PHYSICAL CHARACTERISTICS OF *PLEUROCHRYSIS CARTERAE* IN RELATION TO HARVESTING POTENTIAL FOR BIODIESEL PRODUCTION

By

Marjan Rahbari

School of Chemical Engineering
The University of Adelaide

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Declaration

This work contains no materials which have been accepted for the award of any other degrees or diploma in any university or other tertiary institution, to the best of my knowledge and belief and contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent for this copy of my thesis to be made available in the University Library.

Marjan Rahbari

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Abstract

During the last three decades, microalgae has been suggested as a potential source of biofuel, with a number of advantages over other energy crops. These advantages, including high lipid content, fast growth rate, reduced requirements for land and the possibility of utilizing marine water systems constitute the most likely sources for CO_2 reduction systems. However, no commercial plant has been established to date. This is because of the high capital cost of microalgal systems and the many uncertain aspects of harvesting small cells (<20µm) from dilute cultures (~1g/l). Therefore, this project aims to investigate the physical characteristics of *Pleurochrysis carterae*, a potential biofuel feedstock, with a view to understanding the fundamental characteristics of this species during large-scale harvesting.

The preliminary study of cell growth cycle in the laboratory showed that; *P. carterae* grows quickly in BG11 media with 1.8% salinity. The maximum cell concentration after 20 days was ~0.5 g/l. Also, large flocs (120µm) were observed early in the growth phase (day 4) and the stationary phase (day20).

In order to calculate the theoretical settling rate some parameters, such as cell size distribution and medium viscosity, were measured. The results indicated that cell size grew from 3.8µm at the inoculation to 11.7 µm at the end of the growth cycle. Also, the viscosity of the suspension was independent of cell concentration and is identical to that of seawater (0.001Pa.s). The theoretical settling rate, based on Stokes' law yielded, 1.4×10^{-6} m/s. Screen tests from column settling tests showed that the terminal selling rate of *P. carterae* cells is 4×10^{-4} m/s, which is faster than the predicted rate. It was estimated that 91% of cells, without any pre-treatment, and during 12 hour experiments, settled to the bottom of the columns. It shows flocculation is a potential method for a low cost harvesting in large scale biomass production.

The effect of shear stress on cell viability after centrifugation with various gravity forces was also assessed. The results showed that the number of broken cells

increased at higher shear stress and that maximum cell disruption occurred after harvesting with 11000 × g force.

Results from laboratory in this study indicate that further optimisation need to be done to increase *P. carterae* harvesting efficiency toward the minimum cost, thus achieve economic biodiesel product.

P. carterae is a potential candidate for commercial scale biofuel production with significant advantages including high growth rate, low contamination risk and fast settling velocity. The use of a primary flocculation stage with secondary continuous centrifugation is potentially suitable for low cost commercial harvesting. However, still more work needs to be done to demonstrate the feasibility of large-scale production of biofuel from microalgae.

Chapter 1: Introduction

1.1 Background

Biofuel production from large-scale cultivation of microalgae has been widely regarded as one of the most promising systems that has potential to deal with the problem of global warming (Benemann & Oswald, 1996; Chisti, 2007). The average temperature of the earth, over the last century has increased by approximately 0.76^{o}C and even further warming of about 0.2^{o}C per decade has been predicted for the future. These increases in temperature are attributed to increasing concentrations of CO_2 in the atmosphere. Human activities , particularly fossil fuel use, are the primary causes of increasing atmospheric concentrations of carbon dioxide and thus of global warming (IPCC, 2007). The United Nations Framework Convention on Climate Change (UNFCCC), agreed to by 173 Countries in Kyoto in 1997, required 35 countries to decrease their green house gas (GHG) emissions to determined levels. The individual targets for parties (listed in Kyoto Protocol Annex B) include a total reduction of GHG emissions of at least 5% from the 1990 level in the period 2008 to 2012. In this regard, a great deal of attention has been focused on sustainable renewable sources of energy as one of the ways to reduce green house gases thus the ultimate objective of the UNFCCC is to reduce CO_2 emission, which account the majority of GHG emissions (UNFCCC, 1997).

Different methods, including chemical or physical absorption, membrane processes and biofixation have been proposed for removing and separating carbon dioxide (Benemann, 1993; Kojima, 1995; Pennline *et al.*, 2006; Zhang, 2006). Among these methods, biological systems are a potential alternative to CO_2 fixation. Biological methods enable photosynthetic organisms to capture CO_2, utilize solar energy and act as a reservoir of carbon. Biomass also can be converted to renewable fuels, such as biodiesel, and this will reduce demand for fossil fuels, thus leading to significantly lower CO_2 emissions (Hall & House, 1993).

Animal oil, food crops, and waste cooking oils are the main feedstocks for biodiesel production. These feedstocks are unable to supply large quantities of biodiesel due to their slow growth rate, and the limited availability of freshwater and arable land (Chisti, 2007). Aquaculture resources, particularly marine microalgae, have been recognised as promising alternative feedstocks with a number of advantages including fast growth, high lipid content and higher productivity compared to other crops (Chelf *et al.*, 1993).

Despite this promise, the large-scale production of biofuel from microalgae still has many fundamental limitations. Even though microalgae has significantly higher areal productivities than other plants, commercial production would still require extremely large areas, perhaps >100km^2 per site. Microalgae also have very small cell sizes (in the order of 10 μm) and low concentration (~1g/l). Therefore, any harvesting process is difficult and costly and thus the separation process is typically an important factor in the overall cost of production. Prior harvesting cost analysis has calculated that approximately 20%-30% of the total microalgal biomass production cost should be credited to its harvesting process (Gudin & Thepenier, 1986). The price of algal oil has been estimated at $1.4-$2.8/l which is not competitive with mineral diesel at $0.49/l – $0.79/l (Chisti, 2007). Currently, there are no suitable harvesting methods that can achieve the necessary economic and performance criteria. Therefore, more studies need to be done to investigate economically-feasible harvesting methods for the biofuel production system for the individual types of the algal species.

Successful harvesting techniques are highly dependent on the properties of media and algal species. In particular, properties such as settling rate, culture viscosity and cell concentration as well as the effects of the harvesting process in damaging cells are all important factors for harvesting design and downstream processing (Charles & Ambler, 1979; Svarovsky, 1985; Coulson & Richardson, 2005). The evaluation of these critical factors for each algal species and culturing process is required to develop an economical harvesting system that could lead to a commercial scale process. *Pleurochrysis carterae* has been recognised as a suitable species with high lipid content for biofuel production (Moheimani, 2005). Therefore, this study deals with the physical characteristics of this species and its settling characteristics, these being fundamental requirements for optimizing harvesting methods.

1.2 Aims of this study

Previous work on the use of microalgae for biodiesel production has focused on developing a convenient and highly efficient cultivation system. Among numerous studies which need to be done to improve various aspects of biofuel production and reduce the total price of the final product, this study focused on the harvesting aspect and aimed to:

a) Identify physical characteristics of *Pleurochrysis carterae* during the growth period

b) Investigate settling characteristics of the target particles and compare them with the theoretical prediction

c) Determine cell viability against shear stress applied by high gravity forces

In this thesis, the literature review (Chapter 2) examines the relevant literature on microalgae, biofuel production from microalgae, available methods for measuring cells properties and the most promising methods for harvesting biomass. The materials and methods section (Chapter3) describes the equipments and techniques used in the experiments reported here. The results of these experiments are presented and discussed in chapter 4. Finally, the conclusions arising from this research together with possibilities for future work are discussed in chapter 5.

Chapter 2: Literature Review

2.1 Microalgae and Commercial applications

Microalgal culturing is a modern biotechnology which has existing and potential markets such as food supplements and animal feed. These photosynthetic micro organisms can drive sunlight to their cells and convert carbon dioxide into potential products such as food and biofuels (Chisti, 2007). The first use of micro algae by humans goes back to 2000 years ago while the large-scale commercial culturing of microalgae has more than a 30 year old history (Borowitzka, 1998).

Over the last few decades, the market for microalgal products has been growing and numerous commercial applications have been identified. As a result, the importance of the commercial applications has led to numerous and diverse studies on microalgae systems. Some of the potential commercial applications which have been proposed are:

a) Water treatment (Wilde & Benemann, 1993; Craggs, 1997)
b) Ascorbic acid Production (Running *et al.*, 1994)
c) Renewable fuel production (Benemann & Oswald, 1996; Chisti, 2007)
d) Human nutrients (Seshadri *et al.*, 1991; Otles & Pire, 2001)
e) Animal feed (Martin *et al.*, 1985; Knuckey *et al.*, 2006)
f) Cosmetic industries such as skincare and anti aging (Spolaore *et al.*, 2005).

Although not all of the above projects have resulted in large scale production, due to their high cost and the risk posed by on-site production (Spolaore *et al.*, 2005), several successful commercial systems (Table 2.1) reveal that modern microalgal biotechnology has promise and that more studies and research could overcome or minimize commercial problems.

Table 2.1 Dominant strains in commercial applications (cited from Spolaore *et al*; 2005, Borowitzka, 1998)

Micro algae species	Commercial applications	Advantages	Main producers	Annual biomass product (t)
Arthrospira	Human nutrition, animal feed and nutrition, cosmetics	High protein content, excellent nutrient value, high protein value, positive influence in energy metabolism	China, India ,USA	300
Chlorella	Food additive (i.e. β -1 , 3- glucan), cosmetics(i.e. skin cares)	Active immunostimulator, free radical scavenger, reducer blood lipids	Taiwan, Germany, Japan	2000
Dunaliella Salina	Food additive (i.e. β – carotene)	Rich in vitamin, natural antioxidant	USA, Israel, Australia	1200
Spirulina	Nutraceticals, Aquaculture feeds, Food colouring	Natural food colouring, easy to harvest	USA, Thailand, Japan, China	Unknown
Haematococcus Pluvialis	Aquaculture (i.e. salmon) feed, pharmaceutical markets	Natural astaxanthin such as carp, chicken and red sea bream diet and natural pigments for aquaculture feed	USA, India, Israel	300

The main objective of all commercial production of microalgal is to reduce costs and achieve diversified and economically competitive products. Therefore, several parameters such as biology of the species, cost of the land and production as well as the type of the final product need to be investigated and optimized. These factors will determine the ultimate choice of an economical project (Borowitzka, 1998).

Much of the early work focused on cultivation and the type of the ponds, but there are more difficulties and limitations in some commercial projects. One of the most difficult and costly parts of algal biomass production on a large scale is the harvesting of cells from their dilute suspension (Benemann *et al*, 1980). There has been a strong emphasis in previous studies on the identification of a suitable harvesting technique appropriate for the specific type of species. These studies confirm that an effective harvesting technique is highly dependent on the characteristics of species and the purpose of harvesting the biomass (Shelef *et al.*, 1984; Mohn, 1988; Richmond, 2004). Little information about suitable species and their preferred harvesting method for large-scale biofuel production is available.

There are also more problems in large scale production of microalgal biomass, such as solid and liquid waste by-product streams which are potential environmental pollutors (Benemann *et al*, 1980). Figure 2.1 has been found to show a general model of commercial production of microalgal biomass for different purposes. Some difficulties arising from various parts of the commercial process have been identified in this model. Thus, much effort needs to be exerted to provide a general view of each commercial application and investigate some alternative solutions. It is desirable to study this model of possible commercial applications and to estimate whether the final product suits the potential market. Particular projects must also evaluate different parts of this model to suggest possibilities for overcoming specific and relevant issues.

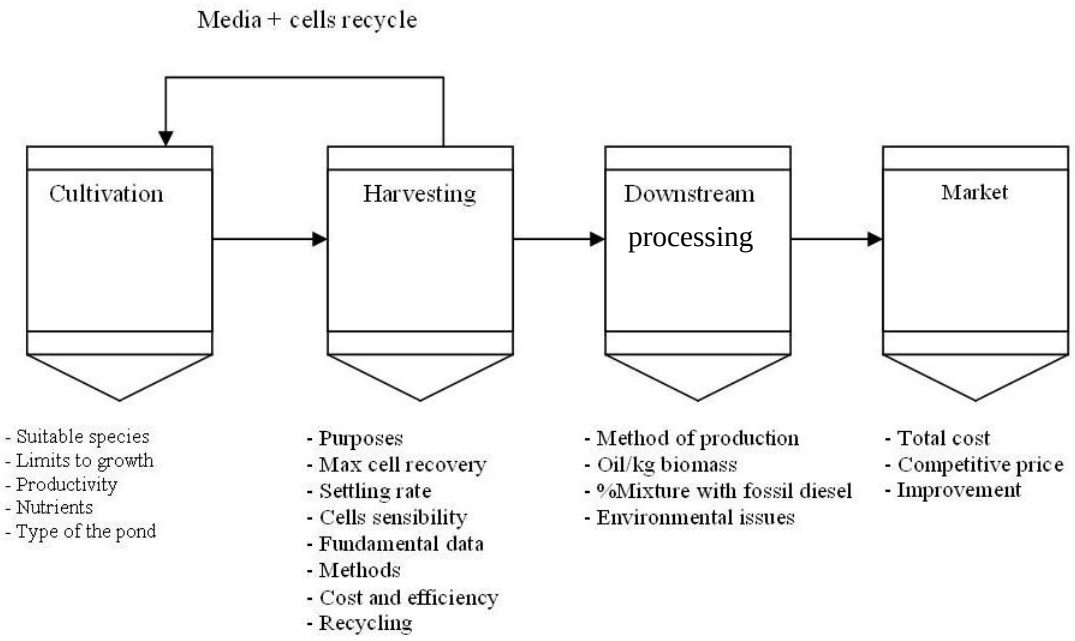


Figure 2.1 model for commercial applications of microalgae

Research on any part of a microalgal system needs primary knowledge of the commercial project from the stage of identifying the type of the species to aspects of downstream processing. Therefore, different steps of biomass production (as is shown in Figure 2.1) are dependent on each other and must be studied from the view point of the whole system. Finally, when the outcomes from each step are integrated the result will be a scheme that could lead to a commercial project.

2.2 Biofuel production from Microalgae

There is a continuously increasing interest concerning biofuel production, mainly because of fossil fuel dependence and an urgent demand for CO_2 capture and utilisation. On the other hand, over the last three years oil prices have been significantly increased from an average $23/barrel in 2002 to more than $100/barrel in 2007 (Bernard & Prieur, 2007) . High prices, unsustainable sources of fossil fuels and carbon dioxide emission from fossil burning create one of the toughest environmental – economical issues in history (Chisti, 2007; Dautzenberg & Hanf, 2008).

Various research relating to biofuel production and market issues, have been undertaken around the world (Faaij, 2006; Bernard & Prieur, 2007; Dautzenberg & Hanf, 2008; Lamers *et al.*, 2008). Europe in 1999 became the most active stimulator and subsequently developer of bioenergy in the world. As a result, in Europe, 4% of the total fossil fuel usage may be replaced by biofuel while a total 10% biofuel contribution has been targeted for 2010 (Faaij, 2006). But currently, in Europe, only 20% of crops are converted to biofuel due to their cost of 2.3 times more than fossil fuel processes (Kondili & Kaldellis, 2006). Meanwhile, a total annual biodiesel production of 1 bbl (billion barrel) in USA and 7.5 bbl for 60 countries have been urged (Lai, 2005). These statistics confirm that effective management of biofuel production may shift the world's dependence on fossil diesel toward sustainable sources. Currently, biodiesel is mainly derived from food crops, animal oils and waste cooking oil. Major problems of the first generation of biofuel sources include slow growth rates, high demand for fresh water and a requirement for large areas of land with consequences for global food needs, while only replacing 0.3% of the transport fuel (Schenk *et al.*, 2008). Therefore, the development of non-food feedstock sources has recently been brought into sharp focus. This so-called second geneneration biofuel, including microalgae, lingo-cellulosic biomass, rice straw and microbial sources are better options when considering the need for food and energy security (Patil *et al.*, 2008). However, details about the lifecycle, growth and biomass production are limited and need to be investigated in details. Biofuel production from microalgae, during the last two decades, has been supported by many environmental organisations (Benemann & Oswald, 1996). These photosynthetic micro-organisms

have a number of advantages including high lipid content and fast growth, thus reducing competition with food crops for land and with the further possibility of growing in marine water and other environmentally efficient systems that can fix CO_2. They have been proposed as a preferred source of biofuel with no net CO_2 contribution (Schenk *et al.*, 2008). Chisti has made a comparison of the main sources of biomass that could produce 50% of all transport fuel needs of the United States (see Table 2.2) (Chisti, 2007). As can be observed from Table 2.2, the land areas needed for food crops are extremely large, while a small area for cultivation of a microalgal biomass will obtain much higher oil yields. This comparison of microalgal biomass with other crops confirms that microalgae (even with 30% oil in wet biomass) is capable of significantly displacing fossil diesel. Taken together these reports provide the most comprehensive evaluation of the importance of these micro-organisms on CO_2 reduction, but to date, no commercial plant has been developed. This is because of the high capital cost of microalgal systems and other uncertain aspects of large scale production, such as the need for CO_2 supplements, the type of the pond and harvesting methods (Benemann & Oswald, 1996).

Table 2.2 Sources of biofuel and comparison details (Chisti, 2007)

Crop	Oil Yield (l/ha)	Land area needed (M ha)	Percent of existing US cropping area
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil Palm	5950	45	24
Microalgae (70% oil in wet biomass)	136,900	2	1.1
Microalgae (30% oil in wet biomass)	58,700	4.5	2.5

Accordingly, a number of reports have attempted to analyse the economic feasibility of biodiesel production from microalgae (Borowitzka, 1992; Chisti, 2007; Schenk *et al.*, 2008; Yanqun *et al.*, 2008). They all have argued that the costs of producing biodiesel from microalgae must be drastically reduced and the quality improved if it is to compete with fossil diesel. In this regard, Chisti (2007) has proposed an economics equation for algal oil as,

$$C_{\text{algal oil}} = 6.9 \times 10^{-3} C_{petroleum} \qquad \text{E.q.2.1}$$

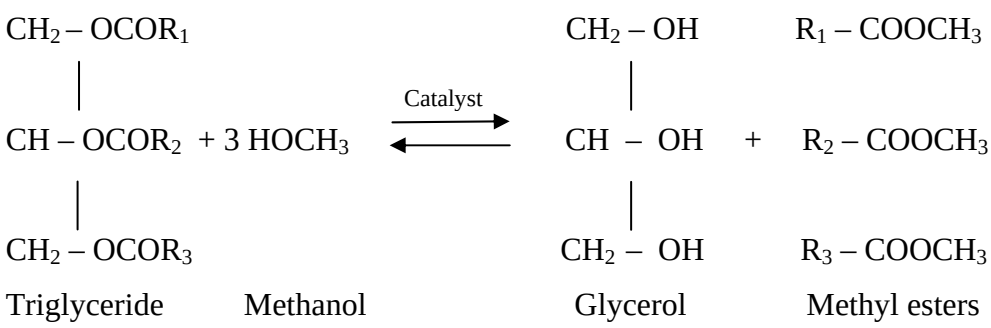
Where,

$C_{\text{algal oil}}$: Microalgal oil in US$ per litre

$C_{petroleum}$: Crude oil in US$ per barrel

In Equation 2.1, Chisti has assumed that algal oil has 80% of the energy content of crude oil. This means that if the initial price of crude oil is US$100/barrel in 2008, the microalgal oil should not be more than US$0.7/litre. The current price of microalgal oil has been estimated to be from US$52-91/barrel, depending on the biomass production methods used (Schenk *et al.*, 2008), whereas palm oil costs approximately US$2.65/barrel (Chisti, 2007). The price of algal oil is not yet acceptable to the market, but undoubtedly, there are some possibilities for reducing costs and improving efficiency with research and development.

As it was discussed earlier, Figure 2.1 represents a general pattern for any type of microalgal system, including biofuel production. The applications of biofuel are proliferating both in terms of quality and price. In practice, a process such as that shown in Figure 2.1 can be improved so as to achieve the aims of cost reduction and increase the industrial feasibility of biofuel production. Therefore, it is necessary to examine what the contributing factors to the cost of the system are and how they might be reduced. By considering some alternatives and the information gathered from previous studies, more projects can be proposed for minimizing cost and increasing commercial feasibility. Even down-stream process such as Transesterification (Box 2.1), which is the common method for all biodiesel production, must be considered and improved through further studies.



Box 2.1 Transesterification process for converting triglycerides to biodiesel diesel (Chisti, 2007)

Among these alternatives, bio-refinery based production, cultivating in open ponds and increasing the productivity by raising annual biomass production and the selection of suitable species and cultivation methods are important factors in the determination of the feasibility and the cost of the final products (Borowitzka, 1992; Becker, 1994; Richmond, 2004). Therefore, the first step in any successful microalgal system is the identification of species and the limits to their growth. Furthermore, results from the cultivation stage and that of identification of the characteristics of the species will be used for the next stages of harvesting and downstream processing. The following literature review focuses on characteristics of the target species, issues concerning harvesting biomass and the most promising harvesting methods.

2.3 Coccolithophores and cultivations factors

Coccolithophores algae are unicellular motile or non motile coccolid cells. They are members of the Haptophyceae family and contain Chlorophyll a and c (Siesser, 1994). Coccolithophores were discovered by Ehrenberg in 1836 in the Baltic Sea. Later in the 19th century different forms of Coccolithophores were observed around the world (Pienaar, 1994). This group of species and specifically *P. carterae* has been noted for its potential application for biofuel production, having a number of advantages including, high lipid content (33% of dry biomass), high productivity (0.23g/l.day) and low contamination risk (Moheimani, 2005).

There are a narrow number of species available in cultures, therefore, knowledge about coccolith formation is based on experimental works and has been focussed on two genera. These are *Pleurochrysis* and *Emiliania* (Siesser, 1994). Figure 2.2 is a micrograph image of a *Pleurochrysis carterae* cell at high resolution. As can be observed in this Figure, the *Pleurochrysis carterae* cell has a spherical surface which is covered by coccoliths.

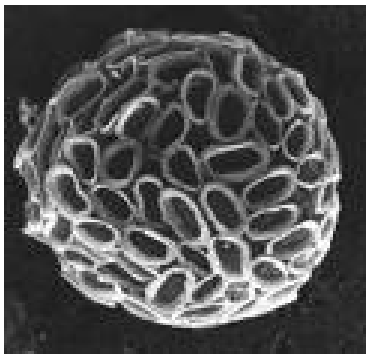


Figure 2.2 Micrograph image of *Pleurochrysis carterae*

The life cycle of *Pleurochrysis* such as *P. carterae* and *P. Scherffelii* (Figure 2.3) is one of the better identified life cycles (Siesser, 1994). Different stages of cells during the growth period, from doubling time to division and filamentous, are shown. These steps can be observed under the microscope during the cultivation process. For instance, the haploid benthic stage (stage 9 in Figure2.3) has been observed by Pineaar (1994) (Figure 2.3) and named the *Apistonema* stage of the *P. carterae* life cycle (Pineaar, 1994).

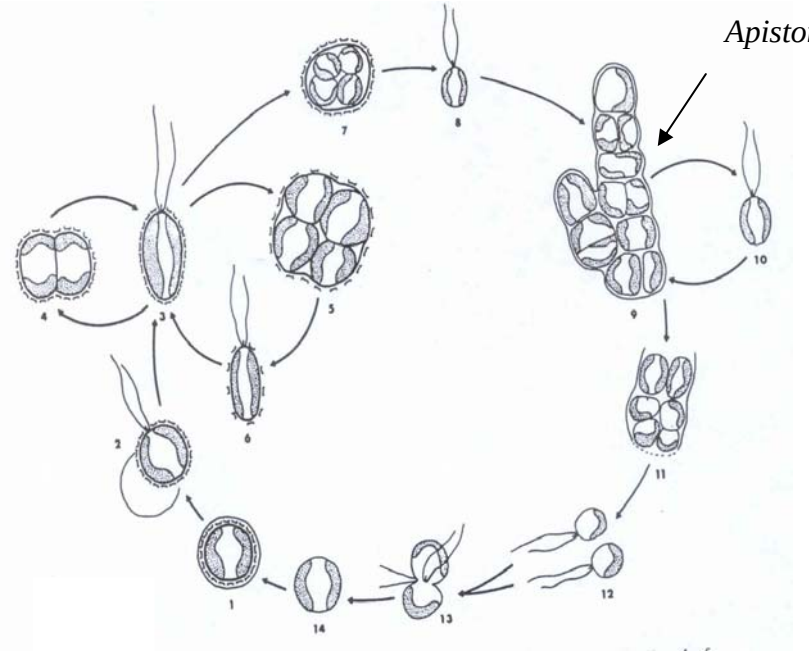


Figure 2.3 A schematic representation of the life cycle of *Pleurochrysis carterae*

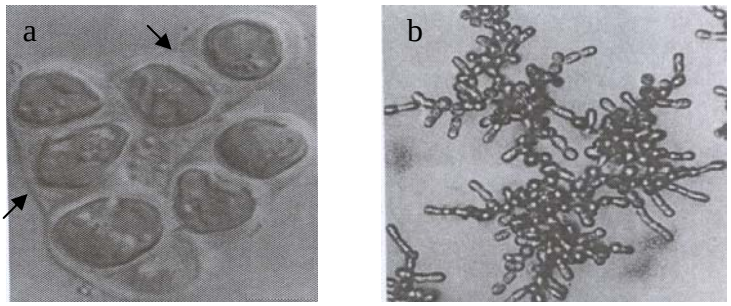


Figure 2.4 Apistonema' stage of *P.carterae* (a) A well defined cell wall (arrowhead) is clear around the cells, (b) Low power view of benthic filamentous stage

Like any higher plants, light, water and inorganic nutrients are the basic elements of the requirements for microalgae (Benemann & Oswald, 1996). In order to establish an effective high value of biomass production it is necessary to optimize the growth parameters (Borowitzka, 1997). These factors have been demonstrated in detail (Moheimani & Borowitzka, 2006b) and are commonly used in preliminary studies for growing and maintaining *Pleurochrysis carterae* species. Below is a brief overview of the growth limits and cultivation issues addressed by previous studies. These fundamental elements provide better understanding of any type of microalgal culture.

2.4 Growth parameters

Each species requires an individual range of physical elements for growth. These needs must be identified at the bench scale then optimised and scaled up to a commercial pond (Borowitzka, 1992). It has to be mentioned that not necessarily all parameters in a raceway pond are similar to a bench scale, as some of them, such as light intensity, temperature, rain and salinity are influenced by the environmental changes. Therefore, a combination of laboratory knowledge and environmental elements has to be evaluated in developing an operational protocol for pond management.

Previous study shows how critical elements affect the productivity of *P. carterae* in a raceway pond. For instance Figure 2.5 demonstrates changes in growth, temperature and daily pH in 12 months of cultivation in a raceway pond (Moheimani, 2005). This information can be used in this study as fundamental data for cultivation. Further more, results from laboratory cultivation can be compared with previous work.

One of the most common problems in cultivating algal cultures is contamination with other micro-organisms (biotic elements) such as fungi, bacteria and viruses (Becker, 1994). In a rich nutrient culture, any other unwanted organisms can contribute and compete with the main species. This will significantly decrease productivity especially in open ponds. Avoiding contamination at a large scale is almost impossible, but ecological knowledge will be useful to maintain an algal culture and choosing the right system and a suitable location will decrease contamination risk. For instance growth in a batch system is less likely to be contaminated with other micro-organisms (Pluz, 2001). It can be seen, therefore, that different systems have been studied for the growth and handling of microalgal in large scale cultivation (Chaumont, 1993; Benemann & Oswald, 1996; Borowitzka, 1998).

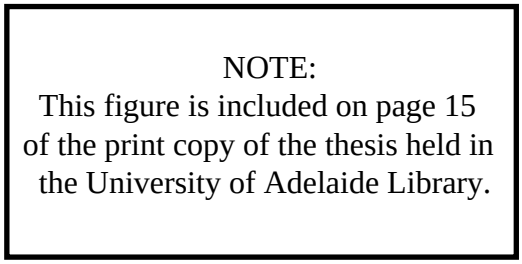


Figure 2.5 Changes in growth versus critical factors in a raceway pond over a year at Murdoch University (Moheimani, 2005)

There are two main types of cultivation systems including open air and closed systems (Benemann & Oswald, 1996; Pluz, 2001). The selection of an individual system is effected by different parameters such as climate, economic condition (water and land) and nature of the species (Benemann & Oswald, 1996).

Open systems incorporate the most popular techniques for the large scale production of microalgae due to their less expensive process. Shallow big ponds, tanks, circular and raceway ponds are currently used in different projects (Borowitzka, 1998). However, low productivity and high contaminate risk make them unsuitable for some commercial applications (Pluz, 2001).

Closed reactors are another type of cultivation systems with higher productivity and low contamination risk. These are very expensive and have only been recommended for individual applications where there must be no contamination risk (Borowitzka, 1998). Pluz (2001) presented a comparison data demonstrating the disadvantages and advantages of each system (Table 2.3). Figure 2.5 shows two examples of microalgal cultivation which have been used on a commercial scale.

Because of concern about the high cost involved in culturing microalgae and cost reduction requirements, more studies need to focus exclusively on issues of the target species. In 1987, Benehann *et al* (1987) estimated US$3.25/kg of dry biomass as the general operating cost of microalgal ponds (Benehann *et al.*, 1987); total costs of cultivation of the *P. carterae* biomass, depending on the type of the pond and harvesting technique, has recently been argued to be between Aus$7.35/kg to Aus$14.17/kg (Moheimani, 2005). These current prices are not competitive in comparison to fossil fuel and more work is needed to make this proposal into a commercial project. As it was mentioned previously, harvesting small microalgal cells is the main problem involved in this costly process. Therefore, separate studies need to focus exclusively on issues relating to the harvesting of the individual species biomass and to also investigate potential harvesting techniques for large-scale recovery. Furthermore, the economies of this biomass recovery should be reported in order to estimate the total cost involved in the design of harvesting methods. Some of the specific harvesting challenges and methods examined in previous studies are discussed in the next section.

Table 2.3 Comparison of open and closed algal cultivation plants (Pluz, 2001)

Parameter	Open ponds (raceway ponds)	Closed systems
Contamination risk	Extremely high	Low
Space required	High	Low
Water losses	Extremely high	Almost none
CO_2 –losses	High	Almost none
Biomass quality	Not susceptible	Susceptible
Variability as to cultivatable species are restricted to a few algal varieties	Not given, cultivation possibilities	High, nearly all microalgal varieties may be cultivated
Flexibility of production	Change of production between the possible varieties nearly impossible	Change of production without any problem
Reproducibility of production parameters	Not given, dependent on exterior conditions	Possible within certain tolerances
Process control	Not given	Given
Standardization	Not possible	Possible
Weather dependence	Absolute, production impossible during rain	Insignificant, allow production during bad weather
Biomass concentration	Low, approx, 0.1-0.2 g/l	High, approximately 2-8 g/l
Efficiency of treatment processes	Low, time-consuming,	High, short-time, relatively small volume flows

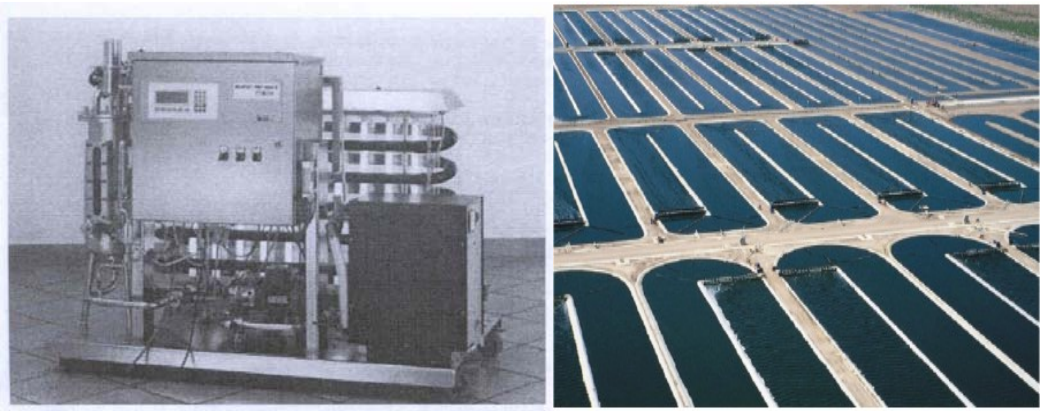


Figure 2.6 Left- A 25 l photobioreactor with tubular sterilisable design , right- Earthrise Farms *Arthrospira* production plant in USA

2.5 Harvesting

Harvesting is the most challenging part of biofuel production. It has been claimed that the cost of harvesting biomass is 20-30% of the total production cost (Gudin & Thepenier, 1986). Thus, the economy of microalgal production is highly dependent on the harvesting technique and investigating an acceptable harvest method at a commercial scale has a considerable effect on total price reduction (Mohn, 1988; Becker, 1994; Benemann & Oswald, 1996; Chisti, 2007).

Nevertheless, one or more solid-liquid separation stages must take place to ensure that microalgal systems can achieve concentrated biomass (Richmond, 2004). Although, there is no lack of harvesting methods, it is difficult to identify economical harvesting technique that is highly efficient at large-scale (Benemann & Oswald, 1996).

Small cell size (3-30 μm), low concentration (~1g/l) and unfavourable density (only slightly greater than media) are all factors that result in an expensive harvesting process and are significant concerns which need to be overcome or minimised through technological development. Of the many harvesting techniques, which were proposed and examined during the previous decades, only a few of them were found to possess potential applications that could be developed for further optimization. These include, flocculation, centrifugation and filtration (Mohn, 1988; Benemann & Oswald, 1996; Molina *et al.*, 2002; Richmond, 2004). Other techniques, including the Jameson cell and bubble column have been tried successfully at a bench scale, but none of them has

been recommended for large scale biomass recovery (Guelcher & Kanel, 1998; Guelcher & Kanel, 1999). Further more, the selection of suitable harvesting techniques depends on the value of the final product, cell concentration, the purpose for which the biomass is used and the type of the species (Becker, 1994; Yanqun *et al.*, 2008).

The next section reviews the most potentially useful harvesting techniques and provides a general discussion of these methods for harvesting the target species.

2.5.1 Filtration

Filtration is a common method for separating particles from a suspension, and is widely used in various processes (Becker, 1994). This technique had been used in water treatment projects but has been suggested as an alternative for harvesting microalgal cells. Collecting filamentous cells such as *Spirulina* using filtration methods seems to be a satisfactory process, but difficulties arise in harvesting unicellular small cells with a spherical shape. Rapid clogging and continuous backwashing are the major problems, which limit throughput rates and continuous harvesting of large volumes (Becker, 1994; Molina *et al.*, 2002).

Many types of filters have been examined and compared but dramatic improvement at a large scale, has yet to be demonstrated. Various filtration methods have been compared quantitatively and it is apparent that some are suitable for certain purposes (Mohn, 1988). The application of cross-flow membrane filtration for the separation of microalgal cells has been widely used recently. This adapted technology needs to be improved and studied in greater detail for each individual commercial application.

These and other early filtration machines have little practical application for harvesting large volumes. Results achieved in the identification of the physical characteristics of *P. carterae* in this work could be used to assess potential filtration techniques. Possible limitations and difficulties for large-scale harvesting of *P. carterae* using filtration should be addressed in a separate study.

2.5.2 Flocculation

Flocculation of algal cells is shown to be a rapid and effective harvesting method (Molina *et al.*, 2002), which has been recognised as an essential step in the harvesting of microalgal cells from their media (Sukenik *et al.*, 1988). This method refers to the increasing of effective cell size and density using chemicals, polymers or organic elements. As a result fine particles clump together and settle to the bottom of the suspension. Flocculation by organic cationic has been widely employed for water purification.

Two common flocculation techniques, bio flocculation and chemical flocculation, have been examined for the purpose of algal recovery (Mohn, 1988; Benemann & Oswald, 1996). Previous studies have shown that bioflucculation is an effective and highly efficient (>70% cell recovery) method for cell recovery from freshwater. However, this method has failed when attempting to collect cells from brackish water (Pushparaj *et al.*, 1992). Bioflocculation is an economical effective method for cell recovery that does not contribute to contamination in the biomass. However, the process is quite complex and unreliable and so more research is if we are to find a suitable bioflocculation method. Chemical flocculation with inorganic flocculants has been widely used in water purification and aquaculture feed production (Pan *et al.*, 1998; Knuckey *et al.*, 2006). The total efficiency of chemical flocculation depends on pH, chemical dosage and media salinity. Bilanovic *et al* (1988) concluded that the optimum dosage of flocculants was markedly higher in marine cultures (Bilanvoic *et al.*, 1988). Thus, although, chemical flocculation was tried successfully in some processes, the remnant chemicals in the harvested biomass, the expense of the chemicals and low efficiency when using saline media suggest it is an uncertain method for biofuel production.

Nevertheless, flocculation is an effective technique for carrying out the primary concentration. In any event, issues relating to recovering cells, their end use, possible cell disruption among the harvesting, settling velocity rates, the economic potential of large scale processing and a suitable method for the individual species require further investigation.

2.5.3 Centrifugation

Centrifugation is an effective harvesting method which can be used for all types of microalgae (Benemann & Oswald, 1996; Molina *et al.*, 2002; Richmond, 2004). Several centrifugal devices have been considered in previous studies (Charles & Ambler, 1979); Coulson & Richardson, 2005; Ladisch, 2001; Shuler & Kargi, 2002; Svarovsky, 1985), but practical procedures for biomass recovery of microalgae on a large scale have not yet been found. Despite the successful recovery and high efficiency (~95%) in harvesting with the centrifugation technique, this method has a number of limitations, including possible cell damage, high input energy, complicated processing and large capital investment (Richmond, 2004). Moreover, greater detail about particle size and distribution, suspension viscosity and the theoretical foundation of particle settling are required to select a suitable centrifuge machine (Becker, 1994; Benemann & Oswald, 1996; Richmond, 2004).

The recovery rate of any particle in a suspension is a matter of the nature of the particles (i.e. size and density) and medium (viscosity and density), as well as the applied by the centrifuge. In microalgal systems, particles are very small (<100μm) and the viscosity of the medium is slightly greater than water. Therefore, the effect of medium viscosity is neglected. Apart from the nature of the particle, which can be identified for microalgal systems, critical parameters for centrifugation also need to be investigated. Some of these factors are available in data sheets provided by manufacturers and some vary depending on the particles and medium characteristics.

Centrifugal force depends on r (radius from centre of rotation to bottom of tube, cm) and speed of centrifugation (N, rpm). The higher gravity forces the greater recovery and higher energy input. RCF or g factor (gravitational force) can be calculated from the equation,

$$RCF = 18 \times 10^{-4} \times r \times (N/1000)^2 \qquad \text{Eq.2.2}$$

The capacity of the centrifuge and quantification of its performance is an initial factor in the design of a reliable machine. This factor is shown as Σ and named centrifugation coefficient (Eq.2.3). Value of Σ is independent of the medium and

particles properties and only depends on the type of the centrifuge. Σ represents the cross- sectional area of a gravity settler with the same sedimentation characteristics as the centrifuge.

$$\sum = \upsilon_p . Q \qquad \text{Eq.2.3}$$

Settling velocity can be calculated as,

$$\upsilon = \upsilon_p . r\omega^2 / g \qquad \text{Eq.2.4}$$

The ratio of velocity in the centrifuge to velocity under gravity is called the centrifuge effect and is shown as;

$$Z = r\omega^2 / g \qquad \text{Eq.2.5}$$

Where,

Q = volumetric flow rate of feed m^3/s

υ_p = particle settling velocity m/s

ω = centrifuge speed, rad/s

g = gravitational force, m/s^2

Z = centrifuge effect (-)

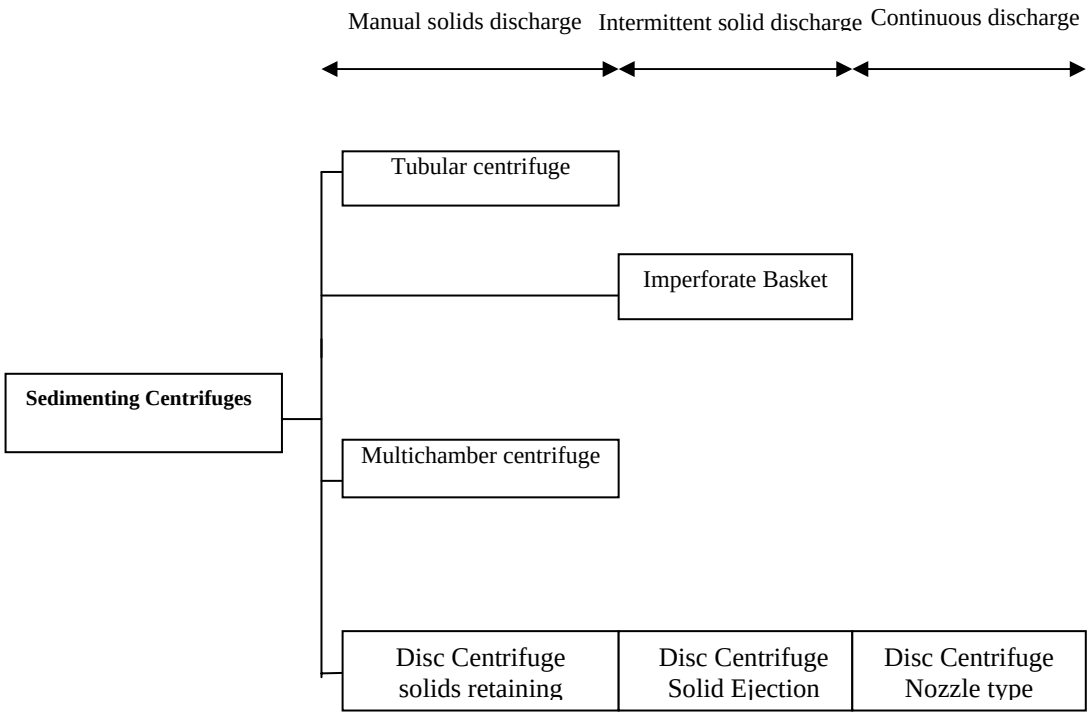
Z factors for industrial centrifuges are 3000-16000 (Molina *et al.*, 2002). From equation 2.3 the settling rate can be increased by increasing centrifuge speed (ω), terminal settling velocity (υ_p) or density differences between particle and liquid ($\rho_p - \rho_w$).

Several centrifugal methods were examined for potential applications in microalgae separation system (Shelef & Soeder, 1980; Mohn, 1988; Becker, 1994), but only some of them were efficient in one step and others needed preconcentration or further steps for maintaining the centrifuge machine, thus resulting in a non continuous process.

Generally, there are two main types of commercial centrifugation machines, including sedimentation centrifuges and centrifugal filters (Charles & Ambler, 1979) which are

the only sedimentation types are suitable for microalgal systems (Shelef *et al.*, 1984). Sedimentation centrifuges are subdivided into chamber, tubular, nozzle, self cleaning disc-stack (solid ejecting disc) and decanter centrifuges. Tubular centrifuges are not continuous as there is no condition for solid discharge in tubular centrifuges. They need to be stopped and cleaned regularly. These types of centrifuges are only desirable in bench scale algal harvesting. The use of chamber centrifuges is very time consuming as they need to be discharged frequently. They are only suitable for small quantities and have been rejected for large scale applications (Svarovsky, 1985; Mohn, 1988; Becker, 1994; Molina *et al.*, 2002). Further optimisation will be achieved by some laboratory tests of centrifugation and gravitational sedimentation. A classification of sedimenting centrifuges has also been shown in Table 2.4.

Table 2.4 Classification of Sedimenting Centrifuges (Svarovsky, 1985)



Among the different type of sedimenting centrifuge machines, a decanters operation is continuous both for feed and discharge solid, needing low maintenance, having a long operation time and achieving 95% recovery (Borowitzka, 1998). Mohn (1980) harvested different types of pre-concentrated microalgae with high reliability but energy consumption was too high. However, Shelef (1984) has reported unsuccessful recovery of flocculated slurry using this technique (Shelef *et al.*, 1984). Decanter centrifuges could be an option for second stage recovery of *P. carterae* cells, but

several uncertain aspects such as recovery rate, acceleration force requirements, energy input and cell viability after centrifugation need to be studied in detail. A continuous decanter centrifuge is shown in Figure 2.7. This type of centrifuge machine is recommended by manufacturer for dewatering biomass after preconcentration (1.5-2% dry solid).

Figure 2.7 Horizontal design of continuous scroll discharge decanter by Westfalia separator AG, Germany

Nevertheless, there is a lack of knowledge about which centrifuge machine is suitable for dewatering *P. carterae* biomass, and whether the energy balance of the system is satisfactory. Previous work has been done on a laboratory scale and has recommended centrifugation recovery as the second stage of dewatering biomass, but to date, no commercial model has been described. Therefore, more studies are necessary to investigate the economics of centrifugation and the effect of this method on cell disruption specifically after flocculation. Furthermore, some bench scale experiments with laboratory centrifuges could result in a better understanding of centrifugation for harvesting *P. carterae* cells.

2.6 Harvesting challenges

As it was discussed earlier, among the various factors relating to biofuel production from microalgae, biomass recovery is an important factor in the determination of the cost of the process. Therefore, it is important to find and optimize harvesting techniques, so as to produce the highest efficiency and also to satisfy the final price requirements. A critical review of previous studies indicate that,

a) A number of studies have been done to improve biomass recovery and estimate the total cost of harvesting process. However, none of them refers to harvesting *P. carterae* for biofuel production.

b) Flocculation is a promising technique for the first stage of cell recovery with lower energy input. However, great care must be taken to not have any unwanted particles or chemicals in the biomass. This will limit further downstream process. Therefore, suitable flocculation method for the specific type of microalgal, *P. carterae* species, must be examined and reported in a separate work. Moreover, the feasibility of this method needs to be estimated for commercial scale biofuel production.

c) Decanter type centrifuges appears to be the most suitable machine with the highest cell concentration (up to 20%), lower energy input and maintenance requirements compared to other machines. They can be used as the second stage of cell recovery after pre-concentration to at least $1.5-2\%$ by flocculation methods. This proposition must be studied in greater detail and must include dewatering *P. carterae* after the initial concentration.

d) Filtration, specifically, cross flow filtration has been suggested for filamentous cells with its resulting high recovery rate and low energy input. But the high cost of filter membranes, backwashing and clogging difficulties suggest it is an unsatisfactory technique for continuous large scale harvesting of non filamentous cells such as *P. carterae*. However, developed filtration technology may offer more possibilities for a filtration technique which satisfies requirements for economy when using continuous processes.

e) One of the strongest challenges in any type of harvesting is the nature of the suspension and particles. Some simple experiments at the bench scale may indicate the minimum requirements for industrial design. Any results will greatly contribute to making the final decision in the selection of a harvesting method. Therefore, a further consideration in selecting a suitable harvest method depends on the algal species, as well as characteristics of the suspension.

In deciding which of the machines and methods described above is to be employed for harvesting *P. carterae*, one should consider; cell size, cell shape, maximum cell concentration and other physical characteristics, which could be used to select an appropriate harvesting method. These parameters have been predicated to play critical roles in designing a suitable filtration system (Benemann *et al.*, 1980; Becker, 1994). Some of these parameters have been addressed in the literature review but further investigation into the *P. carterae* culture including clumping cells, cell size distribution and other physical factors are necessary to design suitable harvesting methods (Charles & Ambler, 1979; Becker, 1994; Molina *et al.*, 2002).

Therefore, the following information must be investigated in this particular case,

a) Nature of the target cell .i.e. type, size and distribution of the cell, motility
b) Settling characteristics of the particles
c) Density and viscosity of suspension at operation temperature
d) Concentration of cells in media
e) Harvesting efficiency
f) Quality of recovered cells
g) Cell damage due to shear rate
h) Pre-concentration requirements
i) Energy input

Industrial designer and manufacturers expect to receive the above information in each specific suspension, if they are to design suitable instruments. Very little of the information about physical characteristics of *P. carterae* and its settling behaviours have been published. Therefore, bench scale screening experiments need to be done to address these characteristics which can be used to investigate suitable harvesting techniques. The next section provides a brief overview of techniques and instruments which have been employed in previous studies to investigate the characteristics of the suspension and cell. Conclusions from this section will illustrate a methodology for laboratory experiments and thus indicate the appropriate analytical procedures.

2.7 Physical characteristics and settling rates

Knowledge of the fundamental factors of density, particle geometry and particle size distribution can be used to describe the settling velocity rate of the suspension. On the other hand effects of harvesting techniques on the target biomass will be critical for examining the next stage of the downstream process. Therefore, the aim of this part of the literature review is to present previous studies of the characterization of the suspension and individual cells, in terms of measuring settling rate and investigating suitable harvesting techniques. Techniques and methods in previous studies as well as bench screening will permit better understanding of physical characteristics of the suspension. This preliminary evaluation of cells and their suspension will lead to an investigation and choice of an appropriate pattern among the possible efficient harvesting methods.

2.8 Cell structure and size distribution

The settling characteristic of a suspension is strongly dependent on the cell size distribution, cell shape and surface area (Metcalf & Eddy, 2003). A theoretical settling velocity rate of the suspension can be calculated. Obtained values will be compared with the actual settling rate to verify the application of the settling prediction thus suggesting an appropriate design of the harvesting system.

Previous studies, mainly in wastewater treatment, show that the characteristics of particles and their terminal settling velocity have a key role in the efficiency of harvesting efficiency (Jun *et al.*, 2007). Therefore, knowledge of species composition, distribution of cell size and its structure are essential factors which should be considered in the selection of the harvesting technique. The relation between the structure and function of cell size, shape, and surface area and the specific purpose of the system requires rapid and specific identification methods. Nevertheless, in the design of any common harvesting methods- centrifugation, filtration and flocculation- preliminary microscopic assessment of cells is required. The traditional method of cell identification, using light microscopy is valuable in regards to cell counting, cell size and shape observation. New technology, using appropriate softwares provides a wide

range of application in microscopic analysis. Further detection of smaller cells and the confirmation results by light microscopy require more accurate methods, such as SEM (Scanning Electron Microscope) (Dawes, 1998).

Since studying cells and dynamic processes in living cells plays a pivotal role in biology, it is common for such data to be obtained by microscopy analysis. This common approach for detecting cells and their events has been used for numerous applications. However, manufacturers offer various types of instruments with different costs and resolution capabilities, but quality, versatility and installed software depend on the applications which are employed. In order to accomplish the task of microalgal study the microscope must be equipped with suitable lenses (magnifications) and analytic software.

The importance of electron microscopy in biological research is confirmed by its exclusive use in medical, biochemistry and biology studies, but the traditional use of dissecting light microscopy is limited to the study of surface features to about 5µm(Dawes, 1998). Thus, the SEM method with higher magnification can be employed to obtain images with higher magnifications, as it is obvious from its 500× magnification in comparison with the dissecting light microscope (Dawes, 1998a). This method with a number of advantages, including great depth of focus, direct observation, ability to switch over a wide range of magnification and easy operation has been used to produce an image and acquire useful information about the species (Hearle *et al.*, 1972).

Both methods can be employed for different purposes. The scanning electron microscope has been used for grain sizing, confirmation of fracture mechanism and observing the presence of other micro-organisms such as bacteria; it has also been used for light microscope cell counting, cell clump and dead colonies which were imaged in day to day study. More details about instruments and software will be explained in the material and methods chapter.

2.9 Particle settling theory

The terminal settling velocity of a rigid particle is a critical parameter in solid-liquid separation (Metcalf & Eddy, 2003). It is needed so as to determine particle velocity rate to simulate and design separation techniques. Settling experiments are commonly used on wastewater samples to demonstrate how the individual particles behave under the influence of gravity as characterized in sedimentation basins (Wong & Piedrahita, 1999). On the other hand, suspension settling under gravity has been reported to be affected by various factors including shape and size of the particles, viscosity and velocity of media (O'Mellia, 1991; Richardson & Zaki, 1954). Terminal settling of any nonflocculating particle depends on Reynolds number and can be explained by Newton or Stokes' equations (O'Mellia, 1991; Metcalf & Eddy, 2003; Crittenden *et al.*, 2005).

$$\upsilon_p = \left(\frac{4g}{3C_d}(Sg_p - 1)d_p\right)^{1/2} \qquad \text{(For Re >1)} \qquad \text{Eq.2.6}$$

$$\text{Re} = \rho.\upsilon_p.d_p / \mu \qquad \text{Eq.2.7}$$

υ_p = particle settling velocity, m/s

d_p = diameter of particle, m

Sg_p = specific gravity of the particle, (-)

C_d = drag coefficient, (-)

Drag coefficient for particles with spherical shape is calculated by Eq.2.8,

$$C_d = \frac{24}{\text{Re} + 3\sqrt{\text{Re}} + 0.34} \qquad \text{Eq.2.8}$$

For Stokes' law yields:

$$\upsilon_p = \frac{g(\rho_p - \rho)d_p^{\,2}}{18\mu} \qquad \text{(For Re <1)} \qquad \text{Eq.2.9}$$

ρ_p = particle density, kg/m^3

ρ = fluid density, kg/m^3

μ = Viscosity of medium, Pa.S

Terminal settling velocity can be calculated using theoretical models but specific conditions such as the type of particle, hydrodynamic conditions and experimental techniques will affect the settling velocity rate. Therefore, the settling velocity of the suspension and its properties must be experimentally determined. With this determination, a comprehensive evaluation of the theoretical calculations with experimental results the next stage of harvesting design will be presented. Young (1994) has developed Stokes' law for the calculation of the settling velocity rate of the Coccolithophores marine suspension.

Metcalf & Eddy (2003) have demonstrated various settling region in a suspension. They have explained that in systems with high concentrations of suspended of solids systems, two different regions affect the settling of particles. These are Hindered (zone) and Compression settling. A high concentration of particles causes the liquid to be more likely to shift up through the interstices of the contacting particles. Thus, contacting particles tend to settle in a similar position to other particles with respect to each other. This phenomenon is called hindered settling. The hindered settling rate depends on the cells concentration and their physical characteristics. Furthermore, cells continue to settle and build a layer of solid on the bottom of the cylinder. This layer is known as compression layer. After the compression layer is formed, lower concentrations of particles extend upward in to the cylinder. Hindered and compression settling are important considerations in any settling rate. However, as in most cases where are an identifiable interface, transition settling develops between the upper phase and the hindered settling region (Figure 2.8).

When the suspension is of low concentration, the affects of mutual interference can be neglected, because the distances between adjacent particles are bigger than the particles' size (Metcalf & Eddy, 2003; Richardson & Zaki, 1954). It is difficult to predict the sedimentation rate as many quantitative factors concerning the surfaces of particles and the presence of an ionised solute are involved in the settling process (Coulson & Richardson, 2005). Therefore, an alternative method of determining the terminal settling based on column settling tests, has been suggested in some wastewater textbooks as a fundamental method to investigate the settling velocity rate (Metcalf & Eddy, 2003; Crittenden *et al.*, 2005).

On the other hand, the significance of different values which contribute to the settling theory has not been investigated for the individual *P. carterae* cells, and so methods for the measurement of the velocity rate must be developed by some laboratory experiments. Furthermore, a comprehensive evaluation of the actual settling rate in conjunction with theoretical results will suggest an effective removal system.

The rate of the settling can be determined by a column settling test. This method has been described and also used to characterize the settling properties of the particle, particularly in wastewater treatment studies (Wong & Piedrahita, 1999a; Metcalf & Eddy, 2003).

A typical settling test involves the collection of an effluent suspension sample, placing it in tall clear columns and observing the particle settling over time, data obtained from the column tests will be plotted to a graph (Figure 2.8) and this will result in an analysis of experiments to verify the applicability of the predictions to actual field conditions (Metcalf & Eddy, 2003).

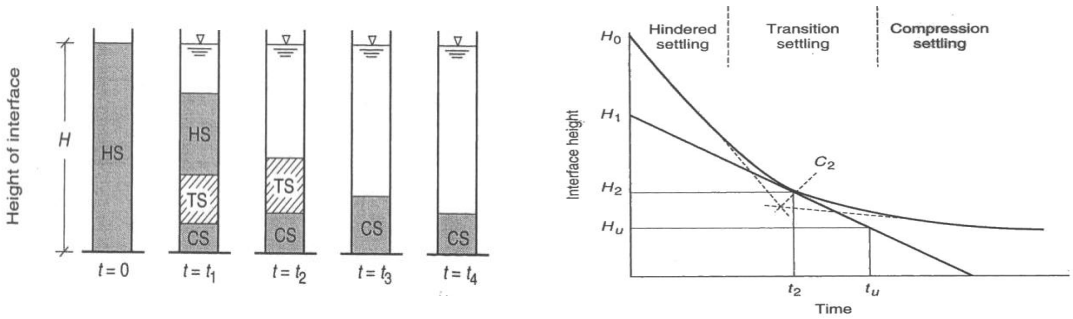


Figure 2.8 Left-settling in column tests through various phases, right-Graphical analysis of settling curves

2.10 Cell viability

As it was earlier concluded, physical forces, particularly shear stress are believed to be an important causative factor in centrifugation which result in damaged cells for the next stage of biomass treatment (Molina *et al.*, 2002). Different studies have verified microalgal cells viability, for various purposes mainly to verify toxicity pollution and biomass quality as aquaculture feed studies have dealt with flowcytometry as a fundamental method for microalgal cell viability assessment

(Franqueira *et al.*, 2000; Hampel *et al.*, 2000; Stauber *et al.*, 2002; Knuckey *et al.*, 2006). On the other hand, previous studies have developed different procedures to asses cells after harvesting using centrifugation and filtration techniques (Vandanjon *et al.*, 1998; Molina *et al.*, 2002; Knuckey *et al.*, 2006). However, the effect of different harvesting methods on *P. carterae* cell viability has not yet been found, but the centrifugation technique has been proposed as the most likely procedure for cell disruption (Benemann & Oswald, 1996; Molina *et al.*, 2002). Therefore, the determination of *P. carterae* cell viability and the assessment of the physiological state of the culture are essential for development of a centrifugation technique. Furthermore, similar assessment methods can be employed for any other harvesting technique.

Cell viability may be estimated using different types of methods depending on the cells or culture. Flow cytometry is an accurate well-known method of cell assessment which has wide range of medical applications (Muirhead, 1984; Zharov *et al.*, 2007) as well as other single cell applications, for example in microalgae studies (Franqueira *et al.*, 2000; Stauber *et al.*, 2002). Employing a flow cytometry technique needs a fundamental understanding of this specialized microscope technology. Over time flow cytometry itself has become a field with numerous applications but this study explains only the basic principles of flow cytometry.

Flow cytometry is an accurate technology which simultaneously measures and analyses multiple physical characteristics of single particles (Stauber *et al.*, 2002). This powerful technique has been used over 30 years to measure various optical parameters for any particular microscopic cells (Sklar, 2005). There are some useful sources of information about the operation systems and how they are employed using this technique (Givan, 1992; Carter & Meyer, 1994; Sklar, 2005). Any particle or cell from 0.2-150 micrometers in size can be carried to the laser intercept in a fluid stream. Flow cytometry is based on analysis of light scatter and staining from a continuous cell sample which are passing through the detector one by one. The distribution of light signals emanating from a single stationary cell while it varies with time under the scrutiny of the detector. A diagram of suspension which is analysed by an optical bench flow cytometer has been showed in Figure 2.9 (Givan, 1992).

Generally, flow cytometers are built on the basis of three major parts including fluidics, optics and electronics. The fluid system of a flow cytometer transports particles of a random three-dimensional suspension one by one through the fluid system. An optical system consists of lasers and filters to illuminate the cells in the sample stream and directs them to resulting light signals and appropriate detectors. Light filters are located in front of each photo detector to produce only the light of the colour transmitted through their detectors. The signal will be continued by the emerging light and photomultiplier tubes or photodiodes convert. Finally, the detectors (electronics system) convert the detected light signals into electronic signals, that can be demonstrated by the computer and appropriate software (Carter & Meyer, 1994). Data gathered from the flowcytometer can be analysed statistically by flowcytometry software so as to describe cellular characteristics such as size, complexity and health. In most cases flowcytometer detect cells between 1-15 µm. The quantum of light which is scattered in the forward direction as laser light striking the cell is forward scatter (FSC). The magnitude of forward scatter is proportional to the cell size therefore this data can be used to quantify the particle size parameter. All cells travelling through the laser beam will scatter light to the side .This is caused by granularity and structural complexity inside the cell. More granular particles will scatter more of the illuminating beam to the side with the resulting intensity being called side scatter (SSC). The signals collected by side scatter and forward scatter detectors will be plotted on one dimensional histogram and analysed by the software connected to the system (Carter & Meyer, 1994).

Figure 2.9 Diagram adapted from Becton Dickinson-optical bench of a flow cytometer

2.11 Viscosity

As argued elsewhere, viscosity of media is one of the major factors for innovation in the harvesting systems. Previous research has been conducted to identify this physical parameter using different methods in respect of microalgal cultures (Bi *et al.*, 2007; Torres *et al.*, 2007). However, viscosity of the *P. carterae* suspension has not been identified yet, and this aspect needs to be addressed properly. The significance of its value can be investigated by viscometer instruments.

Rheology is a well recognized science of the deformation and flow of matter. The knowledge of rheology is used to set out standard methods which are applied to determine rheological properties experimentally (Eirich, 1956). Because the understanding of viscosity is an important offshoot of the measurement of the settling velocity of particles, an accurate estimate of rheological properties (shear stress and shear rate), it is necessary to determine microalgal viscosity. However, various rheological methods have been introduced in text books (Eirich, 1956; Steffe, 1996) but fundamental rheological properties are independent of rheometers and the same results will be achieved from different instruments.

The characteristics of elastic fluids which appear to have single constant viscosity under uniform temperature conditions are referred to as Newtonian flow. This parameter is based on linear-elastic material as shown in a stress-strain diagram (Figure 2.10). Furthermore, viscosity (η) is described by defining the relation between shear stress (τ, Pa) and shear rate (γ, s^{-1}),

$$\eta = \tau / \gamma \qquad Pa.S \qquad \text{Eq.2.10}$$

Plotted data on rheograms will demonstrate an average of viscosity with different cell concentrations (Steffe, 1996)

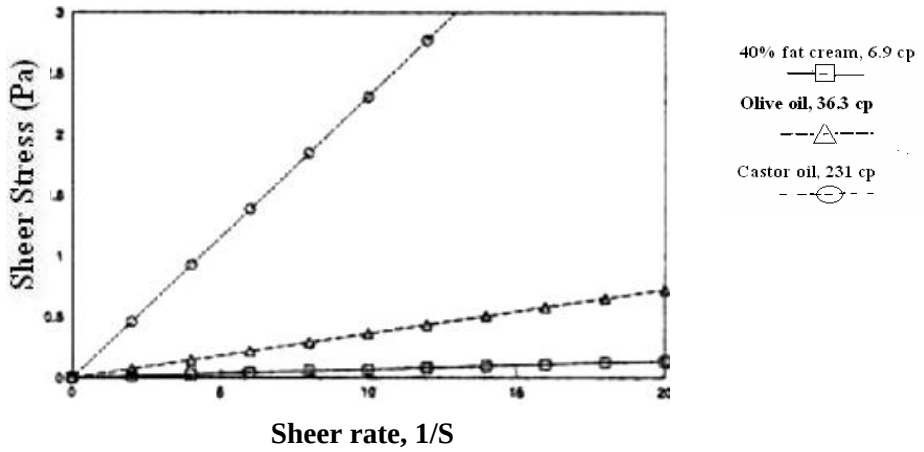


Figure 2.10 Rheograms of some Newtonian fluids

Young (1994) developed a sinking procedure for microalgal culture with a viscosity of $0.001 kgm^{-1}s^{-1}$. In fact he assumed that microalgal culture viscosity is the same as water viscosity. It is expected to behave like Newtonian fluids as found in rheograms of typical Newtonian fluids with a constant value of viscosity. However, there is little prospect of determining whether *P. carterae* cell density may affect the culture viscosity or whether the final rheogram will obey Newtonian fluids rules. Therefore, an experimental procedure using typical reheology methodology must be followed to observe and review *P. carterae* culture viscosity. Obtained results also provide a general view on viscometry techniques and can be applied for any other microalgal culture.

A rheometer usually includes an electrical driven motor which can apply a force to the material and measure its response at various times. The simplest method of obtaining

data involved shear flow and is achieved by shearing a fluid between two plates and analysing output through a graph.

2.12 Summary and remarks

A summary of the literature review indicates that:

a) Optimizing the total cost of the biofuel production from microalgae is critical for the commercial feasibility of biodiesel production from microalgae. However, at the moment, none of the processes are suitable for commercial harvesting but more research upon to selecting suitable species, investigating proper harvesting technique will result in obtaining a low cost system with high efficiency.

b) Before coming to a decision as to which harvesting process will be most suitable for biomass production, the fundamental characteristics of cell-size distribution and geometry and viscosity of the suspension must be determined to calculate particle settling velocity. However, there is undoubtedly considerable overlap between physical characteristics of *P. carterae* and its settling behaviour. Thus, these elements are likely to be significant in order to investigate proper harvesting techniques. Moreover, data from laboratory experiments will propose a comprehensive evaluation of theoretical foundation in comparison with trial observation.

c) Although, various methods have been compared in previous studies, it is apparent that flocculation has more potential to save energy and cost and no commercial harvesting by flocculation has been proposed yet (Benemann & Oswald, 1996). Therefore, sedimentation tests, an important part of characterizing settling behaviours, conducted with column tests, must be carried for the target suspension. The main aims of the column test method which will be examined in this work are: settling test are conducted with column test to characterize how the particulate suspension will behave under influence of gravity; It will perform any possible flocculation method as a

cheap primary harvesting; and comparison of Stokes' settling rate and experimental results will indicate a rational design methodology for settling basins and other primary treatments. This method can be used to perform a similar type of analysis at other suspension facilities.

d) As it has been earlier concluded, centrifugation is a potential harvesting technique in microalgal recovery, after flocculation. Yet, the effect of this method on cell disruption has not been determined for *P. carterae*. Therefore, control of shear flow and investigate the deformation of *P. carterae* cell as well as correlation between the applied shear stress and *P. carterae* cell damage are necessary for various harvesting methods.

e) Finally, results of the screening tests will frequently lead to a preliminary evaluation of particle settling velocity. Furthermore, these results will identify feasibility of pre-concentration through flocculation methods.

Next chapter explains employed techniques in the preliminary work. *Pleurochrysis carterae* was selected as the most suitable species with high oil yield and bacterial tolerance. Most of the material and methods employed in this work are similar to the previous studies in microalgal area. However, some of these methods were limited to the laboratory facilities and project budget.

Chapter 3: Materials and Methods

3.1 Source and maintenance of strain

A species of Coccolithophorid algae, *Pleurochrysis carterae*, was obtained from the Murdoch University microalgae culture collection. This species used in the current study was naked (Coccolith free) *P. carterae* cells. The culture collection number was CCMP 647. Modified BG11 was used to maintain the species.

The artificial seawater was prepared with MilliQ water and 1.8% cooking salt as it was recommended for sea water preparation. To reduce error a stock solution of the first three salts was prepared and each time 1 ml is added to the media.

Stock culture with 3×10^4 cell/ml was maintained in 500/1 ml of the culture media (BG11) in 100 ml conical flasks and subcultured every month with fresh media. The recipe for preparing BG11 media has been shown in Table 3.1. The metal mix is made up of two components and is used in all microalgal media. The first component is the FeEDTA mix (Ferric citrate and Na_2EDTA) and the second component is the trace metals mix. To prepare metal mix, FeEDTA was added to 500ml of MilliQ water. pH was adjusted to 7 and solution boiled to dissolve the salts. Then the trace metals mix was added to 500 ml of MilliQ water and was mixed with FeEDTA. One ml of metal mix per litre was added to BG11 media. After autoclaving, the media was allowed to cool overnight and then a vitamin mix was added.

A store cabinet (20-25°C) was used to maintain the stock sample. Initial cell concentration in the stock sample was 500/1 ml.

The first samples of *P. carterae* culture were filled in 50 ml flasks and put on the shaker screen (70 rpm). White fluorescent light (220v, 500-600 photon.m^{-2}.s^{-1}) was provided 12 hours on/12 hours off cycle. The lights were located in the growth cabinet with 20 cm distance of flasks. Prior to each experiment all cultivation systems and equipment including glassware, drums and tubes were washed with warm water (60-80^{o}C) in the washing machine then autoclaved. All media, glasswares and caps were

sterilised in the autoclave for 20 min at 121°C and 103.35 kPa. Unwanted microalgal cultures were killed with chlorine solution before disposal.

Table 3.1 Modified BG11 medium used to grow *P. carterae*

Compound	Stock Solution	Quantity (ml/l)
K_2HPO_4	30.5mg/l	1
$MgSO_4.7H20$	75mg/l	1
$CaCl_2.2\ H2O$	56mg/l	1
$NaNO_3$	1.5g/l	1
SeO_2	0.013	0.5
Metal mix		1
FeEDTA mix		(For preparing Metal mix add FeEDTA mix to500ml MilliQ water then add trace metal mix to the mixture. Increase total volume to 1000 with MilliQ water)
Ferric citrate	6.7g	
Na_2EDTA	14.9g	
Trace metals		
H_3BO_3	2.86g	
$MnCl_2.4H_2O$	1.81g	
$ZnSo_4.7H_2O$	0.222g	
$Na_2MoO_4.2H2O$	0.390g	
$CaSO_4.5H_2O$	0.079g	
$Co(NO_3)_2.6H_20$	0.049g	
Cooking salt	18g/l	
Vitamin mix		
Biotin	1g/l	1
B12	1g/l	

3.2 Photo bioreactor

Growth of *P. carterae* was investigated using 20 L carboys (see Figure 3.1). The drums and media were sterilized for 20 min at 121^{o}C, 103kPa and cooled one day before inoculation. An air bubbling tube supplied CO_2 and mixed the culture. Cultures

were grown at a temperature of 14-16^{o}C and 300-400µmol photon.m^{-2}.s^{-1} light intensity was provided by a rotation of 12day/12night cycle fluorescents light.

Figure 3.1 Photo-bioreactor carboys used to grow *Pleurochrysis carterae*

3.3 Growth parameters

Critical parameters in growth rate including, pH, temperature, light and number of cells were measured everyday over 40 days of growth cycle. Results from daily measurements were plotted in the graphs for analysis at the end of the growth period.

3.4 Cell counting

A general procedure for Microalgae cell counting by a haemocytometer was used for daily cell counting. A very small sample (1 ml) was transferred into a centrifuge tube. Cells were stained by adding a small aliquot (50 µl) of Evan's Blue stock solution (0.1%v/v) to each centrifuge tube and allowed to stand for 5 minutes. At the same time, a haemocytometer and cover slip with 70% ethylene alcohol was cleaned. Approximately 15µ of the stained suspension was added to each slide using leading notch. The chamber had to be fully loaded with liquid by a pipette, as well as some extra channels beside it; it was then placed on the Olympus IX50 microscope stage. Cells were counted at 40×magnification.

3.5 Cell analysis

Two methods were used for optical analysis including light microscopy and Scanning Electron Microscopy (SEM). The feature of each method has been explained in

literature review (2.6.1). The scanning electron microscope was used for grain sizing, surface observation and observing the presence of other micro-organisms such as bacteria. Also using light microscope cell counting, clump cells and dead colonies were imaged during daily study.

Prior to using SEM, samples were prepared in the following manner:

1. Putting samples in fix tissue and EM fixation solution for 35-40 min
2. Washing samples in Phosphate Buffer solution PBS (2×5 min)
3. Washing samples with successive baths in OSO_4 (Osmium tetroxide) with ethanol using the following steps at 15^oC,

Step No.	Time (min)	Reagent
1.	30	OSO_4
2.	5	70% Ethanol
3.	5	70% Ethanol
4.	5	90% Ethanol
5.	10	90% Ethanol
6.	5	95% Ethanol
7.	10	95% Ethanol
8.	10	100% Ethanol
9.	10	100% Ethanol
10.	10	100% Ethanol

Samples were specimen mounted on subs and gold to be observed by SEM. A microscope connected to the computer scanned the samples. Therefore, Samples were inserted by tweezers supplied inside the chamber. Before opening the chamber the pressure had to be brought to atmospheric pressure. A software programme which could provide both soft and hard images had been installed on the computer. Initially lower magnifications ($5000\times$) provide the better scan.

3.6 Cell isolation

Cells were isolated in agar plate and later were grown in pure culture. Agar plate provides an isolated media with all necessary nutrients for growing and isolating *P. carterae* cells. Agar plates were prepared using the following steps:

1. Measure 18 g agar and add to 1000ml BG11 media
2. Boil suspension and stir medium with a clean glass stir rod as agar is melting
3. While wearing heat resistant hand protection, hold the flask or beaker over the flame. Swish or stir the mixture constantly while heating until agar is dissolved.
4. Pour enough melted agar into each sterile plastic petri dish to cover the bottom about 1/8" to 1/4" deep. Replace the lid immediately. Place agar plates on a counter top to cool and set. The agar medium set like stiff gelatine at room temperature
5. After cooling the plates a drop of microalgal culture was streaked across the agar surface with a thin loop of sterile wire
6. Plates were maintained in a growth cabinet at 14-16^{o}C and 300-400 µmol photon.m^{-2}.s^{-1} light intensity.

3.7 Free settling velocity

The rate of the settling can be determined by a column settling test. This method is described and used to characterize the settling properties of the particle particularly in wastewater treatment studies (Wong & Piedrahita, 1999; Metcalf & Eddy, 2003).

A typical settling test includes collecting an effluent suspension sample, placing it in tall clear columns and observing the particle settling over time. Heights of the interface during the settling time were measured and recorded. Data obtained from the column tests was plotted to a graph (Figure 3.2) forming the basis of the subsequent experiments that were conducted to verify the applicability of the predictions to actual field conditions.

Figure 3.2 Graphical analysis of settling curves (Metcalf & Eddy, 2003)

A simple visualizing method for estimating the settling velocity of *P. carterae* was employed in this study. The settling tests were carried out in column tests. The total sedimentation rate was measured in 3 cultures with the cell densities of 2.1×10^4, 1×10^5 and 2×10^6 cells/ml. Every half-an hour the height of the interfaces were measured visually and recorded until transparent overflow suspensions were obtained. The slope of the interface indicates the settling velocity rate of the *P. carterae* cells. The samples were withdrawn from the overflow at the end of experiments to determine the cell concentration of the supernatant by cell counting.

3.8 Viscosity

The viscosity determination was carried out with a VT 550 viscometer which was operated at a steady shear rate (constant angular velocity). A MV1 geometric sensor (suitable for low viscose suspension, working at a medium shear rate) was installed and a temperature vessel was attached to the system. Five labelled samples including pure media, 5×10^4, 25×10^5 cell/ml, concentrated culture (2.4×10^8 cell/ml) and contaminated media were transferred to the geometric vessel. All data was obtained in a constant temperature of 20-22^{o}C. Eight different shear rates (50-300 s^{-1}) were introduced to the system. The machine measured shear stress versus each individual shear rate within 120 seconds. Plotted data in a graph determine viscosity. As was explained in section 2.11 slope of this graph defines the relation between shear stress and shear rate and determines viscosity.

3.9 Centrifuge

Samples were harvested with a Beckman Coulter J2-21ME-1M with applied acceleration factors of 300, 1500, 6000 and $11000 \times g$ (where g is the acceleration due to gravity). The radius of the rotor was 11 cm with 6×400 ml capacity. Cell densities were determined before concentration by microscopic examination at $400 \times$ magnification. *P. carterae* cultures with 11×10^5 cell/ml initial cell concentrations were introduced to the centrifuge. The temperature was fixed at 18^oC for all samples. During the centrifugation process samples of the supernatant were collected to evaluate harvest efficiency and to determinate concentration of cells retained in the supernatant.

3.10 Cell viability

The aim of this experiment is to develop a methodology that permits frequent tests on *P. carterae* viability and obtain a comparison of results using a flow cytometry method versus light microscopy. In order to achieve this objective, viable cells need to be collected and spun at various gravity forces including 300, 1500, 6500 and $11000 \times g$. The concentrated cells should be prepared for the flow cytometry procedure. A method for sample preparation and using a Dickinson 'Facscalibur' flowcytometer located in Australian Water Quality Centre (AWQC) was demonstrated and followed as below:

1. Cells were stained with bisoxonol dye DIBAC4
2. A total volume of 500 µl of DIBAC4 was used for each sample
3. 2 µl of 1 mM DIBAC4 was used in 500 µl.
4. Samples were centrifuged, and then DIBAC was added and leaved for 10 min before experiments.
5. The machine has been set with no differences in fluorescence of the cells for (green florescence), FL-3 was used as threshold.
6. The side scatter detector was set to 335 V and the green fluorescent detector (FL1) set to 500 V. The fluorescence detector FL3 was used as the threshold and set to a value of 289.
7. Sheath fluid was Milli Q water

8. Samples were examined with a total 5000 events
9. Data were examined on scatter-plots of log FSC vs Log SSC using WinMDI version 2.8 software
10. Histograms were plotted for FSC against total events.

3.11 Cell size distribution

A Mastersizer, Malvern 2000, was used to measure cell size distribution. Results from this machine allow understanding of the basic concepts of particle size, the concept of the equivalent sphere and the differences observed in the scattering of large and small particles.

The mastersizer 2000 system is designed such that equal volumes of particles of different sizes produce a similar measured scattering intensity. The size distribution is reported as a volume distribution as this best reflects the sensitivity of the system. Volume distributions are based on the volume occupied by their constituent particles. It should be remembered that all data in this study were analysed based on the volume distribution.

This accurate instrument measures particle diameters from 0.02 to 2000 microns (dependent on the dispersion accessory). Two dispersion accessories at a time were being connected to the optical unit. Each dispersion accessory was provided with a coded measurement cell cassette. When a cell is inserted into the optical unit it is automatically recognised by the system which configures itself to operate with the dispersion accessory.

The cell is fitted with a laser safety shutter override and a half-turn locking mechanism locks the cell into the correct location with a single action. The system is designed to be operated using Standard Operating Procedures (SOPs) to give consistent results between laboratories and individual users.

Sample of 1 liter BG11 media was introduced to machine as blank sample. Particle size distribution results from this sample were used as background of the experiments. Approximately 100 ml of cell culture were added to 900 ml of pure BG11media and

introduced to mastersizer machine. One machine was turned on the standard procedure was followed.

Chapter 4: Results and discussion

The first critical factor for any successful microalgal system is the process of investigating its cultivation and the determination of growth factors (Richmond, 2004). Results relation to *P. carterae* cultivation are available from a previous study (Moheimani, 2005). The main aims of cultivation experiments presented here were to grow *P. carterae* at a laboratory scale, observe possible changes in cultures and cells during the growth cycle and obtain sufficient cells for bench-scale harvesting tests. The results obtained and visual observations from daily bench scale records can provide useful information to investigate the physical characteristics of *P. carterae* cells. This chapter also presents previous results and analysis of *P. carterae* from other research and comparison with the present study.

4.1 Cell identification

Images from Light microscopy and Scanning Electro Microscopy (SEM) method show that *P. carterae* cells are spherical, with a diameter greater than 5 µm and non-motile (Figures 4.1 & 4.2). As shown in Figure 4.2, the cell surface is covered by a layer of polymer, the $CaCO_3$ sheath is missing thus it does not look like the previous *P. carterae* image shown in the Literature Review (Figure 1.2). This could be because of using Osmium tetroxide prior SEM sample preparation. Osmium tetroxide forms a layer to stabilise and strength on the cell surface (Singh *et al.*, 2003).

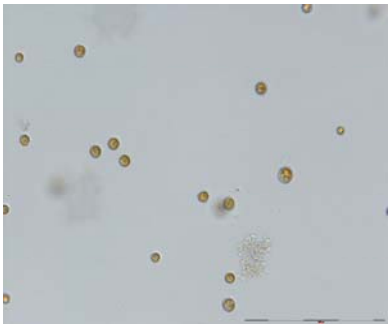


Figure 4.1 Light microscopy image at 400× magnification of *P. carterae* extracted 4 days after inoculation

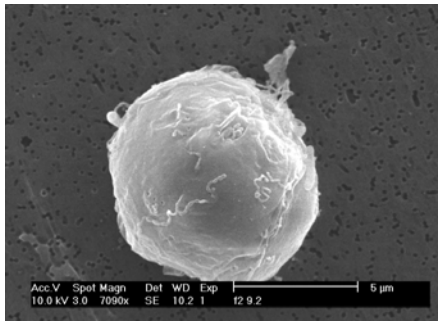


Figure 4.2 High resolution image of *P. carterae* cell (SEM method) from the Murdoch University sample

4.2 Cultivation

Cultures were grown for 15 days in a growth cabinet (14-16ºC, 300-400 µmol photon.$m^{-2}s^{-1}$) until the stationary phase was reached. Results show cells are likely to have a 5-7 day lag phase (Figure 4.3). The maximum cell density of 2×10^6 cell/ml was obtained by the early stationary phase. The colour of the cultures changed from a dark brown at the highest density, to a cloudy light yellow at the end of the death phase. After 30 days, the number of cells had decreased significantly. Once the culture reached the death phase, cells died within 5 days. Therefore, cultures with a high density were transferred to fresh media while they were in the exponential growth phase.

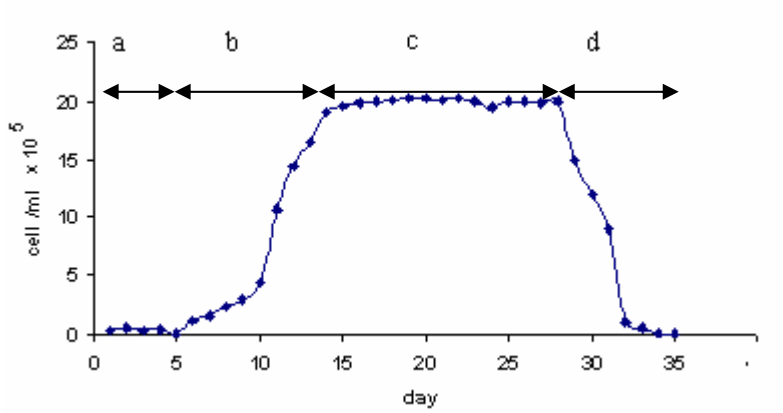


Figure 4.3 Cell density of *Pleurochrysis* cell was measured as number of cells produced per millilitre culture during a light: dark of 12:12. (a) Lag, (b) Exponential, (c) stationary and (d) death phases

The initial pH of the cultures was 7.2 which during the exponential growth phase increased to 9.4, then at the end of the death phase pH dropped to 7.8 (Figure 4.4).

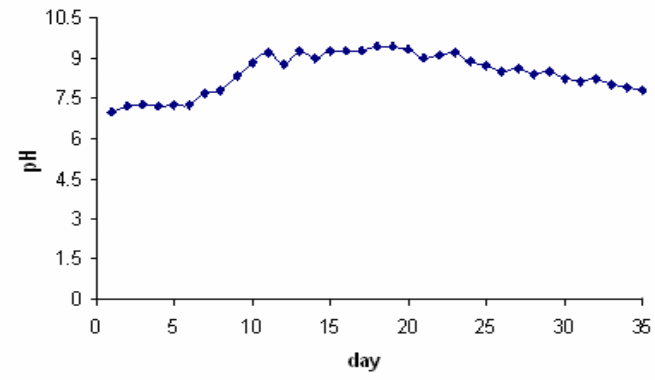


Figure 4.4 pH during the growth cycle

Increasing the initial pH to 8.5 did not produce any significant change in growth rates. As it can be seen in Figure 4.5, this increase only resulted in a longer lag phase of nine days but the final obtained density was the same. At day 17 number of cells in increased pH sample dropped to 1.6 $\times 10^6$ cell/ml. This could be because of cell counting error or clumping cells together.

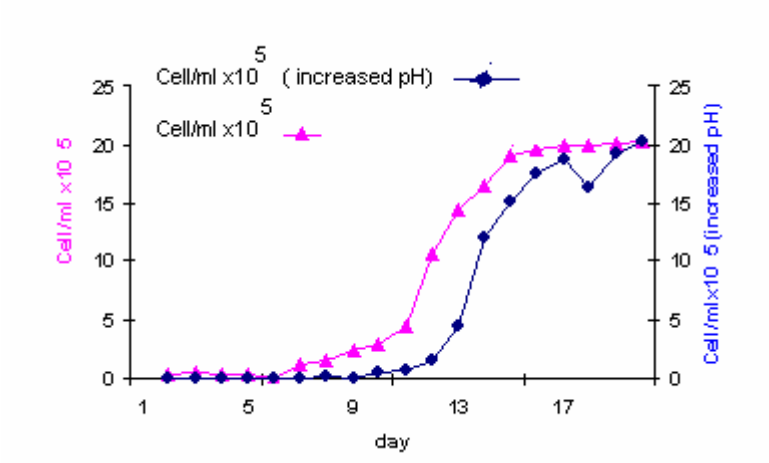


Figure 4.5 Comparison of logarithmic growth after and before increasing initial pH

Five days after inoculation *P. carterae* cells started sticking together, forming colonies (80-120µm) and settled to the bottom of the glassware (Fig 4.6). Cell counting, during this period was difficult. In 20l carboys, the use of air bubbling did not prevent development of clumps, so daily manual stirring was used to re-suspend the cultures. After day five, cells started growing separately. This phenomenon happened again at the end of the stationary phase when the culture had its maximum density.

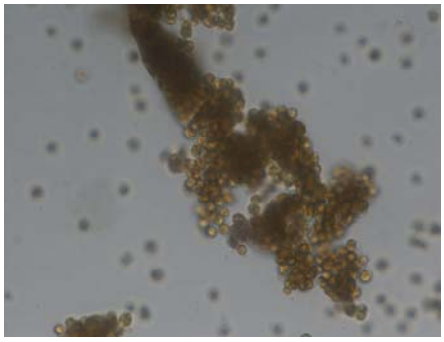


Figure 4.6 Lower magnification of clump cells at day 6

The presence of bacteria did not appear to have any negative influence on the cultures. *P. carterae* cells did not have high tolerance to invasion by other microalgal species and the cells died shortly after being contaminated. Isolated cells were kept on agar

plates. Agar isolation is an accurate method for cell isolation, allowing cells to grow slowly (over about two months). Primary samples of agar cultures showed small micro-organisms growing with the *P. carterae* cells but have not appear to affect their growth.

A previous study showed the maximum cell density obtained from a raceway pond was 1.05×10^6cells/ml (Moheimani, 2005), which was less than laboratory results in this work. As it was mentioned in the literature review (Borowitzka, 1998), closed systems are not suitable for large-scale cultivation of microalgae due to their expensive processes, and provide a less satisfactory basis for commercialisation. Therefore, open cultivation has been recommended for commercial scale work. However, several difficulties in open ponds such as contamination risk and environmental changes will affect the final cultivation productivity. Results from this work confirm that the presence of bacteria did not affect the growth rate, but it should be considered that *P. carterae* cells are competitive against other algal species and continuous maintenance in a large scale cultivation is required to prevent the risk of contamination.

Microalgal cells, during the growth cycle, capture CO_2 from the atmosphere which increase pH of the media (Huretas *et al.*, 2000). Dissolved CO_2, in a *P. carterae* culture, increased the pH of the media from 7.2 to 9.4 and at the end of the growth cycle due to a decreasing number of cells, will reduce pH to 7.8. Similar results have been reported from a raceway pond cultivation (Moheimani, 2005). Increasing the initial pH of the media did not change growth rate, thus indicating that the effect of higher pH on *P. carterae* growth rate is minimal, although the pH range for *P. carterae* growth has been reported between 8-11 (Moheimani & Borowitzka, 2006a). The data shown in Figure 4.3 shows that *P. carterae* has a long stationary phase, although, after reaching the death stage, the cells died within 5 days. Therefore, the cells must be harvested or transferred to a new culture before reaching the death phase.

Pienaar (1994) described the filamentous stage (Apistonema) during the growth of *P. carterae*. He described how cells stick together and make bigger `colonies in the media (Figure 2.3). At laboratory scale of *P. carterae* growth, two filamentous stages

were observed at an early stage after inoculation, and at the end of the growth cycle. These stages could be explained as the Apistonema stage and as a part of the *P. carterae* cycle (after inoculation). Little evidence is available to explain this phenomenon, but it must certainly be considered as a part of the *P. carterae* cultivation process, either on the laboratory or open pond scale.

4.3 Cell size distribution

Results obtained from samples taken during the growth period indicate that the size distribution of *P. carterae* cells depended greatly on the collection time. A shift occurred from small to larger sizes within the growth period. At the beginning of growth, the average cell size and surface area obtained from light microscopy were: cell size, 3.83 µm and surface area 11.7 μm^2. These values increased to 8.8 µm, 61.7 μm^2 and 11.67, 109.46 μm^2 during days 20 and 40. The dramatic changes in average size and surface area are shown in Figure 4.7.

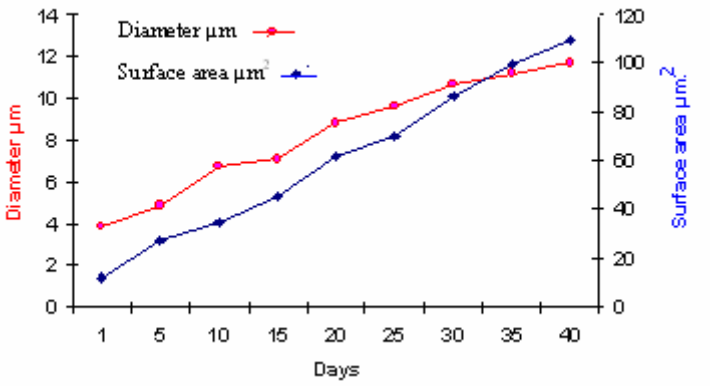


Figure 4.7 Changes of the average cell size and surface area during the growth cycle, using light microscope

Cell size distribution was also assessed by the Mastersizer, during the growth cycle. The results from samples taken at 10 days after inoculation to the end of the life cycle are presented in Figure 4.8. As primary samples with low cell density could not be assessed by the Mastersizer, light microscopy was used to asses the average cell size 10 days after inoculation, when cells grew to about 8 µm in diameter. After 10 days, cultures still contained small cells that were slightly greater than 5 µm. As can be observed in Figure 4.8(a) 10 days after inoculation, the culture contained some

P. carterae cells of 5-11 µm diameter and some particles with diameter sizes between 0.3-2 µm. Sixteen days after inoculation, the cell population of 10 µm diameter size increased. However, some very small particles were still present in the media (Fig 4.8 (b)). Various cell sizes from day 22 and 32 can be seen in Figure 4.8(c). These may include bacteria, immature *P. carterae* cells after division and grown *P. carterae* cells. Thirty two days after inoculation (at the end of the stationary phase), cells started to clump together and make big colonies. These colonies, as was observed with light microscopy, were between 80-120 µm (Figure 4.8(d)).

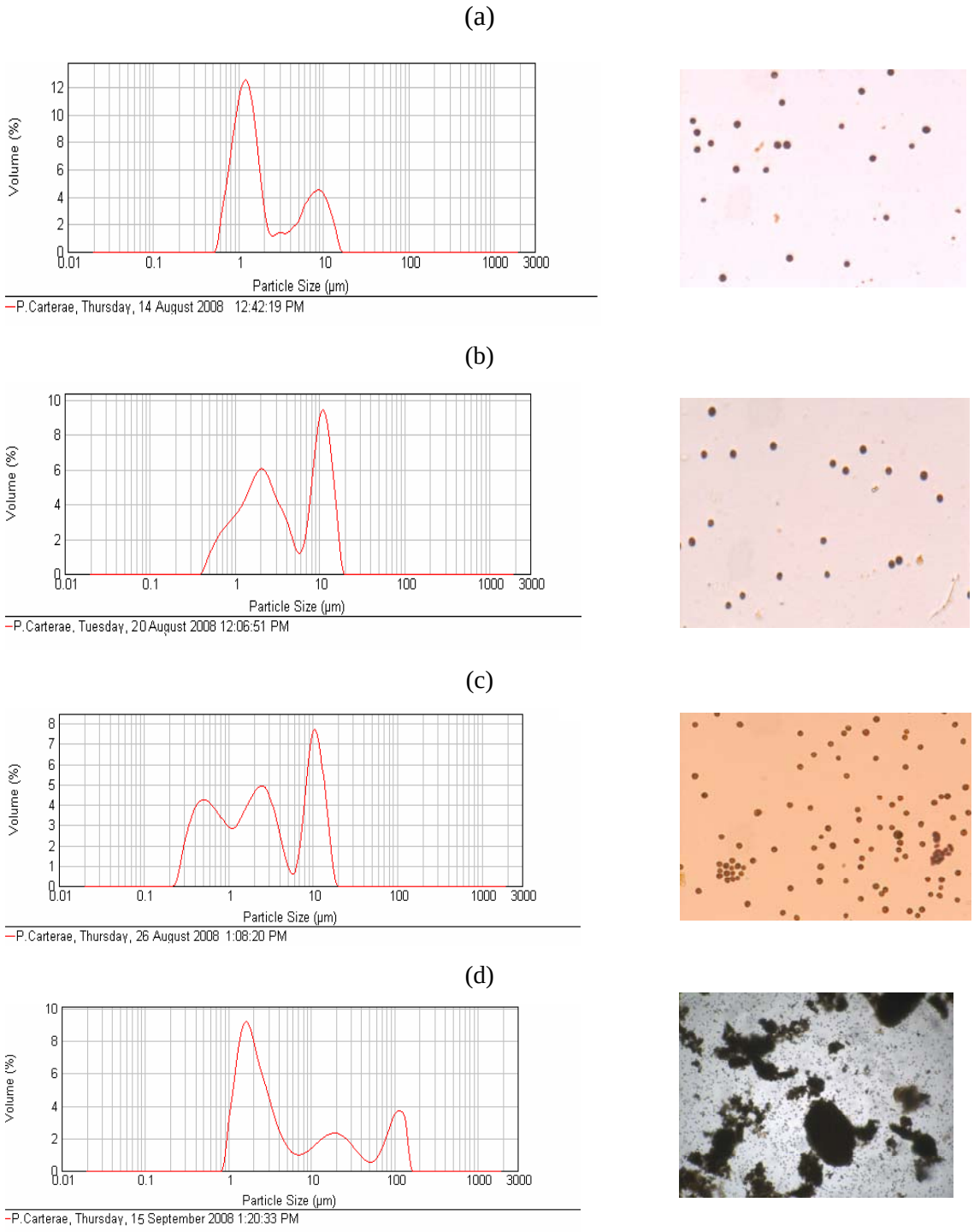


Figure 4.8 Left- Cell size distribution by the Mastersizer during the growth cycle at days (a) 10, (b) 16, (c) 22, and (d) 32 in comparison with, right- light microscopy images

Information available from the literature indicates that the settling velocity depends on the physical characteristics of the suspension and specifically particle size distribution (Eq 2.9). Results from the laboratory cultivation show that *P. carterae* cell diameter increased from 3.5 µm to 11.2 µm during the growth cycle. Furthermore, cell surface area increases dramatically during the growth cycle. These progressive changes in

diameter and surface area could greatly affect the settling rate. Therefore, a different settling rate is expected throughout the various collection times. There is limited information about the settling rate of *P. carterae* suspension in relation to collection times. Metcalf & Eddy (2003) concluded that the settling velocity rate of any kind of suspension is strongly increased by increasing particle size, so an understanding of particle cell size distribution is a key factor in predicting the settling rate. The Mastersizer results emphasise that all samples, depending on differences in growth time, include small particles with size <3 μm. According to the literature review *P. carterae* cells are > 5 μm in diameter (Pienaar, 1994). On the other hand, as was discussed in the cultivation results, agar plates of isolated *P. carterae* cells had some other micro-organisms (more likely to be bacteria). Therefore, the population of small particles in the Mastersizer results could be bacteria present in the culture. These bacteria have been recognised as a potential nutrient for *P. carterae* cells during their growth phase (Moheimani, 2005). Figures 4.7 and 4.8, from light microscopy and the Mastersizer assessment confirm that *P. carterae* cells size and surface area increased over the growth cycle. However, the Mastersizer provides a more accurate method for measuring cell diameter size distribution, not only in the case of a few random samples but also of the majority of cells in the medium. Light microscopy measured size and surface area but a statistically valid analysis of millions particles must be done manually which is not practical.

4.4 Viscosity

During the measurement of shear stress versus shear rate there was no significant difference with increasing cell number. However, high cell concentration after centrifugation and contaminated samples caused a minor change in culture viscosity. Test results obtained with samples at 22^{o}C are shown in Figure 4.9. Data obtained from the viscometer were plotted in graphs where the slope of the function identifies the cultures viscosity. As can be seen in Figure 4.9 cultures displayed Newtonian flow with different densities and have an almost a constant slope of 0.001Pa.S. Increasing cell concentration and presence of other species did not affect viscosity. A summary of obtained results are presented in Table 4.1. As can be seen in Table 4.1 the viscosity of *P. carterae* cultures was a constant and independent of density.

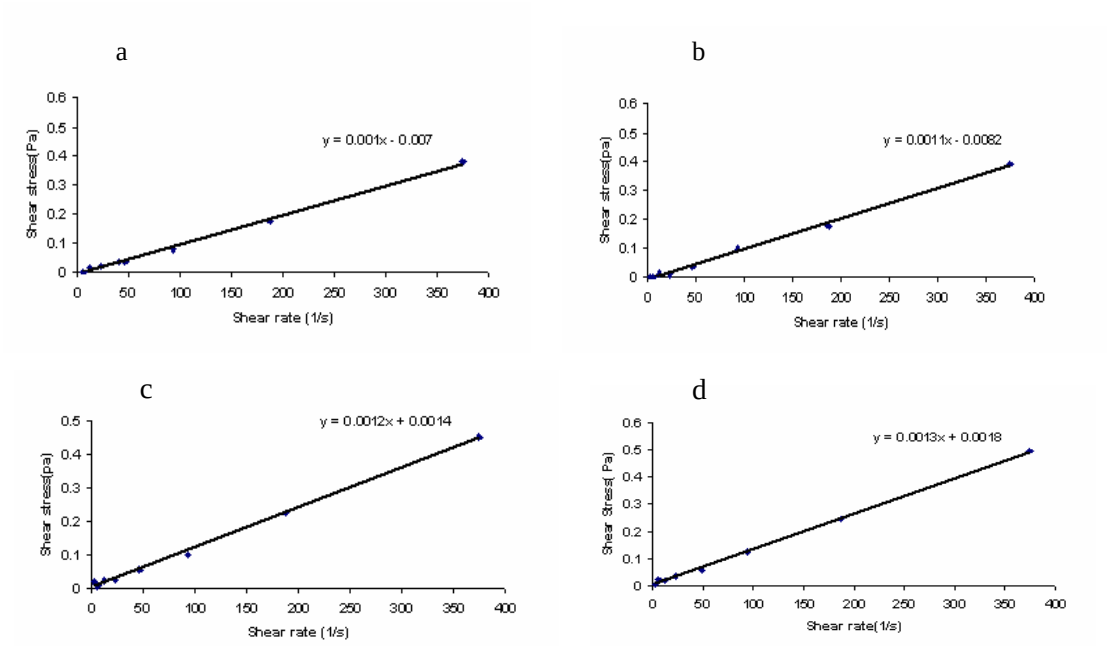


Figure 4.9 Shear rate against shear stress for different cell concentrations (a) pure media, (b) 25×10^5cells/ml, (c) high concentrated cells after centrifugation, and (d) contaminated culture. Slope of the graphs indicates culture viscosity.

Table 4.1 Variation of Microalgal density with cell concentration

Sample	Cells/ml	Viscosity (Pa.S)	Temperature (^oC)	Comment
0	0	0.001	20	Pure media
1	25×10^5	0.0011	20	-
2	centrifuged cells (2.4×10^8)	0.0012	20	-
3	Contaminated (0.2×10^4)	0.0013	20	Culture included Chlorella and bacteria

Viscosity is the quantity that describes a fluid resistance to flow. Viscosity varies with temperature in microalgal cultures, while cell density is the critical factor for measuring medium density (Young, 1994). The literature review (section 2.11) described the proportional relation between shear stress versus shear rate to viscosity. Plotted data from the viscometer indicate Newtonian and non Newtonian fluids. Thus, from the previous results it can be seen that the *P. carterae* suspension, as was

expected from the literature review (Reynolds, 1984; Young, 1994), behaved like Newtonian fluid, with a constant viscosity similar to sea water. This shows that viscosity is a genuine material constant and that the substance is fully characterized by one single numerical. Even highly concentrated biomass, after centrifugation (sample 2), did not display any significant change in viscosity. Microalgae have low dry weight, hence, cell density is similar to that of the surrounding water and likely to have seawater viscosity (Reynolds, 1984; Pienaar, 1994; Young, 1994). Therefore, when selecting any type of separation method, viscosity can be assumed to be seawater viscosity (1Pa.S) independent of cell density or temperature changes.

4.5 Free settling velocity

Results from laboratory experiments and column tests show that *P. carterae* cells flocculate and agglomerate during the settling experiment. This coalescence occurred as the mass of the flocculated cells settled faster. The *P. carterae* cells formed a compressed. Clarification occurred in the upper zone of the columns and the bottom of the columns exhibited both thickening and compression of solids. A middle phase with a lower concentration of cells formed between the clear liquid and the compression zone. This middle phase disappeared and melded with the settled mass at the end of the settling time (after 17 hours). No significant change in settling rate was observed for cultures at different densities. Settling tests and different regions during the experiment are shown in Figure 4.10.

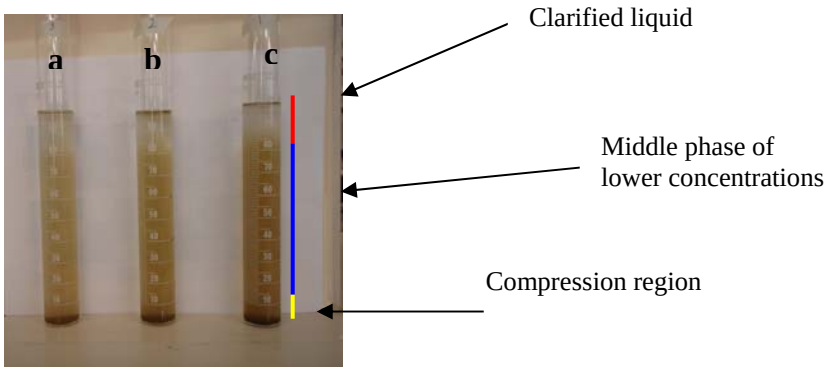


Figure 4.10 settling columns with various cell densities and collection times (a) 2.7×10^5cell/ml, day 7 (b) 1.2×10^6cell/ml, day12 and (c) 2×10^6cell/ml, day 25

At the end of the settling experiment, the cell number of the interface liquid in samples (a), (b) and (c) were 0.5×10^5, 1×10^5 & 1.7×10^5 cell/ml. A general approach

to the graphical procedure for suspension settlement has been set out in text books (Metcalf & Eddy, 2003; Qasim, 1999). This approach was used on *P. carterae* cells using a graduated cylinder as shown in Figure 4.10. The settled depth with a time of sludge settling for various cell densities was also plotted in graphs (Figure 4.11). The slope of the graphs, as it is shown in Figure 4.12, indicates the settling velocity rate of the suspension samples (a) = 1.47, (b) = 1.49 and (c) = 1.5 cm/h.

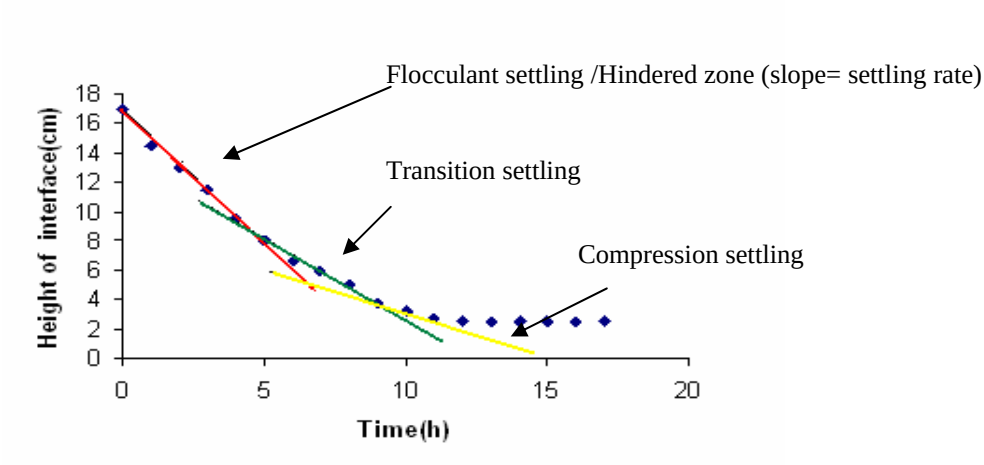


Figure 4.11 Graphical procedure of *P. carterae* suspension settlement. Arrows identify different stages during settling tests. Cited from (Qasim, 1999)

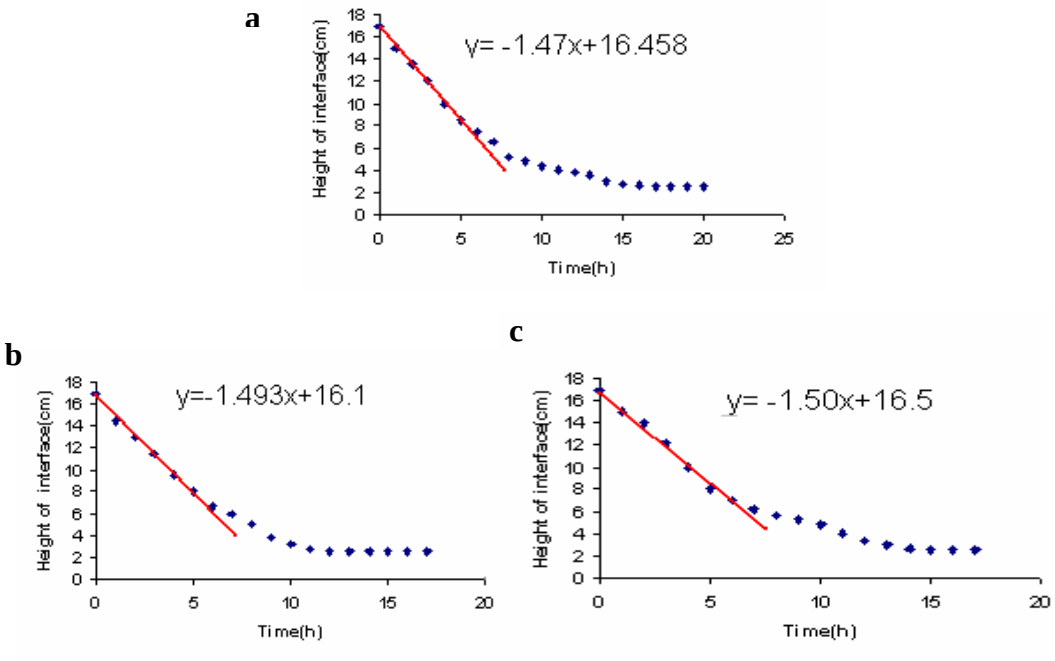


Figure 4.12 Settling curves obtained from settling column tests at different cell concentrations, (a) 2.7×10^5 cell/ml (b) 1.2×10^6 cell/ml and (c) 2×10^6cell/ml

As was discussed in the literature review, the slope of flocculent settling in the hindered zone indicates the settling velocity rate of the suspension. Furthermore, the

settling rate of the interface is a function of particle concentration and their characteristics (Qasim, 1999; Metcalf & Eddy, 2003). Therefore, the settling rate of cultures with different densities can be estimated from Figure 4.12. Little difference was observed in settling rates and the cells settled with almost the same settling rate of 4×10^{-6} m/s at different cell densities. Young (1994) reported the physical parameters of Coccolithophores as follows: average size, 4-20 µm, excess density, 0-50 kg/m^3 and viscosity of medium similar to sea water, 0.001 Pa.s (Young, 1994). The results from *P. carterae* indicate that the average cell size was about 4-12 µm, with a medium viscosity of 0.001Pa.s. If we assume excess density as 25 (Reynolds, 1984) and the average cell size to be 10 µm, the theoretical terminal settling rate of *P. carterae* based on the Stokes' law (Eq.2.9) is :

$$\upsilon_p = \frac{(10\times10^{-6})^2 \times 25 \times 9.8}{18\times0.0001} = 1.36 \times 10^{-6}\ \text{m/s}$$

where,

average cell diameter (d_p) = 10 µm

excess density ($\Delta\rho$) = 25 kg/m^3

viscosity of medium (μ) = 0.001 *Pa.s*

$$\text{Re} = \frac{1000\times1.3\times10^{-6}\times10\times10^{-6}}{0.001} \qquad \text{(From Eq.2.7)}$$

$= 1.3\times 10^{-5} < 1$ Stokes' law yields for this equation

All parameters in the above calculation were measured in this work, except for excess density. As it was mentioned previously, it has been recommended that excess density between medium and *P. carterae* cells be between 0-50 kg/m^3(Young, 1994). On the other hand, settling column test in this study, indicated that *P. carterae* cells settled at the rate of 4×10^{-6} m/s. The average cell size distribution at the end of the growth cycle was 10 µm and viscosity was estimated to be 0.001pa.s. Thus, excess density was calculated as:

$$2.7\times10^{-6} = \frac{(10\times10^{-6})^2 \times \Delta\rho \times 9.8}{18\times0.0001} \longrightarrow \Delta\rho = 73.4\ \text{kg/m}^3$$

Therefore, the excess density between *P. carterae* (naked) and the medium is 73.4 kg/m^3 which was higher than the density reported in the literature review. If we assume the culture has constant excess density and velocity, the only method of increasing the settling rate is to raise the cells' surface area and diameter by flocculation techniques. Previous studies suggested various flocculation methods as the most suitable technique for the initial collection of cells from the dilute media (Mohn, 1988; Benemann & Oswald, 1996; Molina *et al.*, 2002).

4.6 Cell disruption

Light microscopy assessment was carried out to visually examine cell breakage after applying different acceleration forces. Cell disruption is clearly evident in samples (c) and (d) (Figure 4.13) and the number of broken cells is much higher in sample (d). No significant damage was observed in harvested cells after applying acceleration forces of 300 and 1500$\times$g. However, cell damage dramatically increased, after centrifugation at 6500 and 11000$\times g$. A mixture of cells and associated debris can be seen in the sample (Figure 4.13(d)). In this sample, *P. carterae* cells have lost their walls, thus, it is difficult to realize individual cells in suspension and measure the number of damaged cells/ml.

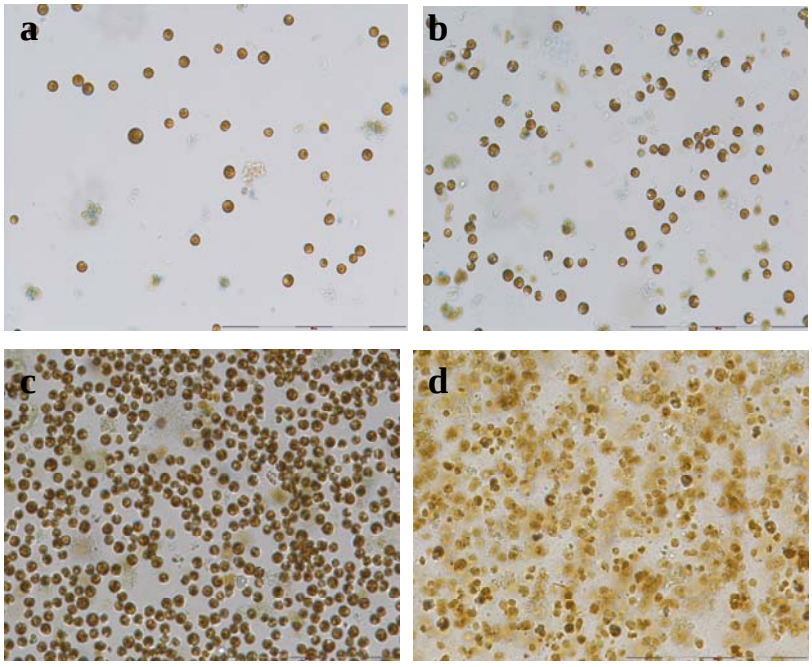


Figure 4.13 Light microscopy slides of *P. carterae* culture at $\times$400 magnification after centrifugation with (a) 300, (b) 1500, (c) 6500, and (d) 11000$\times g$ gravity forces

Differences in cell breakage after the application of acceleration forces, as shown by the results from light microscopy, were evaluated using flow cytometry. Healthy and heat treated cells were selected to be used as controls. Results from flow cytometry showed 94% of cells from the untreated culture were healthy (Figure 4.14(a)) and 87% of cells were damaged after heat treatment (Figure 4.14(b)). It can be observed from the histograms that the cell population moved to the lower side scatter and the left side of forward scatter. The percentage of broken cells increased from 8% after centrifugation at 300×g to 12% at 1500×g then to 13% at 6500×g. Cells concentrated in similar spots to healthy cells (Figure 4.14(a, b &c)). Increasing the acceleration force up to 11000×g, destroyed 31% of the *P. carterae* cells. This breakage transferred the cell population to the lower side scatter and left side of the forward scatter. At 11000×g cells were scattered in different locations and no concentrated population was recorded (Figure 4.14(d)).

As seen from the light microscopy experiments, the number of damaged cells increased by applying higher gravity forces. Surprisingly, higher forces, up to 11000×g did not break cells more than 31%. However, light microscopy images show almost all cells were broken, as a result of 11000×g (Figure 4.13(d)).

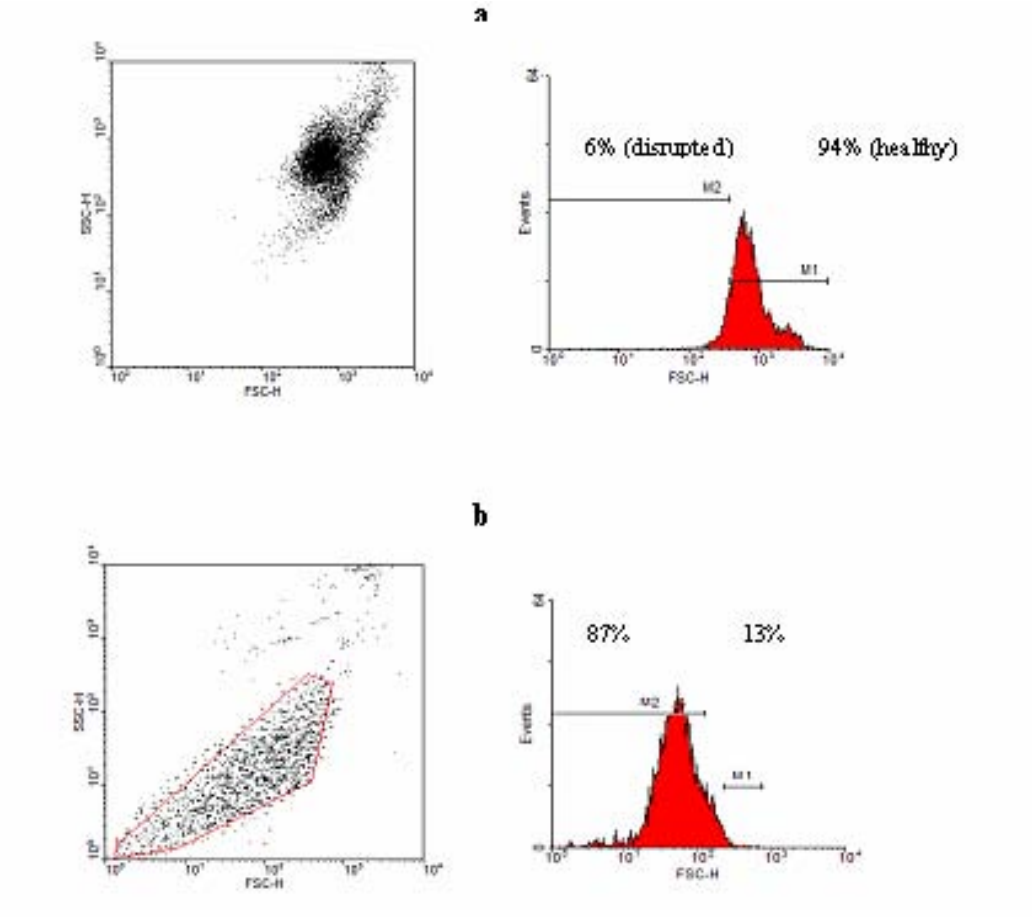


Figure 4.14 Left- Recorded results after assessing samples with flow cytometry, right- representative histograms of (a) healthy sample collected 30 days after inoculation, (b) heat-treated cells

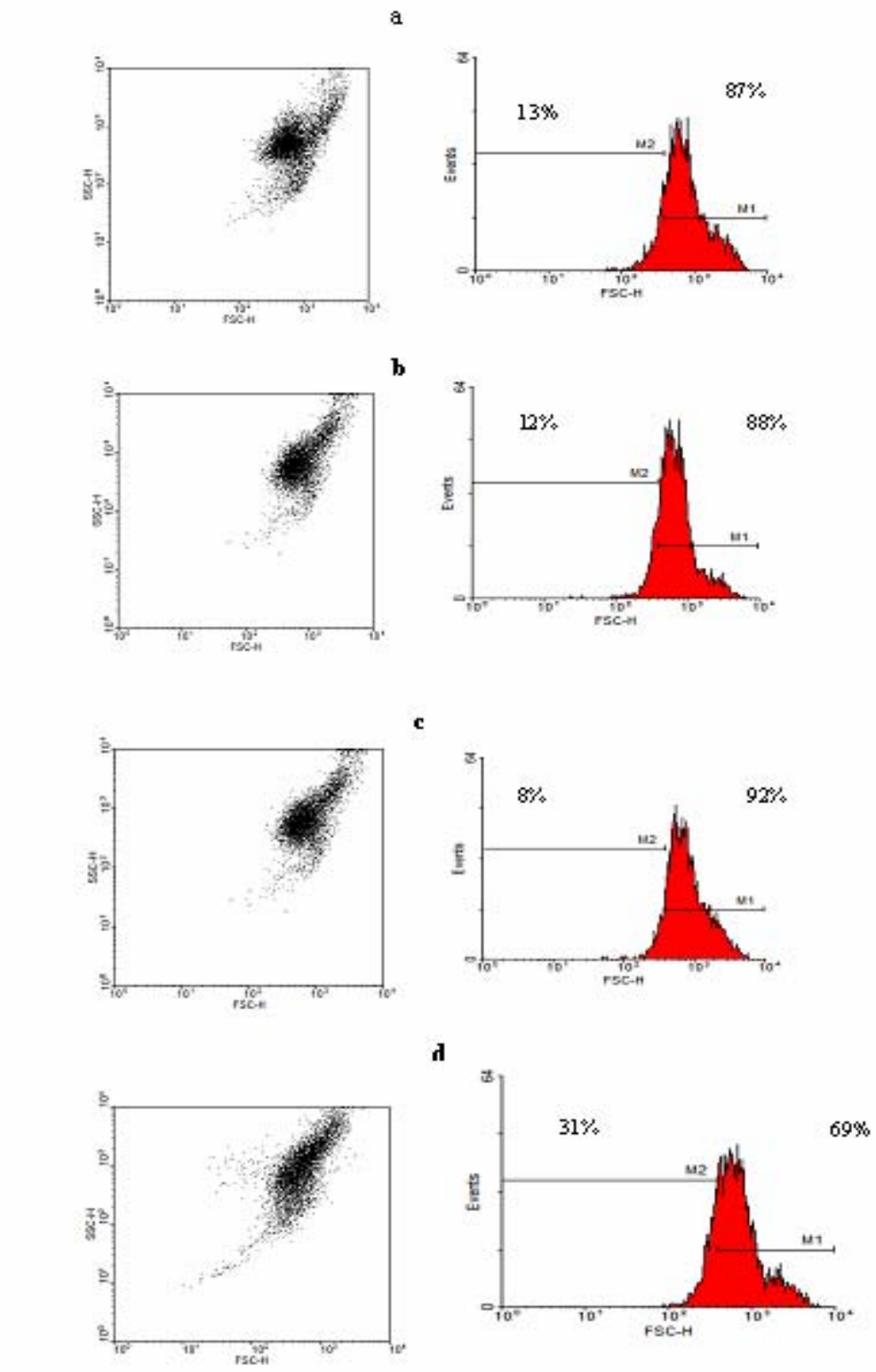


Figure 4.15 Left- flow cytometry records of centrifuged cells, right- histograms analysis of cell damaged after (a) 300, (b) 1500, (c) 6500, and (d) 11000×g

Results from flowcytometry method in assessing the *P. carterae* culture indicated that cells are relatively shear insensitive. High gravity forces, however, apply great shear stress, yet, a remarkable number of healthy cells can be found in the suspension after

the centrifugation process. In the present work, *P. carterae* cellular characteristics, such as cell size and internal complexity, were examined and displayed in histograms using a flow cytometry method (Figures 4.14 & 4.15). The magnitude of forward scatter is proportional to the size of the cell. Small cells provide small amount of forward scatter. These small cells appear toward the left and larger cells move to the right side of the forward histogram. Comparisons of damaged cells after heat treatment with healthy cells from the culture show damaged cells have moved toward the left side of the forward scatter. On the other hand, cells are travelling through the laser beam will scatter light at all angles. This is caused by their granularity and structure. Results from cells with less granularity will be plotted in lower side-scatter histogram (Givan, 1992). Harvested cells under a low acceleration force did not have any significant change in side-scatter or forward scatter, while $11000 \times g$ force damaged 31% cell walls, and made a suspension of cells and their inside structure (as it was shown in Figure 14.3(d)). Therefore, the majority of cells with smaller size and less internal granularity have transferred to the left side of the forward scatter and the lower part of the side scatter histogram (Figure 4.15(d)). Finally, plotted data from cells passed through the laser were analysed in a histogram, revealing 31% cell damage. In regard to the flow cytometry, a smaller percentage of cell viability in comparison to light microscopy was observed.

The critical disruption point which was shown with both methods was $11000 \times g$ and higher. *P. carterae* cells have a high level of sensitivity to shear stress. Light microscopy showed a suspension of dead cells after $11000 \times g$ but only 31% damaged cells have been reported by the flow cytometry method. Viability, was described to be a limiting factor in the use of some harvesting methods, specifically, centrifugation (Mohn, 1988; Benemann & Oswald, 1996).

4.7 Discussion

The comparison of growth results from this work and a previous study (Moheimani, 2005) showed *P. carterae* has a similar growth cycle at a bench-scale and in raceway ponds. The maximum cell concentration obtained from the laboratory study was higher than in a raceway pond. The reason for this difference can be explained by

comparing the condition of the cultures in these two systems. The growth carboys were located in the laboratory, under continuous maintenance and sufficient nutrition. They were operating as small closed reactors, but in an exterior system conditions such as, rain, light and contamination will negatively influence growth rate and maximum cell density. This problem has already been reported as one of the main limitations facing the commercialisation of microalgal projects (Borowitzka, 1998). This study of *P. carterae* cells at bench scale showed that these cells grew well in BG11 media with 1.8% salinity, pH 8-11 with a high tolerance of bacteria contamination. As a result, this marine species with its high growth rate could be a suitable candidate for biofuel production, although cell recovery from the dilute media still present harvesting difficulties and the lipid yield of biomass is unknown.

Moheimani (2005) estimated a final cost of *P. carterae* biomass production, depending on cultivation and harvesting methods, to be Aus$7.35/kg to Aus$14.17/kg. He has suggested flocculation as an efficient method for initial harvesting of heavy Coccolithophorid cells thus reducing dewatering costs (Moheimani, 2005), but no specific screen tests have been done to evaluate the feasibility of different harvesting techniques.

The importance of cell size distribution and surface area of particles in harvesting efficiency and methods of improving these parameters was discussed in the literature review (Benemann & Oswald, 1996; Metcalf & Eddy, 2003). Observing cells under the microscope in comparison with the Mastersizer observations showed that the cell diameter and surface area grow during the life period to 11.67µm and 109.46 μm^2. Screening results from this work showed that the harvesting time was a critical factor in cell size distribution. Results from cultivation and cell size distribution show approximately, between days 15-25 (between stationary and dead stage) as the optimum time for cell recovery. Over this period, the culture has maximum cell density and size distribution was achieved. It must, however, be considered that productivity may vary during the growth phase. Productivity of *P. carterae* cells (at the end of their growth cycle) at maximum high concentration has been measured as 16-33.5$g.m^{-2}.d^{-1}$ in a raceway pond (Moheimani, 2005). Therefore, the lipid content of cells must be measured over different collection times to harvest cells with the optimum productivity as well as cell size distribution.

The settling velocity rate can be predicted theoretically through Stokes' law and can be compared with the actual terminal velocity of particles in column tests. Column test modelling of sedimentation is a common technique in water treatment engineering when designing sedimentation basins (Metcalf& Eddy, 2002). Results from the settling column tests showed the settling rate of *P. carterae* cells at different cell densities was 4×10^{-6}m/s which was faster than the predicted rate of 1.3×10^{6}m/s. Because of the advantage provided by this high excess density (73.4 kg/m^3) and fast terminal settling of the *P. carterae* cells, sedimentation would be a suitable harvesting method for low cost biomass recovery process. Experiments from the settling columns indicate that a number of cells ($\sim1\times10^{5}$cell/ml) remained in the effluent. These cells can be cultivated in fresh media. However, little evidence is available to explain the feasibility of recycling the cells and reusing media. As a result, *P. carterae* cells with their high excess density and fast settling rate can initially be recovered by sedimentation methods. However, concentrated cells at the bottom of the columns had a high water content and high moisture content has been determined as a negative effect on the next stage of oil extraction (Demirbas, 2008), and so there is a need for a second dewatering step to obtain satisfactory water content for downstream processing. Filtration and centrifugation were two potential dewatering techniques after pre-concentration, these techniques have been recommended for the microalgal harvesting process (Mohn, 1988; Benemann & Oswald, 1996; Molina *et al.*, 2002). *P. carterae* cells seem unsuitable for filtration methods due to their size and flocculating behaviour culture which leads to membrane clogging difficulties. These issues associated with continuous back washing and the high costs involved in harvesting on a large scale require detailed studies in order to develop possible filtration techniques with less energy input and a maximum dewatering percentage. Centrifugation has the most potential as a dewatering technique after flocculation, particularly for large scale recovery (Molina *et al.*, 2002). Nozzle centrifuges are not suitable instruments for harvesting *P. carterae*. This is mainly because clumped cells clog nozzles and disrupt the continuous process. To date, little study has been done to investigate any practical centrifuge machine for second stage recovery of microalgal cells after flocculation. Hence, further work needs to be done to investigate the potential of centrifugation machines with their minimum need for energy input need. However, cell damage appears as one of the fundamental problems with centrifugation recovery. Further, cell disruption at a high stirring rate has been discussed as a difficulty prior to the

cultivation of *P. carterae* in a raceway pond (Moheimani, 2005). Results from flow cytometry and light microscopy show *P. carterae* cells are strong enough cells to resist shear stresses up to $11000 \times g$. However, cell disruption caused by centrifugation prior to the harvesting process could be an advantage, because broken cells cause release lipid to the medium, thus assisting the lipid extraction process. More studies are required to examine alternative combinations of harvesting and extraction methods so as to reduce the total cost of biofuel production.

Research on production of biofuel from microalgae has received much recent attention (Demirbas, 2009). Biofuel production from feedstock is presently being limited as a result of global crisis concerning food and water sources. On the other hand, CO_2 emission and global warming are still unsolved environmental issues. To overcome these problems, marine microalgae, with their low demand for agricultural land and water, may be potential sources of biomass for biofuel production. Specifically, countries like Australia with sufficient sea water sources, land and sunlight are suitable candidates for the commercial production of microalgal biofuel. However, previous studies reveal that high costs and uncertainty about some aspects of large scale production limit any alternative commercialization, but results from this work confirm that improving different parts of the system (such as was shown in Figure 2.1) will significantly reduce costs and support the plan to produce biofuel from microalgae in commercial scale as a worthwhile project with CO_2 fixation basis. However, few studies have undertaken to estimate and update the economics of such a system from cultivation to marketing. Results obtained in this study confirm that improving different parts of the process will greatly reduce the total cost of the final product. Therefore, further study is required to analyse alternatives that would improve the feasibility of a realistic commercial product. The results and analysis presented here will constitute a worthwhile basis for future work on microalgal projects, specifically, biofuel production from microalgae. The next chapter presents a general summary of this preliminary work and discusses future direction for biomass recovery and cost reduction of biofuel production on a large-scale.

Chapter 5: Conclusion and future work

5.1 General Conclusion

P. carterae has previously been shown to be a suitable candidate for biofuel production because of its high lipid content, fast growth rate and low contamination risk (Moheimani & Borowitzka, 2006b). On the basis of this study, certain conclusions about harvesting can be drawn from the characteristics of the target species. Several characteristics of *P. carterae* cells were screened and tested with respect to their effect on harvesting and the downstream process in relation to biofuel production.

Bench cultivation has successfully identified the growth cycle of *P. carterae* and any change within this period at the laboratory scale has been recorded. The maximum cell density obtained from laboratory cultivation in this study was 2×10^{6} cell/ml. The growth cycle of *P. carterae* cells, as with other micro-organisms have four growth stages including, lag, growth, stationary and death phases. The presence of bacteria in the media did not considerably influence the growth of *P. carterae* but the occurrence of other species had negative effects on the growth of *P. carterae* cells. Daily pH measurement indicated that the pH of the medium increased from 7.2 to 9.4 during the logarithmic growth phase. Changes in initial media pH were found to have a minimal impact on growth rates. The results presented here from laboratory screen testing confirm that *P. carterae* cells can be maintained and grown in marine media with high pH tolerance. This procedure optimises the use of *P. carterae* marine microalgae with low contamination risk and fast growth rate and indicates their potential feasibility of commercial production of biomass.

One of the most interesting results of this study was the increase in cell size during the growth period. Cell size, under laboratory conditions, increased from 3.8 µm to 11.6 µm during 20 days. Moreover, *P. carterae* cells 5-7 and 30-32 days after inoculation were shown to flocculate resulting in large colonies (80-120µm).

The physical characteristics of the *P. carterae* cell and medium, such as cell size distribution and terminal velocity were used to calculate a theoretical settling rate.

Results from settling columns were compared to predicted terminal settling rate. Examinations of settling curves from column tests confirm that *P. carterae* cells settle faster than the theoretically predicted velocity rate. These results together indicate that, a *P. carterae* culture has a similar viscosity to sea water, and naked cells have excess densities of about 73.4 kg/m^3. Cells are likely to stick together and settle quickly. Therefore, flocculation, as the initial stage of harvesting, has the potential to save a large amount of energy in the recovered *P. carterae* cells.

After cell counting in the culture recovered from column settling tests, it was estimated that 91% of cells, without any pre-treatment, and during the 12 hour experiments, settled to the bottom of the columns. On the other hand, as shown in the growth cycle diagram, during the stationary phase, the culture maintains constant cell concentration for about 12-15 days. Therefore, sedimentation in a reservoir can be a potential harvesting technique for initial cell collection, although the high water content of the biomass at this stage is not acceptable for any downstream process. There is a need for further dewatering techniques to achieve a dry biomass for the next step of oil extraction. A recent study in microbial flocculation, particularly using *P. carterae* culture, suggests the potential of microbial flocculation method for the low cost large scale production of biofuel (Lee *et al.*, 2008).

Continuous centrifugation, has been suggested as the second stage of harvesting after flocculation (Molina *et al.*, 2002). However, cell disruption and expensive processing are the main limitations of large-scale centrifugation. *P. carterae* cells after centrifugation with various gravity forces were examined in this study. Flow cytometry and light microscopy results both showed that *P. carterae* cells have a high tolerance against shear stress. Gravity forces less than $6500 \times g$ did not affect cell viability and only a small percent of broken cells were observed in centrifuged samples, however, higher g forces dramatically damaged >30%. It may be feasible to develop a combined stage of dewatering and oil extraction, but more work is needed to evaluate the feasibility of this potential technique as a means of cost reduction.

Results from this study have shown that a preliminary evaluation of the physical characteristics of the target species and the settling velocity rate of the suspension, are extremely important for the investigation of feasible harvesting techniques. Further

results obtained from laboratory tests of *P. carterae* and its physical characteristics indicate that there are possibilities for further optimisation of harvesting processes, but these approaches must be carried out in outdoor systems under natural conditions. Finally, the methods and analysis presented here can be used to perform similar work in future studies

5.2 Future work

The methods and techniques discussed here reveal aspects of the settling behaviour and characteristics of *P. carterae* in relation to the production of suitable harvesting techniques. These results suggest work in several directions. Firstly, more work is required to optimise the growth of the species in an open raceway pond in individual areas and climate conditions. Although, *P. carterae* was considered as the most likely potential species for biofuel production, different microalgal species must be grown and examined in order to improve productivity. Secondly, similar tests for estimating the settling characteristics of *P. carterae* should be done for raceway pond samples as to find the effect of open outdoor cultivation on their settling characteristics. Flocculation results from outdoor cultivation have the potential to significantly identify an initial stage of harvesting using a low energy input. Furthermore, these results can be scaled up to improve the economic modelling of large scale biomass production. Previous studies have suggested various flocculation techniques, but none of them have been examined out of the laboratory under natural growth conditions. A recent study of microbial flocculation suggests a potential method for microalgal cell recovery (Lee *et al.*, 2008). Optimisation of this technique is necessary to examine the feasibility of a cheap flocculation method in outdoor recovery. As it was discussed previously, a second stage of dewatering is required to reduce biomass water content for downstream processing. Centrifugation as one of the most promising methods for second dewatering stage should be assessed by examining a large volume of recovered cells after flocculation. Various centrifuge options are available in the market. Only continuous systems with minimum energy input and low maintenance are suitable for this system. More studies need to investigate suitable centrifuges and estimate the total cost of recovery. Energy input and viability assessment are the

critical factors in assessing the feasibility of a centrifuge for work at a commercial scale.

In addition, CO_2 fixation in the large scale cultivation of microalgae is widely regarded as one of the most promising alternatives for the supply of a sustainable source of biofuel that can support action against global warming. Yet, there are many unsolved aspects in the commercialisation of biofuel production from microalgae. These issues should be examined in details and addressed in separate studies. The next section suggests some future work which is required to improve this system so as to aim towards a sustainable source of biofuel on an economical basis.

From the above discussion, it is evident that, there is a considerable overlap between laboratory and outdoor cultivation and harvesting results for biofuel production. Therefore, future studies in the use of outdoor ponds need to optimise the growth and lipid productivity of the target species, investigate waste treatment methods and estimate an economical harvesting system for biofuel production from microalgae on a commercial scale.

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