

**CULTURE OF BOTRYOCOCCUS BRAUNII**

by

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## **DECLARATION**

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

## ABSTRACT

*Botryococcus braunii* is a potential source of renewable energy due to high lipid yield and the ability of hydrocarbon excretion. *B. braunii* 807/2 was successfully grown in outdoor paddle wheel driven raceway ponds for over 5 months. The growth of this alga in tubular photobioreactor (Biocoil) was also successful for up to a month, but was inhibited by high cell sticking to the coils. There was no significant difference in growth rate and biomass productivity between these two systems. The lipid productivity *B. braunii* culture in the open pond was  $0.008 \pm 0.004 \text{ g.L}^{-1}.\text{day}^{-1}$  at a specific growth rate of  $0.06 \pm 0.03 \text{ day}^{-1}$ , and the indoor culture was  $0.04 \text{ g.L}^{-1}.\text{day}^{-1}$  at growth rate of  $0.04 \pm 0.03 \text{ day}^{-1}$ . *B. braunii* was found to have highest photosynthesis under  $30^{\circ}\text{C}$  at  $100 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ . Lower temperature ( $10^{\circ}\text{C}$ ) reduced photosynthesis by 58% at  $6 \text{ mg.L}^{-1} \text{ O}_2$  and 84% at  $2 \text{ mg.L}^{-1} \text{ O}_2$ . *B. braunii* was light saturated at  $100 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ , and no significant photoinhibition was observed even under  $1900 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ . Photosynthesis was inhibited by 40 to 80% when  $\text{O}_2$  was increased from  $2 \text{ mg.L}^{-1}$  to  $6 \text{ mg.L}^{-1}$ . Interestingly increasing oxygen concentrations decreased the dark respiration rate. Increasing temperature by  $5^{\circ}\text{C}$  in open ponds increases lipid productivity, but had no effect on biomass productivity and growth rate. However, lowering pond depth by 5 cm (in winter) had no effect on growth and biomass/lipid yield. It was also found that aerating the pond reduces the  $\text{O}_2$  concentration and this can be potentially useful for reducing photoinhibition.

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## CHAPTER 1. INTRODUCTION

### 1 Introduction to algae

The term 'algae' is Latin for "seaweed", and the world of algae contains a very large group of both prokaryotic and eukaryotic, mainly photosynthetic, organisms of different complexity, colour and shape (Borowitzka 2012). Generally, algae are plant like organisms but have no specialised organelles (i.e. roots, stem), but they may also include other features: containing chlorophyll *a*, carbon fixation through photosynthesis, and living aquatically or in moist environment (Borowitzka 2012). Algae are generally divided into microalgae and macroalgae. Microalgae are mostly microscopic. For example, a colony of *Botryococcus* is only 30-500 µm in diameter (Wolf 1983). On the other hand, some algae are macroscopic. For instance, the biggest alga in the world is a brown alga *Macrocystis pyrifera* (kelp), which can be longer than 50 m (Hoek et al. 1995).

In old taxonomic systems, algae were divided into 4 divisions based on pigment: Rhodophyta, Heterokontophyta, Chlorophyta and Diatomaceae (Dixon 1973). But now, the taxonomy of algae has changed a lot due to further understanding of their ultrastructure and their genomes (Borowitzka 2012). In the new system, the eukaryotic algae are divided into: Glaucophyta, Rhodophyta, Cryptophyta, Heterokontophyta, Dinophyta, Haptophyta and Chlorophyta. Prokaryotic algae are the Cyanobacteria which is also known as blue-green algae. The algae not only have taxonomic diversity, but also they contain varieties of useful compounds. In the following section, the application of microalgae will be generally discussed.

### 1.1 The application of microalgae

#### 1.1.1 Nutrients and healthy products for humans

##### Protein source

The same as any other organism, a certain part of microalgae biomass is protein. These microalgae proteins are generally rich in cysteine and methionine (Leveille et al. 1962). Microalgal amino acid composition can be very different depending on

species, growth condition and life cycle (Tindall et al. 1977; Wheeler and Stephens 1977).

Some microalgae such as *Spirulina* can have a very high protein content (Olguin et al. 1994). Therefore, microalgae can possibly be used as a substitute source of protein for human food or animal feed stock (Becker 2007). One advantage of microalgae is that they have higher protein content compared to other sources of biomass (Becker 2007). For example, *Dunaliella salina* protein content can be as high as 57%, which is higher than meat (43%), soybean (37%) and milk (26%) (Spolaore et al. 2006).

To satisfy human food safety requirement, protein from algae must not have any negative side effects on human body. One potential issue with microalgae is the contamination with heavy metals. This is due to the fact that some microalgae can actively absorb heavy metals and accumulate them in the cell (Çetinkaya Dönmez et al. 1999). Luckily, heavy metal was not detected in animal tissue after feeding with microalgae (Yannai and Mokady 1985).

### **Vitamins and derivatives**

Similar to higher plants, some algae can biosynthesize vitamins (Hirschberg 1999). The content and types of vitamins have been previously reviewed by Borowitzka (1988b), and high value vitamins such as B<sub>12</sub> and E, have the potential for commercialization (de Jesus Raposo et al. 2013). Some fresh water microalgae (i.e. *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus obliquus*) can release water soluble vitamins to the culture medium which can be collected and purified easily (Aaronson et al. 1977).

One commercialized algae product is  $\beta$ -carotene, which is a derivative of vitamin A. *Dunaliella salina* has been grown as a commercial source of carotenoids for the last 30 years (Borowitzka et al. 1984). The main uses of carotenoid are: a) strong antioxidants (Murthy et al. 2005; Sachindra et al. 2007; Hu et al. 2008), b) natural pigments in food and feed (Bauernfeind 1972) and c) potential anti-cancer function (Nishino et al. 2002; Hosokawa et al. 2004). The largest plant (lagoon) for  $\beta$ -carotene



production from *D. salina* is located in Western Australia, and the overall cost is very low compared to other microalgae production (Moulton et al. 1987; Borowitzka 1991).

Another commercialized product from algae is astaxanthin, this is a derivate of carotene and produced from *Haematococcus pluvialis* which has about 3% astaxanthin content in dry weight (Lorenz and Cysewski 2000a). Astaxanthin is used as: a) strong antioxidant in healthy products, and b) as additive in aquaculture feed to it improve fresh color of prawn and salmon (Higuera-Ciapara et al. 2006). For commercial production, 25,000L scale outdoor photobioreactor for *Haematococcus pluvialis* has been proposed, and the productivity of *Haematococcus* can be  $9 - 13 \text{ g.m}^{-2}.\text{day}^{-1}$  (Olaizola 2000).

### **1.1.2 Application of lipid from microalgae**

Microalgae contains different lipids (i.e. free fatty acids, triglycerides and hydrocarbons). These compounds have a wide range of potential commercial applications:

#### **Biofuel production**

Some algae such as *Botryococcus* can produce long chain hydrocarbons (Marlowe et al. 1984). These hydrocarbons can be hydrocracked into shorter chain for producing fuels such as kerosene (Lupton and Traynor 2010). However, this is a very slow growing microalga and studies on achieving higher hydrocarbon productivity are still limited. Some growth and lipid productivity figures of this microalga will be introduced later in this chapter.

On the other hand, triglycerides and fatty acids from other algae can be converted into biodiesel (hydrocarbon) by transesterification (de Boer et al. 2012). One of the main limitations for biodiesel production from microalgae is the overall lipid productivity (Griffiths and Harrison 2009). Therefore, there is a worldwide interest in enhance the lipid productivity of targeted species of microalgae. For instance, there

have also studies focused on genetic modification to alter of lipid synthesis metabolism in the microalgae (Sato and Moriyama 2007).

### **Health and medical products**

Fatty acid from algae can be either saturated (SFAs), mono-unsaturated and polyunsaturated (PUFAs), and some of them can be use as health food products. For example, arachidonic acid (AA) and eicosapentaenoic acid (Cepák and Lukavský 1994) are two important PUFAs for human nutrition which contain  $\omega$ -6 and  $\omega$ -3 respectively. Additionally, several other products like docosahexaenoic acid (DHA), palmitoleic acid, oleic acid, linolenic acid, and palmitic acid are also commercially produced (Sousa et al. 2008; Plaza et al. 2009).

Sterols are also useful products from microalgae (Avivi et al. 1967; Patterson 1971). Sterols can be used to synthesis steroid hormones and its derivates. For example, stigmasterol and cholesterol are two sterols which can be obtained from brown algae (Vahouny et al. 1983; Kaniyas et al. 1992) , green algae (Ikekawa et al. 1968) and red algae (Tsuda et al. 1957).

### **Waxes**

Microalgae can be substitute waxe sources. For example, some algae like *Euglena gracilis* can synthesis wax esters from paramylon when shifted from aerobic to anaerobiosis (Inui et al. 1982), and the content can reach 50% of dry weight (Inui et al. 1982, 1983). The green alga *Chroomonas salina* was also found to produce wax ester when grown photoheterotrophically using glycerol (Antia et al. 1974).

## **1.1.3 Application in agriculture**

### **Farming**

Algae can be added into soil as biofertilizers. Nitrogen is one of the most important elements in agriculture production. Furthermore a big proportion of farming is nitrogen limited (Vitousek and Howarth 1991). Usually, nitrogen is supplied as fertilizer like urea, or it can be naturally produced by N-fixing bacteria like *Rhizobium* (Peoples and Craswell 1992). Similarly, heterocystous nitrogen-fixing

blue-green algae (*Cyanobacteria*) can also produce nitrogen fertilizer (Benemann 1979) which can be added into soil in a process known as algalization. Researchers have shown that, the algalization can increase the yield, nitrogen content, and the growth of rice (Aiyer et al. 1972), and even contribute to germination of seeds (Nanda et al. 1991).

Algae can also be used as soil conditioners in agriculture to reduce soil erosion, enhance aeration and water retention (Borowitzka and Borowitzka 1988a). It has also been shown that the crust of blue-green algae covering the surface of soil can keep the moisture and minimize soil erosion (Booth 1941). Also in rice production, the existence of *Azolla* can provide extra oxygen to root system of rice and prevent the rise in pH by releasing more CO<sub>2</sub> (Mandal et al. 1999).

In addition, algae are also used as sources of plant growth regulators (PGRs) affecting the growth of plants. For example, plant hormones were found in a seaweed product (Crouch and Staden 1993). These hormones can adjust the growth similarly in algae and higher plants (Bradley 1991). For instance, it is shown that addition of microalgae biomass can enhance the growth rate and development of orchard (Virág et al. 2011).

### **Aquaculture**

Algae have been used in aquaculture for many years. The application of several microalgae has been reviewed and evaluated by Borowitzka (1997). The algae are used mainly for 2 purposes in aquaculture: a) as a food source and b) as a feed additive.

In natural environments, some fish and shellfish live on microalgae and other plankton. Therefore some microalgae can be grown as feed (e.g. prawn) (New 1990). However, the cost of algae biomass production is still very high compared to natural food resource, thus an economical culture needs to be established for wider application in aquaculture (Borowitzka 1997).

Another application in aquaculture is feed additive, the algae containing carotenoids or other derivatives (e.g. astaxanthin) are fed to prawns (Yamada et al. 1990) and

salmon (Foss et al. 1984; Torrissen 1986) to make color of the meat more "red". One commercialized alga for astaxanthin production is *Haematococcus pluvialis*. The content of astaxanthin can be 1.5 - 3% of dry weight (Borowitzka 1992; Lorenz and Cysewski 2000b).  $\beta$ -carotene from *Dunaliella salina* (Sommer et al. 1991) are also used commercially as pigment in shrimp and other crustaceans industry.

### **Livestock**

In animal production, algae have the potential to substitute the protein supplement from soybean or other grains. For the past decades, some trials on the feeding of chickens, sheep, cattle and pigs have been widely tested. It was found that ruminants like cattle and sheep feed on grass and can digest algae better than chickens because their digestion system can break cell wall easily (Mertens 1993; Wilson 1994).

Various microalgae were used to feed the chickens. However, most microalgae showed a very poor feed efficiency ratio (feed consumed mass / weight gain mass) which was below 5:1. This is possibly due to insufficient digestion of unprocessed algae material (Borowitzka and Borowitzka 1988a). It was also found that microalgae can substitute up to 25% total protein from soybean, and such partial substitution by microalgae would not affect egg production of chicken (Lipstein et al. 1980). However higher proportion of microalgae in the feed that can also limit the growth of chickens (Lipstein and Hurwitz 1980; Lipstein et al. 1980; Mokady et al. 1980).

Additionally, the feed can only be partially substituted by algae biomass (e.g. *Chlorella*), because the digestibility on carbohydrate decreased with higher algae material content in food (Hintz et al. 1966; Davis et al. 1975). One study finally that 20% algae biomass was the highest content in food without a decrease in digestibility (Calderón Cortés et al. 1976).

Pigs are the most successful animal with high algae material content feed (Witt et al. 1962). From the tests with chickens, cattle and sheep, it can be noticed that any content beyond 20% algae biomass in feed will result in lower feed efficiency ratio. During a series of trials in Germany, results showed that there was no negative effect

on growth of pigs when the algae biomass (*Scenedemus*) was as high as 75% (soybean and fishmeal 25%) of total feed (Witt et al. 1962).

#### **1.1.4 Application in wastewater treatment**

During industrial and agricultural processes, waste residues containing heavy metals, nutrients and greenhouse gas are intensively generated. The following will introduce some applications of microalgae in waste treatment.

##### **Metal removal**

Fresh algae culture, pretreated (e.g. dried) algae biomass or immobilized algae biomass can absorb and accumulate heavy metal ions to very high concentrations (Gadd 1988; Gadd 1990). The application of algae in waste treatment including heavy metal, toxicant metal, and organic metal compounds has been summarized and introduced(1998).

Heavy metals: *Chlorella vulgaris* is one of the species which can be used for waste water treatment. This alga can absorb metal ions of Cr, Fe, Cu, Zn, Pb and Hg (Mehta and Gaur 1999; Slaveykova and Wilkinson 2002; Li et al. 2012). Usually, the ion is rapidly absorbed by microalgae which is considered as a passive process, then the ion will be processed by the cell through metabolic activities (Trevors et al. 1986). The absorption is affected by pH, and with each metal having its optimal uptake pH (Harris and Ramelow 1990). Higher cell density can lead to higher absorption efficiency (Mallick and Rai 1993). The cell wall absorption ability of *Chlorella* may due to the sugars, uronic acids, glucosamine and proteins on the cell wall which provide potential binding sites for heavy metals (Blumreisinger et al. 1983; Darnall et al. 1986; Simmons et al. 1995). This means that dried or dead biomass which still contains those compounds can be also used for heavy metal removal. Furthermore, to reduce the need of harvesting the culture and cost for culture maintenance, immobilized culture algae in polyacrylamide matrices has also been proposed, and some studies have shown successful heavy metal removal by immobilized *Chlorella* (Harris and Ramelow 1990; Costa and Leite 1991; Robinson and Wilkinson 1994).

Toxicant metals: As, Sb, Bi are three toxic metals from industry and mining activities. If these metals leak to natural water, they can be accumulated through food chain which further threatens food safety (Reglinski 1994). Some algae can bioaccumulate these metals, for example, *Chlorella pyrenoidosa*, *Chlorella ovalis* (green algae); *Oscillatoria rubescens* (blue-green algae); *Phaeodactylum tricorutum*, *Phaeodactylum* sp. and *Skeletonema costatum* (diatoms) are amongst species which can absorb these ions efficiently (Lunde 1973).

### **Nutrient removal**

Removal of nitrogen and phosphate: The domestic sewage contain high loads of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  which can be used by algae as nutrients (Hemens and Mason 1968). To avoid the harvesting step of the biomass processing, immobilized algae biomass has been proposed to remove N and P from sewage. For example, bead-shaped K-carrageenan as matrix was used (Kierstan and Coughlan 1985) to immobilize microalgae such as *Chlorella vulgaris* (Lau et al. 1997) and *Scenedesmus* (Chevalier and de la Noüe 1985). In application of immobilized *Chlorella vulgaris*, it was found that over 90% of N and P in sewage were removed within 72h which is almost 2 times more efficient than free culture with no apparent inhibition on growth (Lau et al. 1997).

Algal-bacterial BOD removal: In waste treatment, bacteria are grown to degrade organic compounds (Mitsui et al. 1985; Fetzner 1998). In this process, the sewage usually requires aeration to provide adequate  $\text{O}_2$  for bacteria growth. Microalgae can produce  $\text{O}_2$  by photosynthesis, and bacteria can release  $\text{CO}_2$  for algae growth. Therefore, a "photosynthetic aeration" between bacteria and algae can be established to maximise energy efficiency. There are some successful trials with this technique. For instance, several combinations of algae (e.g. *Stichococcus minor*, *Chlorella* sp., *Scenedesmus quadricauda*, *Nostoc* sp., *Phormidium* sp. and *Scenedesmus obliquus*) and bacteria (e.g. alcanotrophic bacteria, *Rhodococcus* sp. and *Kibdelosporangium aridum*) have been tested to remove organic compounds and heavy metals with success (Safonova et al. 1999; Safonova et al. 2004). More importantly, the BOD and COD were reduced dramatically by 97% and 51%, respectively (Safonova et al. 2004).

## **Flue gas**

During most industrial processes (e.g. power station), energy is gained by burning fossil fuel which releases large amount of CO<sub>2</sub> mixed with SO<sub>x</sub> and NO<sub>x</sub>. Therefore people are proposing bioremediation to reduce green house gases in the atmosphere, and microalgae have shown great potential for removing atmospheric CO<sub>2</sub> (Moheimani 2005; Narasimhan 2010; Moheimani et al. 2012). In terms of using flue gas from a power station, dissolved CO<sub>2</sub> and NO<sub>x</sub> can be absorbed by algae as nutrients (Doucha et al. 2005). However, SO<sub>x</sub> can lower the pH of the medium, negatively affecting the medium alkalinity which results in lowered growth (Matsumoto et al. 1997). This means that in some cases, SO<sub>x</sub> will need to be removed before bioremediation (Yeh et al. 1985). CO<sub>2</sub> bioremediation by microalgae has been studied intensively and these studies have shown that additional CO<sub>2</sub> in algae culture with constant pH can increase biomass productivity (Goldman et al. 1974; Moheimani and Borowitzka 2011). Additionally, CO<sub>2</sub> fixation by microalgae can be closely related to biofuel production (Brennan and Owende 2010; Stephens et al. 2010), though the feasibility will depend on the scale (Borowitzka and Moheimani 2013b).

## **1.2 Algae cultivation systems**

In biofuel production, the algae will need to be cultured in large scale bioreactors (Chen et al. 2011b). The main algae cultivation systems are open ponds and closed photobioreactors. In the following sections, several types of open systems and closed photobioreactors will be introduced in detail.

### **1.2.1 Open ponds**

There are several types of open cultivation systems for growing algae; these systems can be natural, artificial, mixed and non-mixed. Those open systems are used for commercial production, because they can provide considerable capacity (large-scale) and low-cost operation. In the following section, several open pond cultivation systems are introduced:

### **Lakes and shallow pond**

Natural lakes, which has abundance in certain algae species, can be used as a natural systems for algae cultivation. For example, in the 1980's, *Spirulina* was harvested from Lake Texococo in Mexico (Ciferri 1983). Also, algae can be cultured in shallow pond. To date, the largest commercial algae production plants (~780 ha) are made of several shallow unmixed ponds in Western Australia (Borowitzka and Hallegraeff 2007). This system is used for producing  $\beta$ -carotene from *Dunaliella salina*, and it is only mixed by wind and water flow through several ponds (Borowitzka and Borowitzka 1989). While such unmixed ponds can only provide very low productivity, but they can be operated at very low cost (Borowitzka 1999b).

### **Inclined ponds**

The inclined ponds was originally developed in 1960s (Šetlík et al. 1970), and those ponds have a slope (large wide surface) for culture to flow. Then the culture is pumped up to circle, and the culture is drained into a tank where the culture is mixed and aerated during the night. The advantage of inclined system is that, firstly it can reduce the mixing cost, secondly it can reduce temperature change during the night, thirdly it has large surface to volume ratio. There has been several successful cultivations of *Arthrospira* (Fournadzhieva and Pillarsky 1993), *Scenedemus* (Becker 1994) and *Chlorella* (Borowitzka 1999a) with inclined ponds, and those cultures can reach a productivity of  $20 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$  on average.

Another inclined system is based on raceway type system, but the bottom is modified to a slope (Heussler 1985). Similarly, the culture at lower point is pumped to higher point to form flow. The advantage of this pond based inclined system is less evaporation, and it is easy to harvest biomass from the lower point of the pond where the culture accumulates.

### **Circular central-pivot ponds and mixed ponds**

Circular central-pivot pond is based on a round pond built of concrete, and the culture is mixed by a rotating arm located at the centre, and the length of the arm can be as long as the radius of the pond. The advantage of this system is that it can provide very big capacity which is up to 50 m in diameter and good performance in



mixing; and there are several successful cultivations of *Chlorella* in Japan and Indonesia (Lee 2001).

Mixed pond is similar to circular central-pivot pond, the main difference is the mixing system. The culture in mixed pond is mixed by aeration, therefore the productivity is usually low due to uneven mixing, and the main application of this system is aquaculture feed production (Borowitzka 1997).

### **Raceway ponds**

Raceway ponds are the most widely used system for commercial algae production; the design and technique requirement have been summarized by Borowitzka (2005). This type of system is very economical for construction and maintenance, and it is more feasible compared to closed photobioreactors and most other open ponds (Jorquera et al. 2010).

Raceway open ponds usually require circulation of 20-30 cm.s<sup>-1</sup> to suspend the algae cells (Borowitzka and Moheimani 2013a). To mix the culture in raceway pond, several systems are commonly used, such as air lifts, Archimedes screws, propellers, pumps, water jets and paddles (Borowitzka and Moheimani 2013a). By far, the most favorable mixing system for raceway pond is the paddle-wheel due to its durability and low energy requirements (from 1-2 kW.ha<sup>-1</sup>) (Boyd 1998), and several paddlewheels can be driven by only one motor at the same time (Dodd 1974).

Paddle wheel-driven raceway ponds have been used extensively for many applications since WWII to now (Boussiba et al. 1988; Tredici and Materassi 1992). To date raceway open ponds have been shown to be suitable for mass cultivation of certain species of algae, for example, *Chlorella*, *Spirulina*, *Tetraselmis*, *Porphyridium*, *Chaetoceros*, *Scenedesmus*, *Pleurochrysis*, and *Dunaliella* are amongst the algae grown successfully in open ponds (Borowitzka 1999a; Borowitzka and Moheimani 2013a).

On the other hand, one disadvantage of all open ponds is the potential of contamination. The main contaminants are other species of algae, fungi, bacteria and

protozoa (Sharma et al. 2007). Due to this issue, it can be hard to culture many species of algae in open ponds reliably for long term. One method for successful cultivation of algae in open ponds is by bioprospecting species which are capable of growth at extreme conditions. For example, *D. salina* can grow at very high salinity (Lee 1986; Borowitzka and Borowitzka 1989), and at such high salinity not many contaminants can grow in *D. salina* ponds. Similar method is also applied on other species, for example, *Spirulina* (high alkalinity) and *Chlorella* (high nutrients) (Borowitzka and Moheimani 2013a).

### **1.2.2 Closed photobioreactors (PBR)**

Unlike open ponds, closed PBRs are mainly constructed using glass or plastic. The algae culture is re-circulated by either pump or airlift, and they can be both indoor and outdoor. The close PBR systems usually have the following advantages:

- 1) PBRs have much larger illuminated surface than open ponds (surface area to volume ratio) which contributes to high volumetric productivity and high cell density (Borowitzka and Moheimani 2013a).
- 2) PBRs have the potential of less contamination when the target is to grow a monoculture of algae (Lee 1986).
- 3) PBRs can provide controlled conditions (e.g. light, temperature) to optimize the productivity of the culture.
- 4) PBRs can reduce evaporation dramatically which will consume less water.

On the other hand, PBRs may also have several disadvantages including:

- 1) PBRs usually have poor mass transfer which can cause accumulation of high concentration of oxygen and limitation on CO<sub>2</sub> supply (Torzillo et al. 1986; Richmond et al. 1993; Pulz 2001).

2) There is commonly over heating issues with PBR systems, this is due to the large illumination surface (Morita et al. 2001). This can alternatively result in using a large volume of fresh water for cooling PBRs.

3) The mixing of PBR systems is limited, and PBR consume several times more energy than open ponds at same flow rate (Weissman et al. 1988).

There are several types of closed photobioreactors: flat-plate photobioreactors (Sierra et al. 2008), tubular photobioreactors (Borowitzka 1999a), bubble column photobioreactors (Choi et al. 2003) and internally-illuminated photobioreactors (Ogbonna et al. 1999).

### **Flat-plate photobioreactors**

In application of flat-plate photobipreactors, the algae are grown in a narrow chamber, and the wall chamber can be constructed with hard (e.g. glass) or soft transparent material (e.g. PVC, PETG). The flat-plate photobioreactors can be set both vertically and inclined, and this feature can allow the system use the light more efficiently. To use defuse and reflect light, flat-plate photobioreactors can be set at different angles which will also contribute to higher productivity (Tredici 2010).

The mixing of flat-plate photobioreactors can be achieved by air bubbling, however, to achieve good mixing, the bubbling rate must be high enough. However, high aeration is energy intensive comparing to mixing system of ponds (Bassi et al. 2010). This system has another disadvantage of overheating which may also happen in other closed systems, therefore, water spray and heat exchangers are needed to adjust the temperature (Rodolfi et al. 2009).

The advantage of flat-plate photobioreactors is the potential of scaling up, and one successful example is Flat Panel Airlift (FPA) photobioreactor (Degen et al. 2001). FPA can be used both indoors (Meiser et al. 2004; Schenk et al. 2008) and outdoors (Schenk et al. 2008). For industrial production, the FPA is modified to larger volume up to 180 L (Borowitzka and Moheimani 2013a). Furthermore, this FPA system cost less due to use of plastic membrane for construction.

### **Tubular photobioreactors**

The tubular photobioreactor is one of the PBRs preferred for commercial production (Tredici et al. 2009). They are usually constructed with plastic or glass tubes, and the circulation is provided by pump or air lift systems. Among several types of tubular PBR's, the tubes are arranged in different ways:

- 1) Horizontal (Gudin and Chaumont 1983; Molina et al. 2001).
- 2) Vertical (Pirt et al. 1983; Borowitzka 1999a).
- 3) Inclined (Tredici and Zittelli 1998; Ugwu et al. 2002).
- 4) Conical (Watanabe and Saiki 1997).

Like other PBRs, tubular photobioreactors have the same advantages as mentioned earlier in this section. However, a tubular photobioreactor also have some limitations on application.

In some small diameter tubular photobioreactors, the final oxygen concentration can be supersaturated as high as  $100 \text{ mg.L}^{-1}$  (Weissman et al. 1988). As a result, the culture of outdoor tubular photobioreator is commonly photoinhibited (Zittelli et al. 2013b).

In some tubular closed photobioreactors with high tube diameter (12.5 cm), the light distribution inside the photobioreactor is not even. This means that the deeper part may receive less light due to the shade. This problem may be resolved by improving the mixing (Ugwu et al. 2003; Ugwu et al. 2005).

### **Bubble column photobioreactors and internally-illuminated photobioreactors**

Airlift photobioreactors are mostly lab scale culture systems where the volume can be from 2L (Rasoul-Amini et al. 2011) to 60L (Sánchez Mirón et al. 2002). The system usually consists two parts: a cylinder container and the air pump, and the air is bubbled through the bottom of the reactor to provide turbulence which can further affect light intensity (Sánchez Mirón et al. 2000). One advantage of this system is the good gas-liquid mass transfer. As a result, the dissolved oxygen concentration can be lower than in other PBRs (Sánchez Mirón et al. 2002).

Internally-illuminated photobioreactors are also indoor facilities. This type of PBR usually has an artificial light source (e.g. light tube, LED) which is adjustable in irradiance (Ogbonna and Tanaka 2000) and light color (wave length) (Ogbonna and Tanaka 2000). The light source is located at the center surrounded by the photobioreactor (e.g. Biocoil) which can provide sufficient irradiance and the main purpose is to establish an high cell density and fast growing culture (Lee and Palsson 1994). Another advantage of this system is that using LED light source can cover the high absorbance spectrum of algae, therefore the light transfer efficiency can be remarkably enhanced (Chen et al. 2011a). Additionally, different wave length can also contribute to more biosynthesis of certain compounds like hydrocarbon (Baba et al. 2012) and astaxanthin (Kim et al. 2009).

### **1.3 *Botryococcus braunii***

*Botryococcus braunii* is a fresh water green alga, and is a member of Chlorophyta, Trebouxiophyceae Class, *Incertae sedis* Order, *Botryococcaceae* Family. This colonial alga widely exists in fresh water (Chisti 1980). In 2004, it is found that *Botryococcus* Race A B and L form a monophyletic group whose closest relatives are in the Trebouxiophyceae (Senousy et al. 2004).

The unique feature of *Botryococcus braunii* is the ability to accumulate hydrocarbons in the cell and release them to the external matrix (Metzger et al. 1990). It has been shown that the hydrocarbons in the cell are shorter in chain length than the hydrocarbons in the matrix (Largeau et al. 1980). As reported, the amount of *B. braunii* hydrocarbon can be as high as 86% of dry weight (Brown et al. 1969). This unique feature of *Botryococcus* made it a subject to many studies for bioenergy production. However, the low growth rate of *B. braunii* can limit its biomass and lipid productivity compared to other fast growing microalgae like *Chlorella vulgaris*, and *Scenedesmus* sp (Yoo et al. 2010).

### 1.3.1 Races and hydrocarbon

There are four main races of *Botryococcus braunii*, and each race produces different hydrocarbons (race A, B, L and S) (Kawachi et al. 2012). In A race, the main product is odd-numbered hydrocarbon trienes and n-alkadienes ( $C_{25}$  to  $C_{30}$ ). However, in B race, main product is botryococcenes ( $C_nH_{2n-10}$ ,  $n=30-37$ ). L race only produces hydrocarbon chains of  $C_{40}H_{78}$  (Chisti 1980). S race produces epoxy-n-alkane and saturated n-alkane chains with carbon numbers 18 and 20. Interestingly, the crude oil we are using today is partly from *Botryococcus* and its ancestors millions of years ago. Furthermore, *Botryococcus* is the source of  $C_{27}$ ,  $C_{29}$  and  $C_{31}$  alkanes found in oil shale (Lichtfouse et al. 1994). In most microalgae, the majority of oil product is either triglycerides or phospholipids. These lipids can be converted to biofuels (e.g. biodiesel) by biochemical conversion (Singh and Olsen 2011). On the other hand, one advantages of *Botryococcus braunii* is that its hydrocarbon can be directly hydrocracked into shorter chains such as octane (gasoline)s, diesel, kerosene (turbine fuel) and residual oil (Hillen et al. 1982).

As *Botryococcus* can release hydrocarbons externally (Metzger et al. 1990), thus those external hydrocarbons can be continuously harvested by heptane solvent (Moheimani et al. 2013) or an aqueous-organic biphasic system (Zhang et al. 2013) which is also named as milking. The advantage of this method is that it is non-destructive and has no effect on growth (Moheimani et al 2013).

### 1.3.2 Medium for culturing *Botryococcus braunii*

Several media are used to culture *Botryococcus braunii*, the content of these media is shown in Table 1. The main differences between these media are N, P, Fe source, trace elements and EDTA. As these media are used in cultivation of different strains of *Botryococcus*, a comparison can be made between them to find the optimal medium for a particular strain of *Botryococcus*.

### 1.3.3 Limits to growth and lipid production of *Botryococcus braunii*

The productivity of any algae is limited by several limiting factors (Borowitzka 1998). As shown in Figure 1, metabolites synthesis is photosynthesis dependent (Poulter and Hughes 1977). Therefore, CO<sub>2</sub>, light, temperature, oxygen and nutrient can be limiting factors of lipid and biomass productivity growth. In this section, potential *Botryococcus braunii* hydrocarbon production limiting factors will be discussed:

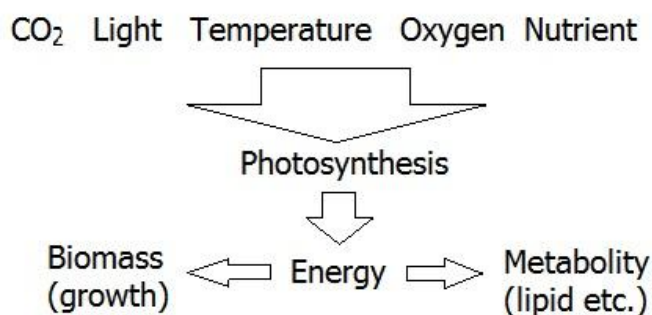


Figure 1. Limiting factors to growth and lipid synthesis of *Botryococcus braunii*.

#### 1.3.3.1 Light

Light is the main source of life on earth through the process of photosynthesis. The photosynthesis in algae is affected by light intensity (Brown and Richardson 1968). In outdoor mass culture, the availability of sunlight is the main growth and biomass productivity limiting factor (Goldman 1979b).

##### Light availability

Light is the photosynthesis driver, and it is the main limiting factor of photosynthesis. As a matter of fact, irrespective of cultivation systems, when algae are grown in with sufficient nutrient and in optimal temperature, light becomes a leading limit to photosynthesis. The concept of light can contain two things: one is the availability of light to the cell, and the other is the light intensity.

In a well mixed culture, each cell is under different irradiance and this due to cell density and the distance of cell to the water surface or vessel wall. Therefore, in a large scale production (open pond) system, the residence time of cells being in

contact with light can be increased by reducing cell density (Myers and Graham 1959) and pond depth (Sukenik et al. 1991).

**Table 1. Media for culturing *Botryococcus braunii*. (Chu 1942; Watanabe et al. 2004; Dayananda et al. 2007)**

	CHU-13	BG11	BBM	AF-6
	mg.L <sup>-1</sup>	mg.L <sup>-1</sup>	mg.L <sup>-1</sup>	mg.L <sup>-1</sup>
KNO <sub>3</sub>	400			
NaNO <sub>3</sub>		1500	250	140
NH <sub>4</sub> NO <sub>3</sub>				22
K <sub>2</sub> HPO <sub>4</sub>	80	40	74	5
KH <sub>2</sub> PO <sub>4</sub>			17.5	10
CaCl <sub>2</sub> dihydrate	107	36	24	10
MgSO <sub>4</sub> heptahydrate	200	75	73	30
Ferric Citrate	20			2
Ferric ammonium citrate		6		
FeCl <sub>3</sub> 6H <sub>2</sub> O				196
Citric acid	100	6		2
NaCl			25	
Na <sub>2</sub> CO <sub>3</sub>		20		
FeSO <sub>4</sub>			5	
EDTA		1	45	
Na <sub>2</sub> EDTA 2H <sub>2</sub> O				1000
CoCl <sub>2</sub>	0.02			4
H <sub>3</sub> BO <sub>3</sub>	5.72			
MnCl <sub>2</sub> tetrahydrate	3.62			36
ZnSO <sub>4</sub> heptahydrate	0.44			22
CuSO <sub>4</sub> pentahydrate	0.16			
Na <sub>2</sub> MoO <sub>4</sub> 2H <sub>2</sub> O	0.084			2.5
MES buffer				400
0.072N H <sub>2</sub> SO <sub>4</sub>	One drop			

However, mixing in photobioreactors may not be efficient enough to guarantee that individual cell can have identical residence of time being exposure to light. In some studies on pond mixing, it was found that longer and the faster rotating paddle-wheel can improve mass transfer and culture mixing a lot, however, the energy cost will

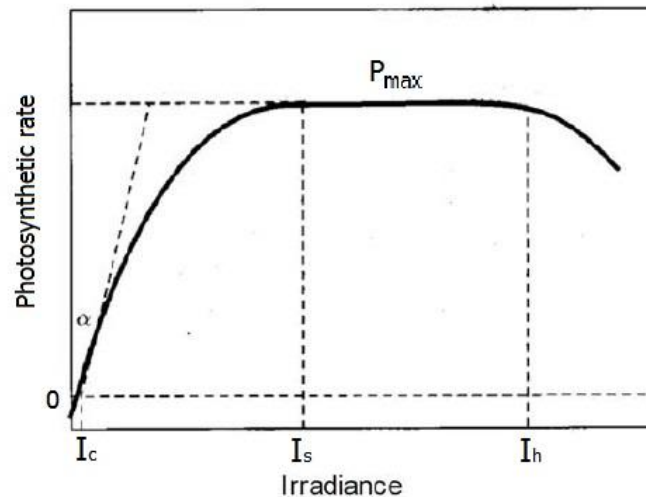


increase and more powerful motor is required (Ahmad and Boyd 1988; Moore and Boyd 1992).

### **Irradiance, O<sub>2</sub> and photoinhibition**

The relationship between irradiance and photosynthesis can be shown by the photosynthesis irradiance (PE) curve (see Figure 2). The increase in irradiance will result in an increase in the photosynthetic rate until photosynthesis reaches the light saturation point (Figure 2). Post light saturation point, any increase in irradiance will not increase the photosynthetic rate. As a matter of fact, at high light photoinhibition can occur (Figure 2). Photoinhibition can especially happen in outdoor cultivation in hot summer, sometime the pond may need to be either covered or the pond depth to be increased or the operating cell density to be increased to reduce light exposure to the cells to prevent photoinhibition.

Photosynthesis is also affected by oxygen concentration (Krause et al. 1994), as oxygen can cause photorespiration especially in C<sub>3</sub> plant like algae (Chollet and Ogren 1975; Lloyd et al. 1977). Oxygen is the by-product of photosynthesis and it can accumulate to high concentration in water (Weissman et al. 1988), therefore it can compete with carbon dioxide to bind with rubisco as rubisco is also an oxygenase (Hatch et al. 1971). Though CO<sub>2</sub> tends to bind easier in this reaction, the high oxygen concentration makes photorespiration inevitable (Lorimer and Andrews 1973). Therefore, it can be also predicted that aquatic plants can have more photorespiration due to accumulation of oxygen in water. On the other hand, absence of photorespiration can even be harmful to plants as this process is considered as protection to C<sub>3</sub> plants from photooxidation (Kozaki and Takeba 1996).



**Figure 2.** The light response curve of photosynthesis (PE curve). The intercept of the vertical axis is the measurement of O<sub>2</sub> uptake due to dark respiration. I<sub>c</sub>= light compensation point, I<sub>s</sub> = light saturation point, I<sub>h</sub> = the irradiance point that photoinhibition occurs (Goldman 1980).

In addition, algae photooxidative damage happens when oxygen captures an electron from NADP<sup>+</sup> to form superoxide radical (O<sub>2</sub><sup>•-</sup>) product which would shifted to H<sub>2</sub>O<sub>2</sub> (Mehler 1951; Strizh 2008). Though O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> are already toxic, according to Fenton reaction, with iron they can further form <sup>•</sup>OH which is extremely toxic (Winterbourn 1995). As those compounds will continuously break photosynthesis membrane and photosynthesis pigments, carbon fixation can be inhibited dramatically (Kaiser 1976).

In some closed photobioreactors, oxygen inhibition is a very serious problem as gas exchange can be very limited within the systems (Weissman et al. 1988). Even in open ponds, dissolved oxygen in the water could possibly inhibit photosynthesis (Sukenik et al. 1987). To date, there has been no feasible solution for reducing of the effect of O<sub>2</sub> on the photosynthesis of algae or any other plant. One possible solution is to culture species with higher tolerance to high O<sub>2</sub> concentrations.

### **Effect of light on *Botryococcus braunii***

In one study, three different *Botryococcus braunii* strains were grown under different irradiances (Li and Qin 2005), and the results showed that, irrespective of light, the specific growth rate and lipid content are very species dependant. Strains have their optimal irradiance for growth or lipid content. For instance, the *Botryococcus* JAP-

836 (N-836 from Japanese National Institute of Environment) has highest specific growth rate under  $300 \text{ W.m}^{-2}$ , however, it has its maximum lipid content at  $100 \text{ W.m}^{-2}$ . (Li and Qin 2005). Except the effect of light intensity on hydrocarbon production, longer illumination period can also increase the hydrocarbon content of *Botryococcus* (Lupi et al. 1994), and this can be easily achieved in an indoor cultivation system. The irradiance can also affect the size of the colony. When the light and nutrient were sufficient, the size of the colony increased; and when light decreased, the size of the colony of *Botryococcus* decreased and the culture produced less external hydrocarbon and polysaccharides (Zhang and Kojima 1998).

The quality of light can also change the shape, hydrocarbon composition and carbon fixation of *Botryococcus* when grown using different light wave lengths (ie. blue = 470nm, green = 525nm, red = 660nm) (Baba et al. 2012). The shape of the alga is more close to grape-shape with red light, and it is least unlike pear-shape under blue light. The hydrocarbon composition under red and blue light is almost identical, and they are both slightly higher than the composition under green light. The culture under blue light had the highest carbon fixation rate, which was 1.8 times higher than the culture under red light. However, *Botryococcus* grown using red light had a higher energy conversion efficiency than blue light.

### **1.3.3.2 Temperature**

Like other organism, every species of algae has its optimum temperature. Growing algae at optimum temperature normally results in higher growth rate. Most algae can survive outside of their optimum growth temperature, but the range of temperature varies in different species. Living at different temperature can affect the alga's metabolic rate, nutrient uptake rate, and chemical contents (Patrick 1969).

Temperature can also influence accumulation of organic compounds. During the day, temperature affects both photosynthesis and respiration rates. Usually, higher temperature can raise the light saturation point, so the algae can transform more light to energy (Moss et al. 1961). Furthermore, within certain range, higher temperature can increase activity of enzymes which related to photosynthesis, sugar transformation and hydrocarbon synthesis. However, higher temperature can also

lead to a higher respiration rate, which means, at higher temperature, organism will consume more organic matter than at lower temperature (Banse 1976; Grobbelaar and Soeder 1985). During the night, and when there is no photosynthetic activity, cells will only respire to release energy for synthesis of other chemical to grow and repair. For instance, there has been some evidence that higher temperature can result in greater biomass loss at night due to more dark respiration (Torzillo et al. 1991).

Another aspect of temperate is fluctuation; different temperature pattern will influence chemical content and growth (Mosser and Brock 1971; Daugherty and Bird 1988). For instance, a bigger diurnal temperature range will benefit sugar accumulation in algae, as the algae produces more sugar during the day and consumes less during the night. While most algae can tolerate a relatively wide range of temperature variation, too much fluctuation may reduce the overall algae biomass productivity.

#### **Effect of temperature on *Botryococcus braunii***

It was found that higher temperature can result in faster growth rate of some *Botryococcus*. *Botryococcus braunii* 807/2 can grow faster at 25°C than 20°C (Ngoon 2011a). In another study, the growth of *B. braunii* 807/1 under 18°C and 25°C are almost identical, but the culture grows much faster under 32°C (Sushchik et al. 2003). On the other hand, the lipid content of *Botryococcus* is higher at lower temperature. For example, in one study, the *B. braunii* 807/2 under 20°C has higher lipid content than at 25°C (Ngoon 2011a). In another study, it was also found that the lipid content of *B. braunii* LB-807/1 at 25°C was 4 times higher than at 32°C (Kalacheva et al. 2002).

Furthermore, temperature can affect *Botryococcus* intercellular lipid composition (Sushchik et al. 2003). This study found that higher temperature can decrease the composition of unsaturated fatty acids (intracellular) in *B. braunii* LB-807/1, but has no effect on extracellular hydrocarbon composition.

### 1.3.3.3 Nutrients

#### CO<sub>2</sub> and pH

CO<sub>2</sub> is the carbon source converted to sugar in all photosynthetic organisms including algae. All algae mass cultures are generally carbon limited and addition of any extra inorganic carbon can increase the biomass productivity and photosynthetic rate (Goldman et al. 1974; Novak and Brune 1985).

However, the CO<sub>2</sub> also affect the medium pH. Uncontrolled addition of CO<sub>2</sub> may most likely result in lowering the medium pH. This low pH can negatively affect algae growth or result in loss of culture (Brock 1973). When carbon dioxide is added to water, it is in a balance of  $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- \leftrightarrow 2\text{H}^+ + \text{CO}_3^{2-}$ , which can be affected by pH (de Rijck and Schrevens 1997). In lower pH, there is more H<sup>+</sup>, and the concentration of H<sub>2</sub>CO<sub>3</sub> and dissolved CO<sub>2</sub> will increase. At higher pH the concentration of HCO<sub>3</sub><sup>-</sup> will increase. Photosynthesis results in an increase in medium pH to as high as pH = 11 during the day due to CO<sub>2</sub> uptake by the algae. This simply means that there is less available "C" for photosynthesis as algae can only utilise CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>. This will also negatively affect the growth of targeted algae as not all algae are bicarbonate users. Furthermore, higher pH can influence precipitation of some of the trace minerals such as Mg, Ca.

#### Effect of CO<sub>2</sub> and pH on *Botryococcus braunii*

In one study, enrichment of 0.3% CO<sub>2</sub> reduced the doubling time of the culture from 6 days to 2 days (Wolf et al. 1985a). That also resulted in shorter chain hydrocarbon production (C<sub>30</sub>-C<sub>32</sub>) rather than long chain hydrocarbons (C<sub>33</sub>-C<sub>34</sub>) (Wolf et al. 1985a). Another trial of adding CO<sub>2</sub> (2%) into *Botryococcus* culture resulted in increasing biomass yield and hydrocarbon content by 100% and 20% respectively (Ranga Rao et al. 2007). In some cases, the added CO<sub>2</sub> can also increase the size of *Botryococcus* colony (Ge et al. 2011).

On the other hand, excessive CO<sub>2</sub> addition can reduce the pH. But in one application, the pH of the culture ranged within  $7 \pm 1$  with additional CO<sub>2</sub> (20%) at an aeration rate of 0.2 vvm (Ge et al. 2011). This means that in the case of adding CO<sub>2</sub> with in low percentage the culture pH does not need to be controlled using a pH stat system.

In an earlier study it was found that the optimal growth pH for *Botryococcus braunii* is pH 8, and the growth rate decreased by 50% at pH 6 (Belcher 1968).

### **Nitrogen, phosphorus and iron**

Nitrogen is a very important element for algae, because it is the main element of protein synthesis (e.g. enzyme). The source of nitrogen can be organic and inorganic; some algae grow better with organic nitrogen but some grow better using ammonium or urea (Antia et al. 1975). Nitrogen uptake can also be effected by other conditions such as temperature (Reay et al. 1999) and light (Macisaac and Dugdale 1972). It is to be mentioned that, though higher concentration of nitrogen can lead to higher maximum cell density, excessive concentration of nitrogen can be toxic to algae (Abeliovich and Azov 1976).

Phosphorus is more related in energy metabolism (e.g. ATP ADP), and also it is needed to synthesise DNA, RNA. Moreover, phospholipids are main part of cell membrane and plasma membrane. Similar to nitrogen, phosphorous uptake by algae is also light dependent (Azad and Borchardt 1970; Nalewajko et al. 1981). Usually, the N:P ratio is more important than concentration, which means, the growth of the alga is more affected by the N:P ratio rather than their individual concentration.

Iron is an essential element involved in photosynthesis and other metabolic activities (Terry and Abadía 1986). The uptake of iron by algae can be affected by the concentration of other metal elements like Zn, Al and Ca (Santana-Casiano et al. 1997). As an example, extra supplemental iron significantly increased the growth rate and biomass yield of *Chlorella* (Liu et al. 2008).

### **Effect of N, P and Fe on *Botryococcus braunii***

Deficiency of nitrogen can increase lipid content (Ben Amotz et al. 1985) and oleic acid concentration (Zhila et al. 2005; Choi et al. 2011) of *Botryococcus braunii*. However, excessive high nitrogen concentration in the medium can also inhibit *Botryococcus* hydrocarbon synthesis (Ohmori et al. 1984). It is also to be noted that some nitrogen sources can be toxic to the culture, for example, the use of  $\text{NH}_4^+$  can decrease the pH of the culture to very low level which causes permanent damage to

the cell (Lupi et al. 1994). Similarly, exposure to  $\text{NH}_3$  can also lead to decreasing hydrocarbon synthesis and carbon fixation. However,  $\text{NH}_4^+$  and  $\text{NH}_3$  can increase the synthesis of some chemicals like alanine, glutamine and amino acids (Ohmori et al. 1984).

Extra P can increase hydrocarbon production; this is not a result of excessive nutrient but the changes of the N:P ratio (Casadevall et al. 1985). Additionally, *Botryococcus* can rapidly take up phosphate and store it intracellularly for later use (Casadevall et al. 1985), therefore this should be taken into account when studying nutrient consumption rates of *Botryococcus*.

In one study, the result showed that high level of iron ion (0.74 mM) can increase the hydrocarbon content of 4 *Botryococcus* strains by 3 - 7% (Yeesang and Cheirsilp 2011). Iron can change the morphology of one *Botryococcus* (BOT-22 race B) strain. Under iron deficiency, the size of the cell is smaller and the shape is conical; with sufficient iron, the size of the cell is bigger and cells stick together to form bigger colonies, and the shape is elliptical (Tanoi et al. 2013).

#### **1.3.3.4 Effect of salinity on *Botryococcus braunii***

*Botryococcus* is a fresh water alga. However at least one strain, showed growth at low salinity (34-85 mM) (Rao et al. 2007). The results showed that small amount of salt can double the content of oleic acid and carotenoids, and slight increase in biomass yield was also observed (Rao et al. 2007).

#### **1.3.3.5 Contamination organism and associated bacteria**

One challenge of algae cultivation for producing any product is to prevent contamination in open and closed cultivation systems (Scott et al. 2010). Contaminants can compete for nutrients, and some microorganism can release chemical compounds like algaecides to degrade other algae (Reim et al. 1974). In general contamination by other organisms lead to the lowering overall productivity of targeted product.

Some algae like *Botryococcus* may have associated bacteria. While the bacterial symbiont can compete for nutrient, those bacteria can potentially provide other compounds which benefit the algae. For example, some bacteria can secrete vitamins and organic chelating compounds which can boost the growth of associated algae (Haines and Guillard 1974). Bacteria can also provide carbon source to algae by releasing CO<sub>2</sub> (Parker and Bold 1961). Therefore, the production of hydrocarbon was considered to be respective to co-existing microorganisms (Murray and Thomson 1977). In one study on *Botryococcus*, the hydrocarbon to biomass ratio was increased from 5.6 to 24.2% with *Corynebacterium* sp (Wang and Xie 1996).

In some studies, it was found that associated bacteria can also negatively affect hydrocarbon accumulation due to the degrading of bacteria (Chirc et al. 1985). For example, *P. oleovorans* is one of the *Botryococcus* associated bacteria which can degrade hydrocarbon (Jones 1972). Therefore, associated bacteria can be positive or negative to hydrocarbon production of *Botryococcus* and if a stable co-existence relationship can be built between the alga and microorganism, the growth, productivity and competitively to other contamination can be increased dramatically.

#### **1.3.3.6 Colony size of *Botryococcus braunii***

Hydrocarbon production is highly related to the size of the *Botryococcus*. This is because there is more need of hydrocarbon when the cells try to stick together to form a colony or become bigger colonies (Zhang and Kojima 1998). Therefore, the factors which can affect colony size will also affect hydrocarbon production.

There are two main factors which possibly affect colony size: one is the light, and the other one is hydrodynamic stress. The colony size will increase if there is sufficient light, nutrients and low cell density. But when the colony size becomes bigger, the hydrocarbon content decreased due to decreasing light availability caused by shading (Zhang and Kojima 1998; Sánchez Mirón et al. 1999).

From the above it can be hypothesised that the *Botryococcus* colonies become bigger when the culture is stressed by strong light or sheer mixing. To accomplish this



protection behaviour, the culture will produce more hydrocarbon as this is the material required to build the extracellular matrix.

#### **1.4 Aims of this project**

As discussed above, due to the high hydrocarbon content *Botryococcus braunii* can potentially be grown for biofuel production. To be grown on large scale *Botryococcus* must be grown in mass cultures in open ponds or closed photobioreactors. Furthermore, the culture must be grown reliably for long term. To date, there is no report on a reliable long term growth of this alga on any culture system. The two main aims of this study are:

- a) To determine the potential of reliable long term cultivation of *B. braunii* in open ponds and closed photobioreactors.
- b) To identify limits to the growth of *Botryococcus braunii*.

In Chapter 3, *Botryococcus* was grown in both open ponds and a tubular closed photobioreactor (Biocoil) to test the reliability of growth and to determine productivity.

In Chapter 4, the limits to growth and lipid productivity (temperature, light and oxygen) of *Botryococcus* were studied.

In Chapter 5, I discuss some possible solutions to contamination and limited productivity of *Botryococcus*. Some further study plans are also discussed.

## CHAPTER 2. MATERIALS AND METHODS

### 2.1 Maintenance and source of strain

*Botryococcus braunii* CCAP 807/2 was obtained from Culture Collection of Algae and Protozoa (CCAP), and maintained at the Murdoch University Algae R&D Centre. This strain was isolated by Jaworski in 1984, and originated from lake Grasmere, Cumbria, England (Hilton et al. 1988). *Botryococcus braunii* 807/2 was cultured using modified CHU-13 medium (Table 2). From the nuclear small subunit (18S) rRNA sequences, CCAP 807/2 has been identified as belonging to the A race of *Botryococcus* (Senousy et al. 2004).

**Table 2. Modified CHU-13 Medium (Yamaguchi et al. 1987)**

Compound	Concentration (mg·L <sup>-1</sup> )	Stock Solution (Concentrated Time)
KNO <sub>3</sub>	400	×1000
K <sub>2</sub> HPO <sub>4</sub>	80	×1000
CaCl <sub>2</sub> ·2H <sub>2</sub> O	107	×1000
MgSO <sub>4</sub> ·7H <sub>2</sub> O	200	×1000
Ferric Citrate	20	×1000
Citric Acid	100	×1000
Trace Element:		
CoCl <sub>2</sub>	0.02	×1000
H <sub>3</sub> BO <sub>3</sub>	5.72	×1000
MnCl <sub>2</sub> ·4H <sub>2</sub> O	3.62	×1000
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.44	×1000
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.16	×1000
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.084	×1000
Adjust pH to 7.5 with KOH solution		

Stock cultures were maintained in 8L carboys and sub-cultured every 21 days, and grown at  $23 \pm 2^\circ\text{C}$ . The cultures were continuously stirred using a magnetic stirrer at 150 rpm. All cultures were also continuously aerated using an aquarium air pump.

Cool white fluorescence lights with a 12h:12h light:dark cycle were used to provide an irradiance of 300  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

250mL conical flasks with a culture volume of 200mL were also used for stock cultures (back up), and all cultures were sub-cultured every 30 days. Cultures were grown at constant 25°C without stirring and aeration at an irradiance of 80  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  provided by cool white fluorescence tube (12h:12h light:dark cycle).

### 2.1.1 Semi-continuous culture

Semi-continuous mode was used to maintain the culture and study the productivity of the culture. In this mode, the culture was maintained at certain volume ( $V_{\text{culture}}$ ) and cell density range between **a**  $\text{cells}\cdot\text{mL}^{-1}$  and **b**  $\text{cells}\cdot\text{mL}^{-1}$  where **b**>**a**.

When the cell density of the culture reached **b**  $\text{cells}\cdot\text{mL}^{-1}$ , a certain volume (**v**) was harvested, and same volume of new fresh medium was added into the culture system resulting in **a** cell density. The harvesting volume **V** was calculated by Eq.1.:

$$V_{\text{Harvesting}} (\text{mL}) = \frac{a (\text{cells}\cdot\text{mL}^{-1})}{b (\text{cells}\cdot\text{mL}^{-1})} \times V_{\text{culture}} (\text{mL})$$

**Eq.1.**

### 2.1.2 Batch culture

A batch culture is a closed system initiated by the inoculation of microalgae under certain environmental conditions (indoor or outdoor). Only a few generations of microalgae can grow before given nutrients are used up. In batch culture, fresh medium was only added at the beginning of the culture, then no more nutrient was added. During the whole period, the culture was mixed constantly by a magnetic stir bar (flask & carboy) or paddle wheel (open pond) at same rate, and cultured at same temperature and irradiance.

## 2.2 Analytical method

### 2.2.1 Specific growth rate

Cell density was determined by using Neubauer haemocytometer. Four replications were carried out for each count. The specific growth rate ( $\mu$ ) was calculated by Eq.2:

$$\mu = \frac{\ln(N_2 - N_1)}{t_2 - t_1}$$

Eq.2.

where  $t_1$  and  $t_2$  were the days that cell density  $N_1$  and  $N_2$  were determined.

### 2.2.2 Biomass determination

The total biomass was measured as dry weight and organic dry weight as follows:

A Whatman GF/C (2.5cm diameter) filter was washed with deionised water three times, then dried at 75°C overnight. The filter was then ashed at 450°C for at least four hours. The filters were then stored in vacuum desiccators over KOH crystals until use.

To measure the biomass yield, the pre-treated filter was first weighed ( $M_{\text{filter}}$ ) on an analytical balance with five decimal places. The filter was used to filter 5mL of well mixed culture. The filter was then dried at 75°C overnight and weighed ( $M_{\text{filter+algae}}$ ). The dry weight was calculated using Eq.3.:

$$\text{Dry Weight (g.L}^{-1}\text{)} = (M_{\text{filter+algae}} - M_{\text{filter}}) \times 200$$

Eq.3.

After weighting, the filter was ashed at 450°C for 5 h and then cooled in a vacuum desiccator to room temperature over night. The filter was then weighed on analytical balance ( $M_{\text{ash}}$ ), and the organic dry weight was calculated using Eq.4.:

$$\text{Organic Dry Weight(g. L}^{-1}\text{)} = (M_{\text{filter+algae}} - M_{\text{ash}}) \times 200$$

**Eq.4.**

### **2.2.3 Total lipid determination**

Total lipid determination was based on Bligh and Dyer (1959), modified by Kates and Volcani (1966) and adapted by Merz (1994). Five mL culture was filtered using Whatman 2.5 cm GF/C filters and stored at -5°C. Prior to determination, the filter was placed in a 4 mL test tube and thawed. About 2mL liquid nitrogen was then added into the test tube. After 30 minutes, a glass mortar was used to homogenize the filter with 1mL solvent mix (methanol : chloroform : DI water 2 : 1 : 0.8 v/v/v). The filter was then transferred to a 10 mL centrifuge tube. Another 4.7 mL extract solvent mix was used to rinse the test tube and glass mortar and also transferred into the centrifuge tube. The extract was then centrifuged at  $1248 \times g$  (3000 rpm,  $r=12.5\text{cm}$ ) for 10 min. The supernatant was then transferred to a 20 mL glass test tube with lid. Then the filter was extracted again with another 5.7 mL extract solvent, and the supernatant was also collected.

Afterwards, the supernatants were combined and transferred into a 20 mL glass tube. 3 mL DI water was added and well mixed by vortex. It was then followed by addition of 3 mL chloroform. The mixed solution was then kept in ~ 5°C refrigerator (dark & cool) over night for phase separation.

The next day, the extract in the glass tube had separated into two layers. The top layer was removed carefully, and several drops of toluene were added to remove any remaining water. The bottom layer was transferred into a pre-weighted 4 mL glass vial, and dried under a stream of nitrogen gas on a heating plate at 38°C for 1 h. The vial was weighed on analytical balance to 5 decimal places.

### **2.2.4 Chlorophyll determination**

The chlorophyll determination was based on the method of Jeffery and Humphrey (1975). Samples were extracted with 90% acetone and determined spectrophotometrically. Five mL culture was filtered onto a Whatman GF/C (2.5

diameter) filter and stored at -5°C in the dark. Prior to extraction, the filter was placed in a 4 mL test tube and thawed to room temperature. Then the filter was homogenized with a glass mortar in 3 mL 90% ice cold acetone in a low light environment. The extract was then transferred into a 10 mL centrifuge tube with lid and centrifuged at  $1248 \times g$  (3000 rpm,  $r = 12.5$  cm) for 10 min at 4°C. The absorbance of the supernatant was measured using a spectrophotometer with 1 cm path length cuvette (20mm  $\times$  15mm, LW) at 664 nm and 647 nm. The content of chlorophyll a and chlorophyll b was calculated from Eq 5 and Eq 6 :

$$\text{Chlorophyll a } (\mu\text{g. L}^{-1}) = 11.93 E_{664} - 1.93E_{647}$$

**Eq 5**

$$\text{Chlorophyll b } (\mu\text{g. L}^{-1}) = 20.36 E_{647} - 5.5E_{664}$$

**Eq 6**

### 2.2.5 Total protein determination

The total protein determination was based on Dorsey et al.(1978). Prior to determination, the following reagents were prepared:

Biuret reagent:     $\text{Na}_2\text{CO}_3$             200 g.L<sup>-1</sup>  
                            $\text{NaOH}$                 40 g L<sup>-1</sup>  
                            $\text{NaK tartrate}$         200 g.L<sup>-1</sup>  
                            $\text{CuSO}_4 \cdot 4\text{H}_2\text{O}$       50 g L<sup>-1</sup>

Folin-phenol reagent: Folin-phenol : dH<sub>2</sub>O 1:1 v

Protein Standard: Bovine serum albumin fraction, 2.5 g.L<sup>-1</sup>

**Table 3. Protein Standard Curve Samples:**

Protein	( $\mu\text{g}$ )	0	50	100	150	200	250	300	350
BSA V	(mL)	0.00	0.02	0.04	0.06	0.08	0.10	0.12	0.14
dH <sub>2</sub> O	(mL)	0.14	0.12	0.10	0.08	0.06	0.04	0.02	0.00
Biuret reagent	(mL)	5	5	5	5	5	5	5	5

5 mL culture was filtered onto Whatman 2.5 cm GF/C filters and stored at -5°C. Prior to determination, filter was placed in 4mL test tube and thawed to room temperature. When the filter reached room temperature, one spoon of liquid nitrogen was added into test tube and left for 30 minutes. A glass mortar was used to homogenize the filter with 1mL Biuret reagent. The content was then transferred to another 10mL centrifuge tube. Another 1 mL Biuret reagent was used to rinse the test tube and this was also transferred to the centrifuge tube. The volume in centrifuge tube was then topped up to 5.5 mL using extra Biuret reagent.

The centrifuge tubes and standard protein samples (range between 0 to 350 µg ) were placed in a water bath at 100°C for 60 min. After incubation, 0.5mL Folin-phenol reagent was immediately added into the tube and mixed well using a vortex stirrer. The centrifuge tubes were then placed in cold water bath (~ 4°C) to cool down and then they were allowed to equilibrate to room temperature. The samples were then centrifuged at  $1248 \times g$  (3000 rpm,  $r=12.5$  cm) for 5 min, and the absorbance of the supernatant was measured at 660 nm. The protein content of samples was calculated using the standard curve.

### **2.2.6 Total carbohydrate determination**

Total carbohydrate was determined based on the method of Kochert (1978) as modified by Ben-Amotz et al. (1985) and Mercz (1994). Five mL culture was filtered onto Whatman 2.5 cm GF/C filters and stored at -5°C fridge. Prior to determination, the filter was placed in a 10mL centrifuge tube with lid and thawed to room temperature. The filter was homogenized with 0.5mL 1M H<sub>2</sub>SO<sub>4</sub> and topped up with another 4.5 mL 1M H<sub>2</sub>SO<sub>4</sub>. Then the centrifuge tube was incubated in a water bath at 100°C for 1 h. When the centrifuge tube cooled to room temperature, it was centrifuged at 3000 rpm for 10 min, and then 2mL of supernatant was transferred to another centrifuge tube.

Six glucose standards were prepared containing 0, 40, 60, 80, 120, 160 and 200 µg of glucose. Each standard was topped up with DI water to 2mL. Then, 1mL phenol solution (50 g.L<sup>-1</sup>) was added to each of the standards and the centrifuge tubes

containing 2mL sample supernatant. The samples were mixed well on a Vortex stirrer immediately. Then 5mL concentrated H<sub>2</sub>SO<sub>4</sub> was added and mixed by shaking three times. When the centrifuge tubes cooled to room temperature, they were mixed again by shaking several times. The absorbance of the sample was measured at 485 nm and the content of the carbohydrate calculated against the standard curve.

### 2.2.7 External hydrocarbon determination

External hydrocarbon determination was based on Eroglu and Melis (2010). 50 mL culture was centrifuged at 1248 *x g* (3000 rpm, *r* = 12.5cm) for 3 min, then the water layer was carefully removed. 8 mL heptane was added into the centrifuge tube, and the centrifuge tube was well mixed by vortex for 3 minutes. The heptane was then separated from the cells by addition of culture medium. The top heptane layer was carefully transferred into a pre-weighted 4mL glass vial. The glass vial was dried under a steam of nitrogen gas on a heating plate at 38°C. After evaporation for 1 h, the vial was weighed on an analytical balance to 5 decimal places.

### 2.2.8 Biomass and lipid productivity determination

In this study, changes in biomass productivity and lipid productivity were used to determine culture's capacity of producing organic compounds and lipid. Usually, productivity was calculated by the equation: Productivity =  $\mu \cdot \text{Yield}$ , where  $\mu$  is the specific growth rate (Eq.2) However, this calculation was not suitable for my study, because the growth rate and productivity sometimes did not show a linear relationship. Also, the culture can accumulate more lipid when the growth rate was zero. Therefore, Eq 7 and Eq 8 were used for measuring biomass and lipid productivities.

$$\text{Biomass productivity (g. L}^{-1}\text{day}^{-1}) = \frac{\ln(\text{organic dry weight}_2 - \text{organic dry weight}_1)}{t_2 - t_1}$$

**Eq 7**

$$\text{Lipid productivity (g. L}^{-1}\text{day}^{-1}) = \frac{\ln(\text{yield}_2 - \text{yield}_1)}{t_2 - t_1}$$

**Eq 8**



### **2.2.9 Measurements of net photosynthetic and dark respiration rate**

A Rank Brothers (UK) polarographic Clark-type oxygen electrode was used to measure net photosynthesis and dark respiration rates. A temperature controlled water bath was connected to the glass chamber of the electrode to provide constant temperature, usually the water bath was turned on 30 min prior to an experiment. A Hanimex 150 slide projector with 150W, Syl-167 quartz halogen bulb was used to provide light to electrode chamber, and the irradiance was adjusted by changing the distance between electrode and projector. The electrode was calibrated by nitrogen gas and air saturated DI water. A Li-Cor model light meter was used to measure the irradiance of the chamber, and the irradiance of the front and back surface of the chamber was measured separately. Then the average irradiance of this chamber is calculated by taking the mean irradiance value of front and back.

For net photosynthetic and respiration rate measurements, a sample in 10 mL centrifuge tube with lid was placed in water bath for at least 10 min. Afterwards, 3 mL of this culture was added into the electrode chamber, and nitrogen gas was gently bubbled into the chamber to reduce oxygen concentration before measurement (usually the culture oxygen was higher than required). A fresh culture was used for each measurement at each temperature, irradiance and oxygen level.

When measuring the respiration rate, two layers of thick black sheets were used to cover the electrode chamber, and all lights surrounding the chamber were turned off.

### **2.2.10 Multi-parameter water quality sonde (YSI)**

Multiparameter Water Quality Sondes from YSI were used to record pH, temperature and dissolved oxygen in the ponds. The models used in this study were 600R and 600XLM, both of them were initially calibrated by YSI company. Prior to each test, each sonde was calibrated with 3 pH buffers (pH 4, 7 and 10) and 100% saturated DI water (25°C).

## 2.3 Cultivation systems

### 2.3.1 Paddle wheel driven raceway pond

In this study, a paddle wheel driven raceway pond (2 m × 0.5 m; L × W) with maximum volume of 200 L was used to culture *B. braunii* outdoors. The 4-paddle paddle wheel was operated at a rotating speed of 28 rpm and generated a flow rate of 20 cm.s<sup>-1</sup>. pH was kept at a range of 7.0-7.5 by a CO<sub>2</sub>-stat system (Roche 8001 pH controller) with a solenoid switch connected to a CO<sub>2</sub> gas cylinder.

Prior to inoculation, the pond was pre-treated with hypochloride bleach for one week. The pond was then filled with tap water for another week to rinse off any chemical residua. Once the stock culture (see 2.1) was inoculated in to the pond with initial cell density of 100 × 10<sup>5</sup> cells.mL<sup>-1</sup> with 80L volume in the pond. The culture was initially kept in batch mode until reached stationary phase. The culture volume was then gradually increase to 200 L with cell density kept between 100 × 10<sup>5</sup> and 150 × 10<sup>5</sup> cells.mL<sup>-1</sup>. Once at 200 L, the culture was operated at semi-continuous or batch mode according to required experiment.

For routine maintenance, tap water was added every morning to compensate the evaporation. Some of the algae stuck (create thin biofilm) to the pond walls and paddlewheel, these biofilm were removed daily using water jet of soft brush.. A fish net was also used routinely to remove dead insect and other large particles from the culture.

### 2.3.2 Biocoil

A vertical helical tubular photobioreactor (airlift) with 40 L total volume was used to culture *B. braunii* in this study, the construction was based on the Biocoil design of Robinson *et al.* (1989). Air was supplied to the airlift from a Binford BLE-20 air compressor. The liquid velocity was regulated by an adjustable regulator at a range of 10-25 cm.s<sup>-1</sup> with an aeration speed between 5-16 L.min<sup>-1</sup>. The water cooling system was set to 30°C during all cultivation period. pH was kept at a range of 7.0-

7.5 by a CO<sub>2</sub>-stat system (Roche 8001 pH controller) and a solenoid switch connected to a CO<sub>2</sub> gas cylinder.

Prior to inoculation, the Biocoil was pre-treated with hypochloride bleach for one week, then filled with tap water for one week to rinse off any chemicals. This step was repeated again as the tube surface is harder to clean. Once cleaned the Biocoil was inoculated using *Botryococcus* grown in open raceway pond. The culture Biocoil volume was 40 L with initial cell density at  $70-80 \times 10^5$  cells.mL<sup>-1</sup>. Afterwards, the culture was grown either in semi-continuous or batch mode according to required experiment.

## CHAPTER 3. OUTDOOR CULTURE

### 3.1 Introduction

*Botryococcus braunii* is a green alga which can accumulate very long chain hydrocarbons in the cell and release them to the external matrix and can accumulate a high concentrations of hydrocarbons (Metzger and Largeau 2005). Due to this feature, *Botryococcus* has a potential to be used as a raw material for biofuel production. However, for *B. braunii* culture to be successful for any purpose, the culture must be reliable and also productive for long periods when grown outdoors (Scott et al. 2010).

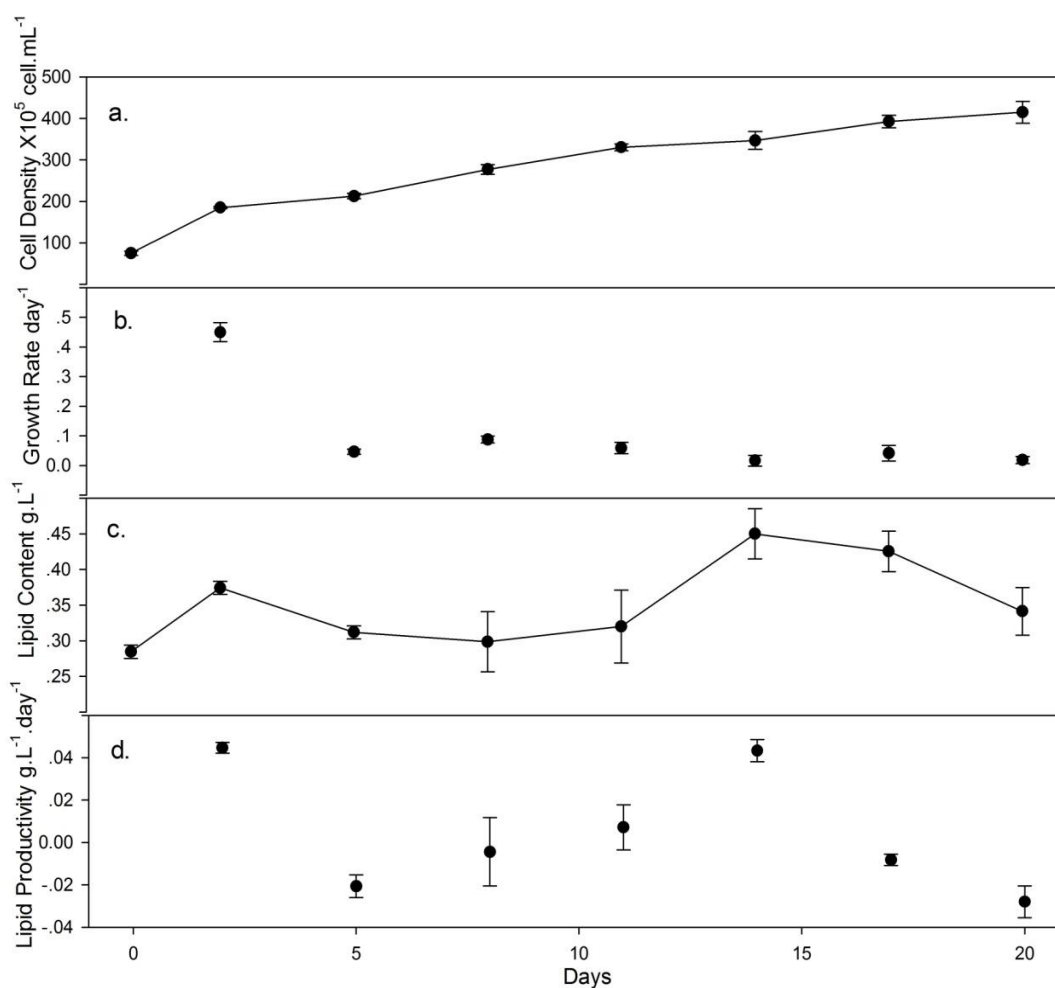
While, there have been many reports on indoor cultivation of several strains of *Botryococcus*, experience in outdoor cultivation is limited to a small number of short-term studies. One study from India showed that *B. braunii* AP103 can be cultured in paddle wheel driven raceway ponds outdoors for 15 days in batch mode (Dayananda et al. 2010). Another study also showed that 2 strains of *B. braunii* (strains LB-572, N-836) can be cultured in open circular and raceway ponds for 18 days in batch mode (Ranga Rao et al. 2012). In Japan *B. braunii* BOT-22 also has been cultured in an outdoor tubular biophotoreactor for 60 days (diluted only once), and this is the longest record of outdoor culture of *Botryococcus* by far (Shimamura et al. 2012). However, these three examples of outdoor cultivation were too short (15-60 days to evaluate the long-term reliability and productivity of outdoor *Botryococcus* culture necessary for any potential commercial application of this alga.

In this Chapter, *Botryococcus braunii* CCAP 807/2 first was cultured indoors in batch mode to study the growth and lipid production under indoor conditions. The same strain then was cultured in a 1 m<sup>2</sup> (up to 200L volume depending on depth) paddle wheel driven raceway pond and a closed helical tubular photobioreactor (Biocoil, 40L) outdoors to test the long-term reliability of the culture in these two cultivation systems. The cultures were periodically harvested at various times during this study to determine the productivity of the culture in semi-continuous culture.

## 3.2 Results

### 3.2.1 Indoor batch culture of *Botryococcus*

*Botryococcus braunii* CCAP 807/2 was cultured in a 5L carboy in batch mode under the growth conditions summarised in Chapter 2.1. The growth curve, specific growth rate, lipid content and lipid productivity are shown in Figure 3:



**Figure 3. Indoor batch culture of *Botryococcus braunii* CCAP 807/2 (a) Growth curve. (b) Specific growth rate. (c) Total lipid content. (d) Lipid productivity. Note: the data points for (b-c) are calculated for the period between sampling. Data are mean  $\pm$  stdev (n=4).**

For this indoor culture the culture was in exponential phase over the whole 20 days as cell density was still increasing by the end of this experiment (see Figure 3). The highest specific growth rate of  $0.45 \text{ d}^{-1}$  was observed immediately after inoculation between day 0 and 2. The specific growth then declined to an average specific growth rate of between day 2 and 15 of  $0.04 \pm 0.03 \text{ d}^{-1}$ . The highest lipid content of

the cultures was on day 15 ( $0.45 \pm 0.01 \text{ g.L}^{-1}$ ), with the highest change in lipid between days 11 and 14, with a lipid productivity between days 11 and 14 of  $0.04 \text{ g.L}^{-1}.\text{d}^{-1}$ .

### 3.2.2 Outdoor culture

The reliability of growth of *B. braunii* CCAP807/2 in a helical tubular biophotoreactor (Biocoil) and a paddle wheel-driven raceway pond (Figure 4) was compared over a period of 2 months under outdoor conditions in Perth Western, Australia (see chapter 2, 2.1). The raceway pond culture was continued for a further 3 months.



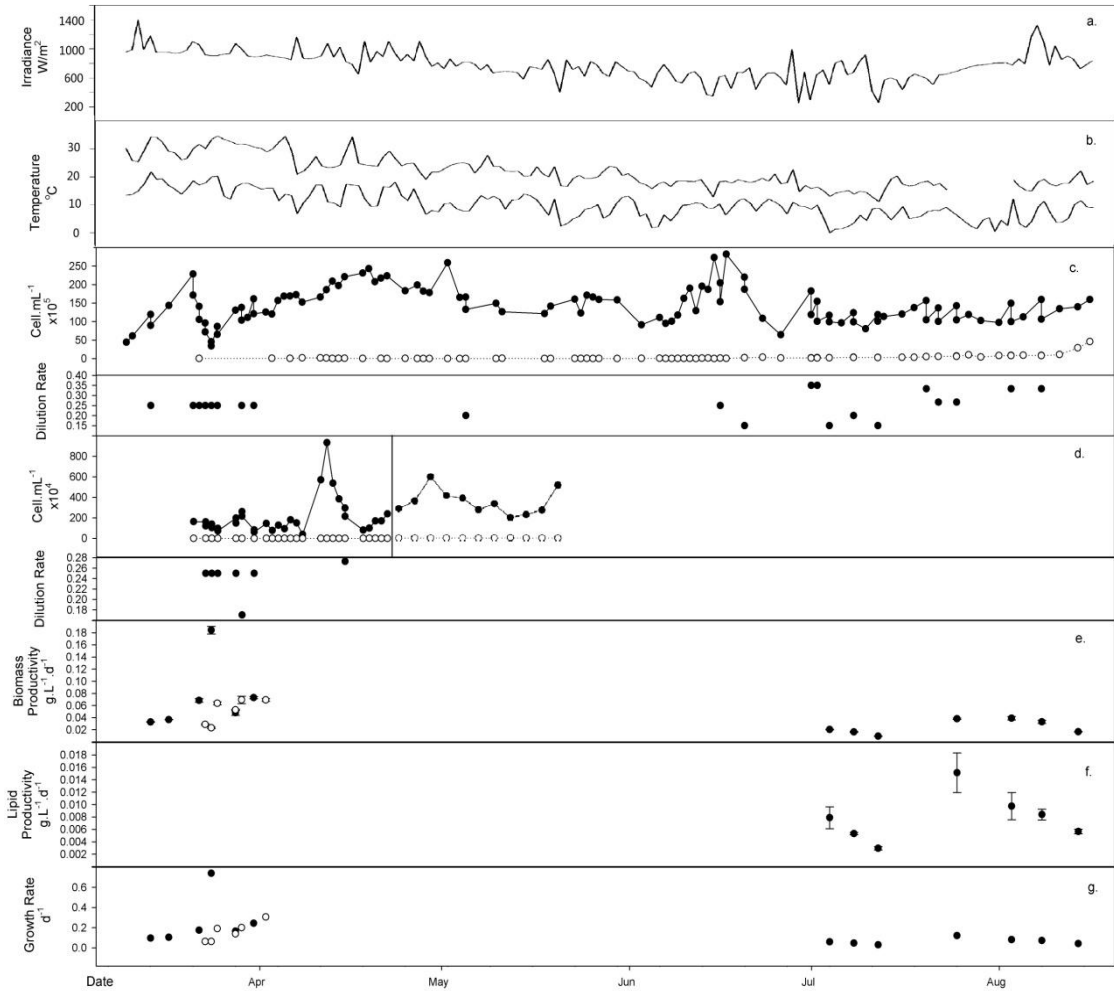
**Figure 4. 40 L Biocoil-type tubular photobioreactor (Left) and 200L paddle-wheel driven raceway pond (Right) used in this study.**

Following inoculation the raceway pond culture initially grew very well with an average specific growth rate of  $0.1 \text{ d}^{-1}$ . Once the raceway pond culture had been fully established in March 2011 the Biocoil-type closed tubular photobioreactor was also inoculated. Growth in the open raceway pond and the Biocoil-type closed tubular photobioreactor between March and August 2011 (i.e. late autumn and winter) is

shown in Figure 5. Both cultures were initially grown in semi-continuous mode and then in batch mode.

*Botryococcus* also grew reasonably well initially for 1 month in the Biocoil (Figure 5 d. left of the vertical line on the graph) until the culture collapsed due to extensive sticking of cells on the tubes of the coil and in the degasser (Figure 6). The sudden high peak in cell numbers during this period of around  $900 \times 10^5$  cell.mL<sup>-1</sup> (Figure 5d) was not a result of growth, but arose because the cells sticking inside the degasser box at the top of the airlift were resuspended when new water and medium were added. Following the culture collapse the Biocoil was cleaned and the culture reinoculated (Figure 5 d. right of the vertical line on the graph). Again the algae grew and reached a cell density of up to  $600 \times 10^5$  cell.mL<sup>-1</sup>, but the culture again collapsed because of cell sticking and no further experiments in the Biocoil were carried out. It also proved impossible to achieve accurate cell counts for the Biocoil culture due to the sticking problem and therefore the calculated growth rates must be considered of very limited accuracy and are likely to be underestimated. This may account for the fact that the calculated productivities in the Biocoil and the open pond were about the same (Figure 5e).

In the raceway pond *Botryococcus* was grown for 5 months (Figure 5c). For a large part of this time the culture was effectively maintained in stationary phase with the main aim being to determine the stability of the culture outdoors. However, in the latter period (July to August) semi-continuous growth was again re-established to obtain data on the specific growth rate and productivities.

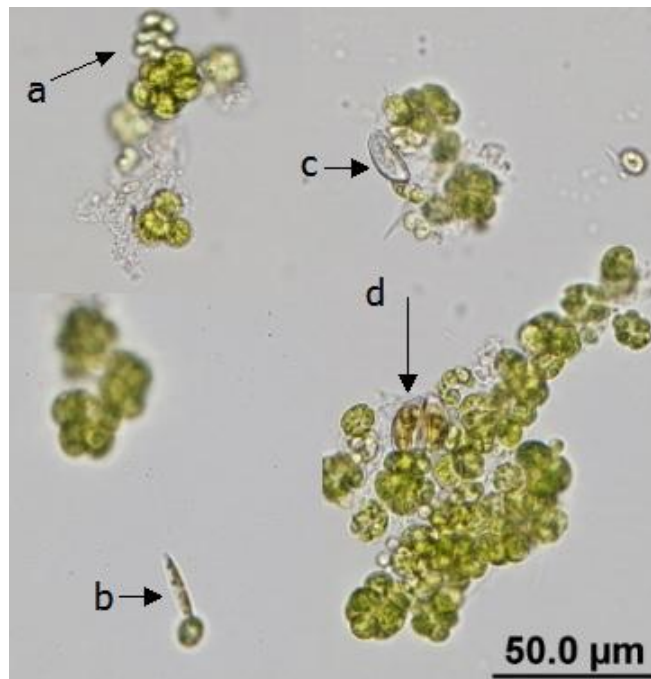


**Figure 5. The long term growth of *Botryococcus braunii* CCAP807/2 in an open raceway pond and a Biocoil-type tubular photobioreactor. (a) air temperature and (b) solar irradiances during this period. (c) Cell densities in raceway pond - (●) *Botryococcus*, (○) contaminating organisms. (d) Cell density in Biocoil - (●) *Botryococcus*, (○) contaminating organisms. (e) Biomass productivity (AFDW) in open pond (●) and Biocoil (○). (f) Lipid productivity in pond. (g) Specific growth rate in open pond (●) and Biocoil (○).**





**Figure 6.** Cells sticking and accumulating inside the Biocoil in the degasser box



**Figure 7.** Contaminating organisms in outdoor culture. (a) *Scenedesmus*. (b) *Amphipleura*. (c) Dead cell. (d) *Tetrallantos*.

While operating the raceway pond, the accuracy of sampling was found to be very important and somewhat difficult to achieve. This was because the cells regularly

adhered to the pond walls and in the corners of the pond. For more accurate sampling for cell counts, the pond wall was washed down regularly using a soft brush. Samples were usually taken in front of the paddle wheel after the culture had been mixed well and after topping up with fresh water to replace evaporative losses.

On days with heavy rain, the paddle wheel was shut down to prevent overflow of the pond and subsequent culture losses. As *Botryococcus* is colonial, the cells/colonies quickly settled to the bottom of the pond when the paddle wheel was stopped and therefore any overflow from the pond was only medium and not algal cells. After rain, and prior to turning on the paddle wheel the excess water was carefully removed by siphoning and fresh nutrients (i.e. N and P) were added to compensate for the medium lost. To minimize contamination in the pond, the dead bodies of insects were removed regularly using a fish net. Also, the culture was maintained at relatively high cell density of  $100 \times 10^5$  cells.mL<sup>-1</sup>. It was also observed that when the percentage of contaminating organisms started to increase, shutting down the paddle wheel for 1-2 days and allowing the cells to settle on the bottom of the ponds had the effect of reducing the number of contaminating organisms. The main contaminating organisms were the green alga *Scenedesmus*, the diatom *Amphipleura* (Figure 7) and few protozoa such as *Vorticella* and *Paramecium*.

From April to June, the culture was grown at batch mode. The cell density firstly increased from  $150 \times 10^5$  to  $240 \times 10^5$  cells.mL<sup>-1</sup> at an average specific growth rate of  $0.03 \text{ day}^{-1}$ , then gradually declined to  $90 \times 10^5$  cells.mL<sup>-1</sup>. At the time of lowest cell density, nutrients were added into the pond to increase biomass resulting in an increased specific growth rate of  $0.09 \text{ day}^{-1}$  until peaking at a cell density of  $270 \times 10^5$  cells.mL<sup>-1</sup>. During this period of open pond cultivation, the culture almost collapsed 3 times in early April, beginning of May and late June with the cell density suddenly decreasing by 84%, 38%, and 82%, respectively. After each collapse, the culture was partially harvested and fresh medium was added and the culture recovered. Interestingly, prior to each collapse, the cell density of the culture was particularly high at  $220 \times 10^5$ ,  $250 \times 10^5$  and  $280 \times 10^5$  cells.mL<sup>-1</sup>, respectively.

Because of the possible relationship between very high cell densities and culture collapse, the cell density was maintained at  $100 - 150 \times 10^5$  cells.mL<sup>-1</sup> when the pond culture was grown semi-continuously during July and August (Figure 5c). During this period, the culture achieved a biomass productivity of  $0.025 \pm 0.012$  g.L<sup>-1</sup>.day<sup>-1</sup> (Figure 5e), and a lipid productivity of  $0.008 \pm 0.004$  g.L<sup>-1</sup>.day<sup>-1</sup> (Figure 5f) at a specific growth rate of  $0.06 \pm 0.03$  day<sup>-1</sup> (Figure 5g). It is noticeable that when the culture was continuously harvested, both biomass and lipid productivity decreased after each dilution. For example, the lipid productivity in July was 0.008, 0.006 and 0.004 g.L<sup>-1</sup>.day<sup>-1</sup> respectively (in date order), although the harvesting interval was the same.

The culture was eventually terminated in late August as a result of a sudden explosion of *Scenedesmus* growth (Figure 5c).

### **3.3 Discussion**

#### **3.3.1 Reliability of *Botryococcus* culture**

*Botryococcus* was grown in open pond for 5 months and the Biocoil for 1 month. This is the longest time that *Botryococcus* has been grown outdoors. The reason for the failure of open pond is the sudden increase of contaminating organisms (mainly *Scenedesmus*), and the Biocoil failed twice due to serious cell sticking on the Biocoil tubes and in the degasser box.

During 5 month of open pond cultivation, the culture partially collapsed three times, but was recovered each time. These partial culture collapses always coincided with very high cell densities and not with any changes in climatic conditions. It is possible that at these very high cell densities the culture became extremely nutrient limited, although light limitation also cannot be ruled out.

On the basis of these results the following pond management strategies to maintain a stable culture are proposed:

- 1) The cell density should be maintained at no more than  $200 \times 10^5$  cells.mL<sup>-1</sup>.

2) When a collapse happens, the culture needs to be partially harvested several times and the nutrient levels should be restored until the cell density increases again. For each partial harvesting step, the dilution rate should be less than 20%. The reason for this is that the culture is very vulnerable at this time, and a sudden decrease in cell density can cause an increase in contamination.

### **3.3.2 Limitations on reliability**

#### **Cell Sticking**

*Botryococcus* is a colonial alga which produces abundant extracellular polysaccharides and therefore cell sticking is normal behaviour of this alga (Wake and Hillen 1981; Fernandes et al. 1989), especially when the environment is unfavourable (Lee et al. 1995). On the other hand, the sticking cells can be removed relatively easily in an open pond, but not in a tubular photobioreactor such as the Biocoil.

In the open pond, the cells adhered on the pond walls and the bottom corner of the pond. As the pond needed addition of water every day to compensate for the evaporative loss of water, the sticking cells on the pond walls could easily be washed off the walls by a water jet. The cells also stuck to and accumulated in the corners of the pond a result of relative slow flow at the corners. This problem can be solved by culturing the alga in raceway ponds with round corners to improve the flow pattern. This design feature is common in ponds used for commercial production (Sanchez et al. 2011). Furthermore, the installation of low rectifiers can improve the flow regime in the corners of the ponds (Shimamatsu 1987). However, it is important to note that the cell sticking in the open pond did not cause any culture collapse in my study.

In the Biocoil however, it is not possible to remove the sticking cells and built-up biomass in the degasser box as the system was closed. As shown in Figure 5 (d) the cell density in the Biocoil had a sudden increase at one time, and it is likely that this extreme high cell density finally led to the culture collapse. Similarly, in the second trial the culture collapsed after a rise in cell density. Therefore, cell sticking was a problem in using the Biocoil for the culture of *Botryococcus*.

It may be possible to overcome this problem by a redesign of the photobioreactor. In the air lift part of the Biocoil and in the degasser box, the flow rate was relatively slower than in the plastic tube of the photostage. Therefore once some cells grow on the wall of the top degasser box (see Figure 6), more cells will stick afterwards. By increasing the flow rate and turbulence the algal cells should have less chance to stick. However, increased shear due to mixing can potentially result in cell damage in some algae (Silva et al. 1987; Cherry 1988; Thomas and Gibson 1990; Hondzo and Lyn 1999; Barbosa et al. 2003). Whether this can be a problem is not known as the sensitivity of *Botryococcus braunii* to shear has not yet been studied. An alternative option may be to change the material of the box and tube, so that it is harder for the algae to stick to the surfaces of the photobioreactor. For example, many tubular photobioreactors are constructed of glass tubes (Zittelli et al. 2013a) and clear Teflon tubing was also trialled in the UK by Biotechna (Borowitzka, personal communication). However, the cost of such materials is extremely high, and hydrocarbon from *Botryococcus* for biofuel production is not a high value product. Closed photobioreactors, in particular tubular photobioreactors are therefore unlikely to be suitable culture systems for this alga.

### **Contaminating organisms**

Contaminating organisms exist both in the open pond and the Biocoil, during the experimental period, the cell density of contamination organism in Biocoil was less than 1% than that of the *Botryococcus*, but due to the short period of Biocoil culture, it is impossible to predict if contamination in Biocoil would increase in long term and present a problem if the culture period could be extended.

In the open pond the concentration of contaminating organisms was very low from March to beginning of August, and then it suddenly increased (Figure 5c). The contaminating organisms in August were the same as in March, which means that for some reason they suddenly grew faster.

A possible explanation for this contamination boom is that the weather conditions in August were less favourable for the growth of *Botryococcus*, making the alga less

competitive. As it can be seen in (Figure 5 g) the specific growth rate in August was lower than in March, and the biomass productivity was also lower. The temperature in August was mostly below 20°C, and the optimum temperature for *Botryococcus braunii* 807/2 growth was 25°C and lower temperatures reduced the growth rate (Li and Qin 2005). On the other hand, the optimal conditions for *Scenedesmus* which is one of the main contaminating organisms in the pond and for biomass productivity (Sánchez et al. 2008) and specific growth rate (Xin et al. 2011) are 20-25°C at high irradiance (1900  $\mu\text{mol photons. m}^{-2}.\text{s}^{-1}$ ). Thus, the temperature in August was also unfavourable to the growth of *Scenedesmus*, however, the higher irradiance may have benefitted its growth. As shown in Chapter 4 (Figure 14), the specific growth rate of *Botryococcus* was not increased above the light saturation point (100  $\mu\text{mol photons. m}^{-2}.\text{s}^{-1}$ ).

One interesting observation of this study was that the contaminating organisms could be reduced by allowing the *Botryococcus* (and the contaminating organisms) to settle on the bottom of the pond for 24 h or longer by turning off the paddle wheel. By effectively concentrating the *Botryococcus* in an unmixed environment is likely to have resulted in a localised increase in hydrocarbons and/or free fatty acids in the water. These hydrocarbons have been shown to have antibiotic activity (Metzger et al. 1989) and free fatty acids from *B. braunii* have also been shown to have an allelopathic effect on phytoplankton (Chiang et al. 2004). In fact, this may be one of the reasons that *Botryococcus* has evolved to secrete hydrocarbons and therefore presents a possible strategy for controlling contaminating organisms in long-term cultures of *Botryococcus*. An alternative might be to grown the alga at a higher cell density (above  $100 \times 10^5 \text{ cells.mL}^{-1}$ ), as higher cell densities should also result in an increase the concentration of hydrocarbons and/or free fatty acids in the medium to levels where they have effective allelopathic activity.

### **3.3.3 Productivity of *Botryococcus* culture**

#### **3.3.3.1 Productivity of indoor culture**

The maximum lipid productivity and specific growth rate of my indoor *Botryococcus* 807/2 (Race A) batch culture was  $0.04 \text{ g.L}^{-1}.\text{d}^{-1}$  and  $0.45 \text{ d}^{-1}$ , no matter

the growth and lipid productivity, the result from my study was high compare to other result on identical and close strain. By far, the highest lipid productivity and specific growth rate of *B. braunii* are reported as  $0.15 \text{ g.L}^{-1}.\text{d}^{-1}$  and  $0.5 \text{ d}^{-1}$  from a *B. braunii* Showa strain (Yoshimura et al. 2013). The following will mainly discuss the effect of growth condition and strain difference on lipid productivity and growth of *Botryococcus*.

### **Growth condition and strains**

In comparison, one culture of *B. braunii* CCAP 807/2 in another study had a specific growth rate of  $0.15 \text{ d}^{-1}$  (Li and Qin 2005) which is 30% of my maximum result. Though the lipid yield was not given in the study, it is predictable that the lipid productivity would also be also lower than my result due to the much lower growth rate. The alga in this study was grown in flask without stirring and aeration, and this is the main difference from my culture. In addition, although the irradiance is different to mine, this result  $0.15 \text{ d}^{-1}$  was the highest growth rate under an optimized irradiance. In this case, it is predictable that culture mixing and aeration are the main factors affecting the growth here.

From another report on a *Botryococcus braunii* which is close to *B. braunii* CCAP 807/2, the lipid productivity of the batch culture is  $0.016 \text{ g.L}^{-1}.\text{d}^{-1}$  on average (Talukdar et al. 2013), and this is about 40% of my result. Also, this culture was grown without constant stirring and the irradiance was  $35 \mu\text{mol photons. m}^{-2}.\text{s}^{-1}$  which is only 12% of the irradiance for my culture. Besides, there was also no aeration to the culture in this study.

In another study, *B. braunii* strain UTEX LB-572 (identical to CCAP 807/1), which is close to CCAP 807/2, showed a lipid productivity of  $0.017 \text{ g.L}^{-1}.\text{d}^{-1}$  (Eroglu et al. 2011) which was about 40% of my maximum result. In this study, the irradiance was  $50 \mu\text{mol photons. m}^{-2}.\text{s}^{-1}$ , and my study used 6 times higher light than this. Besides, the culture was stirred but not aerated.

Reports on this particular strain *B. braunii* CCAP 807/2 are very limited, therefore I can only compare my result to above studies. For all those cultures mentioned above, the temperature and medium are same, and the main differences between those studies and mine are lower irradiance and lack of aeration and stirring. However, it is hard to find out which factor here is the primary one due to limited data. Therefore, further studies on growth conditions in chapter 4 can help to answer this question. On the other hand, the range of lipid productivities from different *B. braunii* strains is huge. In one study, several *B. braunii* strains were cultured at identical conditions, and the lipid productivity can be calculated and shown as follows (Eroglu et al. 2011):

Strain	Race	Lipid productivity g.L <sup>-1</sup> .d <sup>-1</sup>	Lipid content (dry weight)
<i>B. braunii</i> var Showa	B		33.9%
<i>B. braunii</i> UTEX-2629	A	0.038	19.3%
<i>B. braunii</i> Yamanaka	A	0.024	18%
<i>B. braunii</i> Kawaguchi-1	B	0.023	28.4%
<i>B. braunii</i> UTEX-LB572	A	0.017	15.9%
<i>B. braunii</i> UTEX-2441	A	0.010	16.7%

Among those *B. braunii* strains, *B. braunii* var Showa had the highest lipid productivity and this is 4 times higher than UTEX-2441 which is grown under the same condition. Additionally when *B. braunii* var Showa was grown under optimal condition which is 850  $\mu\text{mol photons. m}^{-2}.\text{s}^{-1}$  irradiance and 30°C temperature with 0.2 - 5% CO<sub>2</sub> enrichment, it can have a lipid productivity of 0.15 g.L<sup>-1</sup>.d<sup>-1</sup> and specific growth rate of 0.5 d<sup>-1</sup> (Yoshimura et al. 2013).

Many reports now on *B. braunii* have focussed on the optimal conditions for lipid yield, but a higher lipid yield may not lead to higher productivity because productivity is a function of both lipid yield and specific growth rate (lipid productivity =  $\mu \times$  lipid yield). For example, my strain *B. braunii* CCAP 807/2 has a higher lipid content at 20°C than 25°C, but it grows lower at 20°C than 25°C (Ngon 2011b). Also from above table, although some strains like UTEX-2629 has lower lipid content than *B. braunii* Showa, the lipid productivity is higher and although



some strains like *B. braunii* Kawaguchi-1 have a higher lipid content than UTEX-2629, the lipid productivity is lower.

### **Drop in growth rate and lipid content**

During indoor batch culture period (see Figure 3), the maximum specific growth rate occurred only at the beginning of culture, then the rate decreased to a very low level and remained. At the beginning of the culture, the nutrients and irradiance are both sufficient, therefore the culture can grow faster. When the cell density was doubled, the irradiance for individual cells and nutrient concentration decreased a lot which further decreased the growth rate. Similarly, the highest growth rate of outdoor pond culture in March (see Figure 5) occurred on the day that the cell density of the culture was the lowest in the whole cultivation. In other words, there was more light available for each cell compared to higher cell density.

In addition, if the drop of growth rate is a result of insufficient nutrients, then using more concentrated medium would improve the growth rate and productivity. However, excessive nutrients can be toxic for *Botryococcus*. In one study, the author found that extra high concentration of nitrogen, phosphate and iron can decrease the lipid yield; especially phosphate and iron can decrease the lipid content by half when their concentration was 2 times higher than original (Ruangsomboon 2012).

On the other hand, it can be found that the lipid content drops (see Figure 3 c) from 3<sup>rd</sup>-8<sup>th</sup> day, then it went up again to an even higher content, but then lipid dropped for another time at the end of the batch mode. Similarly to my result, such pattern of lipid composition was also observed in another *B. braunii* strain. In one study of *B. braunii* indoor batch culture, the lipid content had a drop during day 12 - 15, then it increased from day 15 - 24, afterwards, the lipid content dropped for a second time from day 24 - 30 (Ashokkumar and Rengasamy 2012). Also in this study, the outdoor batch *B. braunii* culture also showed the same pattern when the first drop happened during day 6 - 9 and second drop happened during day 12 - 15 (Ashokkumar and Rengasamy 2012).

Unfortunately, due to limited research on *B. braunii*, it is hard to explain the lipid drop, because it cannot be predicted if the missing lipid is a result of excretion or cell respiration. On the other hand, although we cannot find the reason for this phenomenon, if the culture can be harvested before the lipid drop, the culture would have a higher lipid productivity. Therefore, when *B. braunii* strain is grown semi-continuously the harvesting interval should not be too long.

### 3.3.3.2 Productivity of open pond and Biocoil

According to the result shown in Figure 5, there is no statistical difference between overall biomass productivity of the open pond and the Biocoil. The biomass productivity of the open pond and the Biocoil were  $0.07 \pm 0.06 \text{ g.L}^{-1}.\text{day}^{-1}$  and  $0.05 \pm 0.02 \text{ g.L}^{-1}.\text{day}^{-1}$  (t-Test,  $P = 0.19$ ), respectively. However, the dayu-today productivity of the open pond is very variable. For example, the biomass productivity of the open pond on 24 March 2011 was  $0.18 \text{ g.L}^{-1}.\text{day}^{-1}$ , and other productivity data of open pond were all in a range of  $0.03 - 0.07 \text{ g.L}^{-1}.\text{day}^{-1}$ . This extreme value was almost 3 times higher than the average of remaining data, this is because, this data is from the day which additional nutrient was added after a partial culture collapse (see. 3.3.1). Therefore, this productivity was obtained under much higher nutrient than other routine harvesting, so this data should be removed for an equitable comparison.

By removing the extreme data and recalculating, the biomass productivity of the open pond and the Biocoil were now  $0.05 \pm 0.01 \text{ g.L}^{-1}.\text{day}^{-1}$  and  $0.05 \pm 0.02 \text{ g.L}^{-1}.\text{day}^{-1}$ , respectively (t-Test,  $P=0.48$ ). Though there is no difference with cultivation of *B. braunii*, the Biocoil has higher productivity with other algae. In a comparison of *Tetraselmis* grown in the identical open pond and Biocoil in this study, the biomass productivity of the Biocoil was 5 time higher than the open pond (Raes et al. 2013).

In Chapter 1, the main advantage to productivity in a tubular photobioreactor was stated to be the larger illuminated surface area. However, higher irradiance may not benefit *B. braunii* growth. In another two studies on *Botryococcus*, it was found that increasing irradiance at low range ( $30 - 100 \text{ w.m}^{-2}$ ) can improve lipid productivity

and growth rate. However, irradiance higher than  $100 \text{ W.m}^{-2}$  does not bring any higher result and the productivity can be even lower (Li and Qin 2005; Qin and Li 2006a). These two studies have very similar results to my study which the Biocoil with higher irradiance does not lead to higher productivity. Therefore, in Chapter 4, my study on effect of irradiance to growth of *Botryococcus* strain (CCAP 807/2) would help to further understand the relation between irradiance and productivity.

Temperature (see. 2.3.2) and oxygen concentration (see. 2.3.1) are also two important factors that can affect *Botryococcus* growth, and these are further examined in Chapter 4.

### **3.3.3.3 Productivity of outdoor open pond**

#### **Outdoor productivity**

In my study, when race A *B. braunii* CCAP 807/2 was grown semi-continuously, lipid productivity of  $0.008 \pm 0.004 \text{ g.L}^{-1}.\text{day}^{-1}$  (Figure 5f) was observed. In comparison, when another strain of *B. braunii* AP103 was grown in outdoor open raceway pond at batch mode, lipid productivities of  $0.033 \text{ g.L}^{-1}.\text{day}^{-1}$  (day 0 - 3) and  $0.083 \text{ g.L}^{-1}.\text{day}^{-1}$  (day 3 - 6 & 9 - 12) were found (Ashokkumar and Rengasamy 2012). This lipid productivity was much higher than my result, but it is also noticeable that the growth condition of this study is very different from mine. In this study, the culture was grown at average temperature of  $29^{\circ}\text{C}$  and irradiance of  $208 \text{ W.m}^{-2}$  whereas my culture was grown at a range of  $10 - 20^{\circ}\text{C}$  and irradiance of  $300 - 1000 \text{ W.m}^{-2}$  (see Figure 5 a, b) suggesting that the low temperature was the main factor affecting my results. This is very similar to another study on *B. braunii* which found that low temperature and high irradiance both lower the growth rate (Li and Qin 2005). In detail, the specific growth rate of *B. braunii* UK strain under  $20^{\circ}\text{C}$  was 50% lower than at  $25^{\circ}\text{C}$ . Also when this *B. braunii* was grown at  $300 \text{ W.m}^{-2}$ , it grew 17% slower than at  $100 \text{ W.m}^{-2}$ . Of course, strain variation cannot be ruled out.

In another study on *B. braunii* outdoor culture, A race *B. braunii* LB-572 (CCAP807/1) and B race *B. braunii* N-836 were grown in circular pond and raceway pond at batch mode respectively. The average lipid productivity and other parameters

during the whole period was calculated from the result and shown as follows (Ranga Rao et al. 2012):

Lipid productivity g.L <sup>-1</sup> .day <sup>-1</sup>	Raceway pond	Circular pond
<i>B. braunii</i> LB-572 Race A	0.024	0.015
<i>B. braunii</i> N-836 Race B	0.011	0.007
Illumination surface m <sup>2</sup> .L <sup>-1</sup>	0.009	0.007
Culture depth cm	12	14

Due to lack of growth condition data (temperature and irradiance), it is not appropriate to compare this result to my data though this result is higher than my data indeed. But from the above table, it can be found that lipid productivity is very different between two strains. Also, there is big difference in lipid productivity between the two cultivation systems which the raceway pond has 60 - 70% higher result than circular pond. The illumination surface to volume ratio and depth of two systems are very close to each other, which means, the cultures in the two systems have an identical irradiance regime. Therefore, the main reason for the productivity difference is the mixing. In this study, the pond was mixed constantly at 15 rpm, however, the circular pond was only mixed manually every two days. This situation is very similar to the case that mentioned earlier in **3.3.3.1**, which the mixed culture had a much higher productivity than stationary culture.

### 3.3.4 Outdoor and indoor productivity

For outdoor culture (see Figure 5 July-August), lipid productivity under semi-continuous was only 10-45% of the maximum indoor productivity. By considering the growth condition within this period, the nutrient concentration was identical to indoor culture, so temperature, irradiance and mixing regime mainly affect the productivity.

The outdoor temperature during July to August was about 10 - 20°C, in comparison, the indoor culture was grown at constant 25°C. As discussed earlier, the lower

temperature can decrease lipid productivity greatly. Also about the mixing, the indoor culture was aerated and the outdoor culture was mixed by paddle. Though no dissolved O<sub>2</sub> data is available, it can be hypothesis that the indoor culture has better mass transfer rate due to abundant air bubbles.

Also, it was found that there was a decrease in biomass productivity and lipid productivity after each harvesting. This can suggest that the culture may have lag phase in producing new lipid after each harvesting. In one study, the lipid productivity of a batch *B. braunii* LB-572 culture was higher at later days than earlier days (Ranga Rao et al. 2012). Therefore, harvesting interval can also affect lipid productivity where a short interval can lead to lower productivity. According to this, increase harvesting interval to 3-4 days (2-3 days in this study) may increase lipid productivity.

### **3.4 Conclusion**

The reliability and productivity of cultures of *B. braunii* grown in an open raceway pond and a tubular Biocoil-type photobioreactor were compared. The open pond culture was more reliable and could be maintained for a much longer period than the Biocoil in which the culture crashed repeatedly. Cell sticking occurred in both systems but was a much more significant problem in the Biocoil where it could not be managed. In this study *B. braunii* was cultured in the raceway pond for a period of 5 months, which is the longest time achieved for this alga so far by anyone.

The indoor productivity was higher than other studies on closely related *B. braunii* strains, but this may result from differences in growth conditions. The productivity of the outdoor culture was very low when compared to the indoor culture or other outdoor cultures and therefore there is the potential for significant improvement of the operation and management of the outdoor culture to achieve higher productivities. Some of the limiting factors to growth and lipid productivity of *B. braunii* are further studied in the next chapter. Also, studies on outdoor cultures suggest that it is very hard to achieve optimal conditions outdoor as some factors are not controllable.

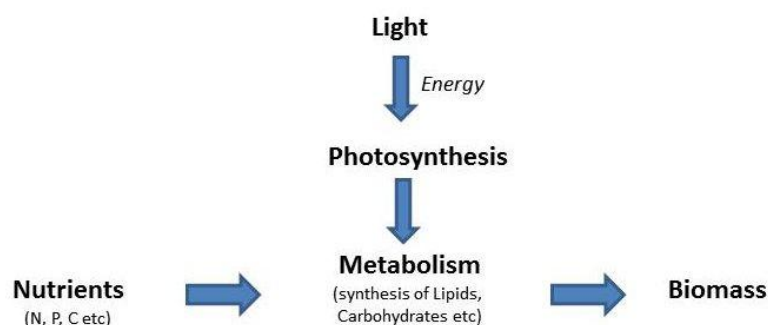
## CHAPTER 4. LIMITS TO GROWTH

### 4.1 Introduction

One aim of this study is to increase the lipid productivity of *Botryococcus braunii* cultures. Considering that productivity (Pr) is a function of specific growth rate ( $\mu$ ) and yield (i.e.,  $\text{Pr} [\text{g.L}^{-1}.\text{d}^{-1}] = \mu [\text{d}^{-1}] \times \text{Yield} [\text{g.L}^{-1}]$ ), slow growth and/or low lipid yield can limit the lipid productivity of an alga. *Botryococcus* is a very slow growing alga with, for example, a doubling time of 6 days, which can be reduced to 2 days with 0.3% CO<sub>2</sub> enrichment (Wolf et al. 1985b).

The production of biomass and lipids on photoautotrophic algae requires energy from light and nutrients (see Figure 8). One potential way to increase biomass production and lipid yield in *Botryococcus* is by adding more nutrients such as N and P (Ruangsomboon 2012). However, the modified CHU-13 medium (see Chapter 2) used in this study already has 4 times the nutrient concentration of the original CHU-13 medium and higher concentrations of N and P could be toxic to the culture (Ruangsomboon 2012). It is therefore unlikely that by adding more nutrient, there will be any further improvement in productivity. Additionally, even under this high amount of nutrients, the lipid productivity of *Botryococcus* outdoor culture was also very low (see Chapter 3, 3.2.2) when compared to indoor cultures.

On the other hand, the provision of extra energy (light) could be one possible way to increase growth and lipid yield of *Botryococcus*. By optimizing the utilization of light energy in photosynthesis, the more the alga can use this energy in biomass production and lipid and hydrocarbon synthesis, especially as lipid and hydrocarbon biosynthesis is an energy intensive process for the alga. According to the literature, *Botryococcus*' hydrocarbon biosynthesis can consume 3% of energy fixed by photosynthesis (Gudin et al. 1984).



**Figure 8. Simple schematic diagram of growth and lipid production in algae.**

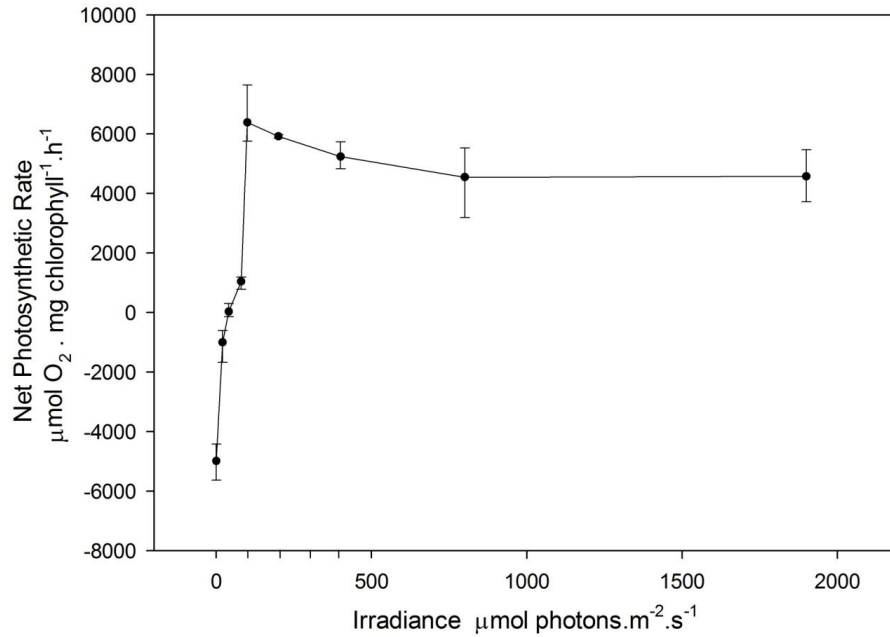
This chapter focuses on selected factors that can affect growth and hydrocarbon formation in the alga. In Chapter 1, the main limiting factors for photosynthesis were shown to be light, temperature, oxygen, nutrient and CO<sub>2</sub>. As mentioned above, further increases in nutrients are likely to have little effect and may even be counterproductive due to toxicities. Furthermore, in this study CO<sub>2</sub> was regularly added using a pH stat system so the culture should not be CO<sub>2</sub> limited. Therefore, this chapter focuses on the effects of light, temperature and oxygen on *Botryococcus braunii* CCAP 807/2. The interaction of these three factors was also investigated. These studies were carried out on both indoor and outdoor cultures.

## **4.2 Results**

### **4.2.1 Laboratory studies**

#### **4.2.1.1 Irradiance and photosynthesis**

Net photosynthetic and dark respiration rates of *Botryococcus braunii* were determined using a Clark-type oxygen electrode as described in Chapter 2. These studies were carried out on cultures in exponential phase (cell density =  $100 \times 10^5$  cells.mL<sup>-1</sup>). Initially a photosynthesis/irradiance (P/E) curve was determined under 4 mg O<sub>2</sub>.L<sup>-1</sup> (48.2% saturation at 25°C) oxygen concentration to determine the light saturation point (E<sub>k</sub>) and the compensation point (E<sub>c</sub>).



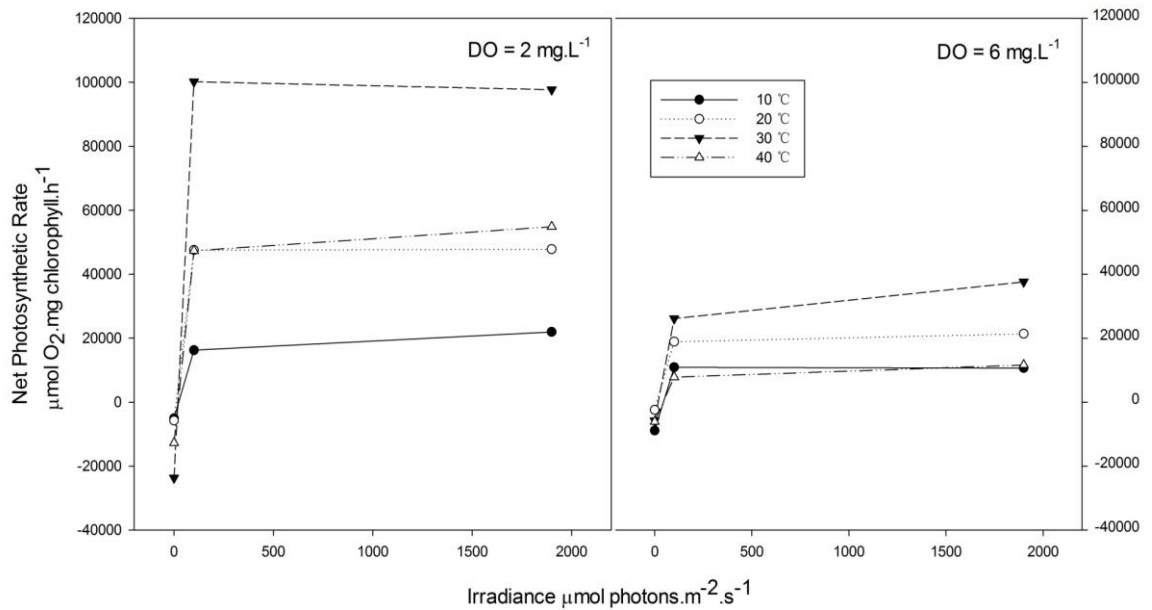
**Figure 9. Photosynthesis-Irradiance (P/E) curve of *Botryococcus braunii*. Data are mean  $\pm$  range, n=3.**

As shown in Figure 9, the light saturation point was  $\sim 100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  and the compensation point is  $\sim 40 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . The  $P_{\text{max}}$  at  $100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  was  $6200 \mu\text{mol O}_2 \cdot \text{mg chlorophyll}^{-1} \cdot \text{h}^{-1}$ . Furthermore, photosynthesis was not significantly inhibited even at  $1900 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  which was an irradiance similar to that outdoors.

#### **4.2.1.2 Interaction of oxygen, temperature and irradiance on photosynthesis**

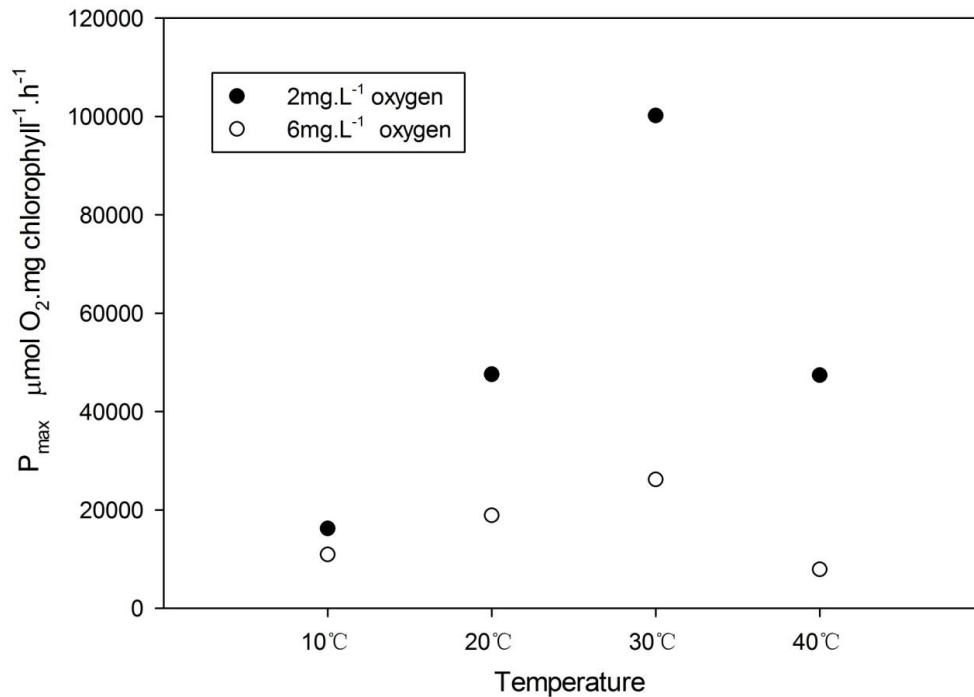
The interaction of irradiance, oxygen and temperature on net photosynthesis was investigated using a Clark type oxygen electrode at 10, 20, 30 and  $40^\circ\text{C}$  at 3 irradiances (dark,  $100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  (saturation point) and  $1900 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  (over saturated), and at a high  $\text{O}_2$  concentration of  $6 \text{ mg} \cdot \text{L}^{-1}$  (72.2% saturation at  $25^\circ\text{C}$ ) and a low  $\text{O}_2$  concentration of  $2 \text{ mg} \cdot \text{L}^{-1}$  (24.1% saturation at  $25^\circ\text{C}$ ). For each experiment, *Botryococcus braunii* was pre-cultured at 10, 20, 30 and  $40^\circ\text{C}$  for 48 h under  $80 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  to fully adapt to the temperature, and the cell density was adjusted to  $100 \times 10^5 \text{ cells} \cdot \text{mL}^{-1}$  by dilution or concentration (using centrifuge).





**Figure 10.** PE curves (net photosynthesis) at two oxygen concentrations of 2 mg.mL<sup>-1</sup> oxygen (left graph) and 6 mg.mL<sup>-1</sup> oxygen (right graph) at 4 temperatures. Data are mean (n=3), the range less than the size of the symbols and so is not shown in the graph.

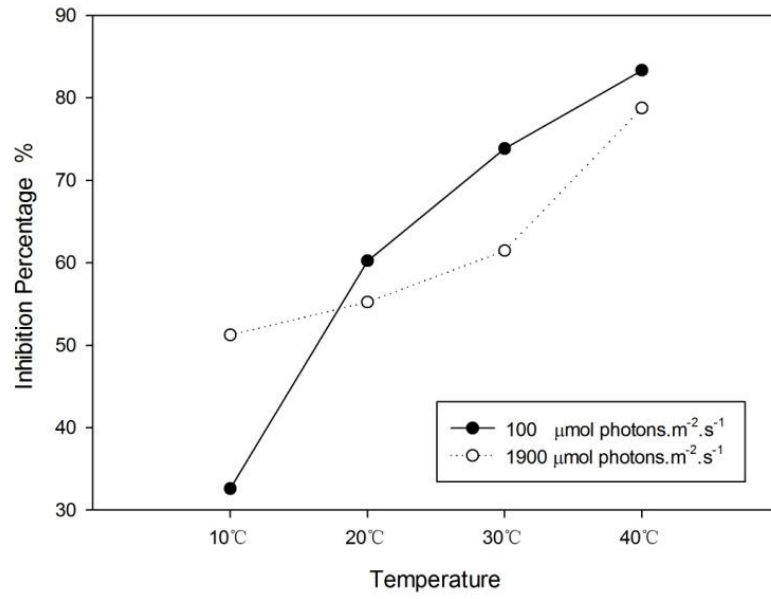
As in the previous experiment, no inhibition of photosynthesis at the very high irradiance of 1900  $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$  was observed (Figure 10). The optimum temperature for photosynthesis was 30°C and photosynthesis was markedly reduced at the higher O<sub>2</sub> concentration (Figure 10). The effects of oxygen concentration and temperature on the P<sub>max</sub> are summarized in Figure 11. Interestingly, increased [O<sub>2</sub>] at temperatures above 20°C decreased the dark respiration rate. At the optimum temperature of 30°C and 2 mg.mL<sup>-1</sup> O<sub>2</sub> the dark respiration rate was -24,000  $\mu\text{mol O}_2.\text{mg chlorophyll}^{-1}.\text{h}^{-1}$  whereas at 6 mg L<sup>-1</sup> oxygen it was only -5,800  $\mu\text{mol O}_2.\text{mg chlorophyll}^{-1}.\text{h}^{-1}$ . This is further discussed in section 4.3.2 below.



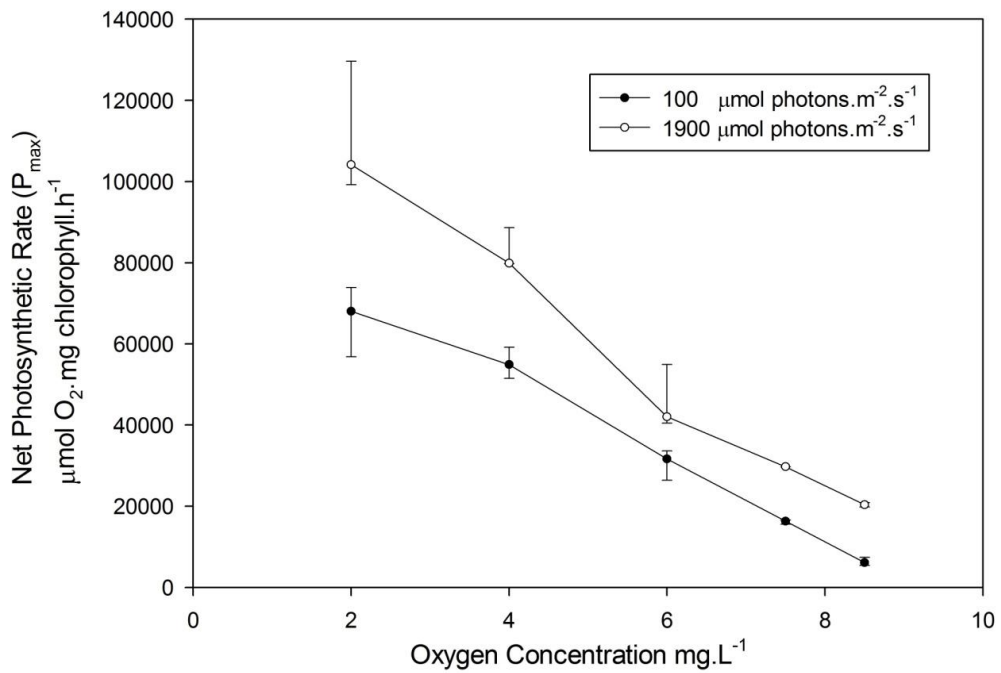
**Figure 11. Effect of temperature on the maximum photosynthetic rate ( $P_{max}$ ) at low and high oxygen concentrations.**

The percent inhibition of net photosynthesis at 6 mg.L<sup>-1</sup> oxygen compared with 2 mg.L<sup>-1</sup> oxygen in low and high light is shown in Figure 12 and clearly shows that the degree of inhibition at high oxygen concentration increases with temperature. At 10°C the combination of high light and low temperature greatly increases the inhibition of photosynthesis by increased oxygen concentration, whereas at temperatures above 20°C the difference between low and high light is small.

The effects of oxygen concentration on the photosynthetic rate of *Botryococcus braunii* ( $100 \times 10^5$  cells.mL<sup>-1</sup>) was further examined at 25°C over a wider series of oxygen concentrations and at 2 irradiances. This experiment confirmed the earlier findings of the inhibition of photosynthesis by increasing oxygen concentrations (see Figure 13):



**Figure 12.** Effect of temperature on percentage inhibition of maximum photosynthetic rate ( $P_{\text{max}}$ ) by an increase of oxygen concentration from of  $2 \text{ mg.mL}^{-1}$  oxygen to  $6 \text{ mg.mL}^{-1}$  oxygen at 2 irradiances.



**Figure 13.** Effect of oxygen concentration on  $P_{\text{max}}(25^\circ\text{C})$ . Data are mean  $\pm$  range (n=3).

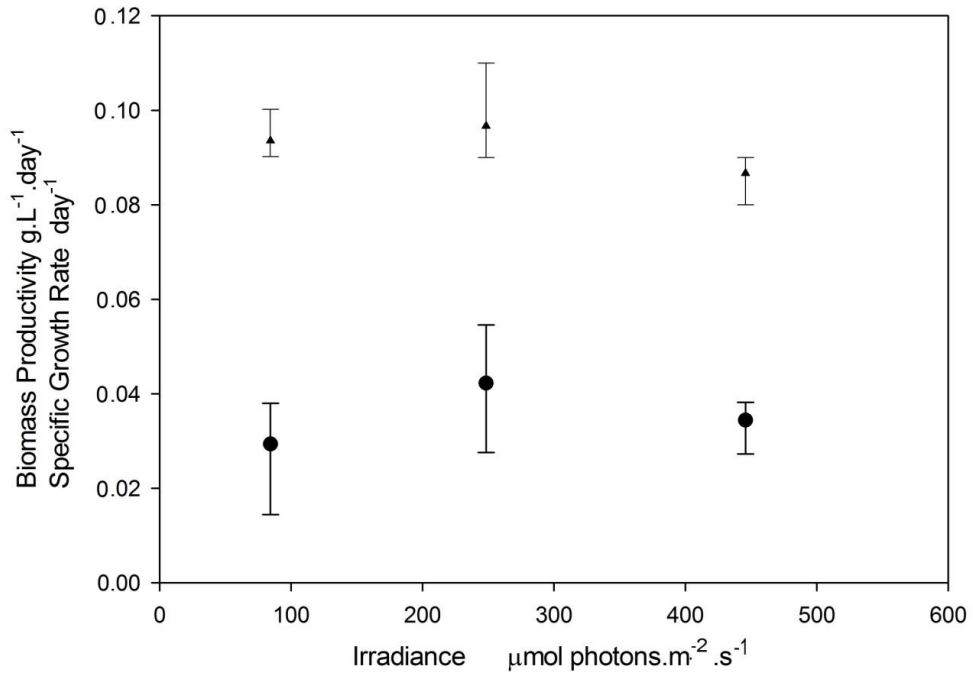
#### 4.2.1.3 Long-term effect of high irradiance

The photosynthesis vs irradiance studies above showed that *B. braunii* photosynthesis was not inhibited at high irradiances. To further examine the effects of high irradiances as would be experienced in outdoor ponds on biomass and lipid productivity. *Botryococcus braunii* CCAP 807/2 was grown in a 1.5 L glass Schott bottles (100 mm diam. × 210 mm high) as a semi-continuous culture with a harvesting interval of 4 days at  $100 \times 10^5$  cells.mL<sup>-1</sup> at 3 different irradiances. The light was evenly provided by cool white fluorescence lights tubes providing irradiances which either saturated photosynthesis or were supersaturating. The average irradiances in the flasks are shown in Table 4.

**Table 4. Irradiances in flasks for semi-continuous culture experiment.** Average Irradiance = (Front Side Irradiance – Back Side Irradiance) × 0.5

Irradiance condition	Average Irradiance μmol photons.m <sup>-2</sup> .s <sup>-1</sup>	Front Side Irradiance μmol photons.m <sup>-2</sup> .s <sup>-1</sup>	Back Side Irradiance μmol photons.m <sup>-2</sup> .s <sup>-1</sup>
Saturated	<b>89</b>	200	22
Over Saturated	<b>250</b>	600	100
Over Saturated	<b>440</b>	1000	120

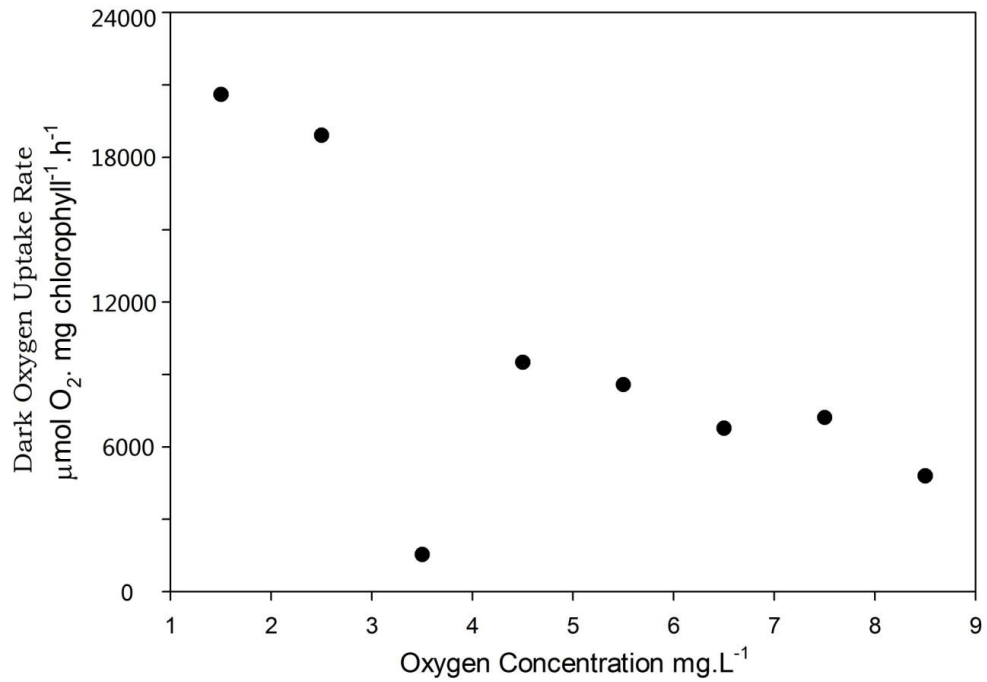
The biomass productivity at 250 μmol photons.m<sup>-2</sup>.s<sup>-1</sup> was slightly higher than the productivity at 89 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>. However, the productivity decreased slightly (but not significantly) at the highest irradiance of 440 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>. The specific growth rate was similar at 89 and 250 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>, but was slightly reduced at 440 μmol photons.m<sup>-2</sup>.s<sup>-1</sup>. One-way-ANOVA showed that there was no significant difference in specific growth rate and biomass productivity under the different irradiances (P=0.42(α=0.01) and P=0.37 (α=0.01), respectively).



**Figure 14. Effect of long term high irradiance on biomass productivity and specific growth rate.** Triangles = specific growth rate, solid circles = biomass productivity. Data are mean  $\pm$  range (growth rate n=3, productivity n=4). For each irradiance, the culture was harvested every 4 days for 3 intervals.

#### 4.2.1.4 Effect oxygen concentration on oxygen uptake (respiration) in the dark

In the experiments above it was observed that high oxygen concentrations appeared to inhibit oxygen uptake (respiration) in the dark. To further examine this unusual finding, the oxygen uptake rate in the dark at different oxygen concentrations was studied.

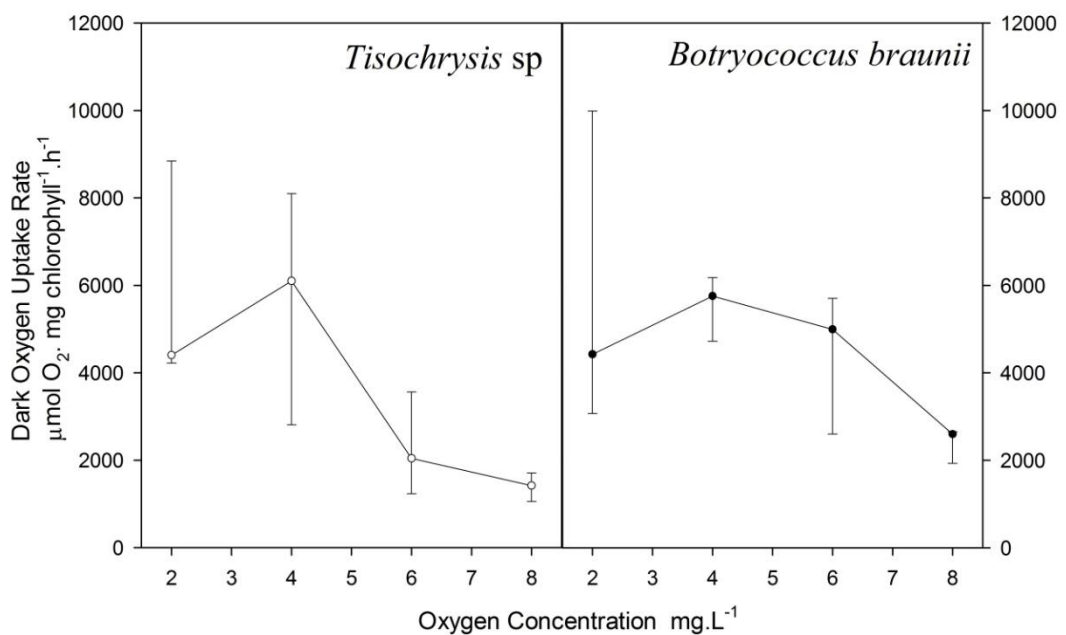


**Figure 15. Dark oxygen uptake curve. The measurement error of oxygen electrode was very small, so no replication for this determination.**

Figure 15. shows that respiration in the dark decreased with increasing oxygen concentrations. In order to exclude possible methodological effects several different ways to adjust the oxygen concentrations were used. Firstly, the measurements were started at a low oxygen concentration and then the oxygen concentration was increased stepwise through photosynthesis by periodically exposing the culture in the electrode chamber to light ( $100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). Alternatively the experiment was started at the highest oxygen concentration which was then decreased stepwise by bubbling with nitrogen gas. Irrespective of the method used to change the oxygen concentration the results were the same.

In order to see whether this effect of oxygen concentration on dark oxygen uptake was peculiar to this *Botryococcus* strain or whether other algae also showed this, the experiment was also carried out with a culture of the prymnesiopyte, *Tisochrysis* sp. (CS-177) (previously known as Tahitian *Isochrysis* (T-iso) - Bendif et al. 2013) from the CSIRO Australian National Algae Culture Collection. The culture was grown in a 250 mL conical flask with f/2 medium (Guillard and Ryther 1962; Guillard 1962; 1975) at 25°C at  $150 \mu\text{mol photons m}^{-2} \cdot \text{s}^{-1}$  on a 12:12 h light:dark cycle.

The experiment was carried out with *Tisochrysis* and *B. braunii* both at a cell density of  $100 \times 10^5$  cells.mL<sup>-1</sup>, at 25°C, with the dark respiration rates measured as above. In both species dark oxygen uptake was similarly inhibited by increased oxygen concentrations (Figure 11). Correlation analysis with  $x = Tisochrysis$ ,  $y = B. braunii$ ,  $r = 1.1$ , showed no significant difference was found between two algae ( $p = 0.23$ , T-test). (Figure 16)



**Figure 16.** Effect of oxygen concentration on oxygen uptake (respiration) in the dark of *Tisochrysis sp.* and *Botryococcus braunii*. Data are mean  $\pm$  range (n=3).

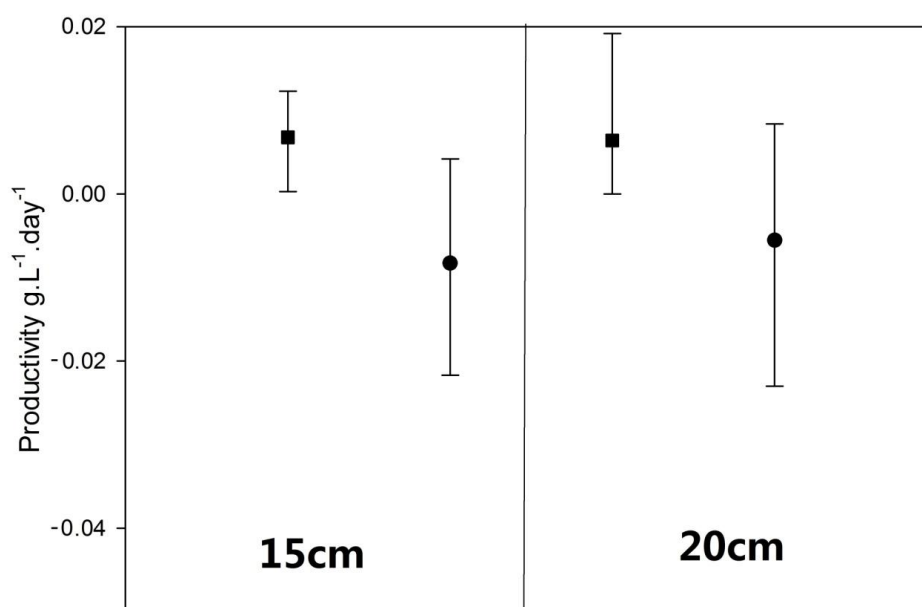
#### 4.2.2 Outdoor experiments

Limits to the growth and productivity of *B. braunii* CCAP 807/2 also were investigated in outdoor cultures to test whether the outdoor productivity of this strain can be improved by reducing potential limits to growth (i.e. the light availability, growth temperature and O<sub>2</sub> concentration).

#### 4.2.2.1 Effect of pond depth

Light affects the productivity of algae cultures and the amount of light received by algal cells in an open pond can be manipulated either by changing the pond depth or by altering the cell density in the pond. The aim of this study was to test how productivity of outdoor cultures of *Botryococcus* can be affected by pond depth (availability of light).

*Botryococcus braunii* was cultured in two identical paddle-wheel driven raceway ponds with same cell density ( $100 \times 10^5$  cells.mL<sup>-1</sup>) and the pH was maintained at 7-7.5 by the addition of CO<sub>2</sub> through a sparger in the pond using a pH-stat system. One pond was maintained at depth of 20 cm as control, and the other pond was operated at 15 cm depth in winter (June-July 2011).



**Figure 17. Effect of pond depth on biomass productivity (squares) and lipid productivity (circles) of *B. braunii* grown as semi-continuous cultures in 1 m<sup>2</sup> raceway ponds in Perth, Western Australia.** Data are mean  $\pm$  range (n=3). This experiment was carried out from 29.06.2011 to 16.07.2011 (Winterbourn) with three harvesting (dilution) intervals. Average daytime irradiance:  $286 \pm 166$  W.m<sup>-2</sup>; Average daytime temperature:  $15 \pm 3$ °C; Average night temperature:  $10 \pm 3$ °C.

Figure 17 shows the biomass productivity and the lipid productivity at the two pond depths. No significant differences were found in biomass productivity ( $p=0.14$ , T-

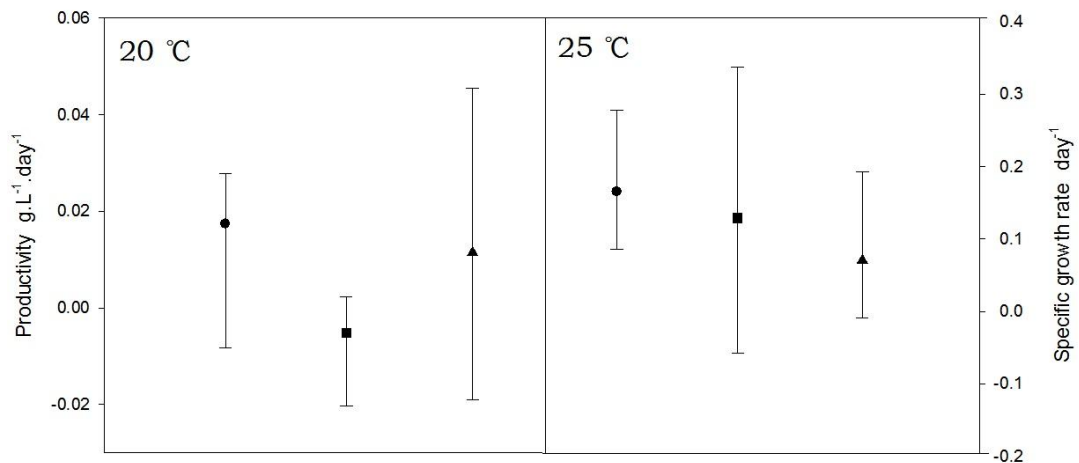


test) or lipid productivity ( $p=0.11$ , T-test) between the cultures grown at 15 or 20 cm depth.

#### **4.2.2.2 Effect of temperature**

Previous studies of algae cultures have shown that pond temperature can affect productivity (Moheimani and Borowitzka 2007; Converti et al. 2009) and that, especially in winter, temperature can be a major limiting factor. Therefore, the purpose of this experiment was to test how temperature affects biomass and lipid productivity in an outdoor pond culture. *Botryococcus braunii* was cultured in one 1 m<sup>2</sup> outdoor race way pond as a semi-continuous culture, a depth of 20cm and a cell density of  $100 \times 10^5$  cell.mL<sup>-1</sup> from the 16.07.2011 to 04.08.2011. CO<sub>2</sub> was added using a pH-stat system to maintain the pH between pH 7 - 7.5, and three aquarium heaters were put in the pond to adjust the temperature. The pond was firstly operated at constant 20°C (24h) and partially harvested 4 times with a harvesting interval of 2 days, then the temperature was increased to 25°C (24h) and the culture was partially harvested another 4 times with the same interval. As the air temperature in this period was below the heater set value, the temperature of the pond was a constant 20°C or 25°C. Pond temperature was continuously monitored with a Tiny Tag temperature logger.

The results show that the biomass productivity between 2 temperatures was not statistically different ( $p=0.27$ ; T-test). The specific growth rates also were not significantly different ( $p=0.47$ ; T-test). However, the lipid productivity at constant 25°C (24h) was slightly higher than the productivity at 20°C (24h) ( $p=0.07$ ; T-test).



**Figure 18. Effect of temperature on biomass productivity (circle), lipid productivity (square), and specific growth rate (triangle).** Data are mean  $\pm$  range (n=4). The average daily total irradiance was 134 W.m<sup>-2</sup>.day<sup>-1</sup> at 20°C and at 81 W.m<sup>-2</sup>.day<sup>-1</sup> at 25°C.

#### 4.2.2.3 Effect of oxygen concentration

The laboratory experiments shown above show that high oxygen concentrations inhibit photosynthesis and thus reduce productivity, but it is impossible to reduce the oxygen concentration in large-scale algae cultures economically.

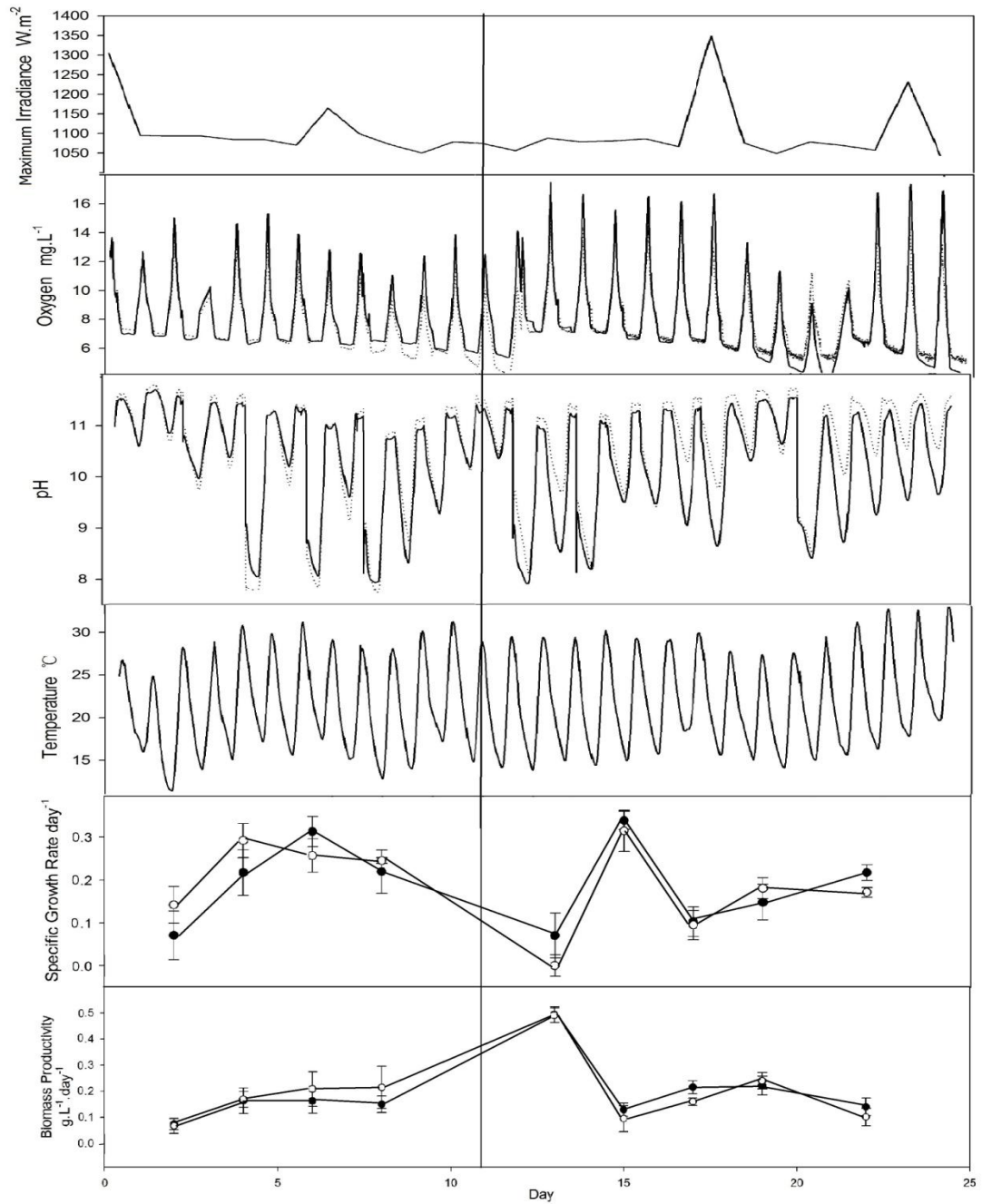
However, the laboratory experiments also showed that that high oxygen concentrations inhibit dark respiration in *Botryococcus* (see 4.2.1.4). As biomass loss due to respiration at night can be very substantial in outdoor cultures (Torzillo et al. 1991; Ogbonna and Tanaka 1996), and the observed reduction on respiration due to high oxygen concentrations can potentially result in an increase in the productivity. Therefore increasing culture oxygen content during night may be a possible strategy for long term cultivation to increase the daily net biomass production.

The aim of this experiment therefore was to attempt to develop a method which can effectively increase dissolved oxygen in the pond during night. Here, 10 m of garden dripping hose (7 mm in diameter) was used as an air diffuser directly connected to an air compressor (BinFord, Model BLE-20, 1100 kPa ). The total surface of the

dripping hose was about 0.22 m<sup>2</sup>, and the compressor pumped air into the pond continuously from 6 pm to 6 am (overnight).

Two 1 m<sup>2</sup> paddle wheel driven raceway ponds were operated in parallel with and without aeration at night. Initially both ponds were kept with no air addition for four harvest intervals of 2 days to stabilize the cultures, and following this air was then pumped into one of the ponds every night between 6pm and 6 am. The other pond was operated without air addition (control).

As shown in Figure 19, before the air was added, the DO, pH and culture temperature (dissolved oxygen) of both ponds were identical. With air addition, the oxygen concentration stayed the same between the control pond and the pond with aeration at night or, in some nights, the oxygen concentration was actually lower in the aerated pond indicating that the aeration process used was ineffective in increasing the night oxygen concentration in the pond. The pH of the pond with added air was a slightly lower than the other pond during the night. The temperature of two ponds was the same over the whole experimental period.



**Figure 19. Effect of aeration during the night on growth of *Botryococcus*.** Oxygen, temperature and pH are from YSI sonde record (see Chapter 2, 2.2.10). At the left side of the vertical line, the two ponds were operated at the same condition without aeration. At the right part of border line, air was pumped into one pond during night. For the oxygen and pH graphs the solid line is the pond with pumped air, and the dotted line is the pond without pumped air. For the specific growth rate and the biomass productivity graphs the solid circle is the pond with added air during night, and the open circle is the control pond without aeration. Specific growth rate data are mean  $\pm$ stdev (n=4), biomass productivity data are mean  $\pm$ range (n=3).

The lower 2 graphs in Figure 19 show the specific growth rate and biomass productivity of the ponds. Not surprisingly, considering the ineffectiveness of the attempt to increase the oxygen content in the pond by aeration at night, there were no significant differences in either the growth rate or the biomass productivity between two ponds (Table 5).

**Table 5. T-test of effect of aeration at night**

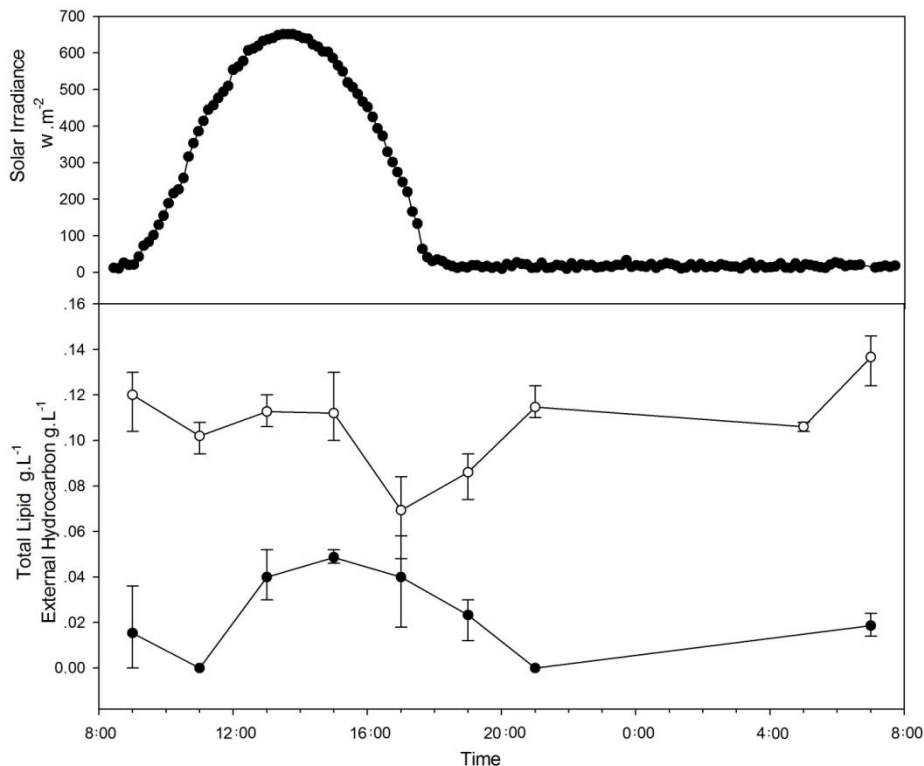
one-tailed distribution, paired	- Air		+ Air	
	Specific growth rate	Biomass productivity	Specific growth rate	Biomass productivity
P Value	0.43	0.09	0.27	0.09

#### 4.2.3 Diurnal lipid synthesis and release

The purpose of this experiment was to determine when in the diurnal cycle *Botryococcus* synthesises and releases lipid and hydrocarbon. This information potentially can be useful for the development of culture management and harvesting strategies for optimising lipid and hydrocarbon productivities.

In this study, one outdoor open pond *Botryococcus* culture at exponential phase was sampled every two hours from 7 am to 9 pm, and another sample was taken at 7 am the next morning and total lipid and external hydrocarbon were determined.

Figure 20 shows that the external hydrocarbon began to increase from 9:00 and peaked at 15:00, the time with the highest irradiance. After 15:00, the external hydrocarbon began to decrease gradually with decreasing irradiance to the same value as at 9:00 in the morning between 17:00 and 19:00, and decreased further after that. The external hydrocarbon increased slightly again by next morning at 7:00 to the same value as at 9:00 on the previous day.



**Figure 20. Diurnal pattern of total lipid content and extracellular hydrocarbon content.** The open circle is total lipid (mean  $\pm$  range (n=3)). The closed circle is external hydrocarbon (mean  $\pm$  range (n=3)). This culture was started with a cell density of  $160 \times 10^5$  cell.mL<sup>-1</sup>.

The total lipid remained almost the same between 9:00 and 15:00 (**Figure 20**) and then fell by  $40 \pm 24\%$  between 15:00 and 17:00. The total lipids then increased to the original values between 17:00 and 21:00 and remained unchanged during the rest of the night.

## 4.3 Discussion

### 4.3.1 Light

When the PE curve (Figure 9) of *Botryococcus* was investigated, it is interesting that there is little inhibition of photosynthesis at an irradiance of  $1900 \mu\text{mol photons.m}^{-2} \cdot \text{s}^{-1}$  (~25% on average). In another study on *B. braunii* photosynthesis, two strains of A race *B. braunii* UTEX 572 and *B. braunii* UTEX 2441 both also showed very little photoinhibition (~8%) under an irradiance of  $2000 \mu\text{mol photons.m}^{-2} \cdot \text{s}^{-1}$  (Yin et al.

2008). Therefore, this indicates that *B. braunii* strains can be grown at locations which have a high irradiance without significant photoinhibition. Secondly, for indoor cultures, using irradiances higher than the saturation point ( $P_{\max}$ ) does not lead to higher growth (Qin and Li 2006a). This is supported by the fact that the specific growth rate and biomass productivity of *B. braunii* were almost identical under saturated and over saturated light in my study (see Figure 14).

Several other algae have also been shown to tolerate high irradiances. For example, high light acclimated *Dunaliella salina*, was photoinhibited only at irradiances greater than 1500  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Baroli and Melis 1996) as does *Spirulina platensis* (Jensen and Knutsen 1993; Vonshak et al. 1996). Similarly the coccolithophorid alga *Pleurochrysis carterae*, when high light acclimated only showed photoinhibition under an irradiance of 2300  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Moheimani and Borowitzka 2007).

One possible reason for *B. braunii* lack of strong photoinhibition at high irradiances is that, the *B. braunii* colony size can become larger when irradiance increases (Zhang and Kojima 1998). As the colony size becomes larger, the average irradiance received by the algal cell will be reduced as has been shown in other studies (Verity et al. 1991; Li and Gao 2004).

Several reports have shown that lower pond depth can improve the growth and productivity of the culture by increasing the average irradiance the algal cells receive. For example, with *Spirulina*, the volumetric biomass productivity at 15 cm pond depth was 28% higher than 20 cm pond depth (Olguín et al. 2003). Similarly, another study on *Chlorella* sp. showed that the volumetric productivity and specific growth rate at 2 cm depth were 280% and 267% higher than the result at 10 cm depth, respectively (Liang et al. 2013). The lack of an effect of pond depth in my study can be attributed to two factors. Firstly, the experiment was carried out in winter (June) and the combined effect of lower temperatures and lower solar irradiation might mean that the increase in the average irradiance received by the cells in the shallower pond was still insufficient to enhance growth. Moheimani & Borowitzka (2007) also observed no effect of reducing pond depth in winter on the

growth of *Pleurochrysis carterae* at the same location as the present study. In summer, however, they did observe a significant stimulation of productivity at lower pond depth. The second possible reason for the lack of an effect of pond depth on *B. braunii* productivity is that the reduction in pond depth was insufficient to provide sufficient light to the algal cells.

### 4.3.2 Temperature

My study found that the maximum photosynthetic rate increased linearly with temperature between 10°C and 30°C, and then declined rapidly at 40°C. Similarly Yin et al (2008) found that *B. braunii* strains UTEX 572 and UTEX 2441 also showed the maximum photosynthetic rates at 34°C and 30°C respectively, and higher temperatures caused dramatic photoinhibition. It should be noted that all these strains (CCAP 807/2, UTEX 572, UTEX 2441) are A race and it is not known whether these findings also apply to other *B. braunii* races.

The decreasing of oxygen production by photosynthesis at higher temperature (40°C) can indicate two things: 1) the activity of PSI was inhibited. 2) oxygen was consumed in other pathways. In one study, when the temperature was increased from 20°C to 35°C, PSI had higher excitation energy (Weis 1985). Though this is a protection mechanism of the plant, PSI is not inhibited. Another possibility is that photorespiration increased at temperatures higher than 35°C. Increased photorespiration at higher temperatures also has been found in other microalgae (Goldman 1979a; Burris 1980) and higher plants (Ku and Edwards 1977; Clark and Menary 1980).

In the outdoor culture study, higher temperature only increased lipid productivity, but there were no significant changes in biomass productivity and specific growth rate (see Figure 18). Kalacheva et al (2002) observed similar results on a B race of *B. braunii* (LB 807/1). They found that: 1) there was no significant difference in biomass productivity between 18°C and 25°C; 2) the intracellular lipid content changed by +50% and -23% under 25°C and 18°C, respectively. 3) at 32°C, although biomass productivity was higher, lipid content was ~75% lower than at the



other two temperatures. In another study, also on *B. braunii* LB 807/1, the specific growth rate was identical at 18 and 25°C, but ~100% higher at 32°C (Sushchik et al. 2003). In another study on the same strain as used in my study (CCAP 807/2) a batch culture grown at 25°C had a slightly higher lipid content than a culture grown at 20°C at the end of the culture period, but the lipid content of the culture grown at 30°C was ~30% less than at 25°C. On the other hand, the optimal temperature for specific growth rate was found to be 25°C, and at 18°C and 30°C the specific growth rate was ~50% and ~40% lower, respectively (Li and Qin 2005).

Therefore according to above studies, it can be hypothesised that for my *B. braunii* (CCAP 807/1): 1) ~30°C was the optimal temperature for maximum specific growth rate or biomass productivity ; 2) ~25°C was the optimal temperature for lipid content and lipid productivity. Suboptimal temperature (i.e 20°C) reduces the lipid yield slightly, but superoptimal temperature (i.e. 30°C) dramatically decreases lipid productivity and yield. However, it needs to be noted that the temperature effect can vary among difference *B. braunii* strains. For example, it is also reported that maximum specific growth rate and lipid content was observed under 23°C and 60 W m<sup>-2</sup> for *B. braunii* CHN 357 (Qin and Li 2006b).

### 4.3.3 Oxygen

As shown in the results, higher oxygen can significantly inhibit net photosynthesis irrespective light and temperature. Also, it was found that high oxygen can inhibit the uptake of oxygen (i.e. respiration) in the dark.

Firstly oxygen can affect the *B. braunii* photosynthetic rate dramatically (see Figure 13). In another study of *B. braunii* photosynthesis, the net photosynthetic rate of a *B. braunii* culture at 25°C was ~1000 µmol O<sub>2</sub>. mg chlorophyll a<sup>-1</sup>.h<sup>-1</sup> (oxygen concentration was not given) (Yin et al. 2008) whereas in the present study it was ~6000 µmol O<sub>2</sub>. mg chlorophyll<sup>-1</sup>.h<sup>-1</sup> at 25°C at an oxygen concentration of 8.5 mg. L<sup>-1</sup> (see Figure 13). Considering the strong effect of oxygen concentration the difference between these two studies can be potentially attributed to differences in oxygen concentration. Although the authors did not provide any data for oxygen

concentration, it can be assumed that the oxygen in the culture was saturated or supersaturated after long time growth. In Figure 6, the relation between  $P_{\max}$  and oxygen concentration was very linear. Therefore when the graph was further extended, it can be calculated that the  $P_{\max}$  of my culture under 25°C, 8.5 mg.L<sup>-1</sup> oxygen and 100  $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$  can be reduced to 1000  $\mu\text{mol O}_2.\text{mg chlorophyll}^{-1}.\text{h}^{-1}$  by increasing the oxygen to ~9.1 mg.L<sup>-1</sup>. Therefore it can be hypothesized that the oxygen concentration in Yin's study was somewhere around 9 mg.L<sup>-1</sup> which was slightly super-saturated under that temperature (100% saturation under 25°C is 8.3 mg.L<sup>-1</sup>). Differences in the irradiance used between the two studies is not likely to be the reason as my culture had almost identical photosynthetic rates under 100 and 400  $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$  (see Figure 10).

Additionally, different algae has different sensitivity towards oxygen concentration. For example,  $P_{\max}$  (25°C) of *Pleurochrysis carterae* under lower oxygen (6 -10 mg.L<sup>-1</sup>) was ~6 times higher than the rate under high oxygen (26 -32 mg.L<sup>-1</sup>) (Shiraiwa et al. 2004; Moheimani and Borowitzka 2007). However, not all algae are sensitive to oxygen. For example, *Spirulina Platensis* was reported that higher oxygen concentration (~20 mg.L<sup>-1</sup>) did not result in apparent inhibition of photosynthesis (Vonshak 1997).

Also, the level of this oxygen induced inhibition changes upon temperature (see Figure 12). With increased temperature the inhibition was stronger, this phenomenon was also observed by another study on *Pleurochrysis carterae* which the inhibition of higher oxygen were 30%, 94% and 96% under the temperature of 10°C, 25°C and 32°C respectively (Moheimani and Borowitzka 2007).

The reason for oxygen induced photoinhibition has already been studied and also introduced in Chapter 1 (see 2.3.1) as O<sub>2</sub> can cause photorespiration especially in C<sub>3</sub> plant like algae (Chollet and Ogren 1975; Lloyd et al. 1977), this is because O<sub>2</sub> can compete with CO<sub>2</sub> to bind with rubisco and RuBP to produce 3-PGA and 2-phosphoglycolate as rubisco is also an oxygenase (Hatch et al. 1971) especially when O<sub>2</sub> level is high (Lorimer and Andrews 1973). In addition, another reason for O<sub>2</sub> induced photoinhibition is that O<sub>2</sub> can also capture the electron from NADP<sup>+</sup> to form

superoxide radical ( $O_2^{\cdot-}$ ) which will shift to  $H_2O_2$  (Mehler 1951; Strizh 2008) and extreme toxic  $\cdot OH$  (Winterbourn 1995), and those compounds can continuously break photosynthesis membrane and photosynthesis pigments (Kaiser 1976). Furthermore, under higher temperature, the activity of oxygenase and PSII can be increased which can further result in stronger photoinhibition and photorespiration (Janssen et al. 2003; Morris and Kromkamp 2003).

To date, there has been no economically feasible solution for reducing of the effect of  $O_2$  on the photosynthesis of algae or any other plant. Also, the absence of photorespiration can even be harmful to alga as this process is considered as protection to  $C_3$  plants from photooxidation (Kozaki and Takeba 1996). Secondly, another very interesting finding of this study was that higher oxygen can inhibit the dark oxygen uptake rate (see Figure 15 and Figure 16), and this was both found in *B. braunii* and *Tisochrysis* sp (CS-177) (previously known as Tahitian *Isochrysis* (T-iso) - Bendif et al. 2013) which means this phenomenon may not only be restricted to *B. braunii*. A study on *Chlorella pyrenoidosa*, *Chlamydomonas reinhardtii*, and *Anabaena flos-aquae* showed that the dark respiration was inhibited by high  $CO_2$  (Bidwell 1977). However, in my experiments  $CO_2$  concentration did not change significantly.

The few available studies of the effects of oxygen concentration on dark respiration in algae and aquatic plants showed that increased  $[O_2]$  results in increased dark respiration (Gessner and Pannier 1958; Owens and Maris 1964; McIntire 1966; Dromgoole 1978). For example, the dark respiration of *Pleurochrysis carterae* was about three times higher under high oxygen (26-32  $mg.L^{-1}$ ) than low oxygen (6-10  $mg.L^{-1}$ ) (Moheimani and Borowitzka 2007) which was just opposite of my study. Also in another study, the author also reported a series of aquatic plants and algae (i.e. *Ranunculus pseudofluitans*, *Hippuris vulgaris*, *Callitriche obtusangula* and *Berula erecta* etc.) had ~2-3 times higher respiration with increased oxygen from 1.5 to 10 $mg.L^{-1}$  (Owens and Maris 1964).

However, almost no reports on the inhibition of respiration by high oxygen concentrations in aquatic organisms can be found. The only one report which found

the same phenomenon to my result was in a nitrogen-fixation bacterium (Dilworth 1961). In this study, it was found that the respiration of the bacterium *Azotobacter vinelandii* was reduced by 50% when oxygen partial pressure was increased from 0.25 to 1 atmosphere pressure. In my study on *B. braunii*, increasing oxygen from 2 mg.L<sup>-1</sup> to 8 mg.L<sup>-1</sup> caused ~70% (see Figure 15) and ~50% (see Figure 16) drop in oxygen uptake rate on average in the dark. Interestingly, the two results had a very similar pattern, i.e. ~4 times higher oxygen can inhibit oxygen uptake by ~50%. The author pointed out that this finding may be peculiar to *Azotobacter vinelandii* (Pseudomonadales) and to date there are no other reports in related or unrelated bacteria.

From above finding, it is tempting to speculate that there was *Azotobacter vinelandii* (or a similar bacterium) in my *B. braunii* CCAP 807/1 culture which was not axenic (the CCAP website also indicates this culture may contain bacteria) and that the observed effect of oxygen concentration was actually on bacterial respiration and not algal respiration. Various Pseudomonadales have been isolated from an A race *B. braunii* (LB-572) culture (Rivas et al. 2010) and included: *Acinetobacter* sp. and *Pseudomonas* sp.. Additionally, another nitrogen-fixation bacterium *Rhizobium* sp. was also isolated. Secondly, one study on co-culturing *Azotobacter chroococcum* and A race *B. braunii* showed that such algal-bacteria relation may exist in natural environment which can contribute to the CO<sub>2</sub> balance in water (Jones 1972). As introduced earlier in Chapter 1 **2.3.5**, *Botryococcus* also may be associated with many other kinds of bacteria which can boost the growth of algae (Haines and Guillard 1974) or increase hydrocarbon production (i.e. *Corynebacterium* sp) (Wang and Xie 1996). Sometime, associated bacteria can also negatively affect hydrocarbon accumulation due bacterial degradation (Chirc et al. 1985).

However, the effect of oxygen on respiration was also observed in *Tisochrysis* sp. and it is less likely that both cultures harbour the same, very unusual, bacterial population. A more probable mechanism for the observed results is likely to involve alternative respiration pathways and/or the action of various oxygenases (Lambers 1985; Millar et al. 2011; Van Dongen et al. 2011; Moore et al. 2013). Time limitation

did not allow this phenomenon to be studied further and at this time no mechanism for the observed results can be proposed.

The attempt to increase the oxygen in the pond during night by aeration to reduce night-time respiration of the culture and thus increase net biomass productivity over 24 h was not successful. The oxygen during night was far from saturation this may be due to too big bubble size and the very short contact time of the air bubbles with the medium.

However, the highest temperature during the day time was from 25°C to 30°C, and the highest oxygen level is from 10-14 mg.L<sup>-1</sup>. As the oxygen solubility (saturated in DI water) at 25°C is 8.3 mg.L<sup>-1</sup>, this means the pond was oxygen super saturated during the day time. It was found that the addition of air in the pond can decrease the oxygen concentration as long as it is over saturated. For example, the oxygen can be decreased from 10 mg.L<sup>-1</sup> to 9 mg.L<sup>-1</sup> within 15 min. Therefore, this may be one economical way to remove oxygen from the water, and thus improve photosynthesis.

#### **4.3.4 Diurnal lipid**

The diurnal study showed that the content of external hydrocarbon and total lipid changed through a day, and this result suggests that the ideal harvesting time for highest lipid yield in a day is between 7:00 and 8:00 am (i.e. soon after sunrise).

Temperature has no apparent effect on *B. braunii* external hydrocarbon content (Kalacheva et al. 2002) nor composition (Sushchik et al. 2003). On the other hand, the pattern of external hydrocarbon may relate to two aspects of solar irradiation: 1) the light intensity, and 2) UV.

Firstly, exposure of *B. braunii* cultures to increased irradiance can lead to an increase in the size of the colony resulting in decreased light available to the individual cells (Zhang and Kojima 1998). As the growth of matrix volume requires extra hydrocarbon increased light can result in a higher content of extracellular hydrocarbon. In Figure 20, it can be seen that the peak of external hydrocarbon was at the time when the solar irradiance reached maximum (13:00-17:00).

Secondly, strong UV can damage photosystem II of algae (Jones and Kok 1966). Generally, algae are more sensitive to short wave UV-B and UV-C, and are less affected by UV-A (Jokiel and York Jr 1984). Interestingly hydrocarbon can absorb UV (i.e. squalene, 190 nm ) (Eroglu and Melis 2010), and different chain length hydrocarbons have different absorbance peaks (Etzkorn et al. 1999). Therefore this may be one possible reason that *B. braunii* released more hydrocarbon externally in the afternoon.

Another noticeable phenomenon is that the total lipid had a sudden drop between 16:00 to 17:00, but the content of external hydrocarbon during this period barely changed from 0.05 to 0.04 g.L<sup>-1</sup> on average, meaning that the drop in total lipid was due to the loss of intracellular lipid. One potential reason for the lipid loss is temperature, as it has been reported that temperature above 25°C can dramatically reduce intracellular lipid in *B. braunii* (Kalacheva et al. 2002). The reduced part are mainly sterols, alcohols and free fatty acids, and the content of polar lipids and diacylglycerides remained the same.

The UV and very high irradiances can lead to photoinhibition, photodamage, and even damage to DNA (Sinha and Hader 2002). When such damage happens together, it activates repair mechanisms to protect the cell (i.e. PSII repair, chloroplast recovery) (Baroli and Melis 1996; Neidhardt et al. 1998) and such activity within the cell requires energy. Therefore, fatty acid are metabolized to gain energy during this process. Additionally, sterol may also take part in other metabolism resulting in their reduction (Heftmann 1971). In conclusion, the drop in lipid during the hottest period of day may indicate that *B. braunii* activated various repair mechanisms during the day.

#### 4.4 Conclusion

In general, increasing temperature from 20°C to 25°C had no effect on biomass productivity and specific growth rate (Kalacheva et al. 2002) (also see Figure 18), but this can increase lipid yield. As a result, the lipid productivity was increased (lipid productivity  $\approx \mu \times$  lipid yield).

On the other hand, though further increasing temperature to 30°C can result in 43% higher growth rate, intracellular and extracellular lipid decreased by 75% and 40% (Kalacheva et al. 2002). Overall, the lipid productivity was still decreased (lipid productivity  $\approx \mu \times$  lipid yield). Besides, it is still not known if this growth rate can be sustainable under this high temperature.

Though the mechanism of change in lipid content and growth rate upon temperature was not clear yet, to optimize the lipid productivity, *B. braunii* should be grown at a temperature no higher than 25°C and as *B. braunii* can tolerate higher irradiances, therefore it can be grown at locations with high irradiances. Although reducing oxygen can potentially improve lipid production, there is still no economically feasible way to achieve this on a large scale.

## CHAPTER 5 CONCLUSIONS

This study clearly showed that *Botryococcus braunii* 807/2 can be cultured reliably in an outdoor paddle wheel driven open raceway pond for as long as 5 months. This is by far the longest time that this alga has been grown under outdoor conditions with almost no contamination.

Proper pond management and operation (see 3.3.1) were responsible for improving the growth performance of *B. braunii*. However, the culture collapsed partially twice in five months. The collapse was mainly due to two reasons: 1) Cell densities higher than  $200 \times 10^5$  cells.mL<sup>-1</sup> which appears to cause nutrient starvation and light limitation. 2) Contamination by other organisms such as *Scenedesmus*. To eliminate contamination organism, the pond can be heated up during allowing *Botryococcus* to outcompete contaminating organisms.

The results of this study also indicated that tubular closed photobioreactors (Biocoil) are not an ideal systems for *B. braunii* culture due to some limitations. 1) massive cell sticking in the degasser box which causes culture collapse and creates difficulties in accurate sampling. 2) The limited mass transfer in the Biocoil which can contribute to extreme high oxygen concentration. As shown in Chapter 4 (see 4.2.2.2), high oxygen can increase photoinhibition which further affects lipid productivity. It was found that the Biocoil culture does not show a higher lipid productivity than open ponds (see 3.2.2), and as it is more costly it is not a suitable system for *B. braunii* culture.

When the outdoor culture in open pond was grown semi-continuously, the culture has a very low lipid productivity compared to indoor culture. This means that there is still a lot room to improve outdoor lipid productivity. After studying limiting factors on photosynthesis of *Botryococcus*, it was concluded that the lipid productivity of my outdoor culture during the test period (Winterbourn) was mainly limited by temperature, high oxygen concentration and less by light.



It is clear that higher temperature can increase *B. braunii* lipid productivity (see 4.2.2) as, for example, by heating the pond to 25°C during winter as 25°C is the optimum temperature for lipid productivity of *B. braunii* (see. 4.3.2). On the other hand, light may not be a major limiting factor in my culture as *B. braunii* photosynthesis saturates at about 100  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (see 4.2.1.1). Also, lowering pond depth by 5 cm in order to increase the average irradiance received by the algal cells did not improve biomass or lipid productivity (see 4.2.2). However it was observed that the oxygen in the pond was super saturated, and that pumping air into pond can remove some oxygen (see 4.2.2). The lipid and hydrocarbon content in the outdoor culture was found to show diurnal variation and the highest lipid content was observed early in the morning between 7:00-8:00am, and therefore this period would be ideal for routine harvesting which would also optimize lipid productivity.

An important finding of this study is that high oxygen can inhibit dark oxygen uptake (i.e. respiration) in *Botryococcus*. The same was also observed with another alga, *Tisochrysis* sp. This has not been previously reported in aquatic plants, but unfortunately time limitation meant that this observation could not be studied further in detail.

In conclusion, to optimize outdoor *Botryococcus* lipid productivity, the following is proposed:

- 1) Heating the pond to 25°C during the cold season.
- 2) Irradiance should be higher than 100  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  ( $100\times 10^5$  cells.mL<sup>-1</sup>).
- 3) Pumping air into pond to reduce the oxygen concentration

## 5.1 Future direction

To further improve the growth of *B. braunii* it would be very interesting to study:

- 1) The growth of *B. braunii* during other seasons (between September (winter) to February (early summer)) to evaluate culture reliability over the whole year.
- 2) The potential of increasing biomass productivity by reducing O<sub>2</sub>, possibly through aeration of the pond.
- 3) To further study the mechanism(s) resulting in the inhibition of respiration (oxygen uptake) in the dark. Reducing the respiration rate may be one way to increase net productivity.

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