells/ml (Rijssel *et al*., 2007), *Alexandrium tamarense* 0.0152×10^7cells/ml (Eschbach *et al*., 2001), *Prymnesium parvum* $0.3\text{-}24.7\times10^7$cells/ml (Fistarol *et al*., 2005; Johansson and Graneli, 1999; Simonsen and Moestrup, 1997) *Fibrocapsa japonica* $1.7\text{-}6.3\times10^7$cells/ml (de Boer *et al*., 2004) and *Chrysochromulina polylepis* $116\text{-}10\times10^7$cells/ml (Simonsen and Moestrup, 1997). Here in this study cell concentration of 1.4×10^6 cells /ml caused 54.85% haemolysis. Beyond a particular cell concentration (2.1×10^6 cells/ml), the haemolytic activity was not increased with increase in the cell number. But here the values are not calculated in saponin units. The results show that the haemolysis caused by this alga is comparable with other toxic algae.

4.5.3.3 Effect of nutrients on the haemolytic property of *Phaeocystis* species

When the nutrients were high in growth medium, the growth rate was high for the *Phaeocystis* and toxins remain intact within the cells and the presence of this toxic algal cells caused haemolysis. When the nutrient concentrations were less, either in condition of N- limiting or P limiting, the haemolytic property is secreted out of the cell into the culture fluid. When the filtrates of both nutrient limited and nutrient sufficient cultures were used, filtrates of nutrient limited cultures showed high haemolytic activity. It shows that nutrient limitation influences the exhibition of toxicity and the secretion of haemolytic component from the cell into the outer culture medium. During the logarithmic phase, when the cells were actively growing with surplus quantity of nutrients, the intact cells showed haemolytic property. During the declining phase or when the nutrients got

limited in culture fluid, alga exhibited toxicity by secreting the haemolytic component to culture medium.

The algal cells didn't exhibit enhanced haemolytic activity under N limiting and P limiting conditions but it might have caused a physiological stress on the algal cells to secrete the toxic principle to outside media. So even after the bloom crashed, due to nutrient limitation, the algal cells secrete out toxin to surroundings, thus enhancing the toxicity of bloom area. These findings were supported by earlier work which showed that the limitation of N and P cause a physiological stress on algal cell (Igarashi *et al*., 1996). Several other environmental stress factors such as light intensity, salinity etc have been shown to have substantial influence on the toxicity of alga like *Prymnesium parvum* (Shilo, 1967; Larsen *et al*., 1993)

The ability of certain species to dominate in a specific natural environment depends on its ability to compete successfully for the growth limiting resources. Thus, from an ecological point of view, the toxin secretion to outside when the nutrients got limited would be of great advantage giving the algae an opportunity to proliferate, where they would otherwise be incapable to competing with competitive superior species of algae. *Phaeocystis* releases large amount of photo-assimilated carbon into the medium when in the colonial stage and the excretion rate appears to increase with irradiance (Guillard and Hellebust, 1971; Verity *et al*., 1991).

Filtered culture fluid from nutrient limited cultures showed significant haemolytic property. So it may be assumed that *Phaeocystis pouchetii* release toxic material into the surrounding water. The release appears to increase during the termination of bloom, since the quantity of active compounds present in culture fluid increases when there is nutrient limitation that mimic

the natural condition occurring in water during the termination of bloom. Estep *et al*. (1990) reported that copepods avoided healthy colonies of *Phaeocystis* while senescent colonies usually present in late bloom were eagerly grazed upon. This indicated that copepod, salmon and cod larvae have reduced appetite at elevated toxin levels of algal cell (Eilertsen and Raa, 1995).

Toxins present at a free state in seawater act as phytoplankton pheromones that promote mating of gametes prior to cyst/spore formation (Wyatt and Jenkinson, 1997; Eilertsen and Wyatt, 1998). The highest haemolytic property values were obtained with live cells, whereas *Phaeocystis* extracts contained only one percent of the total toxicity measured with live sample. Consistent with this, low haemolytic values were reported earlier for the methanol extracts of *Phaeocystis pouchetii* (Stabell *et al*., 1999; Rijssel *et al*., 2007) and extracts of *Phaeocystis pouchetii* cells were not inhibitory to the growth of yeast cell. The bulk haemolytic activity was bound to cell membranes, because substantial part of the cell debris was present in sonicated algal sample. An alternative explanation would be that the live *Phaeocystis pouchetii* cells produce an unstable component and the mechanism that involves physical contact between *Phaeocystis* colony and the blood cell could explain the activity of live *Phaeocystis* (Rijssel *et al*., 2007). Since the haemolytic activity was associated with live cells, the actively growing *Phaeocystis* colonies may inhibit the co- occurring bacteria thus promoting species specific bacterial association during the *Phaeocystis* bloom (Sapp *et al*., 2006). The haemolytic rate indicate that, unprotected cells like blood cells used in this study, will lyse within days during a *Phaeocystis* bloom and live *Phaeocystis* cells are highly haemolytic.

Haemolysis exhibited by *Phaeocystis* sp. in the present study seems to be different from the haemolytic activity exhibited by *Phaeocystis*

globosa isolated from the icthyotoxic blooms in Chinese coastal waters (He *et al*., 1999), prymnesiophyte *Chrysochromulina polylepis* (Yasumoto *et al*., 1990) and *Prymnesium* species (Legrand *et al*., 2003), where, the haemolysis was exhibited more by cell extract. This observation show that the haemolytic mechanism exhibited by the alga in the present study seems to be different from the haemolytic harmful bloom studied so far.

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Chapter 5

BACTERIAL ASSOCIATION WITH *PHAEOCYSTIS* SPECIES

Contents

5.1 Introduction

Bacteria and phytoplankton dynamics are closely linked, and are commonly observed in both freshwater and marine ecosystems. Bacteria are inherent part of the physical environment of microalgae, both in laboratory and in natural environment. The ecological relevance of the algal-bacteria association was studied by many researchers and the association was found to be important for the physiology of both algae and bacteria. Many workers have shown that algae and bacteria influence the growth of each other with both stimulatory and inhibitory effects. Bacterial abundance and community structure greatly depends on species, growth and physiological status of the closely related algae.

Phytoplankton-associated bacteria are found in the phycosphere, an area around the algal cells where bacteria feed on extracellular products of the algae (Bell and Mitchell, 1972). Microalgae need organic substances produced by bacteria such as intracellular phospholipids or extracellular glycolipids for their growth (Riquelme *et al*., 1988). Phytoplankton cells excrete organic compounds, including high proportions of carbohydrates

contributing to the base of the microbial food web (Lancelot, 1983). Bacteria that live attached to algal surfaces and that consume extracellular products consequently participate in biogeochemical cycling and play an important part in the microbial loop. It is known that marine epiphytic bacteria excrete biologically active, beneficial compounds that regulate the ontogenesis of marine organisms, and help them to survive under different types of environmental stresses, including environmental contamination. Biostimulators of cell growth and development, antibacterial substances, toxins produced by marine epiphytic bacteria and other biologically active metabolites are among such compounds. Hence, it is important to elucidate the ecological role of bacteria interacting with phytoplankton.

Recent reports of bacteria lethal to certain harmful algal bloom species (HABs), coupled with a rapidly evolving interest in attempting to minimize the adverse effects of

HAB through various prevention, control and mitigation strategies, have focussed attention on defining the role of algicidal bacteria in bloom termination. Bacteria capable of killing HAB species may affect the phytoplankton population species succession. The association or interaction between bacteria and harmful phytoplankton has been considered as one of the biotic factors that influence the processes of HAB dynamics. These algicidal bacteria exhibit varying degree of specificity for their target algae, ranging from broad spectrum effects across algal classes (Imai *et al.*, 1995) to algicides effective against only one or more closely related species (Fukami *et al*., 1992).

Natural bacterial population influence the bloom dynamics of algal populations. In particular, bacteria with inhibitory effects play an important role in changing the dominant algal species (Fukami *et al.,* 1991). The associated bacteria can produce secondary metabolites which inhibit the settlement of potential competitors, such as invertebrate larvae, and can antagonise other bacteria (Holmstrom and Kjelleberg, 1994). Epibiotic bacteria are, therefore, attracting attention as a source of new natural products. In the present study, culturable bacteria associated with the harmful alga *Phaeocystis* sp. was isolated and identified up to generic level and the effect of associated bacteria on the growth and haemolytic property of the *Phaeocystis* sp. was ascertained.

5.2 Review of Literature

5.2.1 Bacterial groups associated with microalgae

Free-living and phytoplankton associated bacteria are very different from each other and are dominated by distinct phylogenetic groups. Generally, the bacteria associated with algae are gram negative (Simidu *et al*., 1971). About 70% of the bacterial flora associated with the phytoplankton of inshore waters of Japan was *Vibrio* and *Aeromonas,* whereas, the same group of bacteria constitute about 45% in seawater (Simidu *et al.*, 1971). Dodge (1973) reported the presence of bacteria in the cytoplasm of *Amphidinium herdmanii* and *Katadinium glandulum*. The bacterium *Marinobacter hydrocarbonoclasticus,* capable of metabolizing complex or unusual hydrocarbon molecules, such as isoprenoid molecules were found in association with *Alexandrium fundyense* (Rontani *et al*., 1997). *Pseudomonas* and *Moraxella* were found associated with *Amphidinium carterae* and Proteobacteria and Cytophaga group in the cultures of *Alexandrium catenella* (Nayak *et al*., 1997).

In the study of Lu *et al*. (2000) associated bacteria were found both extracellular and intracellular in association with *Alexandrium minutum*. Bacteria were present in various modes on the algae, epiphytic, intracellular, endosymbionts, or with zoospores. Bacterial cells were observed to be attached to the dinoflagellates via the poles, in very close proximity to the outer surface of the dinoflagellate and apparently in the cytoplasm and also in the nucleus (Alavi *et al*., 2001). Bacteria were observed in the cytoplasm and food vacuoles of *Heterocapsa circularisquama* (Maki and Imai, 2001).

Algae harbour distinctly separated bacterial groups belonging to α-Proteobacteria, γ-Proteobacteria, Cytophaga-Flavobacterium-Bacteroides group, Actinobacteria, and Bacillus (Meusnier *et al.*, 2001; Nicholas *et al.*, 2004). Study of bacterial community associated with dinoflagellate *Pfiesteria* showed the presence of diverse group of about 30 bacteria species including *Nocardia, Pseudomonas, Vibrio, Cytophaga, Moraxella, Acinetobacter,* and *Roseobacter* (Alavi *et al*., 2001). Biotechnologically important *Methylobacterium radiotolerans* was isolated from the algal crust formed on electrical insulators of Africa (Zarnowski *et al*., 2002). The diatom associated bacteria mainly belonged to the Flavobacteria-Sphingobacteria group of the Bacteroidetes whereas, the free living bacteria comprised mainly of members of the Roseobacter group of the α-Proteobacteria (Grossart *et al*., 2005). *Caulobacter* like bacterium was found associated with diatom; α-Proteobacteria and γ-Proteobacteria were isolated from *Pseudo-nitzschia multiseries*, and from culture of diatoms and dinoflagellates (Kaczmarska *et al*., 2005; Sapp *et al*., 2006). The cultures of Epilithic cyanobacterium *Leptolyngbya* sp. contains bacterial consortium including *Pseudomonas, Agrobacterium* and *Bacillus* (Bruno *et al*., 2006).

5.2.2 Bacterial association and Toxicity

The symbiotic relationship between the bacteria and toxic flagellates was first described by Silva (1962) who suggested the involvement of bacteria in the toxin production of algae. Later, Kodama *et al.* (1988) and Franca *et al.* (1995) reported the isolation of toxin producing bacteria from different dinoflagellates. The influence of bacteria on the toxin production of algae involves the processes like autonomous toxin production (Tamplin, 1990; Gallacher *et al*., 1997), metabolizing toxins produced by algae (Doucette *et al*., 1998; Smith *et al*., 2002), direct and indirect influence in microalgal toxin production and survival (Bates *et al*., 1995; Nagai and Imai, 1998). Bacteria that were associated with bloom-forming toxic dinoflagellates had been implicated in the production and biotransformation of paralytic shell fish toxins (Tobe *et al*., 2001). Toxin production by bacteria associated with the *Alexandrium fundyense* was studied by Martins *et al.* (2003), *Alexandrium catenella* by Uribe and Espejo (2003), and *Alexandrium tamarense* by Silva (1982) and Dantzer and Levin (1997).

5.2.3 Algicidal effect of associated bacteria

Many bacteria both free-living and associated, which had algicidal effects, had been isolated from phytoplankton including HAB species. The mass production of many food microalgae was affected by the antagonistic effect of associated bacteria, leading to the crash of the entire microalgal population (Baker and Herson, 1978; Suminto and Hirayama, 1993). The artificial bio-manipulation of bacterial assemblages in the algal cultures could stabilize food microalgae mass cultures by improving their reliability. Algicidal bacteria were isolated by several workers (Sakata, 1990; Imai *et al.*, 1991; Sakata *et al.*, 1991; Fukami *et al.*, 1992; Mitsutani *et al.*, 1992; Onji *et al.*, 1995; Yoshinaga *et al.,* 1995). *Flavobacterium* isolated during

the declining stage of the bloom of *Gymnodinium mikimotoi* inhibited the growth of the algae (Fukami *et al*., 1997). 96 bacterial strains isolated from the Hiroshima bay showed lethality towards *Heterosigma carterae* (Yoshinaga *et al*., 1998).

Algicidal effect of *Pseudoalteromonas* on *Chattonella*, *Gymnodinium* and *Heterosigma* was studied by Lovejoy *et al.* (1998.). A yellow pigmented gram negative rod isolated from the waters of west Florida was found to be producing algicidal compound against *Gymnodinium breve* and released it into the growth medium (Doucette, 1999). *Pseudomonas* caused mortality of *Prorocentrum micans* (Lee and Park, 1998) and *Micrococcus* caused mortality of *Cochlodinium polykrikoides* (Park *et al*., 1998). Algicidal microorganisms were identified from the coastal Sea of Japan against *Chattonella antique* and *Heterosigma akashiwo* (Imai *et al*., 1998). The bacteria isolated from a biofilm, degraded the released toxin, microcystin, produced by *Microcystis viridis* (Inamori *et al*., 1998). Bacterial member of Flexibacter-Cytophaga group isolated from the blooms of *Gymnodinium breve*, showed algicidal activity against the same organism in the laboratory test. The activity was very specific, since they didn't show activity against other species of *Gymnodinium* (Doucette, 1999).

Five bacterial associates related to the alpha, gamma Proteobacteria and Firmicutes, isolated from the filaments of *Nodularia spumigena*, affected negatively the cyanobacterial growth, leading to the lower biomass yield upto 38% relative to controls with no bacteria addition (Salomon *et al*., 2003). An algicidal bacterium *Kordia algicida* was isolated from the red tide (Sohn *et al*., 2004). A bacterium *Shewanella* strain had growth-inhibiting effect on three dinoflagellates species- *Pfiesteria piscicida*, *Prorocentrum minimum,* and *Gyrodinium uncatenum* (Hare *et al*., 2005).

The growth of *Heterosigma akashiwo* was strongly inhibited in medium containing rhamnolipids (biosurfactants) produced by *Pseudomonas aeruginosa* (Wang *et al*., 2005).

5.2.4 Stimulatory effect of associated bacteria

Growth of algae may be enhanced by the stimulatory effect of certain associated bacteria. *Pseudomonas* enhanced the growth of *Asterionella glacialis* and *Skeletonema costatum* (Riquelme, 1988; Fukami *et al*., 1991). Bacteria isolated from the deep sea water stimulated the growth of the diatom *Chaetoceros ceratosporum* and *Chaetoceros gracilis*. Film of *Alcaligenes* on the surface of substratum stimulated the growth of *Nitzschia* sp. Natural bacterial community associated during the bloom of *Gymnodinium nagasakiense* was found to be stimulating the growth of *Gymnodinium*, but inhibited the growth of *Skeletonema costatum* (Fukami *et al*., 1991, 1997). The native bacterial flora- *Moraxella* and *Pseudomonas* associated with the dinoflagellate *Amphidinium carterae* were capable of producing vitamins and growth factors required for the growth of the dinoflagellate and thus improve the growth of the algae (Nayak *et al*., 1997).

When bacteria were added to the axenic zoospores, the dinoflagellate population increased two fold (Alavi *et al*., 2001). The native bacterial assemblages of the Bay of Fundy, mainly dominated by Alteromonadaceae were found to be dramatically stimulating the growth of *Alexandrium fundyense* (Ferrier *et al*., 2002). The green alga, *Monostroma oxyspermum* proliferated unicellularly in aseptic culture, but developed into normal folliacious gametophyte in the presence of bacteria within the Cytophaga-Flavobacterium-Bacteroides complex. These bacteria induced the release of spores from the leafy young gametophyte of *Monostroma oxyspermum* and

also exhibited morphogenetic activity against germ free spores of *Ulva pertusa, Ulva conglobata* and *Enteromorpha intestinalis* (Matsuo *et al*., 2003). Catalase enzyme excreted by the associated bacteria *Pseudoalteromonad* associated with marine algae promoted the growth of the algae (Dimitrieva *et al.,* 2006).

5.2.5 Species specificity of algal-bacterial association

Bacteria associated with algae have shown specific association by the specific utilization of algal extracellular products. *Legionella pneumophila* associated with cyanobacterium was found to use the algal extracellular products as its carbon and energy sources (Tison *et al*., 1980). Bacteria have hydrolytic enzymes which could hydrolyze algal exudates and act as remineralizers (Guerrini *et al*., 1998; Obernosterer and Herndl, 1995). Antibacterials produced by the microalgae were active against bacterial flora other than the associated bacteria, which enhances the species specificity in bacterial composition besides the specificity in the algal extracellular products (Naviner *et al*., 1999).

Under oligotrophic conditions, bacteria out compete algae for inorganic nutrients, and bacteria were associated with alga not as a carbon source, but as a substrate for colonization (Rier and Stevenson, 2002). The presence of vitamin B_{12} in marine algae was reported as due to the presence of symbiotic bacterial association (Croft *et al*., 2005). Bacterial populations in the coastal regions showed changes along with the development of dinoflagellate populations (Gasol *et al*., 2005). Bacterial association in the diatom and dinoflagellates cultures showed that bacterial communities were strictly species specific for microalgae (Sapp *et al*., 2006).

5.2.6 Bacterial association in *Phaeocystis* species

Sieburth (1959) showed that *Phaeocystis* sp. produces water soluble compound that inhibit the growth of several bacterial isolates (Sieburth, 1960, 1961). Guillard and Hellebust (1971) and Davidson and Marchant (1987) confirmed that the *Phaeocystis* sp. produce acrylic acid that have antibacterial activity. The bacterial association with *Phaeocystis* was studied by Putt *et al.* (1994). Bacterial community was actively growing with *Phaeocystis* during the initial stages of *Phaeocystis* bloom. Healthy *Phaeocystis* colonies were heavily colonised by bacteria and the growth rates of epibacteria were not negatively affected by the mucilaginous matrix of *Phaeocystis.* The enzymatic activities of the bacteria attached to *Phaeocystis* colonies help in the mineralization of organic compounds secreted by *Phaeocystis* during the bloom termination. Bacterial biomass attached to the colonies increased with the intensity of bloom and at the senescent phase of the bloom the bacterial biomass was at the highest level. The attached bacteria control the bloom dynamics of *Phaeocystis* (Becquevort *et al.*, 1998). Bacteria belonging to Roseobacter group were found to be attached to the dimethyl sulfonio propionate producing North Atlantic bloom (Gonzalez *et al.*, 2000).

5.3 Materials and Methods

5.3.1 Isolation of associated bacteria

5.3.1.1 Algal cultures

Stock culture of *Phaeocystis* sp. maintained in the laboratory was used for the study. Non axenic stock cultures were sub cultured into sterile Walne's medium and were grown at 20^0C, 30ppt salinity, initial pH 8.00 and in 2000 lux illumination with a 12h:12h light:dark cycle. Incubation

was done for 24 days in the environmental chamber. Sampling of algal culture for the isolation of associated bacteria was done at 15th day and 24th day of growth.

5.3.1.2 Bacterial isolation and identification

The *Phaeocystis* sp. culture (100ml) was filtered through 1µm GF/F filter paper using a vacuum pump in sterile condition to separate the associated bacteria from free-living and contaminating bacteria. *Phaeocystis* sp. cells with the attached bacterial biomass were remained in the filter. The algal cells were repeatedly washed using sterile seawater in order to remove all free-living bacteria. The algal cells remained in the filter was backwashed in to sterile seawater and filter paper was put in the sterile seawater and the algal cells were resuspended in seawater through vigorous shaking. 1ml of algal sample was taken and serially diluted using sterile seawater and plated in nutrient agar plates and Zobell's agar (Zobell, 1946) plates. The plates were incubated at 20^{0}C for 7days. The individual bacterial colonies developed were isolated and purified. Isolated colonies were identified upto generic level based on cell morphology and biochemical reactions as per Bergey's Manual of Determinative Bacteriology (2000).

5.3.1.2.1 Media used for the isolation of associated bacteria

Nutrient Agar Medium

Peptone : 5g

Beef extract : 3g

Seawater (25ppt) : 1000ml

Agar : 20g

The pH was adjusted to 7.2 before autoclaving.

Zobells' Agar Medium

Pepton	: 5g
Yeast extract	: 1g
Ferric phosphate	: 0.02g
Seawater	: 1000ml
Agar	: 20g.

The pH was adjusted to 7.2 before autoclaving.

5.3.1.2.2 Tests used for the identification of Bacteria

Gram staining

Bacterial cultures of 12-18hrs old were taken and smears were prepared on clean glass slides for staining. The primary stain ammonium oxalate-crystal violet was added to the fixed smear and allowed to stand for one minute. Then the slides were rinsed in gently running water. The slides were flooded with mordant, Lugol's iodine solution and allowed to stand for one minute. The slides were then rinsed gently in running water. Flooded the slides with the decolouriser (acetone-alcohol) and allowed to stand for 30sec. Rinsed in gentle running water and the counter stain safranine- O was added to the smear and allowed to stand for one minute. Then the slides were washed, air-dried and observed under an oil immersion microscope. Gram +ve bacteria appear in violet colour and Gram-ve in pink colour.

Oxidase Test

Small pieces of filter paper were soaked in 1% aqueous tetramethyl para phenylene diamine dihydrochloride solution and the papers were dried. A small portion of the culture was rubbed on the test paper with a

clean platinum loop and the formation of deep-blue colour within 15 seconds was considered as a positive test.

Catalase Test

On a clean slide a smear of the bacterial culture was prepared. A drop of hydrogen peroxide solution was added on the smear and the reaction was carefully observed. Effervescence or bubbling was noticed in the case of cultures producing catalase enzyme.

Marine Oxidation Fermentation Test (MOF)

MOF Medium (Hugh&Leifson's Medium)

22g dehydrated medium in 1000ml seawater

1% dextrose and 2% Agar.

The pH was adjusted to 8.

Hugh&Leifson's medium was prepared with specified composition, melted and distributed aliquots of 5ml each in culture tubes, plugged with cotton, autoclaved and slants were prepared. The test cultures were inoculated in the hard agar by stabbing and by streaking on the slope, taking care not to allow air bubbles trapped in the agar medium. The tubes were incubated for 48hrs, and observations were made.

Oxidative reaction- change of pink to yellow colour in the slope area.

Fermentative reaction- change of pink to yellow from the slope to the bottom of the butt (entire tube).

Alkaline reaction- Change of pink to deep pink at the slope.

Cracks and bubbles in the medium-gas production.

Mannitol-motility Test

Mannitol Motility Agar

Mannitol : 10g

Beef extract : 3g

Peptone : 5g

Phenol : 0.1g

Seawater : 1000ml

Agar : 4g

pH - 7.2.

The medium was prepared and about 3-4ml was distributed in test tubes. The tubes were sterilized in an autoclave and left for setting in a vertical position. After setting, clear test tubes were selected and the cultures were stabbed straight to the bottom. The tubes were then incubated at room temperature for 48-72 hrs.

Yellow colour in the medium shows the utilization of mannitol. No colour change is negative. Motile bacteria diffuse from the culture and the medium becomes dense. Non-motile forms grow at stab line only with shaft margin.

Arginine dihydrolase test

Peptone : 1g

Potassium hydrophosphate : 0.3g

Agar : 3g

Phenol red : 0.01g

Arginine HCl : 10g

Seawater : 1000ml

pH 7.2

The medium was prepared and autoclaved. About 4-6 ml was dispensed into test tubes and allowed to solidify. Bacteria were stab inoculated. Tubes were covered with a few ml of sterile mineral oil and incubated at room temperature for 48hrs. Development of deep pink colour indicates a positive reaction.

Gelatin Liquefaction

Beef extract : 1.5g

Seawater : 500ml

Peptone : 2.5g

Gelatin : 60g

Ingredients were dissolved, and dispersed about 4-6ml in test tubes. The tubes were autoclaved and kept until cooled. 24hr old bacterial cultures were stab inoculated into the test tubes and incubated at 20^0C. After 48hrs, the test tubes were placed in a refrigerator at 4^0C for one hr until the tubes were chilled. If the medium readily flows as tubes were gently tapped indicated positive reaction and if the medium was solid then it is considered as a negative reaction.

Phosphatase production

Seawater : 100ml

Nutrient broth medium : 23g

Yeast extract : 2g

Agar : 20g

1% filter sterilized phenolphthalein diphosphate tetrasodium salt - 5ml

30% ammonium hydroxide- 1 drop

Agar plates were prepared and spot inoculated the fresh bacterial culture on the plate with a sterile platinum loop. Incubated for 48hrs at room temperature. Placed a drop of 30% ammonium hydroxide in the lid of the petriplate and inverted the plate above it. Allowed approximately 30sec for diffusion of ammonia which caused pH to shift to alkaline level. Phosphatase positive colonies show a pink colour around it.

Citrate utilization

Simmons's citrate agar slants were prepared and bacterial cultures were inoculated by lightly streaking over the slant. Incubated for 48 hrs at room temperature. Positive reaction was indicated by growth with an intense blue colour on the slant (Alkalinity). No colour change indicated negative reaction.

Starch utilization

Starch agar plates were prepared and spot inoculated with the bacterial culture using an inoculation loop. Plates were incubated for 48 hrs and flooded with Gram's iodine solution. A clear zone around the bacterial growth indicates positive reaction.

5.3.2 Preparation of axenic culture

The axenic cultures of *Phaeocystis* sp. were prepared following the method of Andersen (2005). The algal sample was treated with an antibiotic mixture containing Penicillin, Gentamicin and Ciprofloxacin. The antibiotic mixture was prepared by mixing 10mg of Gentamicin, 10mg of Ciprofloxacin and 10mg of Penicillin in 10ml water. The antibiotic solution was filter sterilized and 2ml of this solution was added to 100ml sterile Walne's medium. 5ml of *Phaeocystis* sp. culture from exponential phase was inoculated in to the medium containing antibiotic mixture and kept for

4 days. At the end of fourth day, the culture was centrifuged at 5000 rpm for 10 minutes to separate the algal cells and the pellet was resuspended in sterile sea water. The centrifugation was repeated for three times in order to remove the antibiotics completely. The suspension was added to fresh sterile Walne's medium for algal growth.

5.3.3 Enumeration of total bacterial cell number at different growth phases

Phaeocystis sp. was cultured in sterile Walne's medium for 25 days at pH 8.00, 20^0C, 2000lux illumination, 30ppt salinity and 12h:12h light :dark cycle. The associated bacterial cell number (culturable) was enumerated at 5 days interval to estimate the total bacterial cell number and the number of various bacterial groups associated with *Phaeocystis* sp. 1ml of algal culture was added to 9ml sterile sea water and was serially diluted using sterile seawater and plated in nutrient agar using spread plate technique. The plates were incubated for 7 days at 20^0C to develop individual bacterial colonies. The individual bacterial colonies developed were isolated and purified. Isolated colonies were identified upto generic level through various biochemical tests.

Statistical analysis of the bacterial number at different growth phases was done using one-way ANOVA followed by Duncan's multiple comparison using SPSS 10.0 for Windows. Significant difference was calculated at $p<0.05$.

5.3.4 Antibacterial activity of associated bacteria

Antibacterial activity of the associated bacteria of *Phaeocystis* sp. was assayed following the method of Spragg *et al.* (1998). Activity was tested against ten bacterial pathogens including common human and

aquaculture pathogenic bacteria- *Pseudomonas aeruginosa, Bacillus cereus, Aeromonas hydrophila, Staphylococcus aureus, Escherichia coli, Edwardsiella tarda, Vibrio proteus, Vibrio harveyi, Vibrio parahaemolyticus and Vibrio fluvialis*. Associated bacteria were grown in nutrient broth for 4 days in order to ensure the release of antibacterial compounds into the culture fluid and the culture fluid was separated and filter sterilized using membrane filter. Paper discs (Whatman 6mm) were saturated with the prepared culture supernatant (200µl) and placed onto the nutrient agar plates inoculated with the test pathogenic organism. As a control, filter paper saturated with nutrient broth was used. Plates were then incubated overnight at 37^0C. Antibacterial activity was determined by observing the zone of inhibition around the filter paper discs.

5.3.5 Antimicrobial activity of *Phaeocystis* species

Antimicrobial activity of *Phaeocystis* sp. was also tested following the method of Spragg *et al.* (1998) using the same test organisms. For testing the antimicrobial activity, exponentially growing *Phaeocystis* sp. culture and culture fluid free of algal cells were used and tested as mentioned in section 5.3.4.

5.3.6 Effect of associated bacteria on the growth of alga

The associated bacterial groups maintained in nutrient agar slants in the laboratory were used for the experiment. The individual bacterial strains were sub cultured into fresh sterile nutrient broth and incubated at 25^0C for three days in an orbital shaker to obtain sufficient number of individual bacterial cells.

The *Phaeocystis* culture prepared after the antibiotic treatment was inoculated into sterile Walne's medium (salinity 30ppt and pH 8.00) in

axenic condition to obtain a cell number of 2×10^5 cells/ml. Associated bacteria (Five bacterial strains) were added to the culture media separately to obtain a cell concentration of 10^6 cells/ml. Control flask was prepared without adding bacterial cells. All the experimental flasks were kept in the environmental chamber and incubated at optimum conditions for 24 days. The growth of *Phaeocystis* sp. was calculated by the estimation of chlorophyll-a (Strickland and Parson, 1972) in all culture flasks from the day of incubation till 24th day of growth at three days interval.

The results were statistically analysed by two-way ANOVA using Duncan's multiple comparison of means using SPSS 10.0 for windows. Significant difference was calculated at $p<0.05$.

5.3.7 Haemolytic property of associated bacteria

Haemolytic property of the associated bacterial strains was tested using blood agar. Nutrient agar medium was prepared, autoclaved and made to cool to which fresh human blood was added (5% of the nutrient agar medium). The plates were kept for surface drying at 37^0C overnight. Associated bacterial strains were spotted on the blood agar using a wire loop and kept on incubation at 27^0C overnight. The plates were examined for haemolysis and clear zone (destruction of RBC) on the plate was considered as positive result for haemolysis.

5.3.8 Effect of associated bacteria on the haemolytic property of *Phaeocystis* species

Associated bacteria were isolated, identified and maintained in the laboratory by streaking on nutrient agar slants. Five genera of bacteria were present. *Alcaligenes* I, *Flexibacter, Pseudomonas, Flavobacterium and Alcaligenes* II.

5.3.8.1 Effect of bacterial filtrate on the haemolytic property of *Phaeocystis* species

Individual bacterial stains were inoculated into nutrient broth and incubated at 25^0C in an orbital shaker for 4 days. At the end of 4^{th} day, the bacterial cultures were filtered through 0.2µm pore-size cellulose acetate filter to remove the cell completely. The bacterial filtrate thus obtained was used for the study to investigate the effect of bacterial filtrate on the haemolytic property of *Phaeocystis*. To test the effect of bacterial filtrate, five set of Walne's medium was prepared in triplicate to which *Phaeocystis* sp. cells were added at a cell concentration of 2×10^5 cell/ml and incubated for 15 days. On the 15^{th} day, bacterial culture filtrate prepared was added to the algal culture flask separately in axenic condition and kept overnight and tested for haemolysis.

5.3.8.2 Effect of bacterial cells on the haemolytic property of *Phaeocystis* species

Seven set of experimental flasks were set up with 100ml Walne's medium in artificial sea water. Five strains of bacterial cells (associated bacteria 10^6 cells/ml) were inoculated in to the conical flask separately. *Phaeocystis* sp. cells were added to the culture medium to reach an initial cell density of 2×10^5 cells/ml. To the 6^{th} set of flask only the *Phaeocystis* sp. culture was added which served as the control. To the 7^{th} set both nutrient broth (5ml) and *Phaeocystis* cultures were added. The experiments were done in triplicates and all the flasks were incubated at optimal culture conditions. Haemolytic property was studied on the 15^{th} day when algal cells were in the logarithmic growth phase.

5.3.8.3 Estimation of associated bacterial groups

Associated bacterial groups present in the above experimental flasks were enumerated by plating in nutrient agar and individual colonies were isolated and identified by various biochemical tests as described in section 5.3.1

5.3.8.4 Determination of Haemolysis

Haemolytic property of the *Phaeocystis* sp. from the experimental flasks were analysed by doing erythrocyte lysis assay using the whole culture sample, which include both the algal cell and filtrate. The assay was done as described in section 4.3.4.3

Chlorophyll-a concentration in each experimental flask was calculated to estimate the growth of algae in the presence of bacterial cells and the corresponding bacterial filtrate following the method of Strickland and Parson (1972).

5.3.8.6 Statistical analysis

The haemolytic property of *Phaeocystis* sp. under the influence of bacterial cells and culture filtrate was statistically analysed by doing one way ANOVA followed by Duncan's multiple comparison of the means using SPSS 10.0 for Windows. Significant difference was calculated at $p<0.05$.

5.4 Results

5.4.1 Isolation of associated bacteria

Nutrient agar plates and Zobell's agar plates inoculated with samples from the *Phaeocystis* sp. culture at 15th day and 24th day of growth period showed diverse assemblages of associated bacterial flora. Representative colonies were isolated and identified upto generic level. The bacterial

strains isolated were all gram negative, short and long rods, most of them pigmented brown and yellow and belonged to the genera *Alcaligenes* spp., *Flexibacter* sp., *Flavobacterium* sp. and *Pseudomonas* sp. *Alcaligenes* group was present as two phenotypically different colonies that vary in pigmentation on the agar plate and they are designated as *Alcaligenes* sp. I and *Alcaligenes* sp. II. *Alcaligenes* I was non-pigmented and *Alcaligenes* II was with light brown pigmentation.

5.4.2 Preparation of axenic culture

Antibiotic treatments could not remove completely the associated bacteria from the *Phaeocystis* culture. Antibiotic treated *Phaeocystis* culture showed an extended lag phase compared to untreated culture and at these stage bacteria could not be isolated from the culture. But later, during the logarithmic growth phase of *Phaeocystis*, *Flexibacter* started to grow initially and later all bacterial strains were found to be associated with the alga. This showed that antibiotic treatments could only reduce the bacterial growth on the *Phaeocystis* sp. culture. Higher concentration of antibiotic treatment could kill the bacterial strains but in such conditions, the alga could not grow in the medium. This shows that associated bacteria is essential for algal growth.

5.4.3 Bacterial cell number at different growth phases of *Phaeocystis* species.

Bacterial cell number was calculated from first day to 25th day of algal growth. During the lag phase of algal growth, the associated bacterial count was low, by 5th day and 10th day, the bacterial count increased along with the algal growth and the highest bacterial count was observed on 15th day of growth (logarithmic growth phase). Figure 5.2 shows the growth pattern of *Phaeocystis* sp. at different growth phases. Associated bacterial cell number followed the same growth pattern as the *Phaeocystis* sp. with

lowest number during lag phase and slowly increasing, reaching the highest during logarithmic growth phase and then decreasing towards the stationary phase (Figure 5.1, Table 5.1 of Appendix).

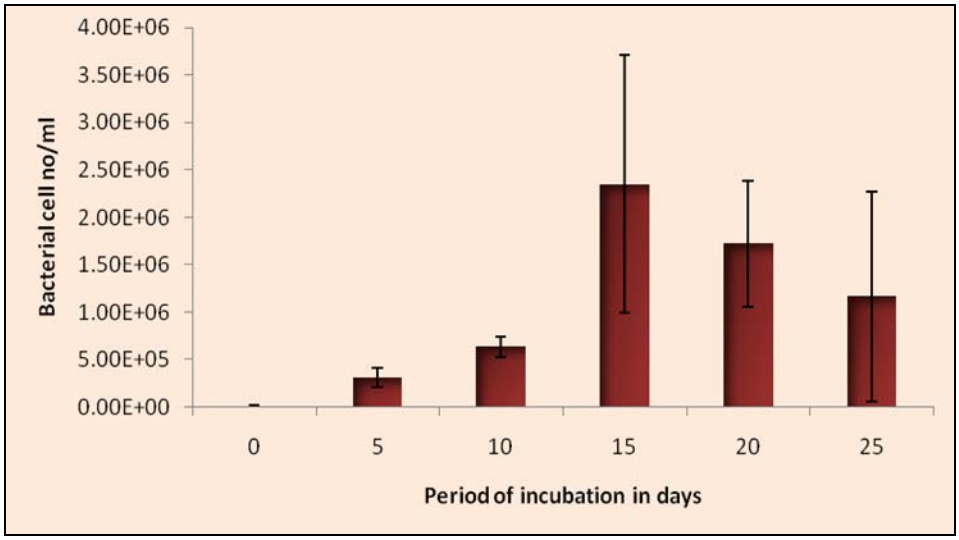

Figure 5.1 Bacterial cell numbers at different growth phases of *Phaeocystis* sp.

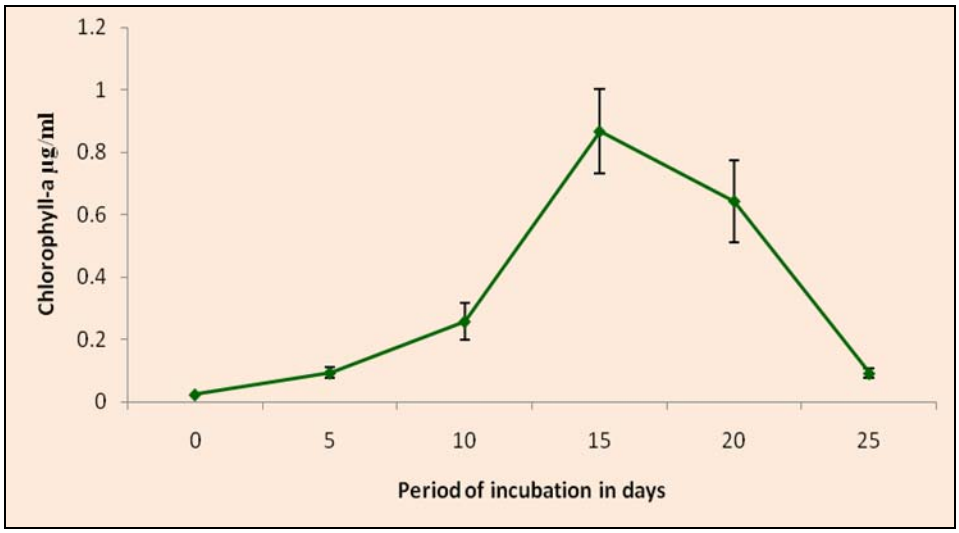

Figure 5.2 Chlorophyll-a concentration at different growth phases of *Phaeocystis* sp.

Statistical analysis of bacterial cell number at different growth phases of *Phaeocystis* sp. showed that no significant difference in bacterial cell number from 0^{th} day to 10^{th} day of growth. By 15th day of growth bacterial cell number significantly varied from other days.

Observation of algal culture under the microscope revealed the growth pattern of the alga. When the bacterial count was low, in the algal culture there was no colony formation, only single non-motile cells were present. By 10^{th} day colony cells increased in the culture, and the cell number in the colony were four and the colony showed a cloud like appearance. Towards the logarithmic phase along with the highest bacterial biomass the colony formation reached the highest stage with majority of algal cells present in the colony. By 25th day of growth, the colonies disintegrated and released single cells from the colony. Flagellated single cells moving in the algal culture could be seen towards the stationary growth of alga.

The different bacterial groups present in the total bacterial consortium associated with *Phaeocystis* sp. also changed along with the growth period of alga and also with the cell polymorphism (Figure 5.3 and Table 5.2 of Appendix). *Alcaligenes* I was highest towards the initial growth period and decreased towards the logarithmic phase. *Alcaligenes* II was highest towards the first half of logarithmic phase, reaching its maximum on 15^{th} day of growth and then the number slowly decreased towards the stationary phase. *Flexibacter* also followed the same pattern with slightly increasing from lag phase towards logarithmic phase and then decreasing. *Pseudomonas* was not detected in the initial growth period, but was detected in the stationary phase, when colonies started disintegrating. The total count of *Flavobacterium* also increased towards the stationary growth period.

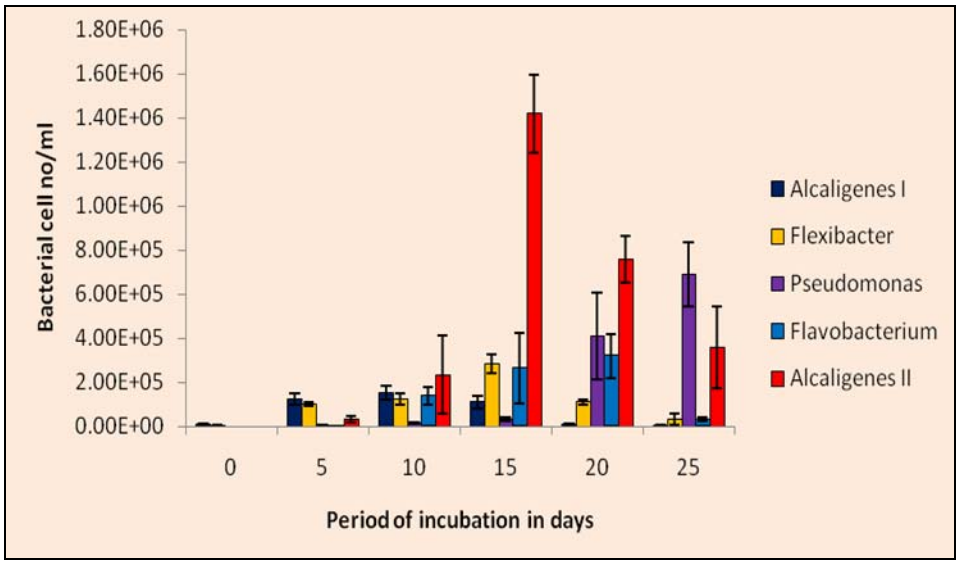

Figure 5.3 Bacterial groups associated with *Phaeocystis* sp. at different growth period

At the Initial growth period, the dominant bacterial flora was *Alcaligenes* I and *Flexibacter*. From the fifth day of the culture, all the five bacterial groups were found associated with the alga and the dominant flora being *Alcaligenes* I. The genus *Alcaligenes* II was the dominant species on 10^{th} day and 15^{th} day when the algal culture was in the logarithmic phase of growth. From the 20^{th} day, when the culture entered the stationary phase, the number of *Alcaligenes* II started to decline and a dominance of *Pseudomonas* was observed and the dominance continued till the decline phase (Figure 5.4 to 5.9)

Flexibacter was present throughout the algal growth with its maximum presence during the initial growth period, whereas *Alcaligenes* I was present only in the initial growth phase of alga. *Pseudomonas* was present in higher number during the stationary phase or during the colony disintegration of alga indicating its possible role in the termination of the bloom. Maximum bacterial

numbers were present during the logarithmic growth phase of alga where all five bacterial strains found associated with *Phaeocystis*.

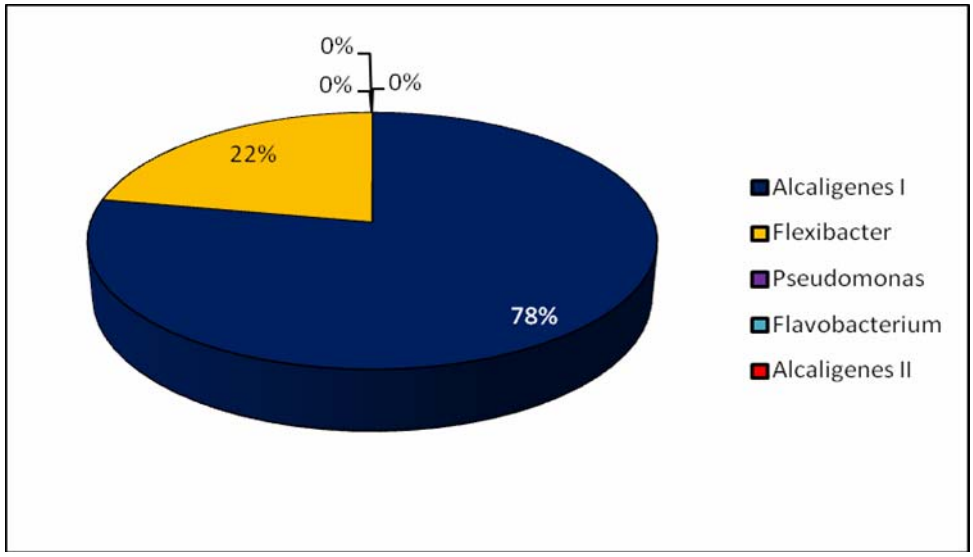

Figure 5.4 Percentage of bacterial groups at first day of growth period of *Phaeocystis* sp.

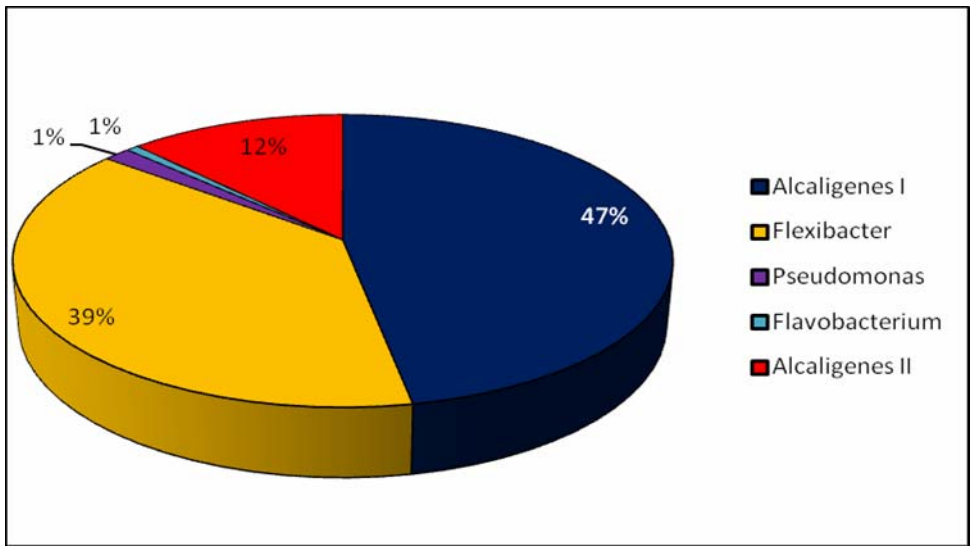

Figure 5.5 Percentage of bacterial groups at 5th day of growth period of *Phaeocystis* sp.

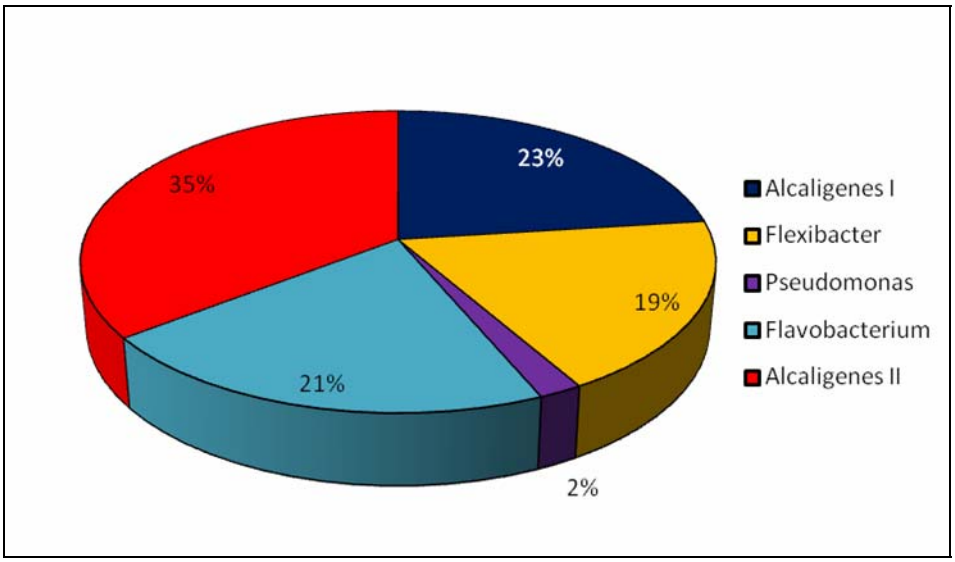

Figure 5.6 Percentage of bacterial groups at 10th day of growth period of *Phaeocystis* sp.

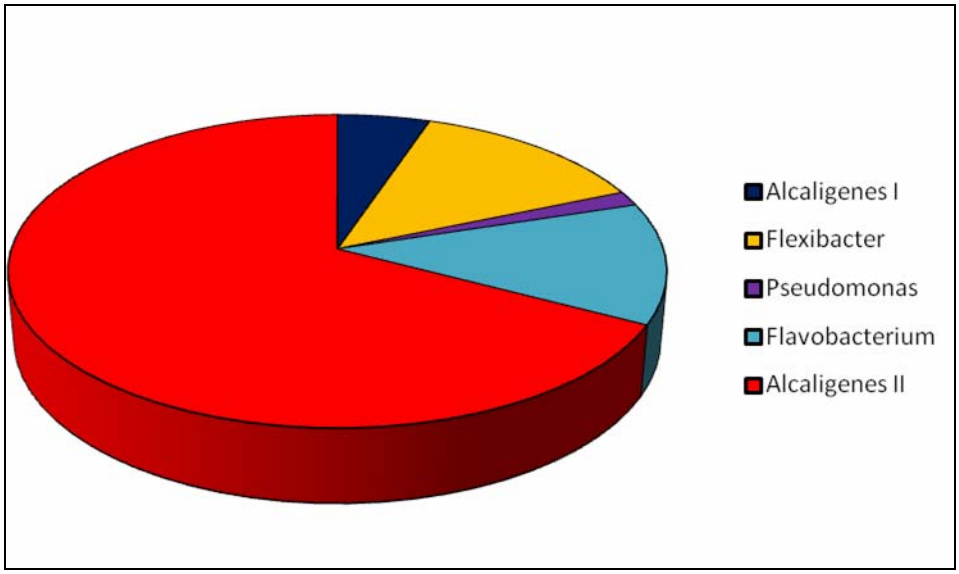

Figure 5.7 Percentage of bacterial groups at 15th day of growth period of *Phaeocystis* sp.

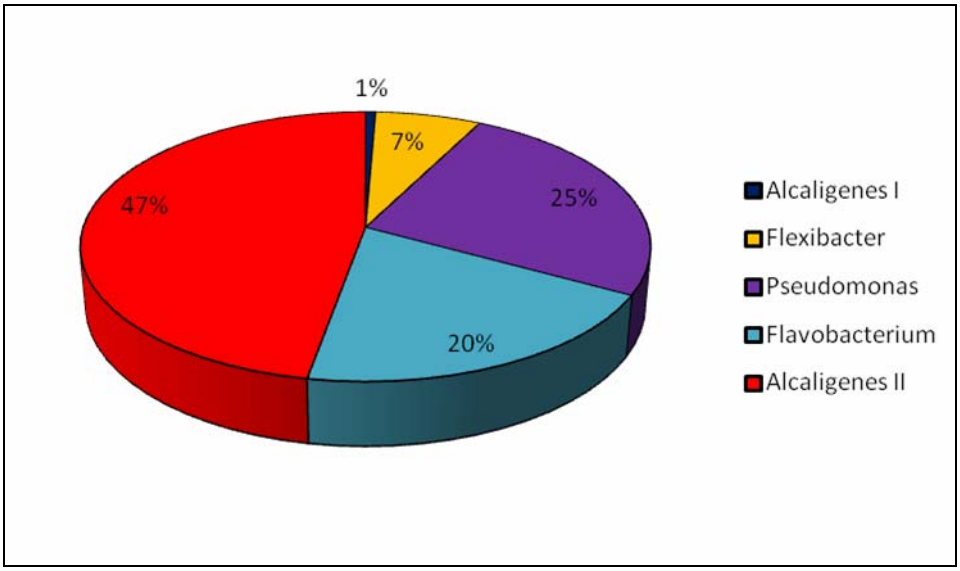

Figure 5.8 Percentage of bacterial groups at 20th day of growth period of *Phaeocystis* sp.

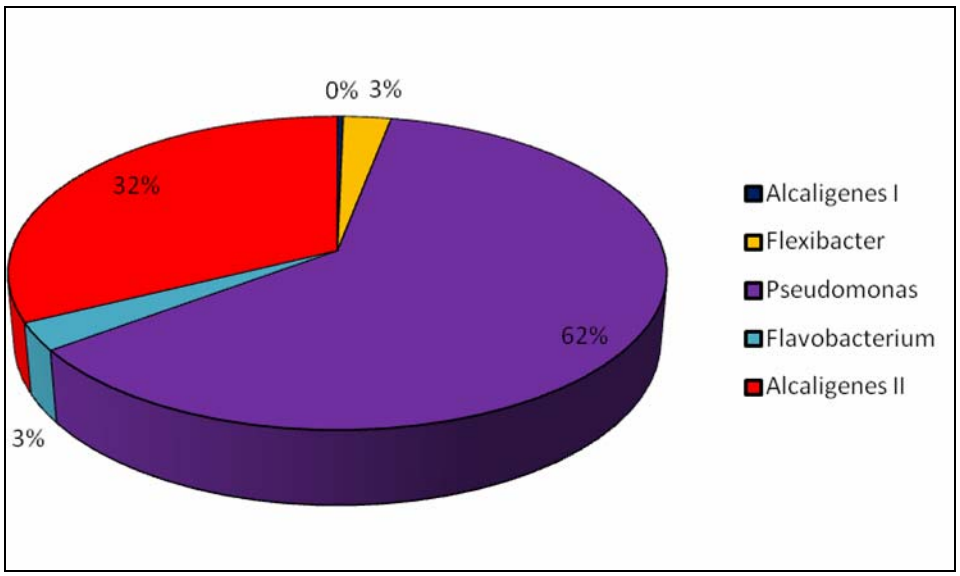

Figure 5.9 Percentage of bacterial groups at 25th day of growth period of *Phaeocystis* sp.

5.4.4 Antibacterial activity

Phaeocystis sp. culture and the associated bacterial strains didn't show any antibacterial activity against the bacterial pathogens tested.

5.4.5 Effect of associated bacteria on the growth of *Phaeocystis* species

Different bacterial groups affected the growth of *Phaeocystis* significantly. In the initial growth period, growth was less in the flask inoculated with *Alcaligenes* I and highest growth was observed in *Phaeocystis* sp. culture inoculated with cells of *Flexibacter,* followed by control culture and *Alcaligenes* II. Growth in the control flask showed the regular growth characteristics of the *Phaeocystis* culture. During the logarithmic growth period, (15th day of growth), the chlorophyll-a concentration in all the flasks were almost same with slight decrease in the culture inoculated with *Alcaligenes* I. The associated bacterial flora seemed to influence the alga only during the initial growth stage. When the *Phaeocystis* reached the logarithmic growth phase, the influence of individual bacteria on the growth of the *Phaeocystis* was reduced since all the associated bacterial strains were well established in the algal culture by then. By 18th day of growth, the growth of *Phaeocystis* in the flask inoculated with *Flexibacter* started decreasing compared to the control flask, due to the nutrient depletion as the rate of algal growth was high in the presence of *Flexibacter* cells from the initial stage onwards. By 21st day of growth, all cultures entered into the stationary phase except the culture inoculated with *Alcaligenes* I, since sufficient nutrients were present in the culture as the growth was less in the initial period (Figure 5.10).

Among the associated bacteria tested *Flexibacter* and *Alcaligenes* II could enhance the growth of *Phaeocystis*. Growth was reduced initially in the presence of *Alcaligenes* I. *Flavobacterium* and *Pseudomonas* didn't show any significant effect on the growth of *Phaeocystis* sp.

Statistical analysis showed that the growth of algal culture inoculated with *Alcaligenes* I varied significantly from all other bacterial groups, whereas the culture inoculated with *Flexibacter* and control did not vary significantly. Significant variation was not observed in the growth of *Phaeocystis* culture inoculated with *Alcaligenes* II and the Flavobacterium (Table 5.3 of Appendix).

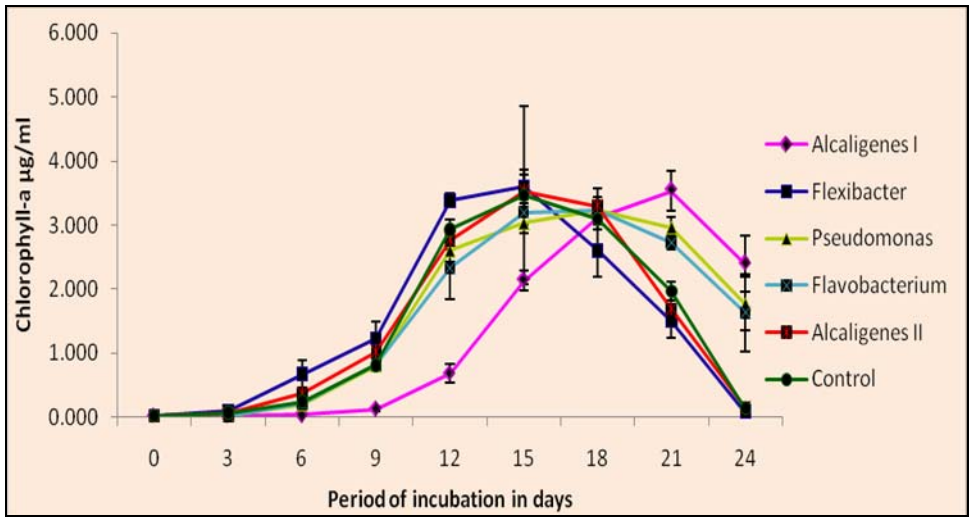

Figure 5.10 Effect of associated bacteria on the growth of *Phaeocystis* sp.

5.4.6 Haemolytic property of the associated bacteria

The haemolytic property of the individual bacterial strains was tested using blood agar. Only *Pseudomonas* showed a zone of clearance in blood agar indicating the haemolytic property. (PLATE 1)

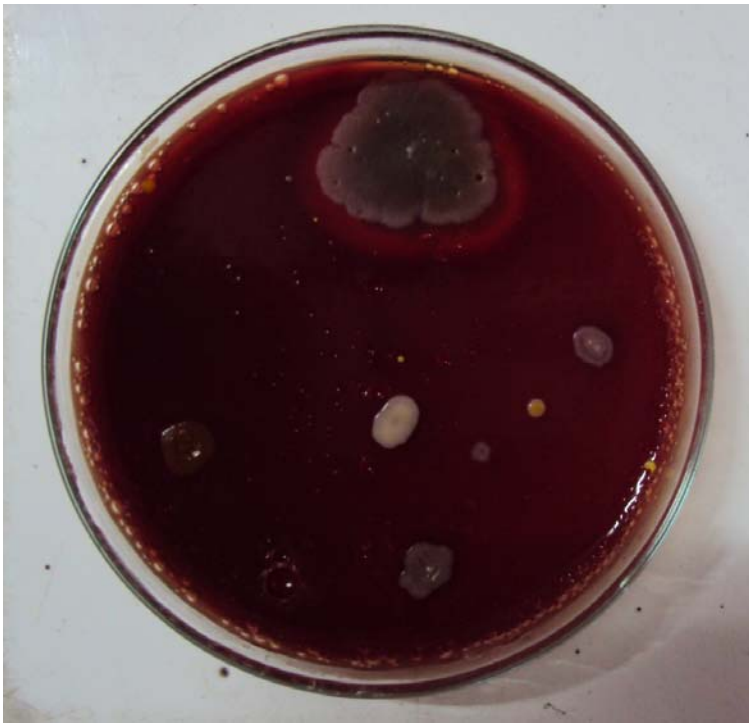

PLATE 1 Haemolysis caused by *Pseudomonas* on blood agar plate

5.4.7 Effect of associated bacteria on the haemolytic property of *Phaeocystis* species

The haemolytic property of *Phaeocystis* culture was tested with and without inoculating associated bacterial cells and culture filtrate. Highest haemolysis was exhibited by algal culture inoculated with *Flexibacter* cell (Figure 5.11 and Table 5.4 of Appendix). The algal culture inoculated with *Alcaligenes* II cells and the nutrient broth added cultures also showed higher haemolytic property which varied significantly with other bacterial strains. Inoculation of the cells of *Alcaligenes* I, *Pseudomonas* and *Flavobacterium* did not influence the haemolytic property of the alga.

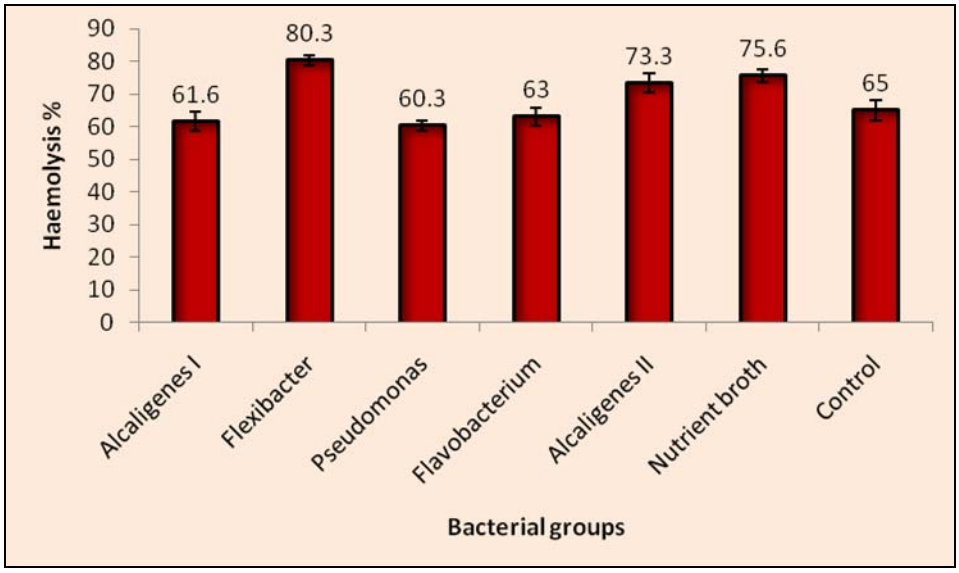

Figure 5.11 Effect of bacteria on the haemolytic property of *Phaeocystis* sp.

However, the haemolysis exhibited by *Phaeocystis* in the presence of bacterial culture filtrate showed variation from the situation where algal culture inoculated with respective bacterial cells. Culture filtrate of *Pseudomonas*, *Flavobacterium* and *Alcaligenes* II significantly enhanced the haemolytic property of the alga (Figure 5.12 and Table 5.5 of Appendix)

Growth of alga in all the experimental flaks were estimated at the same period of haemolysis test to study the relation of algal growth and the influence of bacterial flora in the haemolytic property of the alga. Algal culture inoculated with *Flexibacter* cells showed higher chlorophyll-a compared to other bacterial strains (Figure 5.13 and Table 5.6 of Appendix).The growth of algal culture inoculated with *Pseudomonas, Flavobacterium* and *Alcaligenes* I was almost similar without significant variation and at the same time lower when compared to cultures inoculated with *Alcaligenes* II, *Flexibacter* and the control culture. The chlorophyll-a

value of all algal cultures to which bacterial filtrate were added was almost same without much variation (Figure 5.14 and Table 5.7 of Appendix).

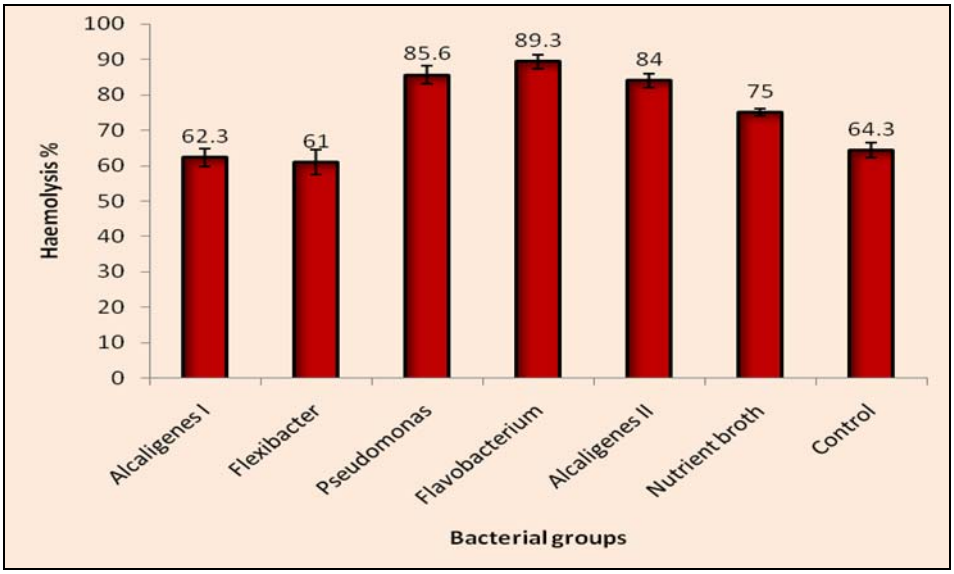

Figure 5.12 Effect of bacterial filtrate on the haemolytic property of *Phaeocystis* sp.

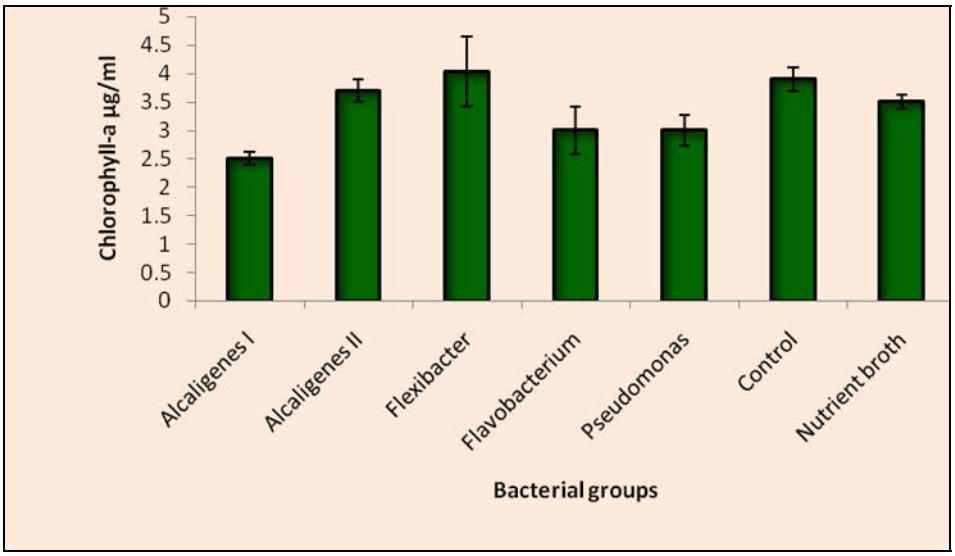

Figure 5.13 Growth of *Phaeocystis* sp. in the presence of individual bacterial cells

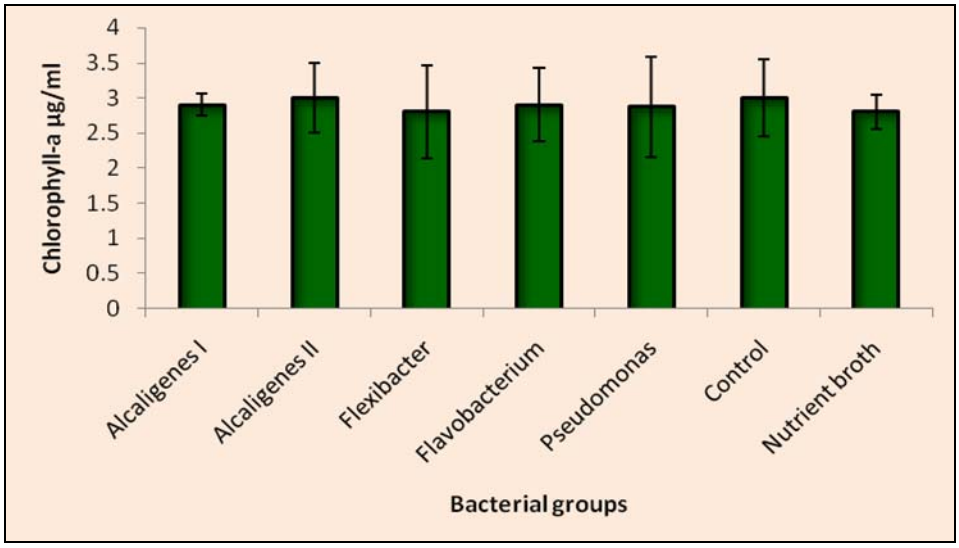

Figure 5.14 Effect of bacterial culture filtrate on the growth of *Phaeocystis* sp.

The associated bacterial cell number were enumerated in all the experimental flasks at the time of haemolysis test (15^{th} day of algal growth) to study the growth pattern of associated bacteria on addition of cells and culture filtrate of individual bacterial strains. In the control group and in the nutrient broth added algal cultures, the bacterial groups were present in the same pattern with *Alcaligenes* II present in higher number followed by *Flexibacter* and *Flavobacterium*. (Figure 5.15 and 5.16) In the nutrient broth added algal culture, a slight increase in the number of each bacterial group was observed compared to control (Table 5.8 of Appendix). The addition of individual bacterial groups and filtrate caused a deviation in the normal growth pattern of bacterial consortium of the alga. In the algal culture, inoculated with the bacterial strains, the cell count of respective bacterial groups was high compared to other bacterial strains except for the algal culture inoculated with *Pseudomonas* cells (Figure 5.17 to 5.20). In algal culture inoculated with *Pseudomonas*, the biomass of *Pseudomonas* was only 4×10^5 cells/ml and the bacterial count of other groups was higher than *Pseudomonas* (Figure 5.21).

In the algal cultures where individual bacterial culture filtrates were added, the number of *Alcaligenes* II was higher than the other bacterial groups (Figure 5.17 to 5.21).

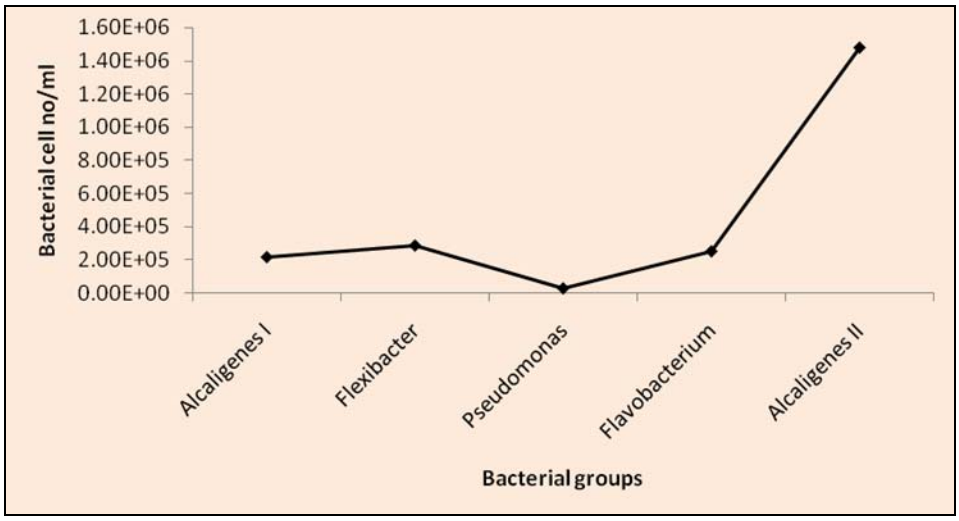

Figure 5.15 Distribution of associated bacterial groups in the *Phaeocystis* sp. in control culture

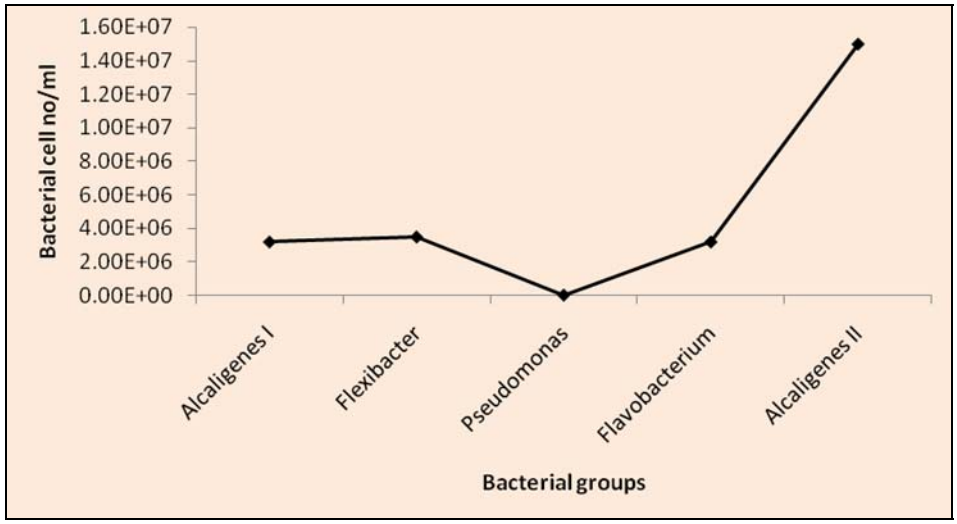

Figure 5.16 Distribution of bacterial groups in the nutrient broth added *Phaeocystis* sp. culture

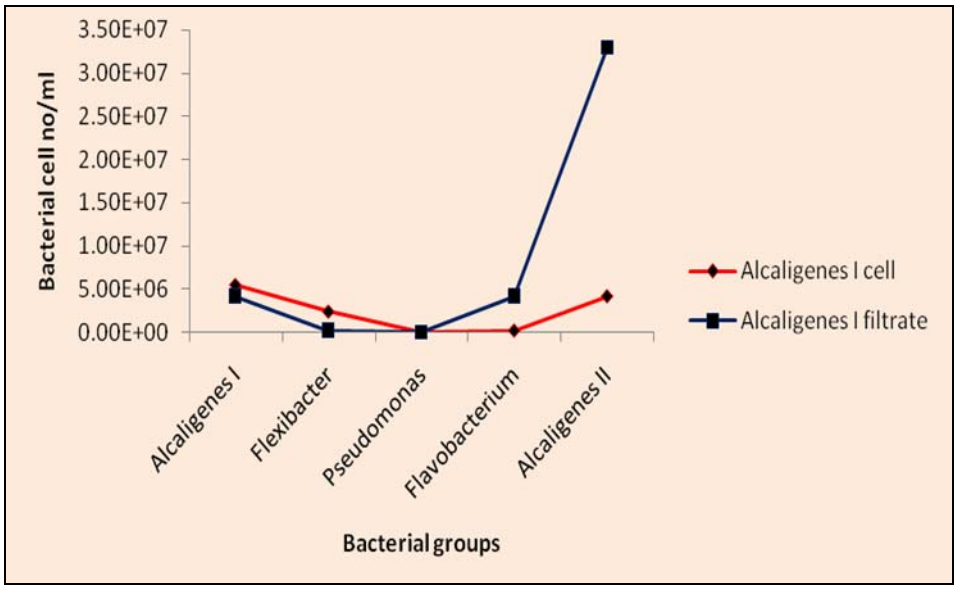

Figure 5.17 Effect of *Alcaligenes* I on other associated bacterial groups

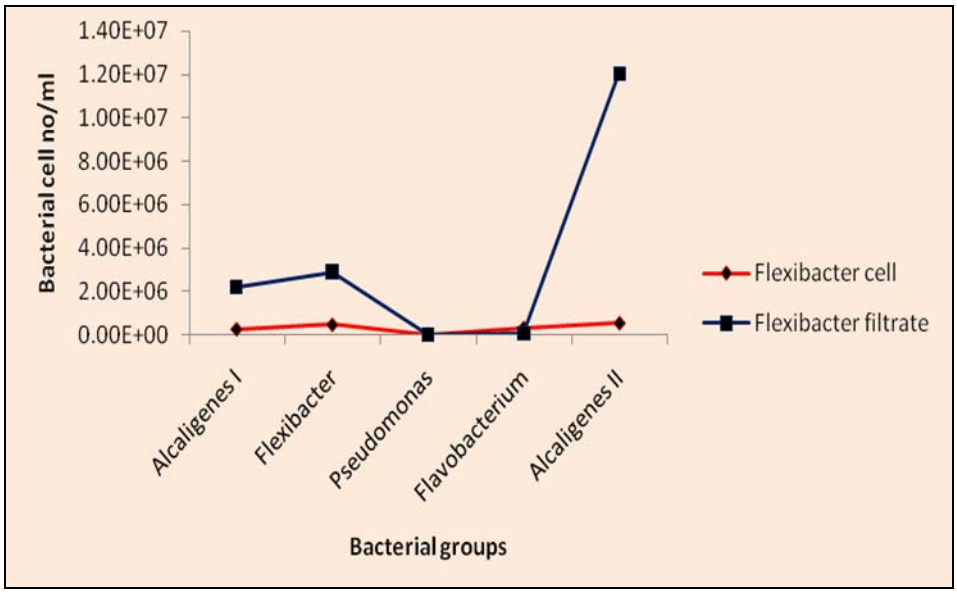

Figure 5.18 Effect of *Flexibacter* on other associated bacterial groups

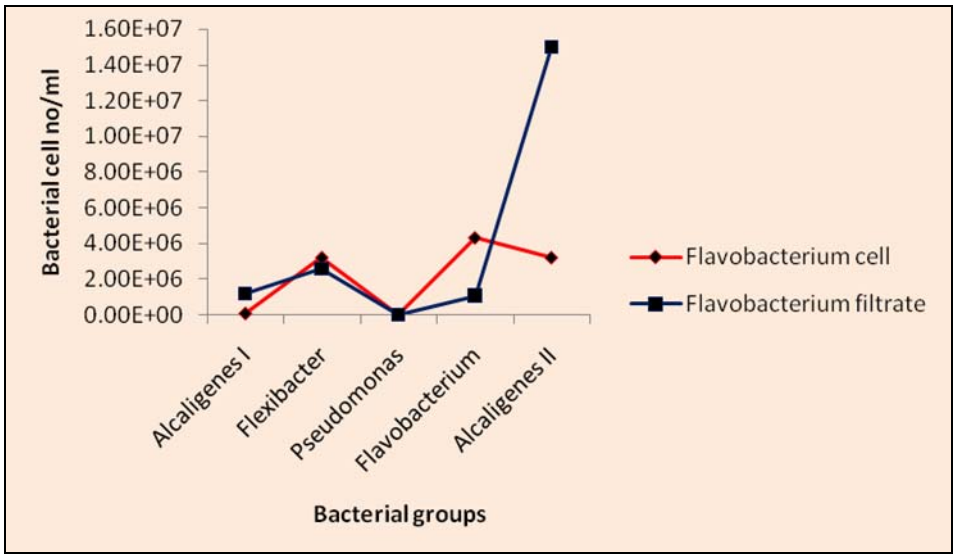

Figure 5.19 Effect of *Flavobacterium* on other associated bacterial groups

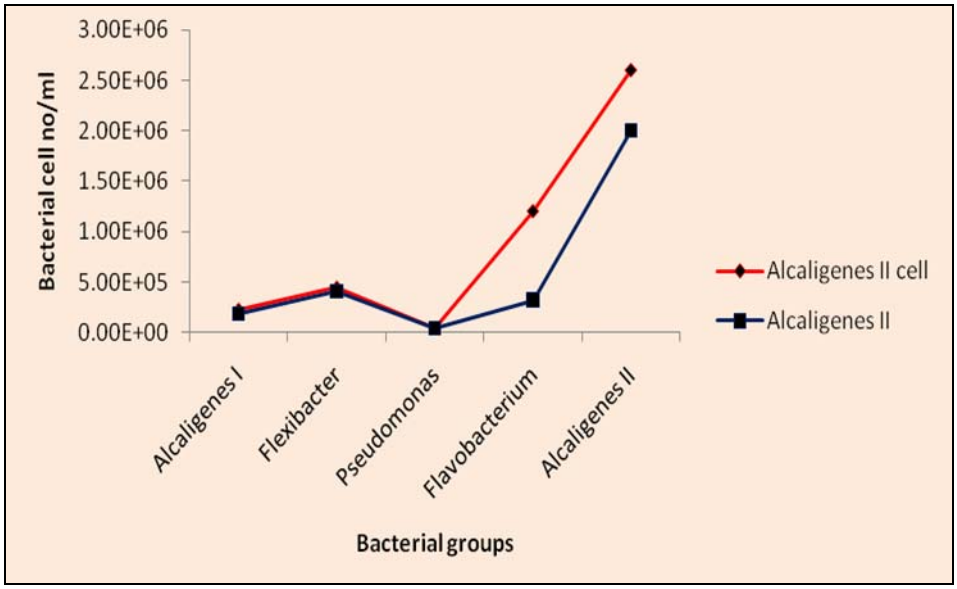

Figure 5.20 Effect of *Alcaligenes* II on other associated bacterial groups

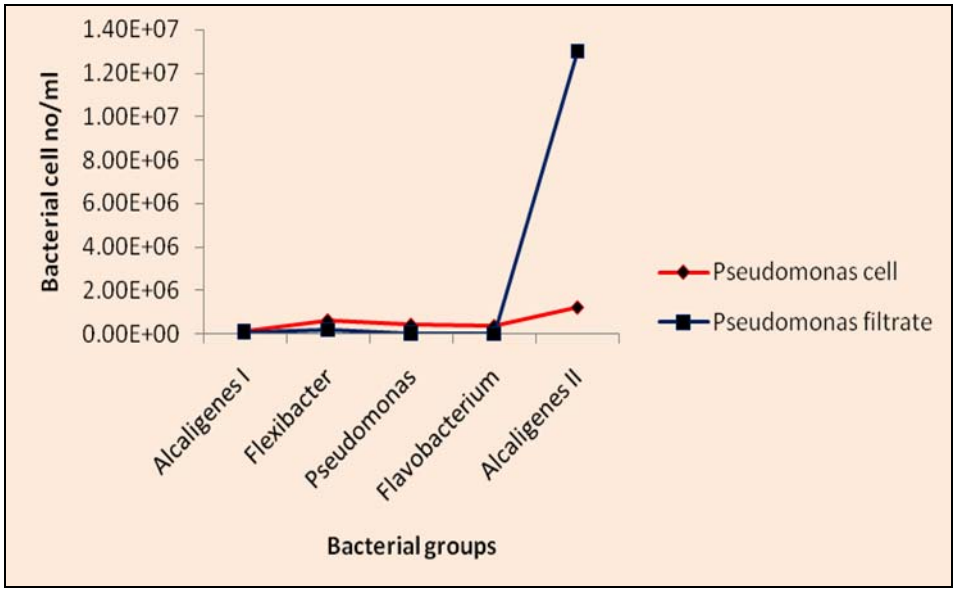

Figure 5.21 Effect of *Pseudomonas* on the associated bacterial groups.

5.5 Discussion

5.5.1 Associated bacteria of *Phaeocystis* species

Generally the bacteria associated with algae were Gram negatives (Simidu *et al.*, 1971). In the present study also the bacteria associated with the *Phaeocystis* sp. culture were found to be Gram negatives and include the groups *Alcaligenes, Pseudomonas, Flexibacter*, and *Flavobacterium. Alcaligenes* was present as two different types that vary morphologically in colour and colony size. In a similar study Nicholas *et al*. (2004) found that the algae harbour distinctly separated bacterial groups belonging to α-Proteobacteria, γ-Proteobacteria, Cytophaga-Flavobacterium-Bacteroides group, Actinobacteria, and Bacillus. Bacteria included in the Cytophaga-Flavobacterium-Bacteroides groups are well adapted for living in close association with phytoplankton. *Flexibacter* along with other bacterial strains were found associated with *Alexandrium* sp. (Jasti *et al*., 2005).

In the present study, the question, whether the *Phaeocystis* sp. cultured in the laboratory harbour specific bacterial communities in their phycosphere was thoroughly analysed. Here the *Phaeocystis* sp. isolated was aseptically cultured in the laboratory for many generations and the associated bacteria were isolated after several washing of the alga. After antibiotic treatments also the same bacterial groups were found associated with the *Phaeocystis*. The result confirms the findings of Grossart *et al.* (2005), Schaffer *et al.* (2002) and Sapp *et al.* (2006), who observed that microalgae harbour specific bacterial communities. Jasti *et al*. (2005) had shown that the various phytoplankton groups grown under same laboratory conditions showed different associated bacterial flora and the same algal species isolated from different geographical areas showed similar bacterial association. Sapp *et al.* (2006) showed that the composition of associated bacterial communities linked to the microalgae was under the influence of the quality or quantity of algal exudates. The antibacterial metabolites produced by some microalgae, inhibit certain bacterial species and at the same time the algal exudates act as organic nutrients which promote the bacterial populations. Thus the algal exudates play an important role in shaping the associated bacterial community by its effect on the growth of the bacterial flora. Myklestad (1995), who analyzed the composition of algal exudates, found out that several microalgae vary in the composition of exudates especially with regard to polysaccharides. But the question of whether the algae producing similar exudates harbour similar bacterial communities needs to be addressed in the future.

5.5.2 Bacterial biomass at different growth phases of *Phaeocystis* species

The community composition of the attached bacteria is highly variable and linked to the physiological state of the algae. In this study the

bacterial biomass was highest during the logarithmic growth phase of alga where the alga was physiologically very active and the extracellular production was high. Diversity of bacterial strains was also high during the logarithmic growth phase. During the stationary phase the bacterial biomass was lower than the logarithmic phase and the bacterial community was mainly dominated by *Pseudomonas* and *Alcaligens* II.

The distribution of bacteria in the algal culture depends on the phase of algal growth. There was a pronounced shift in bacterial community structure during the transition from initial growth stage to the stationary phase. It was well known that the transition in algal growth leads to the changes in the organic matter of the algal origin (Brockmann *et al*., 1979; van Hannen *et al*., 1999; Riemann *et al*., 2000). *Pseudomonas* was not detected along with the *Phaeocystis* sp. during the initial growth period. The number of *Pseudomonas* was found to be increasing along with the growth of alga and the number reached the maximum during the stationary phase where colony disintegration started to operate, which indicated the death phase of alga. But at the same time *Pseudomonas* didn't inhibit the growth of the alga in the experiment in the initial growth phase indicating that the bacteria can utilize the secondary metabolites produced by the alga during the stationary phase and it may act as algicidal only when the bloom reached the maximum growth with formation of colonies in the culture. Accumulation of algal secondary metabolites was predominant in the stationary growth phase and the genus *Pseudomonas* is well known for its ability to metabolize various secondary metabolites. The present study confirms this. Grossart *et al*. (2005) showed that the addition of bacteria to *Thalassiosira rotula* in the stationary phase resulted in rapid degradation of algal cells and a strong increase in bacterial numbers. On the other hand, it

is well known that marine bacteria could produce highly hydrolytic enzymes (Martinez *et al*., 1996) leading to increased algal death and lysis, once algae was nutritionally stressed (Cole, 1982). Here in *Phaeocystis* sp. cultures, during stationary phase, algal cells were in limited nutrient conditions, and hence nutritionally stressed and were more vulnerable to the attack of *Pseudomonas*. Further study is necessary to find out the role of *Pseudomonas* in disintegration of algal colony structure.

Alcaligenes I was found to be present in the algal culture in the initial growth phase along with other bacterial groups but they were diminished in the logarithmic and stationary phase. *Flexibacter* was found to be very closely associated with the *Phaeocystis* sp. Though the antibiotic treatments diminished other bacterial groups; *Flexibacter* survived more effectively than the other bacterial strains and appeared first in the algal culture after antibiotic treatment. The antibiotic treatment could not be able to remove the bacteria completely. When a higher dose of antibiotics was used for the experiment, the alga could not able to survive. Dodge (1973) showed that the bacteria associated with algae could not be easily removed by the treatment of antibiotics, or they were not separated since they enter into an endosymbiotic association with the algae (Nayak *et al*., 1997). Such endosymbiotic association may explain the absence of bacteria immediately after addition of antibiotics but the reappearance after a few days (Dodge, 1973).

5.5.3 Antibacterial activity of associated bacteria

The algal associated bacteria were found to be producing antibacterial compounds against other bacterial strains inorder to inhibit the colonization of non native bacteria on the microalgal surfaces (Spragg *et al*., 1998). The associated bacteria from *Phaeocystis* sp. were screened for the production

of antibacterial compounds against the human and fish pathogens, but none was found to be positive in antimicrobial activity. Current assays for antimicrobial activity are inadequate because some antibiotic producing bacteria may require the presence of an inducer compound produced in the presence of another bacterial species. Since the *Phaeocystis* showed a specific bacterial association, more investigations on the antimicrobial activity has to be done in varying condition, to know whether the microalgae or the associated bacteria might be producing some antimicrobial compounds to maintain the species specificity of the associated bacteria.

5.5.4 Bacterial influence on the growth of *Phaeocystis* species

The results showed that the associated bacterial assemblages obtained from the *Phaeocystis* sp. culture were capable of dramatically influencing the growth of *Phaeocystis* sp. differentially under laboratory conditions. Among the associated bacterial strains, *Alcaligenes* was seemed to influence growth of alga in two different ways; *Alcaligenes* I was found to be inhibiting the growth of *Phaeocystis* sp. initially, where *Alcaligenes* II along with *Flexibacter* were found to be stimulating the growth of *Phaeocystis*. In a similar study, Suminto and Hirayama (1993) observed that the associated bacterial flora contained both the stimulatory bacterial strains and inhibitory bacterial strains at the same time. The stimulatory bacterial strains present here include the *Flexibacter* and *Alcaligens* II and among the two, *Flexibacter* was found to be significantly promoting the growth of *Phaeocystis*. Fukami *et al*. (1989) have shown that *Alcaligenes* sp. showed a significant growth promoting effect on *Nitszchia* sp.

The growth factors produced by bacteria, which were found promoting growth of one phytoplankton species, may suppress the growth

of other species (Fukami *et al*., 1991). The result reveals the capacity for bacterial algal interactions to control the bloom dynamics of *Phaeocystis* along with the abiotic factors. The bloom of the microalgae was characterised by a regular initial growth phase, followed by the peak bloom period where the algae was growing unlimitedly, producing large amounts of extracellular products. This stage of production of extracellular products determines the fate of bloom making it harmful in nature by its toxic effects on other aquatic flora and changing the total ecology of the bloom area. The bloom termination was characterised by the decline of growth and large scale sedimentation of the colonies and cell lysis. The three stage of the algal bloom is specifically influenced by the associated bacterial groups. Here in this study, during the initial growth phase or during the initiation of bloom, *Alcaligenes* I was found to be associated with *Phaeocystis* sp. inhibiting the growth of *Phaeocystis* sp., thus act negatively on the blooming of the algae. Towards the logarithmic phase of the alga, the number of *Alcaligenes* I was reduced and the number of *Flexibacter* and *Alcaligenes* II was increased which promotes the growth of alga thus, stabilizing the bloom of *Phaeocystis*. The inhibitory and stimulatory effect of the associated bacteria appeared to be the result of specific biological interactions between the bacteria and the alga (Ferrier *et al*., 2002). The algicidal bacteria was found to be regulating the dynamic of bloom in a sequential way- bacteria remain in the associated bacterial assemblages in the starting stage, when the algae starts blooming, the bacterial number also increases along with the secretion of algal extracellular material. When the bloom fully develops, a feedback mechanism operates in which the algicidal bacteria associated with the algae start declining the bloom by directly acting on the growth of algae, thus promoting the onset of bloom termination(Fukami *et al*., 1997; Doucette, 1999). In the present study,

Alcaligenes I exert a negative influence on growth during the initial growth stage and its influence was reduced by the increase in the number of other bacterial flora associated with *Phaeocystis* towards the logarithmic period, especially *Flexibacter* and *Alcaligenes* II.

The associated bacterial flora promote the algal growth by the regeneration of inorganic nutrients (Doucette, 1995), production of chelators that enhance the availability of trace metals (Keshtacher-liebson *et al*., 1995) and the bacterial release of organic nutrients, co factors, and vitamins or phytohormones (Ogata *et al*., 1996; Doucette 1995; Maruyama *et al*., 1986). The consortium of associated bacteria with the alga promote the best growth of *Phaeocystis* sp. with specific growth characteristic with a regular lag phase, logarithmic phase and stationary phase. Alteration in the consortium of bacteria by the additional supply of individual bacterial groups shifts the duration of the growth phases such as elongation of lag phase, shortening of logarithmic phase and early stationary phase or a stagnant stationary phase. The effect of bacteria on the growth of *Phaeocystis* sp. was found to be very complex involving the processes such as stimulation and inhibition of growth, encouraging colony disintegration etc.

5.5.4 Bacterial influence on the haemolytic property of *Phaeocystis* species

The effect of associated bacteria on the haemolytic property of the alga was analysed in two ways i.e., the effect of bacterial cells and the effect of filtrate. The effect of bacterial cells revealed that the algal culture grown in presence of *Flexibacter* caused maximum haemolysis. This variation in the results showed that both the algal cell concentration and the associated bacteria have an effect on the total haemolysis exhibited by the

alga. The *Flexibacter* promotes the algal growth more than the other bacterial groups. The inoculation of *Flexibacter* cells resulted in the increase in total algal growth and hence increased the haemolysis. The filtrate of *Pseudomonas* and *Flavobacterium* increased the haemolytic property of the *Phaeocystis* sp. compared to other bacterial groups. *Flexibacter* and *Alcaligenes* II increase the haemolytic property of the alga by promoting good growth and the algal cell number was increased, thus supplying more concentration of algal cells for the haemolysis of RBC. *Pseudomonas* and *Flavobacterium* increased the haemolytic property by supporting the alga in its haemolytic property. This may be either by producing haemolytic substance by the bacteria itself in addition to alga or by physiologically changing the property of haemolytic compound produced by the alga. All the associated bacteria were important in the total haemolysis of alga (Uribe and Espejo, 2003), some members by increasing the algal numbers and some members by directly influencing the physiological property of haemolysis (Dantzer and Levin, 1997; Sivonen, 1990). The studies of Nayak *et al*. (1997) showed that *Ámophidinum cartrae* produced haemolysin more effectively in the presence of associated bacteria.

The screening of the associated bacteria for the production of haemolysin compounds was done inorder to investigate whether the haemolysis of the *Phaeocystis* sp. was supplemented by the haemolytic property of the associated bacteria. Among the tested strains the *Pseudomonas* strain showed haemolysis in blood agar. The filtrate of *Pseudomonas* was also found to be promoting the haemolytic activity of the *Phaeocystis* sp. In the presence *Pseudomonas*, the haemolytic property of the *Phaeocystis* sp. was increased two fold. In a similar study, the paralytic

shell fish toxin was isolated from the associated bacteria of dinoflagellates and the removal of bacteria from the culture of *Alexandrium catanella* caused the activity to diminish to about one fifth of that in non axenic cultures (Martins *et al*., 2003).

The number of associated bacteria in the *Phaeocystis* s. culture was analysed when the bacterial cell and filtrate was added to the algal culture to study the effect of bacterial cell number on the haemolysis exhibited by *Phaeocystis* sp. The addition of bacterial cells into the algal culture caused an increase in the number of respective bacterial groups added. The addition of bacterial filtrate did not cause much change in the bacterial distribution of the *Phaeocystis* sp. In the algal culture where nutrient broth was added, the total bacterial number was increased compared to the control, but the distribution pattern was not altered, with *Alcaligenes* II occurring in high number. The increase in the bacterial flora changed the haemolysis exhibited by *Phaeocystis* sp. also. The haemolysis was high in algal culture added with nutrient broth compared to the control culture.

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Chapter 6

SUMMARY AND CONCLUSIONS

The present study was mainly aimed at isolation of a bloom forming alga from Cochin estuary and to study the physical, chemical and biological parameters leading to algal blooms. Important findings of the study are summarised as follows:

- The spatial distribution of phytoplankton groups along the Cochin estuary was studied. The major groups identified belong to class Bacillariophyceae Chlorophyceae, Dinophyceae, Cyanophyceae and Xanthophyceae. The harmful algae such as *Dinophysis*, *Gymnodinium*, *Protoperidinium* and *Noctiluca* were also observed in the preserved natural algal sample. The major groups identified belong to class Bacillariophyceae.

- Clonal cultures of various microalgae including *Asterionella* sp., *Bacteriastrum* sp., *Biddulphia* sp., *Chaetoceros* sp., *Coscinodiscus* sp., *Gyrosigma* sp., *Navicula* sp., *Pleurosigma* sp., *Rhizosolenia* sp., *Skeletonema* sp., *Ceratium* sp., *Gymnodinium* sp., *Noctiluca* sp., *Protoperidinium* sp. and *Phaeocystis* sp. were developed under laboratory conditions.

- In the light of increased frequency of harmful algal blooms of novel organisms all over the world, the presence of *Phaeocystis* sp. in Cochin estuary had been taken into special consideration and the various aspects of the *Phaeocystis* sp. were studied in detail.

- The hydrographical conditions of the stations (Marine Sciences Jetty and Barmouth) from which the *Phaeocystis* sp. was isolated showed an average temperature of 30^0C, salinity 26ppt and alkaline pH. Nutrients mainly nitrate, nitrite, phosphate and silicate in the stations were found to be higher compared to other stations indicating the signs of eutrophication in these areas.

- *Phaeocystis* regularly forms blooms in the Oceans. It is believed to be entered into our ecosystem as a result of bioinvasion. The selected alga was identified as *Phaeocystis* sp. by observing the cell morphology under microscope and by studying its culture characteristics.

- The alga showed a complex life cycle with three types of cells, non-motile solitary cells, motile flagellated cells and colony cells. Sequential developments of single cell to colonies were observed in the culture with the advancement of incubation period.

- *Phaeocystis* sp. isolated showed close similarity to *Phaeocystis jahnii* mainly in colony structure without a definite colony wall. Moreover, the cells in the colony were not evenly distributed and it appeared in a cloud form with several packets of cells.

- *Phaeocystis* was found to be capable of growing over a wide range of temperature, salinity and pH showing its versatile nature. However, maximum growth of the *Phaeocystis* was obtained at salinity 30ppt, pH 8, and at temperature, 20^0C and 25^0C. This shows that the species could be well established in tropical waters and could form blooms.

- Walne's medium was found to be supporting maximum growth of *Phaeocystis* sp. and the growth was more influenced by the nitrate concentration than the phosphate concentration in the growth medium. High concentration of nutrients, nitrate (>1000µM), phosphate (>50µM) and iron (>5µM) in the medium could lead to blooming of this alga.

- Non-motile solitary cells were observed throughout the culture period with maximum occurrence during the initial and stationary growth period. Colonies started developing in the logarithmic growth phase, when the algal growth was maximum and towards the stationary phase, the colonies got disintegrated liberating the single cells from the colonies. Number of flagellated cells also increased towards the stationary phase.

- The colony formation in the *Phaeocystis* sp. was high in nutrient surplus medium compared to nutrient deficient medium. High concentrations of nitrate, phosphate, and iron were found to cause mass development and colonial growth in this alga.

- Protein content of the algal cell was high during the logarithmic phase than in the stationary phase, whereas, the carbohydrate content was higher in the stationary phase compared to logarithmic phase. No significant variation was observed in the total lipid content between the logarithmic and stationary phases.

- The extracellular release of proteins was higher in the logarithmic phase than in the stationary phase and the secretion of carbohydrates was more during the stationary phase.

- The exudates from *Phaeocystis* sp. have an allelopathic influence on other algal species and the availability of nutrients is an important factor for the regulation of allelopathic substances and allelochemicals are only produced/released under conditions of limited growth.

- The allelopathic effect of *Phaeocystis* sp. on three microalgae, *Chlorella marina, Chaetoceros calcitrans* and *Isochrysis galbana* was studied by using algal cell extract and culture filtrate. The effect was found to be varying from species to species. The culture filtrate of *Phaeocystis* sp. showed an inhibitory effect on the growth of *Chlorella marina* whereas, the growth of *Isochrysis galbana* was promoted by the cell extract and culture filtrate. Growth of *Chaetoceros calcitrans* was found to be unaffected by the *Phaeocystis* sp. cell extract and culture filtrate.

- The *Phaeocystis* sp. was found to be capable of exerting toxicity to animal cells also. It caused 20% to 30% mortality of *Artemia salina*. The toxicity was retained only for a short duration and within 48 hrs, the *Artemia salina* larvae regained the motility and no mortality occurred further.

- The alga expressed haemolytic property also. The cells growing exponentially showed higher haemolytic property than the cells in the lag phase and stationary phase. The haemolytic property was found to be dependent on cell dose and the nutrient status.

- Highest haemolysis was exhibited by algal cells grown in NP non limiting medium whereas, culture filtrate from the nutrient deficient algal culture exhibited higher haemolysis than the

culture filtrate of nutrient surplus culture. It shows that nutrient limitation cause a physiological stress on the algal cells to secrete the toxic principle into the media.

- The algae-bacteria association was found to be very specific and four groups of bacteria were isolated from the *Phaeocystis* sp.- *Alcaligenes, Pseudomonas, Flexibacter,* and *Flavobacterium. Alcaligenes* was present as two different species that varied morphologically in colour and colony size. The bacterial flora associated with *Phaeocystis* sp. was found to be influencing the growth and physiological property of alga.

- The bacteria were found to be very closely associated with the alga. The culture could not be made axenic by the application of antibiotics, and when high concentrations of antibiotics was used which completely eliminated the bacterial flora, the growth of algal cells were seriously affected.

- Regular succession was observed in the bacterial community along with the change in the growth phase of alga. *Alcaligenes* I was observed in higher number in the initial growth stage and by logarithmic growth phase the dominant flora were *Alcaligenes* II and *Flexibacter*. During the stationary phase, *Pseudomonas* was the predominant form. These bacteria would be playing a significant role in the initiation and termination of algal blooms. Bacterial biomass was high towards the logarithmic growth phase of alga than the lag phase and stationary phase

- The associated bacteria and the *Phaeocystis* sp. cells did not show any antibacterial activity towards the bacterial pathogens tested.

- Total bacterial consortium was important for the haemolytic property exhibited by *Phaeocystis* sp. *Flexibacter* and *Alcaligenes* II enhanced the haemolytic property of the alga by promoting good growth. Culture filtrates of *Pseudomonas* and *Flavobacterium* also enhanced the haemolytic activity of the alga. Among these bacteria, only *Pseudomona*s could produce haemolysis in blood agar.

In the present study, the important aspects that result in the bloom (mass growth) of *Phaeocystis* were studied under laboratory conditions. The study reveals the physiochemical conditions at which the alga blooms and the effect of nutrients on the colony formation of the alga. The role of associated bacteria in the growth of the alga was thoroughly analysed along with the allelopathic effect of *Phaeocystis* sp. on other microalgae in our ecosystem.

The study shows that algal blooms are regulated by the hydrographical parameters especially temperature, availability of nutrients such as nitrate and iron and the allelopathic property of alga that help the organism to compete over the co-occurring species. The associated bacterial flora also exerts a significant influence on the blooming of the alga by regulating bloom initiation, mass growth of the algae and termination of the bloom. However, further studies are required to ascertain the role of different bacterial species in algal blooming.

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Appendix

Table 3.1 Growth of *Phaeocystis* sp. at different salinity

Age of culture in Days	10ppt		20ppt		30ppt		40ppt	
	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chla (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})
0	$_{a}0.65 \pm 0.01^{A}$	000	$_{b}0.65 \pm 0.01^{A}$	000	$_{c}0.65 \pm 0.01^{A}$	000	$_{a}0.65 \pm 0.01^{A}$	000
3	$_{a}0.75 \pm 0.04^{A}$	0.047	$_{b}0.83 \pm 0.03^{A}$	0.081	$_{c}0.65 \pm 0.03^{A}$	000	$_{a}0.61 \pm 0.01^{A}$	000
6	$_{a}0.95 \pm 0.06^{B}$	0.063	$_{b}1.11 \pm 0.02^{B}$	0.089	$_{c}1.26 \pm 0.07^{B}$	0.11	$_{a}0.81 \pm 0.00^{B}$	0.036
9	$_{a}3.35 \pm 0.21^{F}$	0.182	$_{b}3.25 \pm 0.05^{F}$	0.178	$_{c}3.57 \pm 0.11^{F}$	0.18	$_{a}2.63 \pm 0.05^{F}$	0.155
12	$_{a}3.40 \pm 0.08^{G}$	0.143	$_{b}3.47 \pm 0.14^{G}$	0.139	$_{c}3.60 \pm 0.10^{G}$	0.14	$_{a}3.07 \pm 0.06^{G}$	0.126
15	$_{a}2.21 \pm 0.12^{E}$	0.081	$_{b}2.13 \pm 0.09^{E}$	0.080	$_{c}2.88 \pm 0.03^{E}$	0.10	$_{a}2.96 \pm 0.06^{E}$	0.101
18	$_{a}1.64 \pm 0.04^{D}$	0.051	$_{b}1.92 \pm 0.03^{D}$	0.060	$_{c}2.24 \pm 0.13^{D}$	0.07	$_{a}2.15 \pm 0.13^{D}$	0.066
21	$_{a}1.46 \pm 0.05^{C}$	0.038	$_{b}1.83 \pm 0.03^{C}$	0.050	$_{c}1.24 \pm 0.04^{C}$	0.03	$_{a}2.07 \pm 0.05^{C}$	0.055
24	$_{a}1.14 \pm 0.09^{B}$	0.022	$_{b}1.33 \pm 0.10^{B}$	0.030	$_{c}1.09 \pm 0.03^{B}$	0.02	$_{a}0.72 \pm 0.02^{B}$	0.004

Table 3.2 Growth of *Phaeocystis* sp. at different pH

Age of culture in Days	6		7		8		9	
	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})
0	$_{a}0.25 \pm 0.01^{A}$	0000	$_{b}0.25 \pm 0.01^{A}$	0000	$_{d}0.25 \pm 0.01^{A}$	0000	$_{c}0.25 \pm 0.01^{A}$	0000
3	$_{a}0.18 \pm 0.00^{A}$	0000	$_{b}0.26 \pm 0.04^{A}$	0.018	$_{d}0.33 \pm 0.01^{A}$	0.088	$_{c}0.31 \pm 0.01^{A}$	0.074
6	$_{a}0.60 \pm 0.01^{B}$	0.145	$_{b}1.04 \pm 0.06^{B}$	0.238	$_{d}1.23 \pm 0.01^{B}$	0.265	$_{c}1.14 \pm 0.01^{B}$	0.252
9	$_{a}0.74 \pm 0.04^{C}$	0.121	$_{b}1.41 \pm 0.05^{C}$	0.192	$_{d}1.64 \pm 0.06^{C}$	0.208	$_{c}1.58 \pm 0.02^{C}$	0.204
12	$_{a}0.60 \pm 0.00^{F}$	0.073	$_{b}2.52 \pm 0.19^{F}$	0.191	$_{d}3.45 \pm 0.03^{F}$	0.218	$_{c}2.07 \pm 0.03^{F}$	0.175
15	$_{a}0.34 \pm 0.00^{F}$	0.019	$_{b}1.35 \pm 0.03^{F}$	0.111	$_{d}4.34 \pm 0.05^{F}$	0.190	$_{c}2.70 \pm 0.02^{F}$	0.158
18	$_{a}0.03 \pm 0.00^{C}$	0000	$_{b}1.29 \pm 0.02^{C}$	0.090	$_{d}4.07 \pm 0.12^{C}$	0.154	$_{c}2.13 \pm 0.03^{C}$	0.118
21	$_{a}0.03 \pm 0.00^{D}$	0000	$_{b}1.14 \pm 0.02^{D}$	0.072	$_{d}3.03 \pm 0.02^{D}$	0.118	$_{c}2.03 \pm 0.02^{D}$	0.099
24	$_{a}0.01 \pm 0.00^{C}$	0000	$_{b}1.22 \pm 0.06^{C}$	0.066	$_{d}2.28 \pm 0.01^{C}$	0.091	$_{c}1.77 \pm 0.01^{C}$	0.081

Table 3.3 Growth of *Phaeocystis* sp. at different temperature

Age of culture in Days	10^0C		15^0C		20^0C		25^0C		30^0C	
	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})
0	$_{a}0.28 \pm 0.02^{A}$	0000	$_{c}0.28 \pm 0.02^{A}$	0000	$_{d}0.28 \pm 0.02^{A}$	0000	$_{d}0.28 \pm 0.02^{A}$	0000	$_{b}0.28 \pm 0.02^{A}$	0000
3	$_{a}0.12 \pm 0.00^{B}$	0000	$_{c}0.42 \pm 0.03^{B}$	0.135	$_{d}0.60 \pm 0.02^{B}$	0.254	$_{d}0.43 \pm 0.01^{B}$	0.142	$_{b}0.28 \pm 0.02^{B}$	0000
6	$_{a}0.08 \pm 0.03^{C}$	0000	$_{c}0.56 \pm 0.07^{C}$	0.067	$_{d}1.05 \pm 0.06^{C}$	0.220	$_{d}1.36 \pm 0.21^{C}$	0.263	$_{b}0.67 \pm 0.04^{C}$	0.145
9	$_{a}0.03 \pm 0.00^{D}$	0000	$_{c}0.63 \pm 0.02^{D}$	0.090	$_{d}1.35 \pm 0.06^{D}$	0.174	$_{d}2.11 \pm 0.12^{D}$	0.224	$_{b}0.18 \pm 0.02^{D}$	0000
12	$_{a}0.01 \pm 0.00^{E}$	0000	$_{c}0.66 \pm 0.05^{E}$	0.071	$_{d}1.46 \pm 0.05^{E}$	0.137	$_{d}2.50 \pm 0.09^{E}$	0.182	$_{b}0.07 \pm 0.03^{E}$	0000
15	$_{a}0.01 \pm 0.00^{E}$	0000	$_{c}0.72 \pm 0.03^{E}$	0.062	$_{d}2.50 \pm 0.04^{E}$	0.145	$_{d}1.47 \pm 0.07^{E}$	0.110	$_{b}0.02 \pm 0.01^{E}$	0000
18	$_{a}0.01 \pm 0.00^{D}$	0000	$_{c}1.10 \pm 0.01^{D}$	0.076	$_{d}2.11 \pm 0.11^{D}$	0.112	$_{d}1.31 \pm 0.10^{D}$	0.085	$_{b}0.01 \pm 0.00^{D}$	0000
21	$_{a}0.01 \pm 0.00^{B}$	0000	$_{c}0.73 \pm 0.07^{B}$	0.045	$_{d}0.80 \pm 0.03^{B}$	0.049	$_{d}0.55 \pm 0.04^{B}$	0.032	$_{b}0.01 \pm 0.00^{B}$	0000
24	$_{a}0.01 \pm 0.00^{A}$	0000	$_{c}0.68 \pm 0.01^{A}$	0.036	$_{d}0.48 \pm 0.03^{A}$	0.022	$_{d}0.36 \pm 0.01^{A}$	0.010	$_{b}0.01 \pm 0.00^{A}$	0000

Table 3.4 Growth of *Phaeocystis* sp. in different growth media

Age of culture in Days	Walne's		f/2		f/2-Si		f/4		f/20		f/50	
	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})
0	$_{f}0.08 \pm 0.00^{A}$	0000	$_{d}0.08 \pm 0.00^{A}$	0000	$_{e}0.08 \pm 0.00^{A}$	0000	$_{c}0.08 \pm 0.00^{A}$	0000	$_{b}0.08 \pm 0.00^{A}$	0000	$_{a}0.08 \pm 0.00^{A}$	0000
3	$_{f}0.14 \pm 0.02^{B}$	0.178	$_{d}0.12 \pm 0.02^{B}$	0.118	$_{e}0.14 \pm 0.01^{B}$	0.178	$_{c}0.13 \pm 0.01^{B}$	0.153	$_{b}0.13 \pm 0.01^{B}$	0.153	$_{a}0.13 \pm 0.02^{B}$	0.153
6	$_{f}0.25 \pm 0.03^{C}$	0.178	$_{d}0.18 \pm 0.01^{C}$	0.131	$_{e}0.19 \pm 0.01^{C}$	0.140	$_{c}0.19 \pm 0.02^{C}$	0.140	$_{b}0.23 \pm 0.01^{C}$	0.171	$_{a}0.09 \pm 0.00^{C}$	0000
9	$_{f}0.30 \pm 0.04^{C}$	0.143	$_{d}0.20 \pm 0.02^{C}$	0.099	$_{e}0.22 \pm 0.02^{C}$	0.109	$_{c}0.25 \pm 0.01^{C}$	0.123	$_{b}0.16 \pm 0.02^{C}$	0.074	$_{a}0.03 \pm 0.00^{C}$	0000
12	$_{f}0.38 \pm 0.04^{C}$	0.126	$_{d}0.25 \pm 0.03^{C}$	0.091	$_{e}0.25 \pm 0.03^{C}$	0.092	$_{c}0.28 \pm 0.01^{C}$	0.102	$_{b}0.04 \pm 0.02^{C}$	0000	$_{a}0.02 \pm 0.00^{C}$	0000
15	$_{f}0.45 \pm 0.03^{E}$	0.113	$_{d}0.47 \pm 0.03^{E}$	0.116	$_{e}0.42 \pm 0.01^{E}$	0.108	$_{c}0.30 \pm 0.01^{E}$	0.086	$_{b}0.03 \pm 0.03^{E}$	0000	$_{a}0.01 \pm 0.00^{E}$	0000
18	$_{f}0.66 \pm 0.06^{E}$	0.115	$_{d}0.44 \pm 0.04^{E}$	0.093	$_{e}0.43 \pm 0.02^{E}$	0.100	$_{c}0.20 \pm 0.01^{E}$	0.049	$_{b}0.02 \pm 0.00^{E}$	0000	$_{a}0.01 \pm 0.00^{E}$	0000
21	$_{f}0.63 \pm 0.03^{E}$	0.096	$_{d}0.42 \pm 0.04^{E}$	0.077	$_{e}0.50 \pm 0.02^{E}$	0.086	$_{c}0.12 \pm 0.02^{E}$	0.018	$_{b}0.01 \pm 0.00^{E}$	0000	$_{a}0.01 \pm 0.00^{E}$	0000
24	$_{f}0.62 \pm 0.03^{D}$	0.084	$_{d}0.23 \pm 0.01^{D}$	0.042	$_{e}0.34 \pm 0.02^{D}$	0.059	$_{c}0.07 \pm 0.02^{D}$	0000	$_{b}0.01 \pm 0.00^{D}$	0000	$_{a}0.01 \pm 0.00^{D}$	0000

Table 3.5 Growth of *Phaeocystis* sp. at different nitrate concentration

Age of culture in Days	2000µM		1500µM		1000µM		500µM		200µM		100µM	
	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})
0	$_{c}0.03 \pm 0.00^{A}$	0000	$_{d}0.02 \pm 0.00^{A}$	0000	$_{c}0.03 \pm 0.00^{A}$	0000	$_{b}0.03 \pm 0.00^{A}$	0000	$_{a}0.03 \pm 0.00^{A}$	0000	$_{a}0.03 \pm 0.00^{A}$	0000
3	$_{c}0.09 \pm 0.06^{A}$	0.366	$_{d}0.03 \pm 0.02^{A}$	0.135	$_{c}0.09 \pm 0.01^{A}$	0.366	$_{b}0.06 \pm 0.04^{A}$	0.231	$_{a}0.06 \pm 0.01^{A}$	0.231	$_{a}0.05 \pm 0.09^{A}$	0.17
6	$_{c}0.37 \pm 0.01^{A}$	0.418	$_{d}0.25 \pm 0.06^{A}$	0.353	$_{c}0.32 \pm 0.09^{A}$	0.394	$_{b}0.13 \pm 0.03^{A}$	0.244	$_{a}0.28 \pm 0.09^{A}$	0.372	$_{a}0.23 \pm 0.07^{A}$	0.339
9	$_{c}0.66 \pm 0.03^{C}$	0.343	$_{d}0.54 \pm 0.06^{C}$	0.321	$_{c}0.42 \pm 0.10^{C}$	0.293	$_{b}0.39 \pm 0.02^{C}$	0.284	$_{a}0.34 \pm 0.15^{C}$	0.269	$_{a}0.16 \pm 0.07^{C}$	0.185
12	$_{c}1.20 \pm 0.14^{EF}$	0.307	$_{d}1.00 \pm 0.46^{EF}$	0.292	$_{c}1.04 \pm 0.37^{EF}$	0.295	$_{b}1.08 \pm 0.30^{EF}$	0.298	$_{a}0.34 \pm 0.14^{EF}$	0.202	$_{a}0.12 \pm 0.03^{EF}$	0.115
15	$_{c}1.43 \pm 0.03^{F}$	0.257	$_{d}1.60 \pm 0.64^{F}$	0.265	$_{c}1.37 \pm 0.20^{F}$	0.254	$_{b}0.85 \pm 0.13^{F}$	0.222	$_{a}0.24 \pm 0.07^{F}$	0.138	$_{a}0.04 \pm 0.03^{F}$	0.019
18	$_{c}0.99 \pm 0.06^{DE}$	0.194	$_{d}1.51 \pm 0.19D^{E}$	0.217	$_{c}1.24 \pm 0.12^{DE}$	0.206	$_{b}0.53 \pm 0.06D^{E}$	0.159	$_{a}0.16 \pm 0.08D^{E}$	0.092	$_{a}0.03 \pm 0.03^{DE}$	0000
21	$_{c}0.70 \pm 0.04^{D}$	0.149	$_{d}1.46 \pm 0.19^{D}$	0.184	$_{c}0.95 \pm 0.80^{D}$	0.164	$_{b}0.45 \pm 0.04^{D}$	0.128	$_{a}0.15 \pm 0.03^{D}$	0.076	$_{a}0.02 \pm 0.01^{D}$	0000
24	$_{c}0.50 \pm 0.06^{C}$	0.117	$_{d}1.19 \pm 0.51^{C}$	0.153	$_{c}0.79 \pm 0.63^{C}$	0.136	$_{b}0.27 \pm 0.09^{C}$	0.091	$_{a}0.11 \pm 0.01^{C}$	0.054	$_{a}0.02 \pm 0.01^{C}$	0000

Table 3.6 Growth of *Phaeocystis* sp. at different phosphate concentration

Age of culture in Days	100µM		75µM		50µM		25µM		10µM		5µM	
	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})
0	$_{c}0.03 \pm 0.00^{A}$	0000	$_{c}0.03 \pm 0.00^{A}$	0000	$_{c}0.03 \pm 0.00^{A}$	0000	$_{b}0.03 \pm 0.00^{A}$	0000	$_{b}0.03 \pm 0.00^{A}$	0000	$_{a}0.03 \pm 0.00^{A}$	0000
3	$_{c}0.09 \pm 0.06^{A}$	0.366	$_{c}0.07 \pm 0.01^{A}$	0.282	$_{c}0.08 \pm 0.01^{A}$	0.326	$_{b}0.06 \pm 0.01^{A}$	0.231	$_{b}0.07 \pm 0.01^{A}$	0.282	$_{a}0.04 \pm 0.01^{A}$	0.095
6	$_{c}0.37 \pm 0.01^{B}$	0.418	$_{c}0.26 \pm 0.04^{B}$	0.359	$_{c}0.31 \pm 0.10^{B}$	0.389	$_{b}0.34 \pm 0.11^{B}$	0.404	$_{b}0.35 \pm 0.05^{B}$	0.409	$_{a}0.32 \pm 0.02^{B}$	0.394
9	$_{c}0.66 \pm 0.03^{D}$	0.343	$_{c}0.49 \pm 0.05^{D}$	0.310	$_{c}0.54 \pm 0.05^{D}$	0.321	$_{b}0.62 \pm 0.11^{D}$	0.336	$_{b}0.50 \pm 0.07^{D}$	0.312	$_{a}0.47 \pm 0.02^{D}$	0.305
12	$_{c}1.20 \pm 0.14^{G}$	0.307	$_{c}1.08 \pm 0.11^{G}$	0.298	$_{c}1.08 \pm 0.07^{G}$	0.298	$_{b}0.76 \pm 0.02^{G}$	0.269	$_{b}0.78 \pm 0.34^{G}$	0.271	$_{a}0.63 \pm 0.04^{G}$	0.253
15	$_{c}1.44 \pm 0.03^{H}$	0.257	$_{c}1.37 \pm 0.07^{H}$	0.254	$_{c}1.34 \pm 0.38^{H}$	0.253	$_{b}0.66 \pm 0.07^{H}$	0.206	$_{b}0.75 \pm 0.06^{H}$	0.214	$_{a}0.53 \pm 0.05^{H}$	0.191
18	$_{c}0.99 \pm 0.06^{F}$	0.194	$_{c}1.07 \pm 0.08^{F}$	0.198	$_{c}1.06 \pm 0.05^{F}$	0.198	$_{b}0.63 \pm 0.09^{F}$	0.169	$_{b}0.69 \pm 0.12^{F}$	0.174	$_{a}0.47 \pm 0.11^{F}$	0.152
21	$_{c}0.70 \pm 0.04^{E}$	0.149	$_{c}0.86 \pm 0.02^{E}$	0.159	$_{c}0.84 \pm 0.18^{E}$	0.158	$_{b}0.48 \pm 0.06^{E}$	0.132	$_{b}0.59 \pm 0.09^{E}$	0.141	$_{a}0.36 \pm 0.03^{E}$	0.118
24	$_{c}0.50 \pm 0.06^{C}$	0.117	$_{c}0.58 \pm 0.03^{C}$	0.123	$_{c}0.58 \pm 0.03^{C}$	0.123	$_{b}0.33 \pm 0.02^{C}$	0.099	$_{b}0.32 \pm 0.08^{C}$	0.098	$_{a}0.30 \pm 0.03^{C}$	0.095

Table 3.7 Growth of *Phaeocystis* sp. at different nutrient concentrations

Age of culture in Days	NP non-limiting		NP limiting		P limiting		N limiting	
	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})	Chl-a (µg/ml)	K' (day^{-1})
0	$_{c}0.03\pm0.00^{A}$	0000	$_{a}0.03\pm0.00^{A}$	0000	$_{b}0.03\pm0.00^{A}$	0000	$_{a}0.03\pm0.00^{A}$	0000
3	$_{c}0.09\pm0.06^{A}$	0.366	$_{a}0.05\pm0.03^{A}$	0.17	$_{b}0.04\pm0.01^{A}$	0.095	$_{a}0.05\pm0.04^{A}$	0.047
6	$_{c}0.37\pm0.01^{C}$	0.418	$_{a}0.16\pm0.01^{C}$	0.278	$_{b}0.32\pm0.02^{C}$	0.394	$_{a}0.23\pm0.04^{C}$	0.227
9	$_{c}0.66\pm0.03^{D}$	0.343	$_{a}0.12\pm0.02^{D}$	0.154	$_{b}0.47\pm0.02^{D}$	0.305	$_{a}0.16\pm0.07^{D}$	0.163
12	$_{c}1.20\pm0.14^{E}$	0.307	$_{a}0.09\pm0.01^{E}$	0.091	$_{b}0.63\pm0.04^{E}$	0.253	$_{a}0.12\pm0.03^{E}$	0.117
15	$_{c}1.44\pm0.03^{E}$	0.257	$_{a}0.04\pm0.01^{E}$	0.019	$_{b}0.53\pm0.05^{E}$	0.191	$_{a}0.04\pm0.03^{E}$	0.044
18	$_{c}0.99\pm0.06^{D}$	0.194	$_{a}0.04\pm0.01^{D}$	0.015	$_{b}0.47\pm0.11^{D}$	0.152	$_{a}0.03\pm0.03^{D}$	0.033
21	$_{c}0.70\pm0.04^{C}$	0.149	$_{a}0.02\pm0.00^{C}$	0000	$_{b}0.36\pm0.03^{C}$	0.118	$_{a}0.02\pm0.01^{C}$	0.019
24	$_{c}0.50\pm0.06^{B}$	0.117	$_{a}0.02\pm0.01^{B}$	0000	$_{b}0.30\pm0.03^{B}$	0.095	$_{a}0.02\pm0.01^{B}$	0.019

Table 3.8 Total carbohydrate content of the *Phaeocystis* sp.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	151.0017	1	151.0017	16.23383	0.015745	7.708647
Within Groups	37.20667	4	9.301667			
Total	188.2083	5				

Table 3.9 Total protein content of the *Phaeocystis* sp.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	374.46	1	374.46	41.45314	0.002994	7.708647
Within Groups	36.13333	4	9.033333			
Total	410.5933	5				

Table 3.10 Total lipid content of the *Phaeocystis* sp.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.426667	1	0.426667	0.046243	0.840254	7.708647
Within Groups	36.90667	4	9.226667			
Total	37.33333	5				

Table 3.11 Extracellular release of protein by the *Phaeocystis* sp.

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	11.53707	1	11.53707	6.566593	0.06247	7.708647
Within Groups	7.027733	4	1.756933			
Total	18.5648	5				

Table 3.12 Extracellular release of carbohydrates by the *Phaeocystis* sp.

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1.29735	1	1.29735	2.207316	0.211544	7.708647
Within Groups	2.351	4	0.58775			
Total	3.64835	5				

Table 3.14 Growth of *Phaeocystis* sp. at different concentration of iron

Age of culture in Days	10µM Chl-a (µg/ml)	5µM Chl-a (µg/ml)	2.5µM Chl-a (µg/ml)	0µM Chl-a (µg/ml)
0	$_{d}0.03 \pm 0.00^{A}$	$_{c}0.03 \pm 0.00^{A}$	$_{b}0.03 \pm 0.00^{A}$	$_{a}0.03 \pm 0.00^{A}$
3	$_{d}0.09 \pm 0.07^{A}$	$_{c}0.06 \pm 0.03^{A}$	$_{b}0.07 \pm 0.01^{A}$	$_{a}0.06 \pm 0.03^{A}$
6	$_{d}0.37 \pm 0.02^{C}$	$_{c}0.35 \pm 0.03^{C}$	$_{b}0.28 \pm 0.11^{C}$	$_{a}0.20 \pm 0.03^{C}$
9	$_{d}0.66 \pm 0.04^{D}$	$_{c}0.57 \pm 0.08^{D}$	$_{b}0.34 \pm 0.08^{D}$	$_{a}0.16 \pm 0.07^{D}$
12	$_{d}1.19 \pm 0.17^{F}$	$_{c}0.97 \pm 0.07^{F}$	$_{b}0.48 \pm 0.17^{F}$	$_{a}0.19 \pm 0.06^{F}$
15	$_{d}1.43 \pm 0.03^{F}$	$_{c}1.30 \pm 0.07^{F}$	$_{b}0.15 \pm 0.06^{F}$	$_{a}0.10 \pm 0.02^{F}$
18	$_{d}0.99 \pm 0.07^{E}$	$_{c}0.89 \pm 0.09^{E}$	$_{b}0.05 \pm 0.02^{E}$	$_{a}0.05 \pm 0.02^{E}$
21	$_{d}0.70 \pm 0.05^{C}$	$_{c}0.51 \pm 0.16^{C}$	$_{b}0.02 \pm 0.00^{C}$	$_{a}0.03 \pm 0.01^{C}$
24	$_{d}0.50 \pm 0.07^{B}$	$_{c}0.30 \pm 0.03^{B}$	$_{b}0.02 \pm 0.01^{B}$	$_{a}0.02 \pm 0.01^{B}$

Table 3.15 Effect of nutrients on the colony formation of *Phaeocystis* sp.

Age of culture in days	Different nutrient conditions							
	NP non limiting		NP limiting		P limiting		N limiting	
	Colony cells/ml	Solitary cells/ml	Colony cells/ml	Solitary cells/ml	Colony cells/ml	Solitary cells/ml	Colony cells/ml	Solitary cells/ml
3	9.00×10^{4}	9.60×10^{4}	0.00×10^{0}	1.17×10^{5}	1.50×10^{5}	9.67×10^{4}	0.00×10^{0}	7.67×10^{4}
6	4.80×10^{5}	1.30×10^{6}	1.20×10^{5}	7.40×10^{5}	4.80×10^{5}	1.78×10^{6}	1.50×10^{5}	7.70×10^{5}
9	2.60×10^{6}	2.30×10^{6}	3.20×10^{5}	2.01×10^{6}	2.30×10^{6}	3.92×10^{6}	2.70×10^{5}	1.02×10^{6}
12	3.60×10^{6}	2.20×10^{6}	2.60×10^{5}	2.12×10^{6}	3.20×10^{6}	6.20×10^{6}	2.50×10^{5}	2.00×10^{6}
15	7.00×10^{6}	2.50×10^{6}	1.70×10^{5}	2.66×10^{6}	4.30×10^{6}	5.60×10^{6}	9.00×10^{5}	1.80×10^{6}
18	1.60×10^{6}	2.80×10^{6}	0.00×10^{0}	1.64×10^{6}	1.40×10^{6}	5.30×10^{6}	1.30×10^{5}	1.61×10^{6}
21	1.30×10^{5}	2.97×10^{6}	0.00×10^{0}	9.43×10^{5}	9.00×10^{4}	3.60×10^{6}	0.00×10^{0}	2.09×10^{6}
24	1.10×10^{5}	1.47×10^{6}	0.00×10^{0}	6.83×10^{4}	8.00×10^{4}	2.30×10^{6}	0.00×10^{0}	1.24×10^{6}

Table 3.16 Effect of iron on the colony formation of *Phaeocystis* sp.

Age of culture in days	Different concentrations of Iron							
	10μM		5 μM		2.5μM		0μM	
	Solitary cells/ml	Colony cells/ml	Solitary cells/ml	Colony cells/ml	Solitary cells/ml	Colony cells/ml	Solitary cells/ml	Colony cells/ml
3	8.67×10^{5}	9.00×10^{4}	8.07×10^{5}	8.00×10^{4}	1.10×10^{6}	0.00×10^{0}	1.03×10^{6}	0.00×10^{0}
6	1.84×10^{6}	1.67×10^{5}	1.64×10^{6}	1.83×10^{5}	1.46×10^{6}	0.00×10^{0}	1.03×10^{6}	0.00×10^{0}
9	1.71×10^{6}	6.43×10^{5}	1.17×10^{6}	6.47×10^{5}	3.80×10^{6}	0.00×10^{0}	1.37×10^{6}	0.00×10^{0}
12	2.05×10^{6}	1.27×10^{6}	2.41×10^{6}	2.10×10^{6}	5.25×10^{6}	0.00×10^{0}	1.12×10^{6}	0.00×10^{0}
15	2.93×10^{6}	8.33×10^{6}	2.91×10^{6}	1.17×10^{6}	1.47×10^{6}	0.00×10^{0}	8.50×10^{5}	0.00×10^{0}
18	4.66×10^{6}	2.93×10^{5}	1.14×10^{6}	1.63×10^{5}	9.23×10^{5}	0.00×10^{0}	7.97×10^{5}	0.00×10^{0}
21	2.07×10^{6}	1.60×10^{5}	6.47×10^{5}	1.17×10^{5}	7.20×10^{5}	0.00×10^{0}	6.87×10^{5}	0.00×10^{0}
24	1.63×10^{6}	3.80×10^{5}	5.07×10^{5}	6.33×10^{4}	3.77×10^{5}	0.00×10^{0}	3.90×10^{5}	0.00×10^{0}

Table 4.2 Effect of cell extract of *Phaeocystis* sp. on the growth of *Chlorella marina*

Time	Chlorophyll-a (µg/ml)	
	Test	Control
0hr	0.012 ± 0.007	0.008 ± 0.00
24hr	0.014 ± 0.005	0.014 ± 0.006
48hr	0.041 ± 0.011	0.069 ± 0.006
72hr	0.076 ± 0.006	0.090 ± 0.002

Table 4.3 Allelopathic effect of cell extract of *Phaeocystis* sp. on *Chlorella marina* at 48hr

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2.99E-05	2	1.5E-05	0.100397	0.908763	19
Columns	0.001127	1	0.001127	7.564632	0.110676	18.51282
Error	0.000298	2	0.000149			
Total	0.001455	5				

Table 4.4 Effect of cell extract of *Phaeocystis* sp. on *Chlorella marina* at 72hr

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	3.1E-05	2	1.55E-05	0.508197	0.663043	19
Columns	0.000294	1	0.000294	9.639344	0.089962	18.51282
Error	0.000061	2	3.05E-05			
Total	0.000386	5				

Table 4.5 Effect of *Phaeocystis* sp. culture filtrate on the growth of *Chlorella marina*

Time	Chl-a (µg/ml) in different Nutrient condition				
	NP non limiting	NP limiting	P limiting	N limiting	Control
0hr	0.05 ± 0.006^{a}	0.04 ± 0.006^{a}	0.05 ± 0.009^{a}	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}
24hr	0.09 ± 0.007^{b}	0.06 ± 0.02^{ab}	0.04 ± 0.007^{a}	0.06 ± 0.01^{ab}	0.12 ± 0.03^{c}
48hr	0.13 ± 0.007^{b}	0.04 ± 0.005^{a}	0.02 ± 0.015^{a}	0.03 ± 0.002^{a}	0.31 ± 0.03^{c}
72hr	0.26 ± 0.001^{b}	0.19 ± 0.005^{a}	0.17 ± 0.001^{a}	0.15 ± 0.007^{a}	0.32 ± 0.02^{c}
96hr	0.39 ± 0.015^{b}	0.37 ± 0.002^{b}	0.36 ± 0.018^{ab}	0.38 ± 0.015^{a}	0.41 ± 0.02^{c}

Table 4.6 Nutrient concentration (µM) in the culture filtrates of *Phaeocystis* sp.

NP non limiting	NO^{3-} (µM)	1233 ± 0.06
	PO_4^{3-}(µM)	6.6 ± 0.011
NP limiting	NO^{3-} (µM)	4.5 ± 5.2
	PO_4^{3-}(µM)	0.65 ± 0.09
P limiting	NO^{3-} (µM)	1232 ± 0.09
	PO_4^{3-}(µM)	0.34 ± 0.04
N limiting	NO^{3-} (µM)	0.73 ± 0.01
	PO_4^{3-}(µM)	11 ± 0.07

Table 4.7 Effect of cell extract of *Phaeocystis* sp. on the growth of *Chaetoceros calcitrans*

Time	Chl-a (µg/ml)	
	Test	Control
0hr	0.10 ± 0.00	0.09 ± 0.007
24hr	0.30 ± 0.032	0.22 ± 0.013
48hr	0.49 ± 0.007	0.41 ± 0.005
72 hr	0.70 ± 0.02	0.70 ± 0.045

Table 4.8 Effect of *Phaeocystis* sp. culture filtrate on the growth of *Chaetoceros calcitrans*

Time	Chl-a (µg/ml) in different Nutrient condition				
	NP non limiting	NP limiting	P limiting	N limiting	Control
0hr	0.18 ± 0.06^{a}	0.19 ± 0.01^{a}	0.19 ± 0.022^{a}	0.19 ± 0.005^{a}	0.19 ± 0.003^{a}
24hr	0.25 ± 0.015^{a}	0.28 ± 0.021^{a}	0.29 ± 0.034^{a}	0.25 ± 0.022^{a}	0.28 ± 0.049^{a}
48hr	0.31 ± 0.004^{a}	0.26 ± 0.052^{a}	0.32 ± 0.005^{a}	0.32 ± 0.092^{a}	0.41 ± 0.026^{a}
72hr	0.51 ± 0.029^{b}	0.47 ± 0.026^{ab}	0.46 ± 0.037^{ab}	0.4 ± 0.054^{a}	0.60 ± 0.054^{b}
96hr	0.53 ± 0.025^{a}	0.61 ± 0.054^{a}	0.62 ± 0.037^{a}	0.60 ± 0.023^{a}	0.58 ± 0.041^{a}

Table 4.9 Effect of *Phaeocystis* sp. cell extract on the growth of *Isochrysis galbana*

Time	Chl-a (µg/ml)	
	Test	Control
0hr	0.23 ± 0.016	0.21 ± 0.030
24hr	0.46 ± 0.025	0.36 ± 0.025
48hr	0.82 ± 0.004	0.63 ± 0.026
72hr	1.25 ± 0.080	0.82 ± 0.088

Table 4.10 Effect of cell extract of *Phaeocystis* sp. on *Isochrysis galbana* at 48hr

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	0.000941	2	0.00047	1.807916	0.356136	19
Columns	0.05462	1	0.05462	209.9263	0.00473	18.51282
Error	0.00052	2	0.00026			
Total	0.056081	5				

Table 4.11 Effect of cell extract of *Phaeocystis* sp. on *Isochrysis galbana* at 72hr

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	0.018633	2	0.009317	1.820847	0.354503	19
Columns	0.273067	1	0.273067	53.36808	0.018227	18.51282
Error	0.010233	2	0.005117			
Total	0.301933	5				

Table 4.12 Effect of *Phaeocystis* sp. culture filtrate on the growth of *Isochrysis galbana*

Time	Chl-a (µg/ml) in different Nutrient condition				
	NP non limiting	NP limiting	P limiting	N limiting	Control
0hr	0.10 ± 0.009^{a}	0.12 ± 0.054^{a}	0.12 ± 0.028^{a}	0.12 ± 0.011^{a}	0.13 ± 0.098^{a}
24hr	0.34 ± 0.010^{b}	0.28 ± 0.023^{b}	0.34 ± 0.021^{b}	0.28 ± 0.031^{b}	0.22 ± 0.098^{a}
48hr	0.53 ± 0.027^{b}	0.47 ± 0.010^{ab}	0.53 ± 0.014^{b}	0.46 ± 0.029^{ab}	0.4 ± 0.023^{a}
72hr	0.78 ± 0.032^{c}	0.67 ± 0.028^{b}	0.78 ± 0.012^{c}	0.77 ± 0.054^{c}	0.61 ± 0.00^{a}
96hr	0.89 ± 0.028^{c}	0.79 ± 0.067^{ab}	0.83 ± 0.048^{abc}	0.86 ± 0.031^{bc}	0.78 ± 0.018^{a}

Table 4.14 Effect of different nutrient conditions on the haemolytic property of *Phaeocystis* sp.

Nutrient condition	Haemolysis % by different samples			
	Whole sample	Filtrate	water extract	Chloroform: methanol extract
NP non limiting	$_{c}70.0 \pm 1.50^{B}$	$_{b}20 \pm 2.0^{B}$	$_{a}1.30 \pm 0.25^{B}$	$_{a}1.0 \pm 0.20^{B}$
NP limiting	$_{c}36.0 \pm 3.05^{A}$	$_{b}19 \pm 1.5^{A}$	$_{a}0.35 \pm 0.05^{A}$	$_{a}0.0 \pm 0.00^{A}$
P limiting	$_{c}65.35 \pm 2.6^{C}$	$_{b}51 \pm 1.3^{C}$	$_{a}0.70 \pm 0.07^{C}$	$_{a}0.5 \pm 0.06^{C}$
N limiting	$_{c}56.0 \pm 3.05^{B}$	$_{b}37 \pm 1.5^{B}$	$_{a}0.70 \pm 0.07^{B}$	$_{a}0.0 \pm 0.00^{B}$

Table 4.15 Haemolysis during different growth phases of *Phaeocystis* sp.

Growth phases	Haemolysis %
Lag phase	19.45 ± 1.5^{a}
Log phase	79.73 ± 1.6^{c}
Stationary phase	42.00 ± 0.5^{b}

Table 4.16 Effect of cell concentration on the haemolytic property of *Phaeocystis* sp.

Phaeocystis Cells/ml.	Haemolysis %
3.9×10^{5}	19.36 ± 1.53
1.48×10^{6}	54.85 ± 0.58
1.52×10^{6}	61.20 ± 1.73
2.11×10^{6}	75.40 ± 1.15
2.57×10^{6}	75.85 ± 1.04
2.75×10^{6}	75.30 ± 1.61
3.4×10^{6}	77.00 ± 1.76
4.7×10^{6}	78.30 ± 0.58
5.8×10^{6}	78.45 ± 2.52
6.5×10^{6}	80.30 ± 0.58

Table 5.1 Associated bacterial number and growth at different growth phase of *Phaeocystis* sp.

Age of culture in days	Bacterial cells no/ml	Chl-a (µg/ml)
0	1.2×10^{4a}	0.024 ± 0.000
5	3.1×10^{5ab}	0.094 ± 0.078
10	6.3×10^{5c}	0.258 ± 0.197
15	2.3×10^{6bc}	0.867 ± 0.541
20	1.7×10^{6bc}	0.643 ± 0.377
25	1.1×10^{6abc}	0.092 ± 0.204

Table 5.2 Bacterial groups associated with *Phaeocystis* sp. at different growth period

Age of culture in days	Associated bacterial groups				
	Alcaligenes I **(Cells/ml)**	***Flexibacter*** **(Cells/ml)**	***Pseudomonas*** **(Cells/ml)**	***Flavobacterium*** **(Cells/ml)**	***Alcaligenes II*** **(Cells/ml)**
0	1.06×10^4	3×10^3	0.00	0.00	0.00
5	1.20×10^5	1.01×10^5	4.0×10^3	1.80×10^3	3.20×10^4
10	1.50×10^5	1.24×10^5	1.3×10^4	1.40×10^5	2.36×10^5
15	1.10×10^5	2.83×10^5	3.2×10^4	2.64×10^5	1.42×10^6
20	1.05×10^4	1.11×10^5	4.1×10^5	3.20×10^5	7.60×10^5
25	3.66×10^3	3.00×10^4	6.9×10^5	3.20×10^4	3.60×10^5

Table 5.3 Effect of associated bacteria on the growth of *Phaeocystis* sp.

Age of culture in days	Chl-a (µg/ml) of ***Phaeocystis*** in the presence of associated bacterial groups					
	Alcaligenes I	***Flexibacter***	***Pseudomonas***	***Flavobacterium***	***Alcaligenes II***	**Control**
0	$_a0.02\pm0.001^A$	$_c0.025\pm0.01^A$	$_d0.025\pm0.000^A$	$_b0.025\pm0.00^A$	$_b0.026\pm0.010^A$	$_c0.025\pm0.00^A$
3	$_a0.02\pm0.001^A$	$_c0.097\pm0.02^A$	$_d0.033\pm0.005^A$	$_b0.034\pm0.002^A$	$_b0.060\pm0.020^A$	$_c0.067\pm0.02^A$
6	$_a0.045\pm0.01^B$	$_c0.673\pm0.22^B$	$_d0.197\pm0.080^B$	$_b0.213\pm0.032^B$	$_b0.377\pm0.035^B$	$_c0.243\pm0.015^B$
9	$_a0.127\pm0.04^C$	$_c1.230\pm0.25^C$	$_d0.807\pm0.025^C$	$_b0.820\pm0.040^C$	$_b1.020\pm0.107^C$	$_c0.813\pm0.096^C$
12	$_a0.683\pm0.15^D$	$_c3.383\pm0.10^D$	$_d2.603\pm0.295^D$	$_b2.333\pm0.493^D$	$_b2.767\pm0.152^D$	$_c2.933\pm0.152^D$
15	$_a2.133\pm0.15^F$	$_c3.600\pm0.26^F$	$_d3.033\pm0.152^F$	$_b3.200\pm0.208^F$	$_b3.533\pm0.251^F$	$_c3.467\pm1.380^F$
18	$_a3.133\pm0.20^G$	$_c2.600\pm0.40^G$	$_d3.233\pm0.208^G$	$_b3.233\pm0.115^G$	$_b3.300\pm0.264^G$	$_c3.100\pm0.10^G$
21	$_a3.533\pm0.30^H$	$_c1.497\pm1.13^H$	$_d2.967\pm0.152^H$	$_b2.733\pm0.602^H$	$_b1.693\pm0.251^H$	$_c1.967\pm0.152^H$
24	$_a2.400\pm.435^E$	$_c0.087\pm0.03^E$	$_d1.767\pm0.416^E$	$_b1.633\pm0.050^E$	$_b0.157\pm0.030^E$	$_c0.133\pm0.051^E$

Table 5.4 Effect of bacterial cell on the haemolytic property of *Phaeocystis* sp.

Associated bacterial groups	Haemolysis %
Alcaligenes I	61.6 ± 2.89^a
Flexibacter	80.3 ± 1.53^c
Pseudomonas	60.3 ± 1.53^a
Flavobacterium	63.0 ± 2.89^a
Alcaligenes II	73.3 ± 2.89^b
Nutrient broth	75.6 ± 2.08^b
Control	65.0 ± 3.00^a

Table 5.5 Effect of bacterial filtrate on the haemolytic property of *Phaeocystis* sp.

Associated bacterial groups	Haemolysis %
Alcaligenes I	62.3±2.51[a]
Flexibacter	61.0±3.60[a]
Pseudomonas	85.6±2.51[cd]
Flavobacterium	89.3±2.08[d]
Alcaligenes II	84.0±2.00[c]
Nutrient broth	75.0±1.00[b]
Control	64.3±2.08[a]

Table 5.6 Growth of *Phaeocystis* culture inoculated with bacterial cell

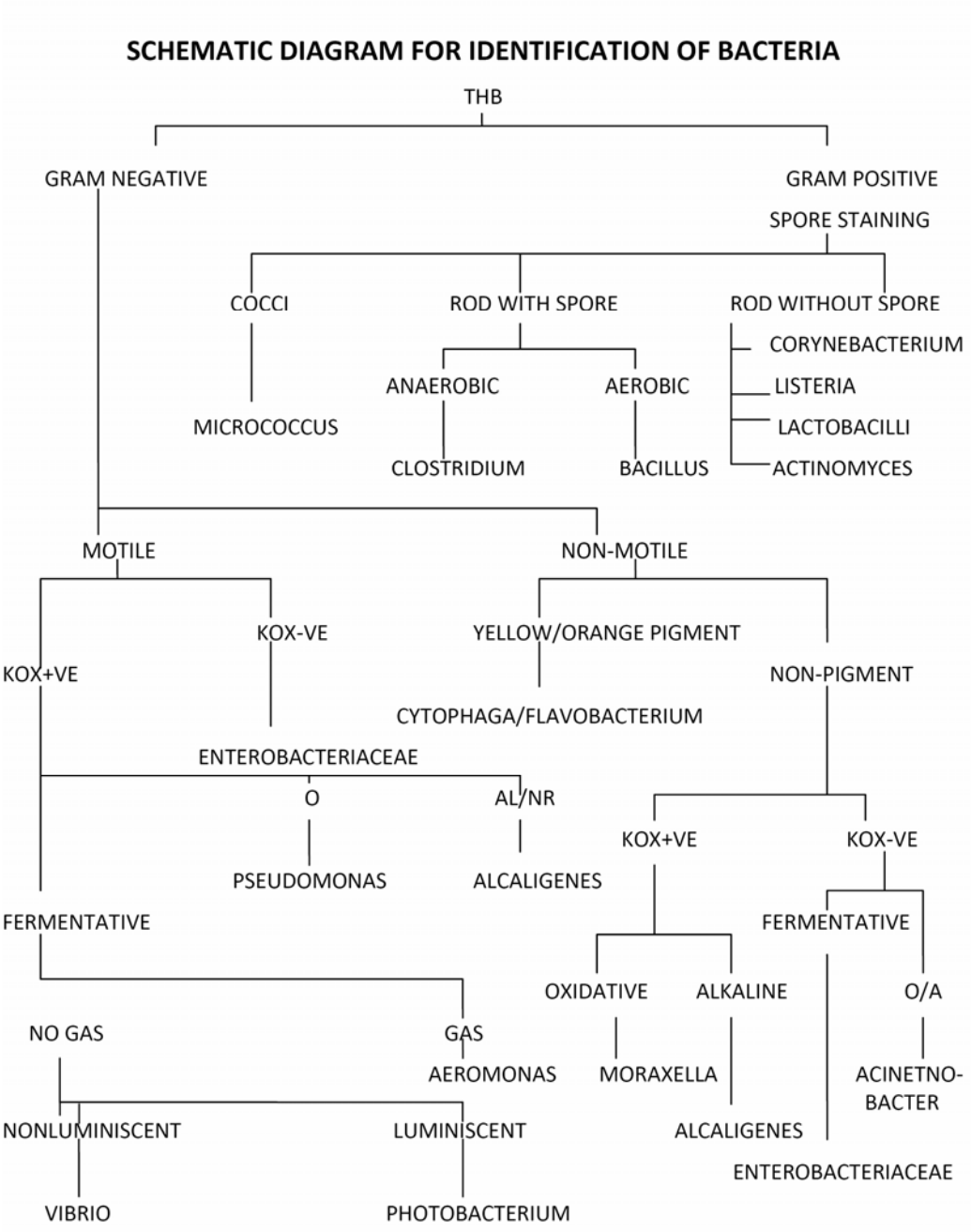
Associated bacterial groups	Chl-a (µg/ml)
Alcaligenes I	2.5 ± 0.12[a]
Alcaligenes II	3.7 ± 0.20[c]
Flexibacter	4.03 ± 0.61[a]
Flavobacterium	3.0 ± 0.42[a]
Pseudomonas	3.0 ± 0.26[bc]
Control	3.9 ± 0.21[b]
Nutrient broth	3.5 ± 0.13[a]

Table 5.7 Growth of *Phaeocystis* culture inoculated with bacterial filtrate

Associated bacterial groups	Chl-a (µg/ml)
Alcaligenes I	2.90 ± 0.15[a]
Alcaligenes II	3.00 ± 0.50[a]
Flexibacter	2.80 ± 0.67[a]
Flavobacterium	2.90 ± 0.53[a]
Pseudomonas	2.87 ± 0.71[a]
Control	3.00 ± 0.56[a]
Nutrient broth	2.80 ± 0.25[a]

Table 5.8 Effect of individual bacterial strains on the associated bacterial flora of the *Phaeocystis* sp.

Associated bacterial groups	Distribution of bacterial groups in algal culture inoculated with individual bacterial strains												Nutrient broth
	Alcaligenes I		*Pseudomonas*		*Flexibacter*		*Flavobacterium*		*Alcaligenes II*		Control		
	Cell	Filtrate	Cell	Filtrate	Cell	Filtrate	Cell	Filtrate	Cell	Filtrate			
Alcaligenes I	5.5×10^{6}	4.2×10^{6}	1.2×10^{5}	1.0×10^{5}	2.6×10^{5}	2.2×10^{6}	1.0×10^{5}	1.2×10^{6}	2.2×10^{5}	1.8×10^{6}	2.2×10^{5}	3.2×10^{6}	
Flexibacter	2.4×10^{6}	2.0×10^{5}	6.0×10^{5}	2.0×10^{5}	4.6×10^{5}	2.9×10^{6}	3.2×10^{6}	2.6×10^{6}	4.4×10^{5}	4.0×10^{5}	2.9×10^{5}	3.5×10^{6}	
Pseudomonas	3.0×10^{4}	2.0×10^{4}	4.0×10^{5}	6.0×10^{4}	3.5×10^{4}	2.0×10^{4}	4.0×10^{4}	4.0×10^{4}	4.0×10^{5}	3.9×10^{5}	3.2×10^{4}	3.0×10^{4}	
Flavobacterium	2.0×10^{5}	4.2×10^{6}	3.4×10^{4}	3.0×10^{5}	3.2×10^{5}	8.0×10^{4}	4.3×10^{6}	1.1×10^{6}	1.2×10^{6}	3.2×10^{6}	2.5×10^{5}	3.2×10^{6}	
Alcaligenes II	4.2×10^{6}	3.3×10^{7}	1.2×10^{6}	1.3×10^{7}	5.3×10^{5}	1.2×10^{7}	3.2×10^{6}	1.5×10^{7}	2.6×10^{6}	2.0×10^{6}	1.4×10^{6}	1.5×10^{7}	


SCHEMATIC DIAGRAM FOR IDENTIFICATION OF BACTERIA
THB
GRAM NEGATIVE
GRAM POSITIVE
SPORE STAINING
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ROD WITH SPORE
ROD WITHOUT SPORE
CORYNEBACTERIUM
ANAEROBIC
AEROBIC
LISTERIA
MICROCOCCUS
LACTOBACILLI
CLOSTRIDIUM
BACILLUS
ACTINOMYCES
MOTILE
NON-MOTILE
KOX-VE
YELLOW/ORANGE PIGMENT
KOX+VE
NON-PIGMENT
CYTOPHAGA/FLAVOBACTERIUM
ENTEROBACTERIACEAE
O
AL/NR
KOX+VE
KOX-VE
PSEUDOMONAS
ALCALIGENES
FERMENTATIVE
FERMENTATIVE
OXIDATIVE
ALKALINE
O/A
NO GAS
GAS
AEROMONAS
MORAXELLA
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