Chapter 3

CULTURE CHARACTERISTICS OF PHAEOCYSTIS SPECIES

## 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, CO₂, 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 used 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°C (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°C and 25°C, lipids and carbohydrates were higher than at 30°C. Protein was not significantly affected by the temperature, but a tendency for lower values was observed at 25°C (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.
Culture Characteristics of Phaeocystis Species

(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°C and reported the growth of culture at temperature of even 27°C. 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°C. 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 CO₂ 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 pouchehtii, 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 microzooplankton 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 phaeocystis pouchehtii in mesocosm in western Norway. In both
environments, mass development of the small needle shaped diatom, *Pseudonitzchia* species, occurred on *Phaeocystis* colonies 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, (Kamermans, 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$^{-3}$ occurred 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°C (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°C (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°C 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°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°C 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.

\[
\text{Chlorophyll-a (µg/ml)} = (Ca \times \nu) / V \times 1
\]

Where:

\( \nu \) = Volume of acetone

\( V \) = volume of water sample filtered (L)

\( l \) = 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°C, 15°C, 20°C, 25°C and 30°C. 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°C, 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$_2$SiO$_3$. 9H$_2$O 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°C, 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).

\[
K'(\text{day}^{-1}) = \frac{\ln \left( \frac{N_i}{N_0} \right)}{t_1 - t_0}
\]

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 15th day and 30th 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°C 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°C 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°C 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 15th day and 30th 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 ($10^7$ cells/ml) and incubated in the environmental chamber for a period of 24 days.

**Composition of Artificial Seawater**

<table>
<thead>
<tr>
<th>Anhydrous salts</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>24.54</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>4.09</td>
</tr>
<tr>
<td>KCl</td>
<td>0.7</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.2</td>
</tr>
<tr>
<td>KBr</td>
<td>0.1</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.003</td>
</tr>
<tr>
<td>NaF</td>
<td>0.003</td>
</tr>
<tr>
<td>Hydrous salts</td>
<td></td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>11.1</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>1.54</td>
</tr>
<tr>
<td>SrCl₂.6H₂O</td>
<td>0.017</td>
</tr>
</tbody>
</table>

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.
3.3.1 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 (3.6 µg/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 3.4 µg/ml on 12th day. Highest chlorophyll-a concentration was 3.47 µg/ml at 20 ppt. At 40ppt, maximum chlorophyll-a concentration was 3.07 µg/ml. Growth of Phaeocystis sp. in different salinity varied significantly ($p<0.05$) between 20ppt and 30ppt.

![Figure 3.2](image)

**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).
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 12\textsuperscript{th} day and there was no growth from 15\textsuperscript{th} day onwards. Cells at pH 8 and 9 remained in exponential phase till 15\textsuperscript{th} day whereas at pH 7 the culture started declining from 5\textsuperscript{th} 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).
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°C, 15°C, 20°C, 25°C and 30°C. *Phaeocystis* sp. showed maximum growth at 25°C on 12th day and at 20°C on 15th day. No growth or negligible growth was observed at 10°C. At 15°C, growth of *Phaeocystis* sp.
seems to be almost steady from 3\textsuperscript{rd} day to 24\textsuperscript{th} day, with maximum growth on 18\textsuperscript{th} day of experiment. At 25\textdegree C, maximum growth was on 12\textsuperscript{th} day, after that chlorophyll-a concentration decreased drastically from 15\textsuperscript{th} day on wards. The turnover rate was high at 25\textdegree C for \textit{Phaeocystis} sp. Growth was observed at 30\textdegree C till 6\textsuperscript{th} 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\textdegree C (0.263 day\textsuperscript{-1}). The maximum specific growth rate at 20\textdegree C was 0.254 day\textsuperscript{-1} and at 15\textdegree C was 0.135 day\textsuperscript{-1}. At 30\textdegree C, maximum specific growth rate was 0.145 day\textsuperscript{-1} (Fig 3.7 and Table 3.3 of Appendix).

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

![Figure 3.6 Effect of temperature on the growth of Phaeocystis sp.](image)
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 0.66 µg/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 (0.23 µg/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 (0.178 day⁻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 \textit{Phaeocystis} sp.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.8}
\caption{Effect of different growth media on the growth of \textit{Phaeocystis} sp.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.9}
\caption{Specific growth rate of \textit{Phaeocystis} sp. in different growth media}
\end{figure}
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 6th day the growth was almost similar in all media. By 9th 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 6th 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.
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 $9 \times 10^4$ 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 $10.5 \times 10^5$ and $2.9 \times 10^5$ 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 ($1.6 \times 10^6$ cells/ml) over the number of solitary cells ($14.4 \times 10^5$ 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 $20.8 \times 10^5$ cells/ml and $2.5 \times 10^6$ cells/ml respectively. The average size of the solitary cells was found to be 8µm in diameter. The maximum number ($8 \times 10^6$ 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 ($3.52 \times 10^6$ cells/ml)
increased over the number of colony cells \((1.6 \times 10^6 \text{ cells/ml})\). Motile cells were also present in the growth medium. The size of the colony was 86.6\(\mu\)m 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 40.6\(\mu\)m in diameter. The size of the solitary cells also decreased from 7.2\(\mu\)m to 3.2\(\mu\)m 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 19.5\(\mu\)m to 33.3\(\mu\)m and the number of colony cells were found to be \(1 \times 10^5 \text{ cells/ml}\), whereas, the number of solitary cells was about \(2.4 \times 10^6 \text{ cells/ml}\). (Figure 3.16).

![Figure 3.16 Number of colony cells and solitary cells of Phaeocystis sp. in Walne’s medium.](image-url)
Plate 1 *Phaeocystis* sp. on the 3\textsuperscript{rd} day of growth

Plate 2 *Phaeocystis* sp. on the 6\textsuperscript{th} 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
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
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 24th 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 24\textsuperscript{th} 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](image1)

![Figure 3.22 Number of colony cells and solitary cells in NP limiting medium](image2)
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

<table>
<thead>
<tr>
<th>Time period</th>
<th>4th day</th>
<th>8th day</th>
<th>12th day</th>
<th>16th day</th>
<th>20th day</th>
<th>25th day</th>
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<tbody>
<tr>
<td>Nitrate (µM)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>NP non-limiting</td>
<td>1843±40</td>
<td>1585±30.4</td>
<td>1233±57.8</td>
<td>1042±79.1</td>
<td>605.3±22.4</td>
<td>271.3±25.8</td>
</tr>
<tr>
<td>Phosphate (µM)</td>
<td>34±4</td>
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<td>6.6±3.05</td>
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<td>0.82±0.46</td>
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<td>Nitrate (µM)</td>
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<tr>
<td>NP limiting</td>
<td>92.3±2.5</td>
<td>80.3±5.5</td>
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<td>Phosphate (µM)</td>
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<tr>
<td>P limiting</td>
<td>1843±106</td>
<td>1486±80.3</td>
<td>1152±131</td>
<td>838±53.9</td>
<td>716±28.8</td>
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<tr>
<td>N limiting</td>
<td>48.3±2.08</td>
<td>18±1.7</td>
<td>8.2±1.5</td>
<td>4.2±0.5</td>
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<td>0.73±0.05</td>
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<tr>
<td>Phosphate (µM)</td>
<td>58±2.5</td>
<td>27±1.5</td>
<td>14.6±0.58</td>
<td>12.6±0.5</td>
<td>11.6±0.55</td>
<td>10.03±1.05</td>
</tr>
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</table>

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 2.5\(\mu\)M

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°C to 34.52°C with minimum and maximum values in January and May. Sea surface temperature varied from 23.3°C to 31°C. 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°C and the strain was found to be able to grow at temperature ranging from 15 to 30°C with maximum growth at 20 and 25°C. 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°C. At 25°C, maximum growth was obtained on 12th 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°C for Phaeocystis globosa (Jahnke, 1989), whereas the Phaeocystis pouchetii could grow even at temperature 1.5°C (Verity et al., 1988). Many scientists could successfully culture Phaeocystis species at temperatures ranging from 20 to 27°C (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 throughout the growth period of 24 days 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 6th 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 re-appearance 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 swarmer 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
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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 limiting culture. But when the phosphate alone was limiting 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 limiting 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 limiting 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 swarmers 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).