The Effects of Light Quality on the Morphology
and Hydrocarbon Production
of *Botryococcus braunii*

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I certify that all material in this thesis which is not my own work has been identified
and that no material has previously been submitted and approved for the award of
a degree by this or any other University.

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ACKNOWLEDGMENTS

ABSTRACT

Botryococcus braunii is a green, colonial microalga that can produce up to 75 % of its dry weight as liquid hydrocarbons that can be converted to a sustainable biofuel. Until now, no studies have been performed on the effects of light quality on *B. braunii* cell morphology and hydrocarbon production. This investigation focused on the effects of white light (WL), blue light (BL), green light (GL) and red light (RL) on cell morphology and hydrocarbon synthesis in *B. braunii*, strain Guadeloupe (race B). *B. braunii* dry biomass increased in WL and RL, but in BL and GL there was no growth. The average cell size was significantly larger ($P < 0.05$) in WL than the average cell size in RL and BL and GL. The amount of hydrocarbons synthesised was not affected by light quality, with a relative hydrocarbon of 38 % to 42 % per dry biomass across the different light regimes. Moreover, there was no change to the composition of hydrocarbons produce by *B. braunii* in the different light treatments.

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%	Percentage
μm	Micrometer
B. braunii	*Botryococcus braunii*
BL	Blue light
DB	Dry biomass
g l^{-1}	Grammes per litre
g l^{-1} d^{-1}	Grammes per litre per day
GC – FID Detector	Gas Chromatography – Flame Ionization
GC – MS	Gas Chromatography – Mass Spectrometry
GL	Green light
LFD	Light Flux Density
m/z	Mass to charge ratio
mg g^{-1}	Milligrammes per gram
mg l^{-1}	Milligrammes per litre
RL	Red light
SE	Standard Error
UV	Ultra-Violet
WL	White light

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CHAPTER 1

INTRODUCTION

The unicellular, colonial, photosynthetic microalga *Botryococcus braunii* (Kützing, 1849) is a member of the Trebouxiophyceae. Recent 18s rRNA sequences of strains of the three different chemical races of *B. braunii* established that these strains form a monophyletic group with closest relatives in the genus *Choricystis* (Senousy et al. 2004 and Weiss et all. 2010).

B. braunii is widespread in fresh and brackish waters throughout the globe including continental, temperate, tropical and alpine zones on all continents (Wolf *et al*., 1985; Metzger *et al*., 1985). *B. braunii* is regarded as a potential source of hydrocarbons because of its ability to produce oil up to 75% of algal dry weight (Maxwell et al, 1968). These oils are predominantly botryococcene (Metzger et al. 1983).

Paleobotanical studies suggest that *B. braunii* is one of the major sources of hydrocarbons in a many oil shale deposits dating from the Ordovian period (Cane, 1977). The occurrence of botryococcane, $C_{34}H_{70}$, the hydrogenated derivative of botryoccenes, at levels of 0.9% and 1.4% in two Sumatran crude oils have been reported (Moldwan and Seifert, 1980). These concentrations are the highest levels ever reported for a single complex fossil biological marker in petroleum (Moldwan and Seifert, 1980). Few oil shales contain botryococcene, although *B. braunii* was abundant during the formation of those shales this difference is explained by degradation of botryococcenes in the oxic condition of early diagenesis (Derenne *et al*. 1988). The outer wall of *B. braunii* is a biopolymer comprising 9% to 12% of dry cell biomass. *B. braunii* is thought to have a role in the formation of torbanites (Cane, 1969; Wake and Hillen, 1981). Coorongite, which is a recently formed deposit of *Botryococcus* is regarded as the precursor of torbanite (Cane and Albion, 1971) which is a fine-grained coal.

B. braunii has been reported to convert 3% of the solar energy to hydrocarbons (Gudin *et al*, 1984). The thermal value of biomass-derived hydrocarbons ranges between 30,000 and 42,000 kJ/kg (Held *et al*, 1985). Converting a 100 MW thermal power plant from using coal to using liquid fuel derived from *B. braunii*, could reduce CO_2 emissions by 1.5 x 10^5 tons/yr

(Sawayama et al, 1999). Potentially the CO_2 emissions values could be reduce further if CO_2 fixation efficiency of algae could be improved.

Races of *Botryococcus braunii*.

In an attempt to classify *Botryococcus*, 13 species were proposed based on morphological differences (Komarek and Marvan 1992). Later studies however, showed that a single *Botryococcus* specie could resemble more than one specie depending on the growth conditions (Plain et al. 1993) and therefore this classification has not been widely use.

Genus *Botryococcus* Kützing are noted for their lipid content, especially hydrocarbons. The most known specie is *B. braunii* which has a worldwide distribution and the different strains differ from the hydrocarbons they produce independently of its physiological state (Metzger et al. 1985). For this reason, *B. braunii* has been classified into 3 distinct races depending on the type of hydrocarbons they produce.

Race A produces odd-number C_{25}-C_{31}, alkadienes and trienes (two double bonds and three double bonds). Race B produces triterpenoid hydrocarbons known as botryococcenes (C_nH_{2n-10}, n = 30 - 37). Race L produces a single hydrocarbon a $C_{40}H_{78}$, a tetraterpene, known as lycopadiene (Metzger *et al*., 1990).

B. braunii Race A produces odd number alkadienes and trienes hydrocarbon structures derived from the fatty acid oleic acid through elongation by successive addition of C_2 units via malonate (Templier et al. 1984, 1987) with decarboxilation as the final step (Chang Yong et al. 1986). This pathway is similar in the synthesis of cuticular hydrocarbons of certain higher plants (Kolattukudy et al. 1976).

B. braunii Race B produces botryococcenes, which are triterpenoids with acyclic and cyclic compounds, which can be converted into high octane gasoline, kerosene and diesel fuels (Banerjee et al. 2002). Of the 50 botryococcenes identified, only about 15 have the structures elucidated. The first botryococcene described was C_{34} isolated from a wild sample (Cox et al. 1973) and was isolated from a culture strain (Metzger and Casadevall 1983). The B race *B. braunii* is estimated to produce botryococcene between 10 – 38 % of dry biomass and can

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exist as isomers within the same strain (Okada et al. 1995; Eroglu et al. 2010). The Botryococcene C_{30} has been shown to be the precursor for the higher homologues compounds (Metzger et al. 1987). In addition, algae of race B are found to synthesise squalene and the higher homologues C31 – C34 methylated squalenes (Huang and Poulter 1989; Metzger and Largeau 1999).

Botryococcene C_{30} is synthesised through the isoprenoid pathway by condensation of two molecules of C_{15} farnesyl diphosphate with a 1' – 3 connection of the two farnesyl molecules which produces a central branch with C = C^2 bonds at C-11 and C-26. *B. braunii* must also produce isopentenyl diphosphate (IPP) as well as dimethylallyl diphosphate (DMAPP), as they are the required precursors for isoprenoid biosynthesis. A labelling experiment showed that botryococcenes and methylated squalenes arise from the non-mevalonate pathway (Sato et al. 2003). C_{30} is further metabolized by methylation at C_3, C_7, C_{16} and C_{20} to produce C_{31}, C_{32}, C_{33} and C_{34} botryococcenes and even higher homologues as C_{36} and C_{37} in some B races strains (Metzger et al. 1985; Okada et al. 2004) and botryococcenes are secreted to the extracellular matrix as they elongate to C34 compounds (Metzger et al. 1987; Wolf et al. 1985).

The majority of botryococcenes are secreted into the extracellular matrix and it has been estimated that only 7 % of the botryococcenes are intracellular (Wolf et al. 1985). Largeau et al (1980) estimated that 95 % of the hydrocarbons produced by *B. braunii* are located in the extracellular matrix.

Cell Morphology Physiology.

B. braunii colonies of semi-transparent cells are embedded in a hydrophobic matrix with threads keeping numerous clusters together (Banerjee et al. 2002; Papa et al. 2008). The colonies can vary in size from 30 µm up to 2 mm with. The individual cells have a pyriform shape with a size of 7 x 14 µm. The *B. braunii* cell wall has a layer made of polysaccharide in external successive layers with hydrocarbons present in between the cell walls. Upon cell division, each cell secrets new cell walls within the mother cell. Thus, the extracellular matrix appears to be composed of successive cell walls saturated with oil (Banerjee et al. 2002).

During cultivation, *B. braunii* can change colour due to an accumulation of secondary carotenoids caused by stress, such as nutrient deficiency or high light intensity (Banerjee et al. 2002). The carotenoid molecules are more abundant in races B and L. The main carotenoid present in the extracellular matrix is a ketocarotenoid named echinenone. The color change is mainly caused by the secretion of echinenone inside the cell and a simultaneous decrease in the abundance of intracellular pigments (Okada *et al.*, 1998).

Botryococcus braunii and Bacterial Symbiosis.

Bacteria can stimulate algal growth by the release of substances such as vitamins and nitrogen derivative. Also bacteria can influence the pH by producing CO_2 and compete for the nutrients available. Several bacteria can have an influence on growth and hydrocarbon production of *B. braunii* (Chirac et al. 1985). The precise effects depend on culture conditions and the microbial species involved. In one study, the presence of *Corynebacerium sp.* increased the amount of hydrocarbon per algal biomass from 5.6% to 24.2% (Wang and Xie, 1996). The presence of *Pseudomonas oleovorans* can inhibit the flourishment of *B. braunii*. However, the association with microorganisms is not essential for the alga to produce large quantities of hydrocarbons (Banerjee et al. 2002).

Culture Conditions.

The production of hydrocarbons in *B. braunii* appears to be growth associated, irrespective of the culture conditions used (Banerjee et al. 2002). *B. braunii* slow growth rate are not because of a limited supply of nutrients but partly due to the limit diffusion of CO_2 and inorganic molecules into the cell through a narrow gap. Another reason for the slow growth is probably as a result of the production of energetically expensive hydrocarbons (Banerjee et al. 2002).

Photosynthetic cultures of *B. braunii* require CO_2. It has been shown that *B. braunii* can grow well in CO_2 concentrations from 2 % up to 20% without any increase in the medium pH. The highest dry biomass was seen in the 20 % aeration in a photobioreactor cultivation (Ge et al. 2010). On the other hand CO_2

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limitation showed a detrimental effect in the growth of *B. braunii* and in the production of hydrocarbons (Chirac et al. 1985).

Although a deficiency of nitrogen, such as nitrogen source nitrate, favours lipid accumulation (Shifrin, N. S., 1981), nitrogen is required for growth. A higher nitrogen concentration resulted in a longer exponential phase (Brenckmann et al. 1985). Ammonia is not suitable nitrogen source for *B. braunii* as the addition of 1M NH_4Cl to the medium results in a decrease of hydrocarbon production, respiration and photosynthetic activity (Ohmori et al. 1984). Study on nutrients show that phosphate is not limiting (Casadevall et al. 1985).

B. braunii growth in various organic carbon sources added to the medium was increased when exposed to glucose or mannose in illuminated cultures or in continuous darkness. *B. braunii* exposed to glucose showed larger colonies and liposomes in the intracellular space than *B. braunii* cultured without glucose (Tanoi et al. 2010).

Studies on light intensity show that a broad range of light intensity is not detrimental to *B. braunii* (Cepak and Lukavsky 1994). Under intense illumination (140 μmol m^{-2} s^{-1}) algal cells that have been adapted to a high light intensity achieved a higher biomass concentration and hydrocarbon content (50% of dry biomass) compared with cells adapted to low light intensity (42 μmol m^{-2} s^{-1}) (Kojima and Zhang, 1999).

A regular increase in pH is partly due to the consumption of dissolved CO_2 for photosynthesis. Similar changes in pH are commonly observed in CO_2 – enriched cultures during exponential growth. The optimum temperature for growth seems to change according to the different *B. braunii* strains (Li and Qin 2005).

It has been shown that *B. braunii* can adapt to low levels (17 mM to 85 mM) of salinity while increasing biomass, hydrocarbon and other metabolites (Rao et al. 2007).

The Nature of Light

Light is a form of electromagnetic radiation (Fig. 1.1). The segment of the electromagnetic radiation most important to life is the band from about 380 nm to 750 nm in wavelength, known as visible light.

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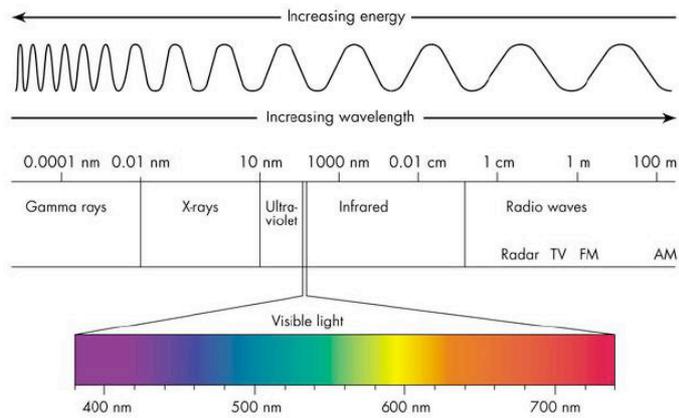
Fig. 1.1: The electromagnetic spectrum

Light has two properties and is described as travelling as a wave and interacts as a particle, called photons. Photons are not tangible objects, but they act like objects in that each of them has a fixed quanta of energy. The amount of energy is inversely proportional to the wavelength of the light: the shorter the wavelength, the greater the energy of each photon of that light. Although the sun radiates the full spectrum of electromagnetic radiation, the atmosphere acts like a filter, allowing visible light to pass through while screening out a substancial fraction of other radiation. Picture taken from: http://www.antonine-education.co.uk/physics_gcse/Unit_1/Topic_5/topic_5_what_are_the_uses_and_ha.htm

When light passes through water, it is subject to both absorption and scattering, which reduce the overall irradiance of light in a process called attenuation. The attenuation coefficient of pure water is smallest at about 465 nm, and increases towards both ultra-violet and infra-red radiation (Fig. 1.2). The main radiation for photosynthetic organisms is in the red light region and therefore it is critical that marine photoautotrophic organism can access this light zone.

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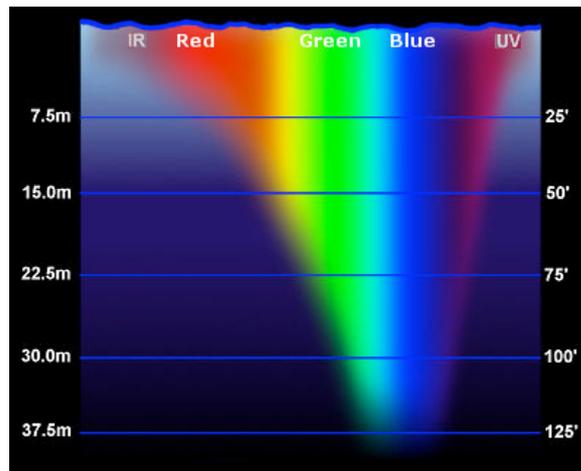
Fig. 1.2: Visible light penetration in water.

Light is attenuated firstly in the red light region and does not penetrate further than 5 metres in clean water, and blue light penetrates further down in the water column. Picture taken from:
http://www.seos-project.eu/modules/coralreefs/coralreefs-c05-p04.html

The Effect of Light on Plant Cells

Light has two main effects on plants; first autotrophic carbon fixation into sugars by photosynthesis, second, photomorphogenesis. Photosynthesis is the process by which plants convert the solar energy into chemical energy stored sugar and other organic molecules. There are two stages to photosynthesis, the light reactions which occur in the chloroplast and where water is split providing a source of electrons and protons, and the dark reaction (Calvin cycle) where CO_2 is fixed and carbon is reduced to sugars.

Light absorbed by chlorophyll excites the electrons and transfers hydrogen ions from water to an acceptor called $NADP^+$ (Nicotinamide Adenine Dinucleotide Phosphate) and $NADP^+$ is reduced to NADPH by adding two electrons and an H^+ and generating ATP. Thus, the solar energy is initially stored in the form of two compounds: NADPH and ATP, which will then be oxidised in the Calvin cycle to reduce the carbon molecules into sugars, in a process called carbon fixation.

The effects of light on plant morphology are what plant biologists call photomorphogenesis. Plants can detect not only the presence of light but also its

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direction, intensity and wavelength, notably red light and blue light.

Blue light responses in plants include phototropism, light induced opening of stomata and the light-induced slowing of hypocotyls elongation that occurs when a seedling breaks ground.

Plants use at least three types of pigments to detect blue light: *Cryptochromes* which are molecular relatives of DNA repair enzymes and involved in the blue-light-induced inhibition of stem elongation. *Phototropins* are a protein kinases involved in mediating phototropic curvatures and chloroplast movements in response to light. *Zeaxanthin* is a carotenoid-based photoreceptor involved in blue-light-mediated stomatal opening and may be related to the phototropins.

Phytochromes are red light photoreceptors. A *phytochrome* has two identical subunits, each consisting of a polypeptide component covalently bonded to a nonpolypeptide *chromophore*, the light absorbing part of the subunit. The *chromophore* of a *phytochrome* is photoreversible reverting back and forwards between two isomeric forms, depending on the colour of light provided, red light (λ 660 nm) or far red (λ 730 nm). In its P_r form, phytochrome absorbs red light and in its P_{fr} form, which is the active form, it absorbs far-red light. The $P_r \leftrightarrow P_{fr}$ interconnection is a switching mechanism that controls various light-induced events in the life of plants. Plants synthesize *phytochrome* as P_r, and if stayed in the dark, the pigment remains almost in the P_r form. Sunlight contains both red light and far-red light, but the conversion to P_{fr} is faster than the conversion to P_r, therefore the ratio of P_{fr} to P_r increases when, for example, seeds are exposed to adequate light, and the production of P_{fr} triggers germination.

Rationale and Aims of the Project

At the present moment the use of petroleum sourced fuels is unsustainable because of depleting supplies and the contribution of these fuels to the accumulation of carbon dioxide in the environment. Recently, microalgae has been receiving a lot of attention because of the potential high production of hydrocarbons that can be use as biofuel, it is carbon neutral and because it does not compete for land with food crops.

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B. braunii is a prodigious organism in producing hydrocarbons with reported amounts of 76 % of the dry weight of the alga (Maxwell et al, 1968). Currently it is accepted that production of high lipid content in *B. braunii* is growth associated, but hydrocarbon content can vary from 0.2 % to 61.0 % of its dry weight (Metzger and Largeau, 2005) in association with the growth conditions. The biological reasons for such high levels of hydrocarbon production remain unknown. Plausible hypotheses include defence mechanisms against abiotic stresses such as UV, or against biotic challenges from bacteria, or a adaptation to maintaining optimal buoyancy in the photic zone of the water column.

A preliminary study suggested that blue light could trigger lipid production in *B. braunii* race B alga (Fig. 1.3). In the preliminary study, with atmospheric CO_2 supply, *B. braunii* race B showed a higher lipid content per dry biomass in blue light conditions compared to algae grown in white light from day 13 to day 34.
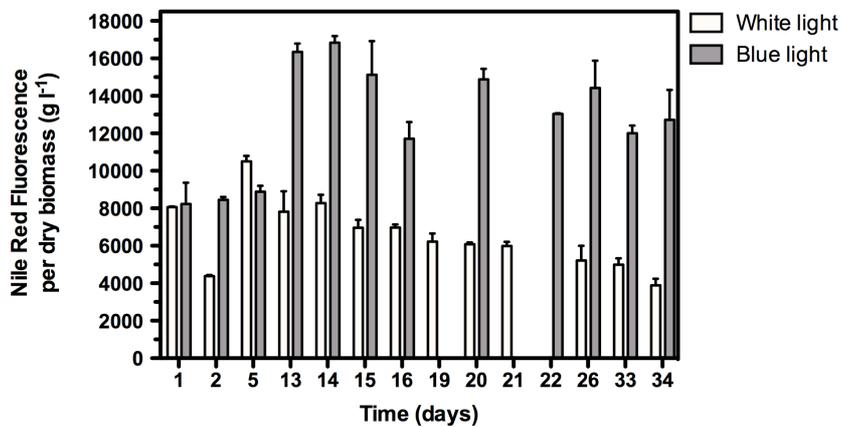
Fig. 1.3: Lipid production per dry biomass (g l^{-1}) accordingly to Nile Red assay in *Botryococcus braunii* grown in white and blue light.

This project aims to investigate that observation further by addressing the hypothesis that light quality affects lipid production in *B. braunii*. The physiological monitoring of *B. braunii* with different light colours over time include biomass determination, measurements of chlorophyll content, monitoring of cell size (width

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and length) and quantity and quality analysis of the lipids produced. In the end of this study, it will be possible to conclude if light quality induces lipid production in *B. braunii* B race.

CHAPTER 2

MATERIALS AND METHODS

2.1 – Algal Culture.

Botryococcus braunii race B was obtained from Pierre Metzger (Laboratoire de Chimie Bioorganique et Organique Physique, Ecole Nationale Supérieure de Chimie de Paris). Algae were grown in modified Chu13 medium supplemented with vitamins, citric acid and sodium selenate (MCV): 400 mg l^{-1} KNO_3, 200 mg l^{-1} $MgSO_4.2H_2O$, 200 mg l^{-1} citric acid, 108 mg l^{-1} $CaCl_2.2H_2O$, 104.8 mg l^{-1} K_2HPO_4, 20 mg l^{-1} $Fe-Na_2EDTA$, 2.86 mg l^{-1} H_3BO_3, 1.8 mg l^{-1} $MnSO_4.4H_2O$, 1.1 mg l^{-1} thiamine, 220 μg l^{-1} $ZnSO_4.7H_2O$, 135 μg l^{-1} cobalamin, 90 μg l^{-1} $CoSo_4.7H_2O$, 80 μg l^{-1} $CuSO_4.5H_2O$, 60 μg l^{-1} $Na_2MoO_4.2H_2O$, 25 μg l^{-1} biotin, 10 μl l^{-1} H_2SO_4, 9.4 μg l^{-1} Na_2O_4Se, pH 7.5 with NaOH.

Algae were grown in Infors HT Multriton incubators in 300 ml MCV, in 1 l conical flasks, at 23°C, with 5% atmospheric CO_2 and shaking at 90 rpm. Illumination was provided by Sylvania GroLux 15W T8 lamps wrapped in one layer of acetate 444 eighth C.T. straw filter (Leefilters, UK). The light : dark photoperiod was 18 h : 6 h. Flasks were inoculated with algae from a 5-day old, actively growing culture of *B. braunii* race B (Guadeloupe) such that the OD_{680} of new cultures was 0.2. Samples were harvested immediately (day 0) and then every 3 days after transfer to the fresh medium. At least 3 independent replicates were performed for each experiment and standard error of the mean was calculated. The 1 l conical flasks were capped with aeraseal sterile (Alphalabs) allowing for sufficient aeration.

Illumination with specific wavelengths was achieved by wrapping the incubator lamps and the front panel of the incubator in coloured acetate filters (Leefilters, UK) and the spectra determined using an ASD HandHeld field photoradiometer (Fig. 2.1). The filters used were a 716 Mikkel blue filter for blue illumination, a 124 dark green filter for green illumination and a 778 Millennium Gold filter for red illumination. The aim was to achieve an equal radiance at the specific bands of wavelength in the different light colours accordingly to the white light spectrum.

Total light flux density (LFD) was measured for each light treatment using a Hansatech Quantitherm Light Meter Thermometer. For white light (WL) illumination, the LFD was 70 μmol m^{-1} s^{-1}, for blue illumination (BL), the LFD was 11 μmol m^{-1} s^{-1}, for green illumination (GL) the LFD was 11 μmol m^{-1} s^{-1} and for red illumination (RL) the LFD was 54 μmol m^{-1} s^{-1}.
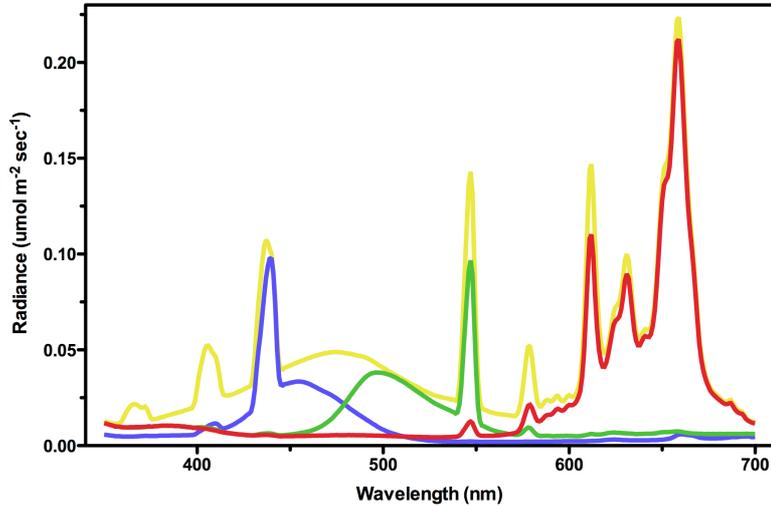

Fig. 2.1: Spectra of light for *Botryococcus braunii* culture.

The spectra and radiances of light treatments in which *B. braunii* were cultured. The yellow line represents the white light (WL) spectrum, blue line represents the blue light (BL) spectrum, the green line represents the green light (GL) spectrum and the red line represents the red light (RL) spectrum.

2.2 – **Absorbance of the Algal Cultures.**

The absorbance of 1 ml of algal culture was quantified at 680 nm (Abs_{680}) using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer.

2.3 – **Dry Biomass.**

10 ml aliquots of *B. braunii* cultures were filtered onto pre-weighed 2.7 μm GF/D glass-fibre membranes (Whatman). The GF/D filters were dried at 75°C for 48 h and the biomass determined.

2.4 – **Chlorophyll Content.**

Chlorophylls a and b were extracted in 10 ml methanol after collecting 10ml of algal culture by filtration through a 2.7 μm GF/D glass-fibre membranes (Whatman). The GF/D filter was sonicated twice, for 10 min, inverting the filter between sonications, until all the chlorophyll was dissolved in the methanol. After sonication, the chlorophyll extract was centrifuged at 2,000 g for 2 min to pellet the cell debris and the remains of the glass-fibre membrane. Chlorophyll absorbance was measured at 650 nm and at 665 nm using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer. Chlorophyll concentrations were calculated according to the following formula: Total chlorophyll (mg l^{-1}) $=25.8Abs_{650}+4.0Abs_{665}$ (Hipkin and Baker, 1986).

2.5 – ***Botryococcus braunii* Cell Size Measurements.**

Confocal microscopy was performed with a Zeiss LSM 510 confocal microscope equipped with a meta detector. The meta detector was used to meet the following requirements: Lipids stained with Bodipy 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) Cooper et al, (2009) with 5 μM final concentration were excited at 488 nm, 4 % transmission and an emission band between 507 – 529 nm was collected in Channel S1 (ChS1). Chlorophyll fluorescence was excited at 458 nm, 10 % transmission and emission between 604 – 754 nm was collected in ChS2. A transmitted differential interference contrast (DIC) was also collected with 458 nm laser. *B. braunii* was imaged after 24 days growth under the different light colours. *B. braunii* individual cells were imaged from three different samples taken from one flask. *B. braunii* cell length and width were then randomly measured manually from the 3 different samples using the LSM 95 software.

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2.6 – *In Vivo* Lipid Assay by Nile Red Fluorescence.

Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one; Sigma UK) was used for rapid determination of lipid production by *B. braunii* (Lee, S. J., et al. 1998). 250 µl of *B. braunii* culture were pipetted into a 96 well plate and the fluorescence measured in a Tecan Infinite M-200 plate reader. After staining the algae with 20 µl of 40 µM Nile red reagent in acetone, 5 s shaking was applied followed by 10 min incubation and a final 5 s shaking. The Nile red fluorescence was measured using an excitation wavelength of 490 ± 5 nm and a emission wavelength 560 ± 20 nm.

2.7 – *In Vitro* Hydrocarbons Purification.

100 ml of algal culture were filtered through 2.7 µm GF/D glass-fibre membranes (Whatman). Algae were collected from the filter, snap-frozen in liquid N_2 and freeze-dried (Scanvac coolsafe) for at least 48 h. Samples were stored at -80°C.

Accelerated solvent extraction 150 (Dionex) was used to purify the hydrocarbons using HPLC grade hexane as the solvent. 20 mg of freeze dried tissue was grounded with diatomaceous earth. The mix was then added to a 5 ml chamber for extraction using the following parameters: Temperature 100°C; Static Time 5 min; Rinse Volume 100%; Purge Time 60 sec; Static cycle 2. The hexane extracts were then transferred to glass vials and purged with N_2. Hydrocarbon samples were analysed at Shell Research Ltd. and quantified using Gas Chromatography – Flame Ionization Detector (GC–FID) with the following parameters: Oven setup - Initial temperature 45 °C, increasing temperature by 8 °C min^{-1} to 320 °C and a holding time of seven minutes, front detector setpoint at 345 °C, oven track on. Column setup ZB1HT column, capillary length 30 meters, He as carrier gas with a constant pressure of 10 PSI and 2.2 ml min^{-1} gas flow. FID was setup with the following parameters: heater at 345 °C, H_2 flow at 34 ml min^{-1}, air flow at 340 ml min^{-1}. Hydrocarbon values are expressed in mg per litre of extracting solvent. Hydrocarbon samples were also analysed qualitatively using Gas Chromatography – Mass Spectrometry (GC–MS) under method TMS496 and

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LIMS number 23026. Samples were analysed as received and, diluted in heptane by a factor of 5. The hydrocarbon time point samples analysed were: WL day 6, day 12 and day 21; BL day 21; GL day 21; RL day 21.

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CHAPTER 3

BOTRYOCOCCUS BRAUNII GROWTH IN DIFFERENT LIGHT REGIMES

3.1 – Methods for Growth Analysis.

The aim of these experiments was to determine the growth response of *B. braunii* to the different light colour. *B. braunii* was cultured in different light conditions with different light intensities as specified in materials and methods. To measure growth, dry biomass and absorbance at 680 nm was quantified. From the dry biomass the growth rate of *B. braunii* was calculated by using the following equation:

Growth Rate = $\dfrac{DB_{t2} - DB_{t1}}{t_2 - t_1}$

Where, DB_{t2} is the dry biomass at t_2, DB_{t1} is the initial dry biomass at t_1. The units of the growth rate are in grams per litre per day (g l^{-1} d^{-1}).

The dry biomass comprised all insoluble material collected by the GF/D glass filter membrane which include living and non living algae cells. Measuring the Abs_{680} provided with a second way of quantifying growth of *B. braunii*.

Analysis of the *B. braunii* growth curve (Fig. 3.1, same data as Fig. 3.3) under WL suggests a linear growth with dieauxic pattern, which is seen when two sources of carbon are available. Our modified Chu media has citric acid and it could have been used by the algae as a source of carbon. It has been considered that *B. braunii* population is actively dividing and increasing biomass nearly up to day 12 and afterwards *B. braunii* population was both dividing and increasing biomass as well as senescing.

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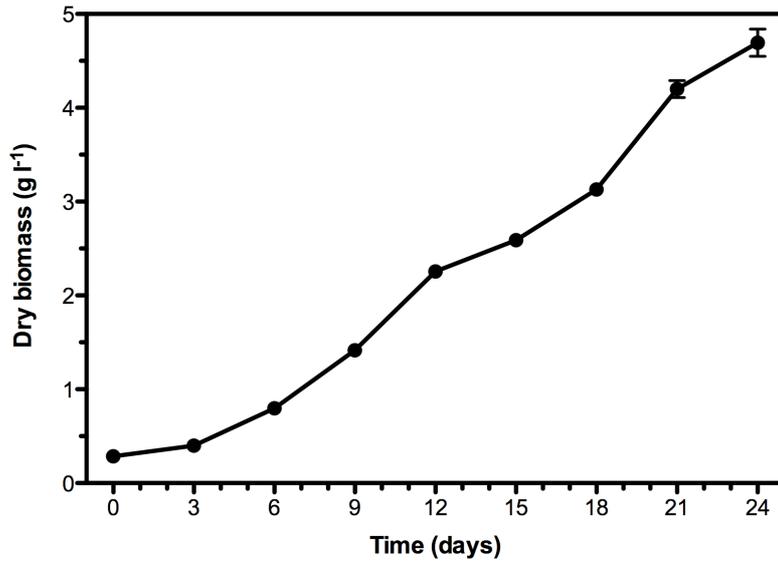
Fig. 3.1: Growth of *Botryococcus braunii* according to dry biomass in WL
showing a diauxic curve.

B. braunii were grown over 24 days in WL. Points represent the mean of three independent replicates with standard errors of mean bars. When error bars are not seen, they are masked by the symbol.

Dry biomass and Abs_{680} from WL data were plotted against each other and a positive linear correlation was observed between both types of measures (Fig. 3.2A). The slope was 0.5367 ± 0.01172 with 95% confidence intervals of 0.5132 to 0.5601 and an r^2 value of 0.9815 clearly stating a very good correlation between dry biomass and Abs_{680}. Consequently dry biomass only was chosen to represent the growth of *B. braunii* as it facilitates future parameter analysis. The correlation between dry biomass and chlorophyll was linear under 2 g l^{-1}, but not significant above that figure (Fig. 3.2B). This is ascribed to the aging of cultures and it was considered that during the linear correlation up to 2 g l^{-1} *B. braunii* was a "young" culture, followed by a transition to a "mature" culture from 2 g l^{-1} and above.

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$r^2= 0.9815$
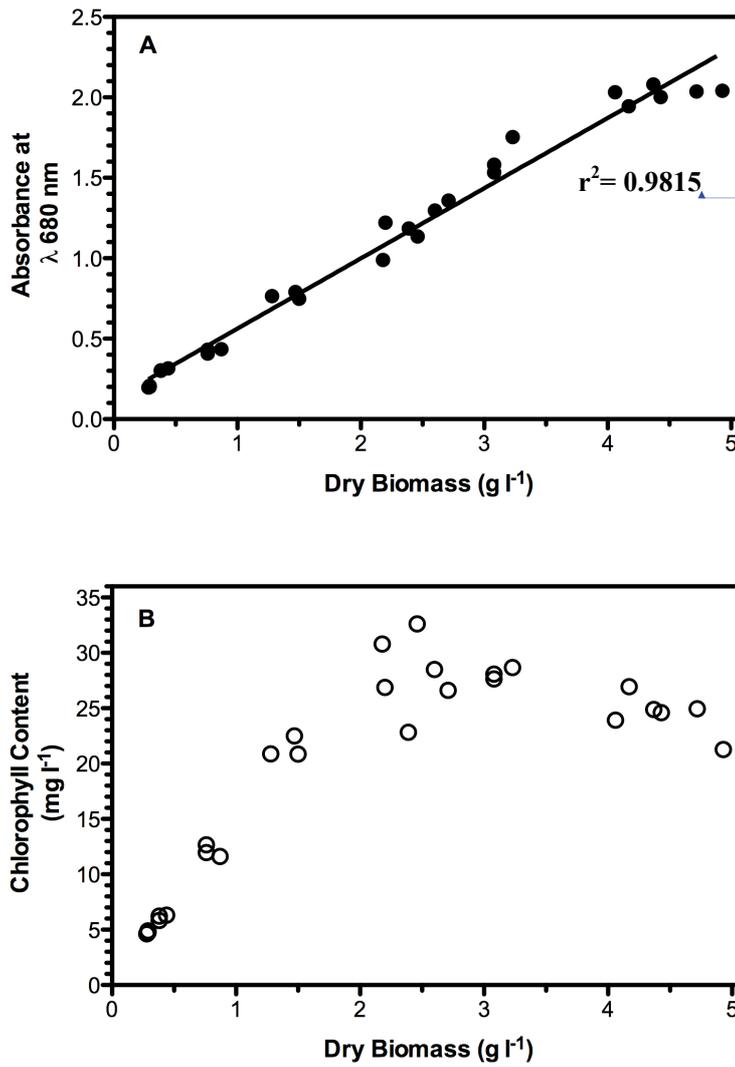
diogo 22/2/11 12:11
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Fig. 3.2: *Botryococcus braunii* correlation between both absorbance at 680 nm (A) and chlorophyll content (B) with dry biomass.

Correlation between methods for determining *B. braunii* growth. **A:** Close circles indicate the correlation between absorbance at 680 nm and dry biomass of *B. braunii* in WL; **B:** Open circles represent chlorophyll content correlation with dry biomass for *B. braunii* in WL.

Therefore *B. braunii* cultures were subsequently grown until the formation of mature cultures at approximately 12 days before exposing the algae to the different light regimes (cf. 3.3). The terms day 12 and 2 g l^{-1} dry biomass will be considered to be equivalent when referring to the transition point at which *B. braunii* populations mature.

3.2 – **Biomass and Growth Rate in White and Blue Light.**

In an initial experiment that mirrored that of the pilot study, *B. braunii* was transferred to a new innoculum and grown in WL or in BL for a period of 24 days (Fig. 3.3).

Algae grown in WL had an average dry biomass of 0.287 g l^{-1} ± SE 0.006 n= 3 at day 0 and by day 24 it was recorded an average dry biomass of 4.693 g l^{-1} ± SE 0.251 n= 3 achieving an increase of 16 fold the initial weight. In contrast *B. braunii* grown in BL only achieve an increase of 3 fold the initial DB with the same starting dry biomass of 0.287 g l^{-1} ± SE 0.006 n= 3 at day 0 (inoculation day) and recording by day 24 0.863 g l^{-1} ± SE 0.081 n= 3 (Fig. 3.3).

The first doubling of dry biomass seen in the growth of *B. braunii* in WL was approximately at day 6 with 0.797 g l^{-1} ± SE 0.037 n= 3, whilst in *B. braunii* grown in BL the first doubling happened at approximately day 12 with 0.630 g l^{-1} ± SE 0.032 n= 3. In WL *B. braunii* average growth rate was 0.184 g l^{-1} d^{-1} whilst *B. braunii* growing in BL average growth rate was 0.024 g l^{-1} d^{-1} over the duration of the experiment.

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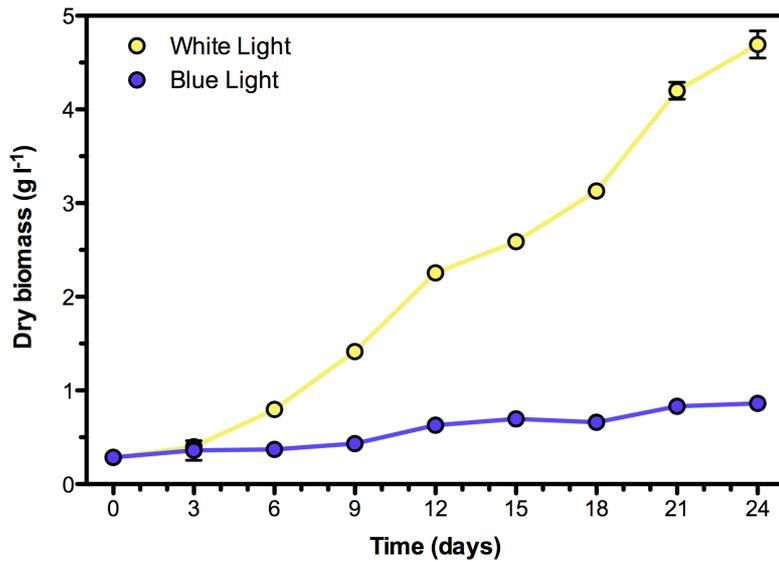
Fig. 3.3: *Botryococcus braunii* growth in white and blue light.

B. braunii were grown over 24 days in WL (yellow circles) and BL (blue circles). Points represent the mean of three independent replicates with standard errors of mean bars. When error bars are not seen, they are masked by the symbol.

Algae grown in BL had an average growth rate approximately 7 times lower than that observed in WL, generating a low biomass that could present erroneous results. Consequently to obtain a larger biomass to generate more precise data, *B. braunii* were grown for 12 days in WL until maturation of the population, before exposing the algae to the different wavelengths of light.

3.3 – Biomass Growth Rate in Different Light Regimes

In all subsequent experiments *B. braunii* was grown in WL for 12 days (reaching a mature population) then transferred to BL, GL and RL for 15 days (from day 12 to day 27). GL is not photosynthetically active, so represents a negative control. *B. braunii* was harvested at day 6 (young cultures) and 12 (mature cultures) and every 3 days afterwards until day 27. The cultures in BL or GL regime were then put back into WL to observe any consequences to having been exposed to BL or GL treatment. As a positive control, *B. braunii* was also maintained in WL for the entire growth period.

Results no longer show the initial diauxic curve pattern observed in Fig. 3.1. Results show the young and mature *B. braunii* population in WL and the mature *B. braunii* in RL in linear growth phase, whilst *B. braunii* population in BL and GL showed no growth (Fig. 3.4) from day 12 to day 27. *B. braunii* grown under WL recorded the highest growth of all the light regimes with a dry biomass of 2.867 g l^{-1} ± SE 0.142 n= 3 at day 15 and 5.230 g l^{-1} ± SE 0.125 n= 3 at day 27. Second highest growth was observed in mature *B. braunii* exposed to RL with a dry biomass of 2.400 g l^{-1} ± SE 0.035 n= 3 at day 15 and 4.253 g l^{-1} ± SE 0.235 n= 3 at day 27. *B. braunii* dry biomass in BL was 1.901 g l^{-1} ± SE 0.086 n= 3 at day 15 and 2.010 g l^{-1} ± SE 0.031 n= 3 at day 27, and mature *B. braunii* dry biomass in GL was 1.723 g l^{-1} ± SE 0.018 n= 3 at day 15 and 1.807 g l^{-1} ± SE 0.089 n= 3 at day 27.

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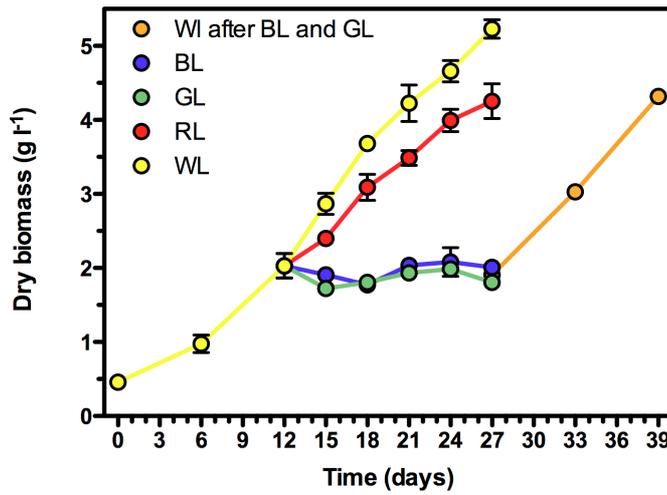
Fig. 3.4: *Botryococcus braunii* linear growth accordingly to dry biomass (g l^{-1}) in the different light colour treatments.

Growth in WL is represented by yellow circles, in BL by blue circles, in GL by green circles, in RL by red circles and in WL after BL and GL is represented by orange circles. Points represent the mean of three independent replicates (except day 0, 6 and 12 points which represent the mean of six independent replicates) with standard errors of mean bars. When error bars are not seen, they are masked by the symbol.

The growth rate of each replicate at each time point of experiment was determined and all values averaged (Table 3.1).

B. braunii grown in WL (positive control) had the highest growth rate followed by *B. braunii* grown in RL. Both *B. braunii* grown in WL and RL show no significant difference (P > 0.05). There is a significant difference in algae grown in BL and GL compared to *B. braunii* grown in WL and RL (P < 0.05). Algae grown in GL recorded the lowest growth rate. There was no significant difference in growth rate between *B. braunii* grown in BL and GL (P > 0.05).

Light Quality	*B. braunii* mean growth Rate (g l^{-1} d^{-1}), standard error, n= number of samples)		
White light	**0.175** g l^{-1} d^{-1}	± SE 0.013	n= 57
Red light	**0.125**	± SE 0.029	n= 15
Blue light	**0.023**	± SE 0.009	n= 39
Green light	**0.009**	± SE 0.011	n= 15

Table 3.1: Growth rate of *Botryococcus braunii* expressed in grams per litre per day according to dry biomass measurements in the different light colours during the period of 39 days.

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CHAPTER 4

EFFECT OF LIGHT COLOUR ON *BOTRYOCOCCUS BRAUNII* CELL MORPHOLOGY AND CHLOROPHYLL CONTENT.

4.1 – *Botryococcus braunii* Colony Morphology.

   B. braunii were grown in 250ml conical flasks in each light regime for 24 days. Bodipy dye which is a lipophyllic stain was added to a 1ml sample of each culture and imaged by confocal microscopy. Images clearly show the extracellular hydrocarbons and intracellular liposomes of mature *B. braunii* colonies (Fig. 4.1). Colony morphology was compared between the different light regimes in relation to clumping and dispersion of the oily extracellular matrix and no difference in colony morphology was observed between *B. braunii* grown in WL, BL, GL and RL. *B. braunii* individual cells were measured in length and width to observe any changes in cell size.
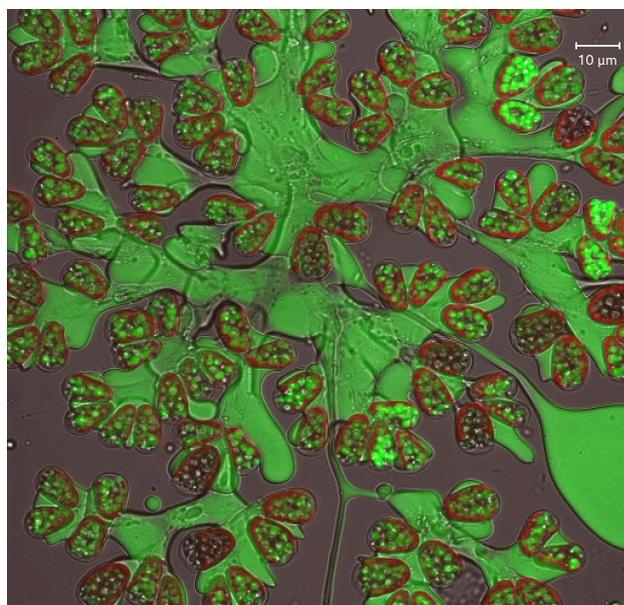
Fig. 4.1: Image of a 24 day old mature *Botryococcus braunii* colony grown in WL.

Mature *B. braunii* shows its pyriform shape with chlorophyll fluorescing in red and the lipids stained with Bodipy dye fluorescing in green. The scale bar represents 10 μm.

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4.2 – *Botryococcus braunii* Cell Size

Although in all light treatments mature *B. braunii* cells remained pyriform shape and with colonies present, light colour did affect the size of individual cells. *B. braunii* grown in WL were significantly larger than algae grown in BL, RL and GL. More specifically, there was a statistically significant difference ($P < 0.05$) in the width of *B. braunii* grown in WL compared to *B. braunii* grown in BL, RL and GL (Fig. 4.2 A). The mean of *B. braunii* width in WL was 9.120 μm ± SE 0.084 n= 95, in BL was 8.811μm ± SE 0.065 n= 95, RL was 8.857 μm ± SE 0.078 n= 95 and in GL was 8.142 μm ± SE 0.092 n= 95. We also observed a statistically significant difference ($P < 0.05$) in the length of *B. braunii* grown in WL compared to *B. braunii* grown in BL, RL and GL with means of 13.22 μm ± SE 0.093 (n= 95), 12.69 μm ± SE 0.081 (n= 95), 12.78 μm ± SE 0.092 (n= 95), and 11.97 μm ± SE 0.115 (n= 95) respectively (Fig. 4.2 B). Both the mean width and length of *B. braunii* cells grown in GL were statistically different ($P < 0.05$) in comparison with the cell size of *B. braunii* cultured in WL, RL and BL. There was no significant difference in cell size between *B. braunii* exposed to BL and RL in both width and length measurements.
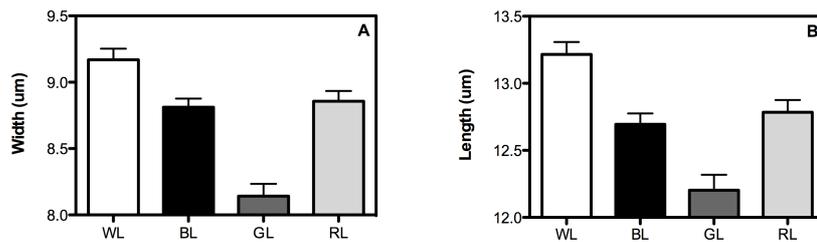
Fig. 4.2: *Botryococcus braunii* cell size measurements in μm accordingly to width (**A**) and length (**B**).

Value shows mean, standard errors of mean bars and n= 95 (n=number of samples). The columns are labeled as WL (white light), BL (blue light), GL (green light) and RL (red light).

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4.3 – Effect of Light Colour on *Botryococcus braunii* Chlorophyll Content.

Chlorophyll analysis was used to monitor the effect of experimental procedures on the photosynthetic pigments of the algae as a measure of health of *B. braunii* cultures in the different light regimes (Fig. 4.3).

B. braunii chlorophyll content increased during the first 12 days in WL whilst young *B. braunii* population increased in biomass. After the day 12 it was seen a decrease in chlorophyll in the mature *B. braunii* population in WL and RL regimes until day 24. Mature *B. braunii* chlorophyll in BL and GL conditions remained the same over time to day 27.
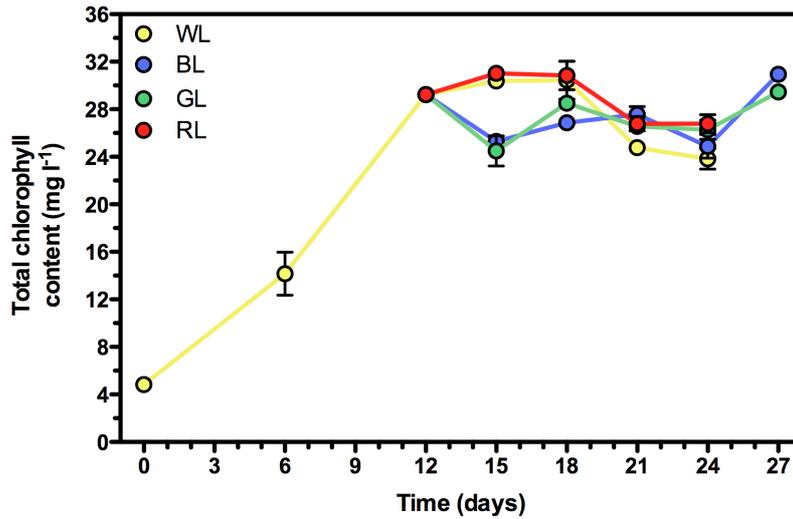
Fig. 4.3: Total chlorophyll content of *Botryococcus braunii* in the different light regimes

Total chlorophyll in WL is represented by yellow circles, in BL by blue circles, in GL by green circles, in RL by red circles and WL after BL and GL is represented by orange circles. The points represent the mean of three independent replicates (except day 0, 6 and 12 points which represent the mean of six independent replicates) with the standard errors of mean bars. When error bars are not seen, they are masked by the symbol.

There was no effect of light in the chlorophyll content of young and mature *B. braunii* population (Fig. 4.4). Young *B. braunii* chlorophyll content in BL and WL regime increased as did biomass up to day 12. Mature *B. braunii* content in WL

and RL decreased as the population of *B. braunii* continue to increase. Mature *B. braunii* chlorophyll content in BL and GL remained the same for the period of 15 days while there was no increase in biomass. Mature *B. braunii* from BL and GL were then transferred to WL and a decrease in chlorophyll was observed as the mature *B. braunii* population increased biomass replicating the response observed in *B. braunii* in WL and RL.
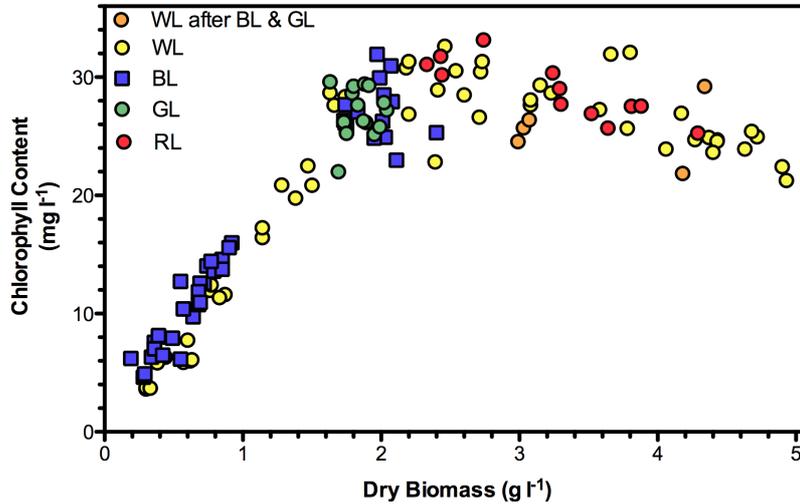
Fig. 4.4: *Botryococcus braunii* correlation between chlorophyll content with dry biomass in the different light treatments.

WL is represented in yellow circles, BL in blue squares, RL in red circles, GL in green circles and WL after BL and GL is represented by orange circles.

Due to the variation seen in *B. braunii* growth, total chlorophyll was normalized against dry biomass. Chlorophyll content per dry biomass increases in young *B. braunii* population for the first 12 days in WL and from day 12 onwards, mature *B. braunii* chlorophyll content per dry biomass decreased until day 24, whilst mature *B. braunii* chlorophyll per dry biomass in BL and GL remained the same during the 15 days of exposure (day 12 to day 27). Chlorophyll concentration per dry biomass observed in *B. braunii* cultures exposed to WL showed the lowest values from day 15 to day 24 compared with *B. braunii* grown

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in the other light conditions (Fig. 4.5). In WL *B. braunii* recorded an increase of chlorophyll per dry biomass in the first six days while a young population, with 10.897 mg g^{-1} ± SE 0.492 n= 6 at day 0 and 14.438 mg g^{-1} ± SE 0.457 n=6 at day 6, followed by a marginal increase until day 12. From day 12 to day 24 the ratio of chlorophyll per dry biomass in mature *B. braunii* in WL decreased substantially with a ratio of 14.801 mg g^{-1} ± SE 0.988 n= 6 at day 12 and 5.127 mg g^{-1} ± SE 0.275 n= 3 at day 24. *B. braunii* mature population in GL recorded the highest chlorophyll per dry biomass from day 15 to day 24 with 14.200 mg g^{-1} ± SE 0.626 n= 3 and 13.223 mg g^{-1} ± SE 0.284 n=3 respectively. *B. braunii* mature population in BL showed a similar results in the ratio of chlorophyll per dry biomass over time with 13.343 mg g^{-1} ± SE 0.845 n= 3 at day 15 and 12.220 mg g^{-1} ± SE 1.508 n= 3 at day 24. *B. braunii* mature population in RL showed a decrease in the ratio of chlorophyll per dry biomass at a similar rate as seen for mature algae in WL, but with higher chlorophyll per dry biomass ratio with 12.930 mg g^{-1} ± SE 0.289 n= 3 at day 15 and 6.740 mg g^{-1} ± SE 0.427 n= 3 at day 24.

The chlorophyll content per dry biomass at day 27 in *B. braunii* under GL and BL was statistically different ($P < 0.05$) from *B. braunii* grown under WL and RL. *B. braunii* mature population exposed to 15 days in BL and GL once exposed to WL, showed an immediate response in chlorophyll content per dry biomass, with a decrease to 5.845 mg g^{-1} ± SE 0.455 n= 3 at day 39 from 15.896 mg $^{-1}$ ± SE 0.491 n= 6 at day 27. If WL after BL and GL can be considered that day 39 is equivalent to the 24th day in WL conditions, it is seen a similar chlorophyll content per dry biomass between *B. braunii* in WL during the same period of 12 days. *B. braunii* chlorophyll content per dry biomass at the 24th day in WL after BL and GL (experiment 2) was 5.845 mg g^{-1} ± SE 0.455 n= 3 and in experiment 3 *B. braunii* grown in white light recorded at day 24 a chlorophyll content per dry biomass of 5.127 mg g^{-1} ± SE 0.275 n= 3.

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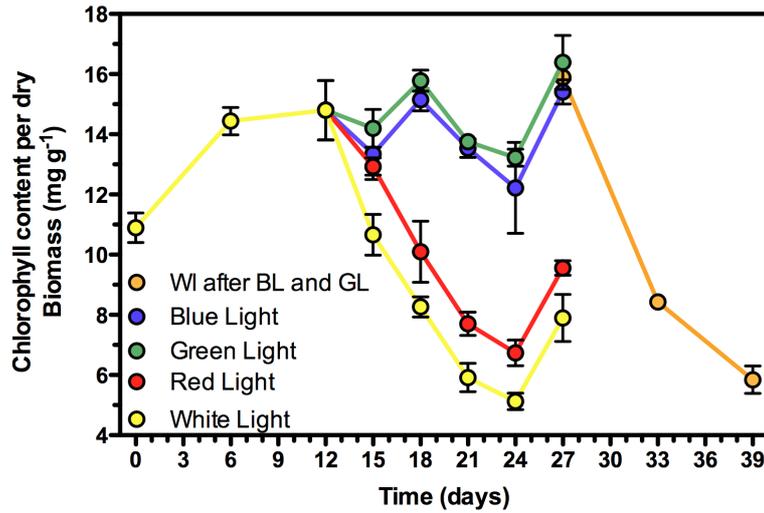
Fig. 4.5: *Botryococcus braunii* chlorophyll content per dry biomass
in the different light regimes.

Chlorophyll per dry biomass in *B. braunii* in WL is represented by yellow circles, in BL by blue circles, in GL by green circles, in RL by red circles and in WL after BL and GL is represented by orange circles. The points represent the mean of three independent replicates (except day 0, 6 and 12 points which represent the mean of six independent replicates) with the standard errors of mean bars. When error bars are not seen, they are masked by the symbol.

The increase seen in chlorophyll per dry biomass at day 27 was unexpected and it is unclear the reason why this increase was seen in *B. braunii* mature populations exposed to WL and RL. Due to the lack of time points following day 27 we are not able to determine if the increase in chlorophyll per dry biomass is due to a sampling error or a type of physiological response from *B. braunii*. *B. braunii* mature population in BL and GL showed renewed growth once exposed to WL. Algae maintain their photosynthetic potential although there was not much energy available.

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CHAPTER 5

EFFECT OF LIGHT COLOUR ON *BOTRYOCOCCUS BRAUNII* HYDROCARBON SYNTHESIS.

B. braunii mature population cultured in WL and RL showed an increase in lipids compared to the static lipid amount of the mature *B. braunii* population in BL and GL as determined by the Nile red assay (Fig. 5.1). On day 27, which represents 15 days of exposure to the BL, GL and RL, *B. braunii* had a value (arbitrary units per 250ul algae culture sample) for lipid production of 12177 ± SE 208 n= 3 in WL compared to 10504 ± SE 237 n= 3 in RL, 8713 ± SE 250 n= 3 in GL, and 8012 ± SE 141 n= 3 in BL. *B. braunii* lipid content in BL and GL remained similar from day 15 to day 27, whilst *B. braunii* cultures in WL and RL showed an increase in the lipid production during the same period. When *B. braunii* grown in BL and GL were transferred to WL, an increase in lipid content according to the Nile red assay was observed, together with an increase in algal biomass (Fig. 3.4) within a period of 12 days. The average lipid content was 8363 ± SE 203 n= 6 in day 27 and an increase to 14411 ± SE 449 n= 3. The fact that lipid content accordingly to Nile red assay did not decrease in the mature *B. braunii* population during the 15 days of exposure to BL and GL, suggests that the oil is not being metabolised by the algae for energy purposes.

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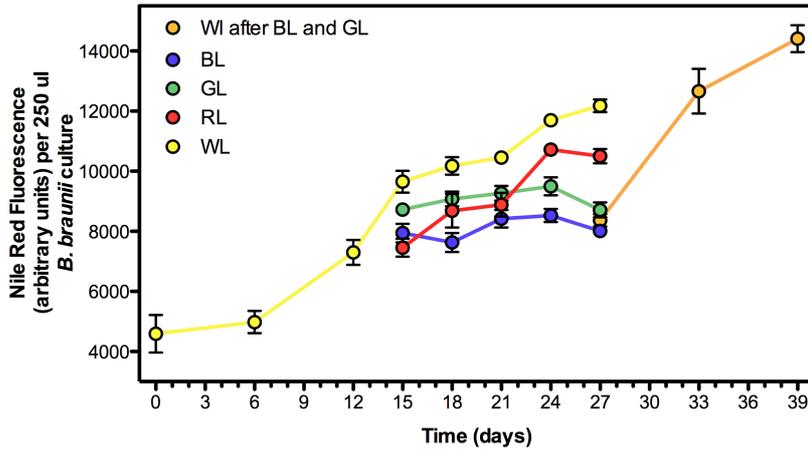
Fig. 5.1 Lipid content, determined by the Nile red assay, of *Botryococcus braunii* population in the different light regimes.

Lipid content of *B. braunii* in WL is represented by yellow circles, in BL by blue circles, in GL by green circles, in RL by red circles and in WL after BL and GL is represented by orange circles. Points represent the mean of three independent replicates (except day 0, 6 and 12 points which represent the mean of six independent replicates) with standard errors of mean bars. When error bars are not seen, they are masked by the symbol.

The measurements of lipid production, determined by Nile red fluorescence, were plotted against dry biomass (Fig. 5.2). We observed a strong positive correlation ($P < 0.05$) between lipid production and dry biomass in all cultures of *B. braunii* grown in the different light colours. The correlation seen in WL has a slope of 2004 ± 158.7 with slope 95% confidence intervals of 1687 to 2321. *B. braunii* grown in WL, BL, RL and GL show a similar amounts of hydrocarbons produced relative to biomass and within the 90 % prediction band of WL linear regression.

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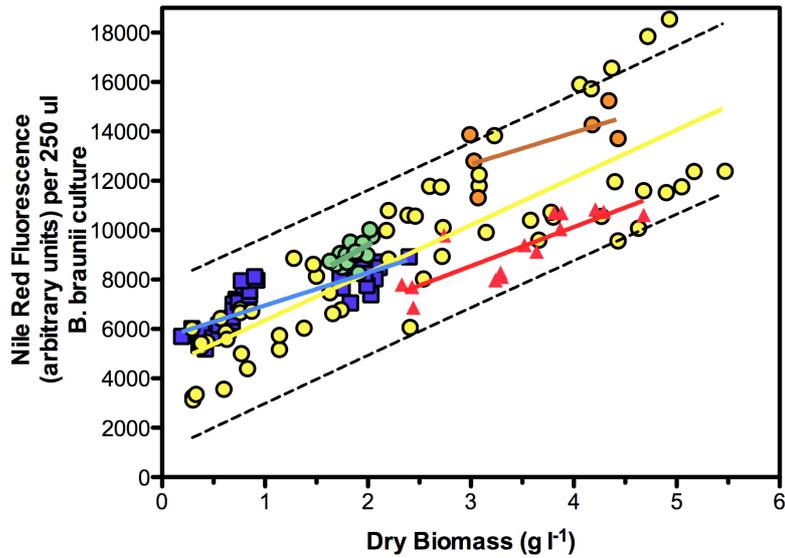
Fig. 5.2 Correlation between lipid production, determined by nile red assay, and dry biomass of *Botryococcus braunii* in the different light colours.

Points represent correlation for: WL (yellow circles), GL (green circles), BL (blue squares), RL (red triangles) and WL after BL and GL (orange circles), with respective colour for linear regression. 90 % prediction band for white light is shown (black dash line).

Nile red assay only gives an approximate quantification of hydrocarbons. Consequently hydrocarbons extracted from 20 mg of freeze dried *B. braunii* biomass grown in all light conditions from day 6, 12 and 21 were analysed by Gas Chromatography – Flame Ionization Detector (GC-FID) to separate the different hydrocarbon molecules and to more precisely quantify their abundance. All hydrocarbon samples from the different light regimes showed an identical chromatogram profile with four main peaks (Fig. 5.3).
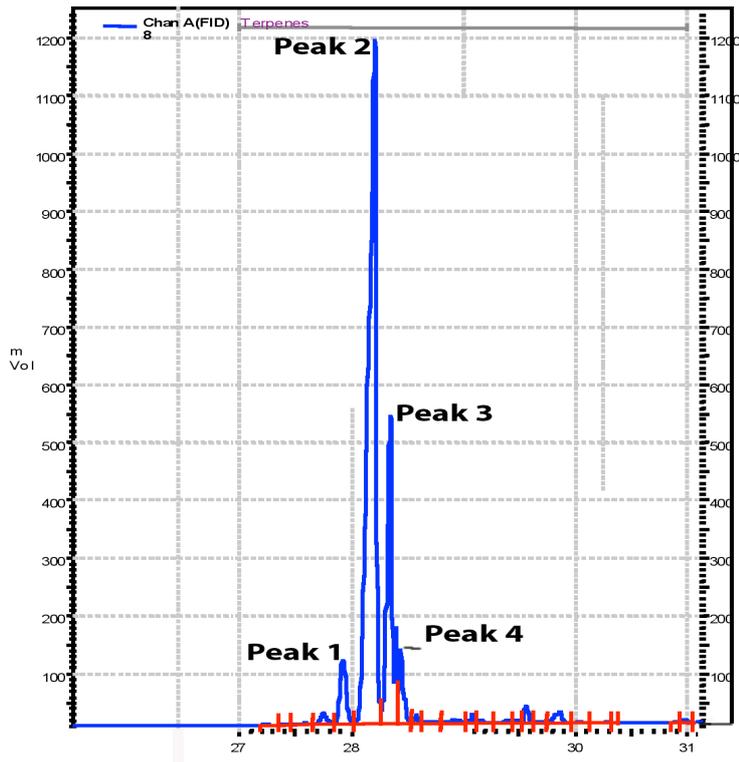
Fig. 5.3: Chromatogram of hydrocarbons present in a mature *Botryococcus braunii* population in WL at day 21.

Represented in the figure are the four main peaks present in all hydrocarbon samples across the different light regimes according to GC-FID.

There were no significant difference between the quantity of hydrocarbon extracted from *B. braunii* grown in WL samples at the different time points (day 6, day 12 and day 21), therefore only hydrocarbon results from WL day 21 are shown in comparison with the other light treatments (Table 5.1). Peak 1, 2, 3 and 4 (Fig. 5.3) correspond to the peaks in table 5.1 with retention times of 27.928, 28.220, 28.345 and 28.397 respectively.

The most abundant hydrocarbon compound had a retention time of 28.220 min (Peak 2) with some variation between samples. *B. braunii* grown in GL had the highest quantity of this hydrocarbon with 398.84 mg l^{-1} ± SE 26.33 n= 3 and algae grown in BL with the lowest amount at 325.53 mg l^{-1} ± SE 72.76 n= 3. The second most abundant component had a retention time of 28.345 min (Peak 3) with some variation between samples with the highest amount recorded in hydrocarbons extracted from *B. braunii* in WL at 115.60 mg l^{-1} ± SE 4.28 n= 3 and the lowest amount in hydrocarbons extracted from *B. braunii* in GL at 84.71 mg l^{-1} ± SE 6.98 n= 3. Hydrocarbons extracted from *B. braunii* in GL also revealed lack of the compound with retention time 28.397 min (peak 4), which was present in WL, BL and RL. The hydrocarbon sample from *B. braunii* grown in RL, had a unique compound with a retention time of 28.460 min at a significant quantity of 59 mg l^{-1}, although it was only present in one of the replicates. At retention time 27.928 min a hydrocarbon compound was detected in similar quantity across all samples. At 29.843 min there was a compound with significant variation, where hydrocarbons from *B. braunii* grown in WL had an average of 5.64 mg l^{-1} ± SE 0.43 n= 3, hydrocarbons from BL algae had 14.69 mg l^{-1} ± SE 3.25 n= 3, green light samples had 35.68 mg l^{-1} ± SE 1.31 n= 3 and in RL an amount of 4.19 mg l^{-1} ± SE 0.11 n= 3.

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Ret. Time	White Light			Blue Light			Green Light			Red Light		
	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
27.233	0.07	± 0	1	n.d.			0.06	± 0	1	n.d.		
27.423	0.32	± 0.03	3	0.34	± 0.11	3	0.18	± 0.02	3	0.58	± 0.05	3
27.555	0.66	± 0.12	3	0.42	± 0.11	3	0.54	± 0.09	3	0.57	± 0.05	3
27.753	4.17	± 0.60	3	2.46	± 0.60	3	3.22	± 0.40	3	3.51	± 0.21	3
27.928	**25.74**	**± 0.71**	**3**	**23.10**	**± 5.12**	**3**	**28.61**	**± 1.91**	**3**	**25.59**	**± 0.88**	**3**
28.220	**357.38**	**± 5.15**	**3**	**325.53**	**± 72.76**	**3**	**398.84**	**± 26.33**	**3**	**358.40**	**± 11.57**	**3**
28.345	**115.60**	**± 4.28**	**3**	**91.84**	**± 27.18**	**3**	**84.71**	**± 6.98**	**3**	**111.57**	**± 7.40**	**3**
28.397	**21.44**	**± 0.60**	**2**	**15.06**	**± 0.00**	**1**	n.d.			**37.83**	**± 7.19**	**3**
28.460	n.d.			n.d.			n.d.			59.89	± 0.00	1
28.583	3.41	± 0.77	3	4.47	± 0.98	3	3.78	± 0.30	3	4.73	± 0.97	3
28.670	2.30	± 0.16	2	n.d.			1.32	± 0.24	3	2.68	± 0.00	1
28.967	3.42	± 0.08	3	4.16	± 0.95	3	7.68	± 0.28	3	3.49	± 0.23	3
29.092	2.84	± 0.11	3	5.24	± 1.20	3	9.36	± 0.73	3	1.98	± 0.12	3
29.160	1.42	± 0.01	3	1.6 4	± 0.42	3	2.49	± 0.00	1	1.84	± 0.16	3
29.302	n.d.			n.d.			0.50	± 0.00	1	0.59	± 0.00	1
29.418	1.45	± 0.13	3	1.48	± 0.38	3	1.66	± 0.16	3	1.38	± 0.05	3
29.488	0.90	± 0.06	3	0.77	± 0.21	3	0.89	± 0.14	3	0.92	± 0.08	3
29.575	4.84	± 0.10	3	4.43	± 1.01	3	5.93	± 0.42	3	5.27	± 0.29	3
29.707	1.25	± 0.06	2	3.41	± 0.84	3	9.19	± 0.06	3	0.85	± 0.08	3
29.843	5.64	± 0.43	3	14.69	± 3.25	3	35.68	± 1.31	3	4.19	± 0.11	3
30.045	0.54	± 0.01	3	0.50	± 0.17	3	0.30	± 0.06	3	1.26	± 0.10	3
30.103	n.d.			n.d.			0.18	± 0.04	3	n.d.		
30.210	n.d.			n.d.			n.d.			0.43	± 0.11	3
30.285	0.35	± 0.04	3	1.38	± 0.37	3	4.58	± 0.63	3	0.10	± 0.00	1
30.340	0.14	± 0.03	3	0.71	± 0.18	3	2.07	± 0.04	2	0.05	± 0.00	1
30.423	0.08	± 0.00	1	0.32	± 0.09	3	0.96	± 0.01	3	n.d.		
30.542	n.d.			n.d.			0.17	± 0.00	1	n.d.		
30.662	n.d.			n.d.			0.15	± 0.05	3	n.d.		
30.837	n.d.			n.d.			n.d.			0.28	± 0.00	1
30.902	0.25	± 0.02	3	0.24	± 0.07	3	0.20	± 0.02	3	0.58	± 0.23	3
30.992	0.82	± 0.12	3	0.30	± 0.09	3	0.39	± 0.04	3	1.25	± 0.00	2

Table 5.1: GC-FID results of WL, BL, GL and RL hydrocarbon samples of mature *Botryococcus braunii* population from day 21.

Table shows retention times of the different molecules, the mean of the hydrocarbons present in the extractable hydrocarbon samples (expressed in mg l^{-1}), the number of replicas (N), and n.d., not detected. The highlighted rows represent the four main peaks. A full analysis is in appendix 4.

The relative hydrocarbon content per dry biomass was determined, and results show no significant difference across the different light colour treatments (Fig. 5.4). In *B. braunii* grown in WL the hydrocarbon percentage of the dry biomass did not change significantly over a period of 21 days with 42 % ± SE 1.1 n= 3 as young *B. braunii* cultures at day 6, 41 % ± SE 1.1 n= 3 as mature *B. braunii* at day 12 and 38 % ± SE 0.7 n= 3 at day 21. Hydrocarbon content in BL at day 21 of the experiment was of 33 % ± SE 7.8 n= 3. This value is misleading as one of the replicas gave an unusual low value attributed to an error of sampling. In GL hydrocarbon percentage was 39 % ± SE 2.4 n= 3 and in RL was 39 % ± SE 1.3 n= 3. At day 21 there is no significant difference in the hydrocarbon content in *B. braunii* grown in different light colours.
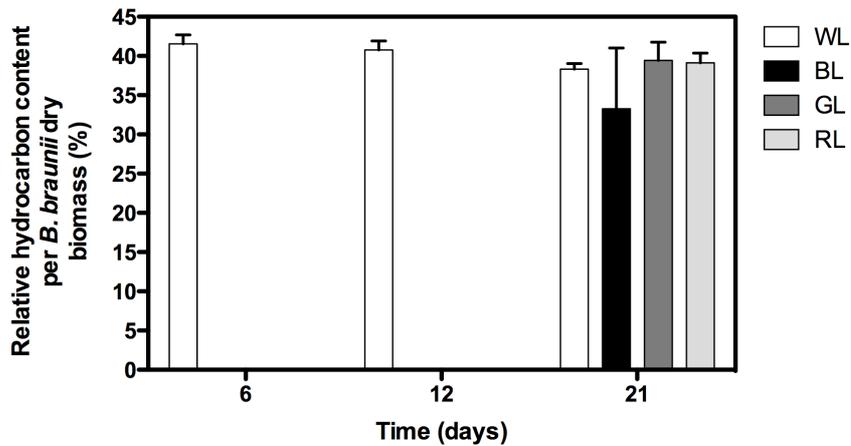
Fig. 5.4: *Botryococcus braunii* percentage of hydrocarbons per dry biomass at day 6, 12 and 21 for WL and day 21 for GL, BL and RL hydrocarbon samples.

Columns represent the mean of three independent replicates with standard errors of mean bars for white light (WL), blue light (BL), green light (GL) and red light (RL) extracted hydrocarbon fraction.

The average percentage of the main molecules present in the samples analysed by GC-FID (Table. 5.1) was determined for mature *B. braunii* population in WL, BL, GL and RL from day 21 (Table. 5.2). It was observed that the peak 1 ranged from a 4% to 5% in the total hydrocarbon samples for WL, BL, GL and RL, whilst the peak 3 varied between 14 % to 21 % in the different light colours. The main hydrocarbon molecule represented by peak2 contributed between 61 % to 66 % of the total hydrocarbon sample for all light conditions.   The hydrocarbon present with retention time of 29.843 varies between 1 % in WL and RL, to 3 % and 6 % in BL and GL respectively. In all light colours, with exception of GL, the hydrocarbon molecule peak 4 was present with a percentage of 4 % to 6 %, whilst the hydrocarbon with retention time of 28.460 was present in one replica of the RL hydrocarbon sample and had a 10 % of the total hydrocarbon sample.

Retention Time (min.)	27.928 Peak 1	28.220 Peak 2	28.345 Peak 3	28.397 Peak 4	28.460	29.843
White light	5% ± SE 0.0 n= 3	65% ± SE 0.2 n= 3	21% ± SE 1.1 n= 3	4% ± SE 0.1 n= 2	n.d.	1% ± SE 0.1 n= 3
Blue light	5% ± SE 0.0 n= 3	66% ± SE 0.3 n= 3	18% ± SE 2.0 n= 3	6% ± SE 0.0 n= 1	n.d.	3% ± SE 0.1 n= 3
Green light	5% ± SE 0.0 n= 3	66% ± SE 0.1 n= 3	14% ± SE 0.3 n= 3	n.d.	n.d.	6% ± SE 0.2 n= 3
Red light	4% ± SE 0.0 n= 3	61% ± SE 0.6 n= 3	19% ± SE 1.4 n= 3	6% ± SE 1.2 n= 3	10% ± SE 0.0 n= 1	1% ± SE 0.0 n= 3

Table. 5.2: Percentage of the main compounds present in *Botryococcus braunii* extracted hydrocarbon from the different light colours at day 21.

Values presented by mean, standard errors of mean and n (number of samples).

CHAPTER 6

EFFECTS OF LIGHT COLOUR ON *BOTRYOCOCCUS BRAUNII* HYDROCARBON COMPOSITION

Hydrocarbon samples analysed by the Gas Chromatography – Mass Spectrometry (GC-MS) from *B. braunii* grown in all light colours showed similar chromatographs, but with some notable variations between the different samples (Fig. 6.1). Peaks 1-4 are present in all samples with relative similar retention times. WL samples from day 6, day 12 and day 21, show a similar hydrocarbon composition over time with the main peaks (Peak 1-4) being conserved. Samples from WL, BL, GL and RL treatments at day 21 also show an identical spectra profile of the hydrocarbons produced.
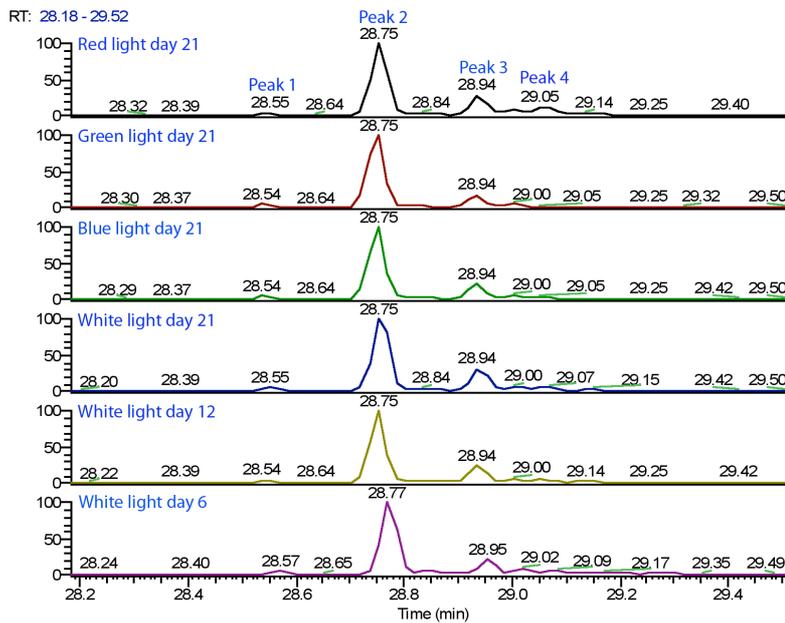
Fig. 6.1: Analysis of *Botryococcus braunii* hydrocarbon composition by GC-MS from the different light regimes.

Chromatograms of hydrocarbon samples extracted from *B. braunii* in the different light colours at day 21, plus white light samples from day 6 and 12. Peaks 1, 2, 3 and 4 are illustrated in the chromatograms with the respective retention times.

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The mass spectra of the main peaks, 1, 2, 3 and 4 (Fig. 6.1) indicate these compounds are identical shown by the high degree of similarity of the peaks spectra (Fig. 6.2) as all peaks have indicative *m/z* of 55, 69, 81, 95, 109 and 121 fragments.

A search in the National Institute of Standards and Technology (NIST) library of the main four peaks returned no positive identification of these hydrocarbons. A manual search of essential oils libraries also resulted in no definite fit to the structure obtained by the mass spectrometer.
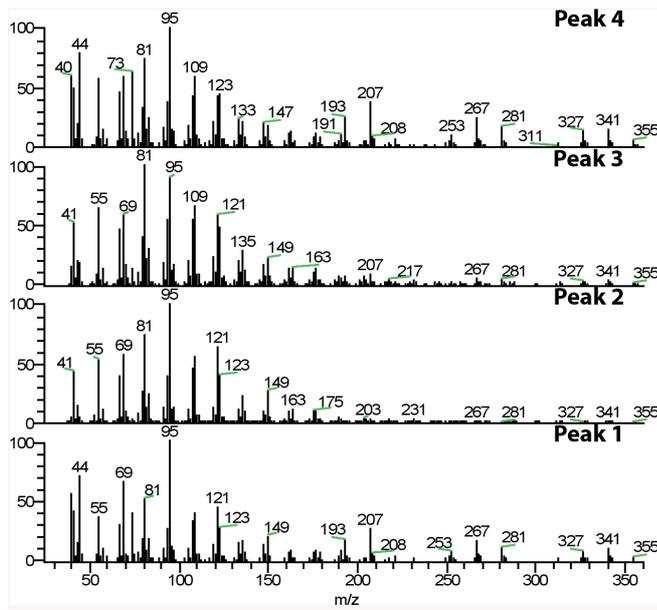
Fig. 6.2: Mass spectra of peak 1, 2, 3 and 4 from *Botryococcus braunii* grown in white light for 21 days.

Peak 1, 2, 3 and 4 correspond to the peaks seen in the chromatogram in Fig. 6.1.

CHAPTER 7

DISCUSSION

The results from this investigation suggest that light quality could have an effect on the growth of *B. braunii* but further experiments are needed to reach reliable conclusions. Light quality did have an effect on the morphology of *B. braunii* Guadeloupe (race B) alga, and had no effect on the quantitative and qualitative aspects of hydrocarbon synthesis and the chlorophyll content.

The growth patterns of *B. braunii* in Fig. 3.4 shows a very different response to the previous growth pattern seen in Fig. 3.1. In Fig. 3.1 it seems that we are looking at a diauxic curve which is common when two carbons sources are present. The media used in these experiments did have citric acid and this could be metabolized by *B. braunii*, as other organisms, such as the green algae *Chlamydomonas reinhardtii,* have been show to metabolize citric acid (Heifetz et al. 2000). In Fig. 3.4 though we saw a linear growth pattern instead and it is thought that it is because there are more independent replicates leading to the suggestion that the diauxic curve seen in Fig. 3.1 had a sampling error. Experiments done in other projects within the department have shown that there is no growth differente of *B. braunii* in media with or without citric acid (personnal communication) suggesting that citric acid is not being metabolized, but more experiments are need to clarify this point, such as long time series growth of *B. braunii* with increasing concentration of citric acid.

B. braunii grew best under WL and RL with a linear growth increase in dry biomass. WL provided the best light quality for *B. braunii* growth as it reached the highest dry biomass increase compared to algae in RL. In contrast to WL and RL, mature *B. braunii* population stopped growing and remained in a dormant state whilst in BL and GL. No signs of degradation were seen in *B. braunii* exposed to 15 days in BL or GL as it recovered to normal growth when treated back with WL. This is an important observation and raises the question on how the algae are obtaining their energy. *B. braunii* has been shown to be heterotrophic (Tanoi et al. 2010) and therefore careful planned experiments need to be designed to elucidate where do the algae get energy from whilst in an hibernating state, such as during GL exposure.   Through this experiments, we are not able to say if biomass

increase is due to light intensity or light quality, as there are large differences in the LFD delivered for each light treatment (See materials and methods pg. 12). Future experiments should look at *B. braunii* growth in different monochromatic lights with equal light intensity. These future experiments would be more easily achieved using LEDs as it gives more control over light intensity and quality. Although we cannot attribute the differences in *B. braunii* growth to light quality in these study, other studies done with higher plants have also showed that plants under monochromatic light had reduced growth compared with plants grown in WL (Wang et al. 2009).

Previous studies of *B. braunii* have found growth rates ranging from 0.06 g l^{-1} d^{-1} to 0.30 g l^{-1} d^{-1} (Eroglu et al. 2010; Tran et al. 2010) and our results fit well within that range. Although there was a significant difference ($P < 0.05$) in dry biomass between *B. braunii* grown in WL and RL, no significant difference ($P > 0.05$) was observed in growth rate between these two conditions. A possible explanation to the low growth rate in *B. braunii* exposed to RL compared to *B. braunii* exposed to WL is that the RL exposed algae had to respond to the change in light quality at day 12 and the reduction of light intensity from 70 μmol m^{-1} s^{-1} to 54 μmol m^{-1} s^{-1}. This reduction in light intensity and the change in light quality is probable to have caused a stress response and a reduced final dry biomass compared to *B. braunii* grown in WL. In hindsight, it would have been useful to do some irradiance response curves for each light colour as it would allowed us to reach a more solid conclusion in the effects of the different light colours in the growth of *B. braunii*. But one needs to remember that the hypothesis of this study was that BL could trigger hydrocarbon production in response to depth. This study aimed at mirroring natural light conditions in the water environment.

Because *B. braunii* is considered a potential microalgae for hydrocarbon production (Casadevall et al. 1985; Dayananda et al. 2007), such results are of great importance to maximizing *B. braunii* biomass productivity as means of yielding higher amounts of metabolites and reducing the overall cost. The uses of *B. braunii* metabolites are not only potentially useful as a biofuel source, but other metabolites may be used in other industries such as fatty acids for pharmaceutical industry as a drug carrier via the skin (Fang et al. 2004). A better understanding of light effects on the growth of microalgae as source of biofuels and other high value

products are important as the knowledge can be use in the developments of photo-bioreactors technology.

Our results have shown that light quality had an effect on the morphology of mature *B. braunii* cells. *B. braunii* cells grown in WL were significantly larger (P < 0.05) in length and width compared to algae grown in RL, BL or GL. *B. braunii* grown in GL was significantly smaller than algae grown in the other light regimes, which was expected as green light is not photosynthetically active and therefore there was insufficient energy to invest in growth. Both *B. braunii* grown in BL and RL showed similar cell size which raises further questions into the how light quality could effect cell size. There was an approximate 5 fold difference in light intensity between algae in BL and RL (see Materials and Methods chapter) and no significant difference was observed between the different light regimes, whilst compared to algae grown in WL, approximately 1/3 more light intensity than RL, both algae in BL and RL showed significant smaller cell sizes. A combination of different light qualities could have a role in *B. braunii* cell morphology but more studies are needed to elucidate such a suggestion. And in terms of future research it opens possible bioengineering routes, which could improve the yield of microalgae growth both in bioreactors and open pounds. Further reasons for future research is the carrying capacity of hydrocarbons per dry weight. *B. braunii* race B have shown to produce between 30 % to 40 % of triterpenoid hydrocarbons per dry weight (Metzger et al. 1985) as such the size of the cell becomes important as maximizing *B. braunii's* size could in turn maximize hydrocarbon production.

Some microalgae have the ability to survive heterotrophically, such as *Chlorella*, and naturally metabolize sugars, whilst other species harvest most of their energy from the sun. Again, *B. braunii* has been shown to be able to be heterotrophic (Tanoi et al. 2010), therefore investigating the effects of light quality in the growth and size of cells can be misleading if *B. braunii* are exposed to other sources of carbon. Therefore it is recommended that future experiments investigating light quality, be in a growth medium without carbon source.

Light quality showed no effect on the chlorophyll content of *B. braunii*, whilst showing dependence on growth. The young *B. braunii* in WL and BL, showed a correlation in increase of dry biomass and chlorophyll content (Fig. 4.4). Chlorophyll content of mature *B. braunii* in BL and GL remained the same and

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photosynthetic activity started once algae in BL and GL were exposed to WL. These results lead to the conclusion that photosynthetic apparatus of *B. braunii* did not degraded during the 15 days exposed to BL and GL. Similar chlorophyll trend in WL has also been previously documented (Bailliez et al. 1986) and Grung et al. (1986) in examining caratenoids noticed that more are produced during the linear and stationary phase possibly to protect the algae from photoinhibition. These phenomena could in turn explain the decrease of chlorophyll content per dry weight during the linear and stationary phase.

From our results we conclude light quality has no effect in the amount of hydrocarbons synthesized by *B. braunii*. Both the Nile red fluorescence assay and GC-FID tests show a correlation between hydrocarbons produced and dry biomass.

In WL and RL *B. braunii* showed an increase in lipid production, whilst algae in BL and GL showed no production of lipids. Parallel to the lipid production, *B. braunii* was actively growing in WL and RL, compared to the inactive *B. braunii* in BL and GL that showed no increase in biomass. When *B. braunii* lipid production was plotted against the dry biomass, a significant correlation appeared for WL (Fig. 5.2) with BL, RL and GL lipid per dry biomass correlation falling in the 90 % prediction band of WL. Preliminary studies of the Nile red fluorescence, have shown that its fluorescence intensity can vary with time, which makes the determination of lipid content more difficult. Also, the colony forming morphology of *B. braunii* could cause the diffusion of Nile red dye in a nonhomogenous diffusion and reducing the accuracy of lipid determination. Therefore GC-FID analysis was also taken.

Further analysis of the hydrocarbon fraction by GC-FID showed the hydrocarbon content of *B. braunii* to be between 38 % and 42 % dry weight which is considered the normal for a race B *B. braunii* (Dayananda et al. 2007; Okada et al. 1995). This hydrocarbon content was similar for *B. braunii* in all different light qualities, suggesting that light quality has no effect in the production of hydrocarbons, even though we had different light intensities between the different light colours. These results also confirm what was observed by the Nile red assay that hydrocarbons are growth associated. Although there was a difference in growth rate between *B. braunii* grown in WL and RL and no growth in *B. braunii* in

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BL and GL, the hydrocarbon percentage per dry biomass is conserved throughout the different light treatments. *B. braunii* grown in white light also showed similar hydrocarbon content per dry biomass over time, with a marginal decrease of 3 % hydrocarbon per dry weight by day 21 which corresponds to similar results reported (Largeau et al. 1980; Metzger et al. 1985). The small decrease of hydrocarbon percentage per dry weight seen in WL over time was attributed to the increase of cell debris within the culture, since when harvesting for analysis it is not possible to separate the cell debris and the active mature *B. braunii* cells. The initial observations from the pilot study that light could trigger hydrocarbon production, has been shown to be false as hydrocarbon synthesis is growth associated.

When comparing the composition of hydrocarbons by GC-MS it also appears that light quality had no effect on the type of hydrocarbons produced. The chromatograms of the hydrocarbon samples from the different light conditions were identical (Fig. 6.1) and the mass spectra of the four main peaks were very similar. The main peak, which constitutes 66 % of the total hydrocarbon fraction (Table 5.2), has been identified as C_{34} in similar proportions (Metzger et al, 1985).

There was some variation in the amount of the main hydrocarbons compounds present in the different light regimes, but it is not clear if such a difference is light quality related as we have concluded by other results that hydrocarbon synthesis is growth associated. This leads to the suggestion that natural solar light quality is sufficient for hydrocarbon production in outdoors ponds.

GC-FID analysis of the hydrocarbons and the lipid content per dry biomass, accordingly to the Nile Red assay, show that the hydrocarbon content per dry weight of *B. braunii* did not vary in quantity and composition in the different light qualities, although there were significant physiological changes observed including, slower growth rate and smaller cell sizes in *B. braunii* grown in red, blue and green light, suggesting strongly that hydrocarbons production is associated with growth, which has also been suggested (Kojima and Zhang, 1999; Metzger and Largeau, 2005).

It is probable that the ratio of lipid content per dry biomass in *B. braunii* has an optimum value in upward force and therefore giving advantage to *B. braunii* as

it does not need to respond to the need or not of having to produce lipids in function of depth or light colour. It is likely that the lipids have other functions besides providing buoyancy, such as protection against photo-bleaching, harmful radiation, other organisms or chemicals in the surrounding environment and desiccation.

This study has shown that light quality does not affect lipid production in the green alga *B. braunii*. This study was done due to the observations in the preliminary study where it was recorded double the amount of lipids, accordingly to the Nile Red assay, in *B braunii* grown in BL in comparison to algae grown in WL. The reason why we observed such effect was because *B. braunii* was not growing and therefore the ratios of lipid per dry biomass stayed as they were, whilst in WL the biomass increased and therefore the ratio of lipids per dry biomass decreased. If we take the data from this study and plot the lipid production, using the nile red assay, against dry biomass we will see the same pattern, but it does not reflect what is actually happening within the cells. This point became very clear when GC-FID results were analysed.

Many questions are still unanswered of why *B. braunii* produces such a high amount of lipids, and triggers it's production. Is it simply a life cycle response to cell size and growth, could it be triggered by nutrient limitation. Another aspect of depth is also pressure. When observing algae with on a microscope the cover slip pressure causes the oil also to be secreted out of the cells and a similar response could be happening as the pressure builds up due to depth.

Future work in the area of *B. braunii* biotechnology is suggested as this prodigious oil producing algae is a potential candidate for mass production. Lipid production seems to have the primary function of providing buoyancy to the organism and an interesting experiment would be to compared *B. braunii* that are floating with *B. braunii* on the bottom of the flask, which could shed some light in the variation of hydrocarbon content seen in the literature, due to perhaps a mix culture of living cells and cell debris. Further experiments to induce stress in *B. braunii*, such as long dark periods, colder temperatures and relate to lipid content would help to investigate if lipids have other function besides providing buoyancy, such as nutrient and carrier molecule.

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CHAPTER 8

CONCLUSION

The best light to grow *B. braunii* is the full visible spectrum WL as it increases biomass at a faster rate producing the largest individual cells. RL also allows *B. braunii* to grow but at a slower rate than WL and the individual cells are smaller compared to WL. In BL and GL *B. braunii* did not grow with individual cells in BL being the same size as in RL, and in GL individual cells were the smallest. Light quality has no effect in the quantitative and qualitative aspects of hydrocarbons produced in *B. braunii*, and it seems that it is growth associated with 40 % hydrocarbon per dry biomass. What this study has showed is that WL provides the best light condition as it maximizes growth and size of individual *B. braunii* cells, which offer the best conditions to higher yields of hydrocarbons from the perspective of biodiesel exploitation.

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APPENDIX 1

Nile Red versus Dry Biomass Correlation

	White Light	BLue Light	Green Light	Red Lifght
Best-fit values				
Slope	2004 ± 158.7	1324 ± 143.1	2370 ± 871.5	1581 ± 287.6
Y-intercept when X=0.0	4418 ± 476.2	5631 ± 183.2	4668 ± 1617	3804 ± 1011
X-intercept when Y=0.0	-2.204	-4.254	-1.969	-2.406
1/slope	0.0004990	0.0007554	0.0004219	0.0006325
95% Confidence Intervals				
Slope	1687 to 2321	1034 to 1614	487.9 to 4253	959.8 to 2202
Y-intercept when X=0.0	3466 to 5370	5260 to 6002	1176 to 8160	1621 to 5987
X-intercept when Y=0.0	-3.140 to -1.514	-5.761 to -3.285	-16.71 to -0.2767	-6.209 to -0.7394
Goodness of Fit				
R square	0.7200	0.6982	0.3627	0.6992
Sy.x	1995	610.7	395.5	773.9
Is slope significantly non-zero?				
F	159.4	85.59	7.398	30.22
DFn, DFd	1.000, 62.00	1.000, 37.00	1.000, 13.00	1.000, 13.00
P value	< 0.0001	< 0.0001	0.0175	0.0001
Deviation from zero?	Significant	Significant	Significant	Significant
Data				
Number of X values	64	39	15	15
Maximum number of Y replicates	1	1	1	1
Total number of values	64	39	15	15
Number of missing values	69	94	118	118

B. braunii Cell Width

	Parameter
Table Analyzed	Bbraunii width

One-way analysis of variance

P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	28.88
R square	0.1873

Bartlett's test for equal variances

Bartlett's statistic (corrected)	12.15
P value	0.0069
P value summary	**
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	53.26	3	17.75
Residual (within columns)	231.2	376	0.6148
Total	284.4	379	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
WL vs BL	0.3576	4.445	Yes	*	0.06150 to 0.6537
WL vs GL	1.027	12.77	Yes	***	0.7313 to 1.323
WL vs RL	0.3125	3.885	Yes	*	0.01645 to 0.6086
BL vs GL	0.6698	8.326	Yes	***	0.3737 to 0.9659
BL vs RL	-0.04505	0.5600	No	ns	-0.3411 to 0.2510
GL vs RL	-0.7148	8.886	Yes	***	-1.011 to -0.4188

B. braunii Cell Length

Parameter			
Table Analyzed	Bbraunii length		

One-way analysis of variance

P value	< 0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	18.74		
R square	0.1301		

Bartlett's test for equal variances

Bartlett's statistic (corrected)	12.41		
P value	0.0061		
P value summary	**		
Do the variances differ signif. (P < 0.05)	Yes		

ANOVA Table	SS	df	MS
Treatment (between columns)	49.15	3	16.38
Residual (within columns)	328.7	376	0.8741
Total	377.8	379	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
WL vs BL	0.5209	5.431	Yes	**	0.1679 to 0.8740
WL vs GL	1.012	10.55	Yes	***	0.6593 to 1.365
WL vs RL	0.4312	4.495	Yes	**	0.07813 to 0.7842
BL vs GL	0.4914	5.123	Yes	**	0.1383 to 0.8444
BL vs RL	-0.08979	0.9361	No	ns	-0.4428 to 0.2632
GL vs RL	-0.5812	6.059	Yes	***	-0.9342 to -0.2281

GC-FID Full Table

Ret. Time	DAY 6 White Light Rep 1	Rep 2	Rep 3	DAY 12 White Light Rep 1	Rep 2	Rep 3	DAY 21 White Light Rep 1	Rep 2	Rep 3	Blue Light Rep 1	Rep 2	Rep 3	Green Light Rep 1	Rep 2	Rep 3	Red Light Rep 1	Rep 2	Rep 3
27.233							0.07								0.06			
27.423	7.05	16.96	27.66	1.02	1.52	0.55	0.26	0.32	0.37	0.43	0.12	0.46	0.14	0.21	0.20	0.49	0.58	0.68
27.555	0.89	1.08	1.18	0.58	0.58	0.50	0.44	0.86	0.68	0.54	0.20	0.53	0.38	0.53	0.70	0.47	0.65	0.60
27.753	3.54	3.37	3.19	4.22	3.60	3.36	3.00	4.56	4.96	3.13	1.26	2.99	2.51	3.88	3.27	3.09	3.79	3.65
27.928	31.15	35.99	42.10	23.41	23.27	24.97	24.33	26.48	26.42	29.10	12.91	27.30	24.87	31.12	29.84	23.90	26.84	26.03
28.220	358.05	327.21	306.52	310.03	304.68	351.24	347.57	365.00	359.56	411.96	180.92	383.72	346.78	431.78	417.96	336.11	374.92	364.17
28.345	68.77	61.50	57.89	93.97	79.10	125.36	124.11	112.04	110.64	119.64	37.48	118.39	71.19	94.48	88.46	110.63	124.82	99.25
28.397	45.43	55.80	60.47	18.33	80.17			20.84	22.03		15.06					40.88	48.48	24.14
28.460																		59.89
28.583	2.50	2.17	2.08	1.89	1.82	2.34	2.58	2.71	4.95	5.46	2.51	5.45	3.19	4.14	4.01	2.78	5.65	5.75
28.670	1.89	1.78	1.17	1.55	1.91	1.83	2.14	2.45					1.80	1.09	1.07	2.68		
28.967	5.87	5.78	4.96	3.04	2.11	3.19	3.54	3.46	3.26	5.38	2.28	4.83	7.16	8.14	7.73	3.11	3.89	3.48
29.092	5.04	4.62	3.84	2.54	0.49	2.79	2.91	3.00	2.62	6.71	2.86	6.15	8.61	8.64	10.82	1.83	2.22	1.89
29.160	1.89	2.16	1.06	1.27	0.23	1.28	1.44	1.42	1.40	2.12	0.81	1.98		2.49		1.79	1.60	2.13
29.220					0.14													
29.302	0.28														0.50		0.59	
29.418	1.81	2.07	1.77	1.63	0.78	1.66	1.54	1.61	1.19	1.87	0.72	1.84	1.45	1.97	1.55	1.29	1.46	1.39
29.488	0.98	0.89	0.66	1.01	0.62	1.09	0.82	1.02	0.85	1.05	0.35	0.92	0.64	0.88	1.14	0.77	1.05	0.94
29.575	4.71	4.30	3.71	4.30	3.88	4.78	4.73	5.04	4.76	5.61	2.42	5.27	5.09	6.34	6.35	4.81	5.80	5.21
29.707	2.44	2.32	1.74	1.05	0.61	1.19	1.31	1.19		4.54	1.78	3.91	9.13	9.31	9.13	0.70	0.98	0.88
29.843	16.29	13.98	12.32	5.60	2.88	6.96	6.25	5.85	4.81	19.22	8.40	16.45	33.06	37.12	36.86	4.16	4.40	4.02
30.045	0.14	0.15	0.26	0.86	0.53	1.14	0.56	0.52	0.54	0.69	0.17	0.64	0.19	0.38	0.34	1.10	1.43	1.24
30.103													0.10	0.24	0.20			
30.210																0.22	0.58	0.48
30.285	0.83	0.73	0.67	0.34		0.58	0.42	0.37	0.27	1.85	0.66	1.63	5.85	3.98	3.92	0.10		
30.340	0.47	0.41	0.43	0.11		0.20	0.18	0.16	0.09	0.96	0.36	0.80		2.11	2.03	0.05		
30.423	0.20	0.25	0.46			0.10	0.08			0.45	0.15	0.36	0.97	0.98	0.94			
30.542					3.27								0.17					
30.662	0.16	0.36	0.53										0.13	0.25	0.07			
30.837		0.15	0.13													0.28		
30.902				0.21		0.17	0.21	0.26	0.27	0.32	0.11	0.30	0.16	0.22	0.23	1.04	0.33	0.38
30.992	2.46	2.50	2.73	1.20	6.57	0.90	0.59	0.94	0.93	0.40	0.12	0.38	0.31	0.41	0.44		1.25	1.24
Total (mg l^{-1})	562.84	546.53	537.53	478.16	518.76	536.18	529.01	560.17	550.6	621.43	271.65	584.3	523.88	650.69	627.82	542.28	611.31	607.44