

Article

Australian Strains of *Botryococcus braunii* Examined for Potential Hydrocarbon and Carotenoid Pigment Production and the Effect of Brackish Water

Kim Jye Lee-Chang ^{1,*}, Elisabeth Albinsson ¹, Lesley Clementson ¹ , Andrew T. Reville ¹, Ian Jameson ² and Susan I. Blackburn ²

¹ CSIRO Oceans and Atmosphere, Castray Esplanade, Hobart 7000, Tasmania, Australia;

Lisa.Albinsson@tassal.com.au (E.A.); Lesley.Clementson@csiro.au (L.C.); Andy.Reville@csiro.au (A.T.R.)

² Australian National Algae Culture Collection, CSIRO National Research Collections, Castray Esplanade, Hobart 7000, Tasmania, Australia; Ian.Jameson@csiro.au (I.J.); Susan.Blackburn@csiro.au (S.I.B.)

* Correspondence: Kim.leechang@csiro.au

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Abstract: The green alga *Botryococcus braunii* produces abundant hydrocarbons, in the form of drop-in biodiesel, which promoted interest in the species as a renewable fuel. However, despite the observation of dense populations in the wild, *Botryococcus* grows very slowly in culture, severely limiting its potential for development as a bioresource. Undertaking a biodiscovery program, we found new strains of *Botryococcus* in locations ranging from tropical to temperate Australia and from both fresh and brackish waters. As part of the ecophysiological characterisation of this new biodiversity, lipid and pigment compositions were studied for six new strains from six different locations. The strains were inoculated in either freshwater or brackish (salinity of 4)-based medium and maintained over 150 days. The growth of cultures was studied continuously, while lipid and pigment composition were analysed at final harvest on day 150. No significant differences in growth rate between fresh and brackish media were observed. Some strains were more tolerable of brackish conditions than others with a link between salinity tolerance and original location. The use of lower salinity (4 ppt) had a minimal effect on lipid composition, with only two of the six strains showing a different hydrocarbon profile in comparison to the other strains; pigment composition showed only minor variations for fresh and brackish water cultures, although the concentrations varied significantly with the freshwater cultures containing higher pigment concentrations.

Keywords: biofuel; *Botryococcus braunii*; hydrocarbon; pigment

1. Introduction

Botryococcus braunii is a green algae (Trebuxiophyceae) that is characterised by its ability to produce up to 61% of its dry weight as a hydrocarbon-rich oil that is composed of non-oxygenated hydrocarbons [1–3]. Historically, the interest in studying *B. braunii* has been due to its geochemical significance in a variety of oil-rich deposits throughout the world dating from the Ordovician (485.4–443.7 mya). When the hydrocarbon-rich oil from *B. braunii* was “cracked” (the catalytic and thermal degradation of long chain hydrocarbons to form useable shorter chain hydrocarbons), it yielded 67% petrol, 15% aviation fuel, and 15% diesel distillate, which equivalent to “gas–oil fraction of crude oil” and therefore has attracted significant interest as a potential species for producing drop-in biofuels [4,5].

B. braunii Kützing is widely distributed globally in freshwater and saline environments including continental, temperate, tropical, and alpine climatic zones [1]. The species tolerates desiccation

treatment of 6–8 months and extreme temperature shifts ranging from -20 to 40 °C, which may account for the worldwide distribution of *B. braunii* [6]. A key feature of the physiology of *B. braunii* is the hydrocarbon-rich oil that is excreted in the form of long-chain (defined as $\geq C_{23}$) aliphatic hydrocarbons from the cells into the extracellular matrix, which in turn is encased in the retaining wall [3]. Based on the types of hydrocarbons that *B. braunii* produces, it is classified into three chemical races—A, B, and L [1,7]. Additionally, Kawachi et al. described a new race S strain producing a different group of hydrocarbons [8]. Strains of race A are characterised by *n*-alkadienes and/or *n*-alkatrienes and their derivatives with an odd carbon number (C_{23-33}); Race B produces a range of specific botryococcones that are C_nH_{2n-10} triterpenes (C_{30-37}) and methylated squalenes (C_{31-34}); Race L produces lycopadiene—a single tetraterpenoid hydrocarbon not formed by other strains of *B. braunii* ($C_{40}H_{78}$); Race S produces shorter chain epoxy-*n*-alkanes and saturated *n*-alkanes with 18 and 20 carbons, respectively [8].

B. braunii is capable of producing large amounts of hydrocarbons that are excreted from the cells and retained extracellularly to maintain the colonies' buoyancy. Furthermore, the intracellular lipids (primarily the fatty acid bearing triacylglycerols) can be extracted directly from living *B. braunii* cells with suitable organic solvents. Moheimani et al. demonstrated the non-destructive "milking" approach that can harvest the oil without killing the cells [9]. However, the slow-growing nature of *B. braunii* has to date hindered the commercial development of biofuel production from this microalgae. In addition to the potential biofuel production, *B. braunii* are also capable of forming bioactive compounds such as high antioxidant activity carotenoid pigments, especially the botryoxanthins and exopolysaccharides, the latter of which can be used as scaffolds for polyesters production for the potential of nanoparticles synthesis [10].

The potential advantage of cultivating freshwater *B. braunii* in low salinity/brackish water evades the problem of water scarcity if the commercial-scale culture of the microalga is considered for hydrocarbon production; such an approach also reduces the contamination risks by other freshwater-living organisms in the open pond [11]. Furuhashi et al. demonstrated that the Showa strain of *B. braunii* was able to be cultured in the modified Chu13 medium by supplying artificial seawater for three culture periods, and a hydrocarbon recovery rate exceeding 90% was reported by simply mixing wet microalgae biomass with *n*-hexane without any pre-treatments; this indicates the potential for hydrocarbon milking from the intact cell [11]. In addition, Rao et al. reported different levels of salinity that can influence the growth rate of *B. braunii* (race 'A') and accumulation of hydrocarbons, carbohydrate, fatty acid, and carotenoids [12].

In this study, we examined the effect of brackish (salinity of 4) media on the growth, pigment, and lipid composition in newly isolated Australian strains of *B. braunii*. In the first part of the study, we screened six different Australian *B. braunii* strains using a 24-well plate to study growth; in the second part of the study, the six cultures were scaled up (from 3 mL multi-well plate to 75 mL tissue culture flasks) in order to harvest enough biomass to examine the effect of salinity on the secondary metabolites, including hydrocarbon and pigment composition, of the cultures.

2. Materials and Methods

2.1. Botryococcus Braunii Strains and Culturing Conditions

The strains used in this study (Table 1) are maintained in the Australian National Algae Culture Collection (ANACC), CSIRO, Hobart, Tasmania (www.csiro.au/anacc). Henceforth, strains are referred to by their descriptive strain name rather than unique strain number. The strains were originally isolated from Australian freshwater environments by horizontal net tows (5×2 m in surface waters) followed by micropipetting and washing in sterile AF-6 medium (Kasai et al., 2004). Then, single colony isolates were maintained under conditions most likely to support growth in the cultures, i.e., conditions similar to those at the sites from which the respective strains were isolated. These were at 20 °C in sterile AF-6 at approximately $80 \mu\text{mol PAR m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent illumination with a 12:12 h

light: dark cycle. The experimental cultures were each established from a 0.5 mL inoculum into 2.5 mL of either fresh or brackish (salinity of 4) AF-6. For each strain, 6 experimental cultures were established, 3 in freshwater media and 3 in brackish media, adding up to a total of 36 experimental cultures. The experimental cultures were maintained in 24-well multi-well plates (Falcon, Thomas Scientific USA) for a period of 51 days, during which they were microscopically examined every 7 days. During the examination, the whole water column of the well was photographed through the ocular using an 8 MPix camera, and the resulting picture was then enlarged, and colonies were counted by hand. Biovolumes were calculated using the formula $V = 4/3 \pi r^3$, assuming the colonies to have a spherical shape, or if required, using several spheres to cover the shape of the colony. After 51 days in the multi-well plates, the cultures were each transplanted into 75 mL tissue culture flasks containing 30 mL of either fresh AF-6 or brackish AF-6, depending on the original media. Then, the cultures were left for approximately 100 days before being harvested and analysed for pigment and hydrocarbon profiles.

Table 1. Australian *Botryococcus braunii* strains investigated in this study.

Strain Name	CSIRO CS Number	Original Location (Latitude, Longitude in Degrees Decimal)	Race	Genebank Accession Number
BBUL	CS-952/04	Upper Lily Dam, Musk, VIC (−37.384723 144.204083)	B	KP071393
WML	CS-1046	Wind Mill Lagoon, TAS (−41.330275: 148.311401)	A	KP071389
LCRW	CS-1052	Lake Crescent Raceway, TAS (−42.170028: 147.149106)	B	KP071385 ^a
LCM	CS-1055	Lake Crescent Marsh, TAS	Unknown	KP071385 ^b
LOL	CS-1057	Lagoon of Islands, TAS (−42.110106: 146.93385)	A	KR869723 ^c
LHD	CS-1062	Lower House Dam, Musk, VIC (−37.386125: 144.204302)	B	KP071384 ^d

CS number refers to strains deposited in the Australian National Algae Culture Collection. In total, 18S sequences from this study were submitted to the GenBank database (accession numbers) and other similar strain's genebank accession numbers from the same locality were provided for reference purposes; ^a CS-1049, ^b CS-1051, ^c CS-1056, ^d CS-1063.

2.2. DNA Extractions, PCR, 18S and *rbcl* Gene Sequence Alignments

Total DNA was extracted from 8–10 mL from each strain of Australian *Botryococcus* cultures using the DNeasy Plant mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNA was eluted in 200 µL of Buffer AE and quantified using a Nanodrop ND1000 vers 3.0 (Nanodrop Technologies Inc., Wilmington, DE, USA). DNA ranged from 1.5 to 70 ng/µL. Following the extraction and quantification of DNA, the 18S gene (1630 base pairs) was amplified using primers of Kawachi et al. [9], 63F (5'–ACGCTTGTCTCAAAGATTA–3') and 1818R (5'–ACGGAAACCTTGTTACGA–3') at Macrogen Inc. (<http://dna.macrogen.com/eng/index.jsp>; Korea) with the following conditions (94 °C for 3 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min). Sanger sequencing was undertaken and PCR

products were purified using a Montage PCR clean up kit from EMD Millipore (Darmstadt, Germany). Sequencing was undertaken using Big Dye Terminator chemistry v3.1 (Life Technologies, Carlsbad, CA, USA) on an Applied Biosystems 3730XL DNA Autosequencer (Applied Biosystems, Foster, CA, USA at Macrogen Inc., Seoul, Korea).

Remaining archival DNA was stored at $-80\text{ }^{\circ}\text{C}$ at the CSIRO Marine Laboratories. The 18S sequences were submitted to GenBank and outlined in Table 1. The six strains were analysed for hydrocarbon, pigment, and only 2 stains were successfully sequenced for genes, with the remaining strains not DNA extracted due to culture crash and limited quantities at the end of the experiment. Therefore, the other similar strain's Genbank accession number from the same locality was provided for reference purpose.

2.3. Pigment Extraction and Analysis

Pigments were extracted from three mL of *Botryococcus* culture by filtering through a 47 mm diameter Whatman[®] GF/F glass fibre filter (Sigma-Aldrich, St. Louis, MO, USA) and ground in a glass mortar on ice with 100% acetone (3 mL). The extract was quantitatively transferred into a 10 mL centrifuge tube and vortexed for about 30 s. Samples were sonicated in an iced water bath for 15 min in the dark and kept in the dark at $4\text{ }^{\circ}\text{C}$ for approximately 15 h. The filter paper was removed by centrifugation, and extracts were transferred to a 10 mL volumetric flask.

One mL of water was added to the sample extract, and 100% acetone was used to make up 10 mL of the volumetric flask with final extract mixture of 9:1 acetone/water (*v/v*). The final extract was filtered through a $0.2\text{ }\mu\text{m}$ membrane filter (Whatman[®], anatope). The analysis was conducted on the high-performance liquid chromatography (HPLC), which comprises a 2695XE separations module with a column heater and refrigerated autosampler coupled with a 2996 photo-diode array detector (Waters Cooperation, Milford, MA, USA). The extracts were analysed using a C_8 column and binary gradient system with an elevated column temperature following the protocol described in [13]. The separated pigments were detected at 436 nm and identified against standard spectra using Empower[™] software (Waters Cooperation, Milford, MA, USA). Concentrations of chlorophyll a, chlorophyll b, β , β -carotene, and other pigments in sample chromatograms were determined from standards (Sigma-Aldrich, St. Louis, MO, USA; DHI, Hørsholm, Denmark).

2.4. Hydrocarbon Extraction and Analysis

In total, 36 cultures of *Botryococcus* were analysed for excreted hydrocarbons, i.e., those recovered by a liquid–liquid extraction with hexane. Each culture (8–10 mL) was extracted by shaking with 16 or 20 mL respectively of hexane and then allowed to separate overnight. Then, the upper hexane layer was transferred to a round-bottom flask, and after the addition of 100 mg of anhydrous sodium sulfate, the hexane was removed by rotary evaporation. The lipid-containing residue was transferred with hexane into a vial for subsequent analysis by gas chromatography (GC) and gas chromatography mass spectroscopy (GC–MS). The lipid nomenclature used throughout is in the form $\text{C}_{n,x}$, where *n* is the number of carbon atoms and *x* is the number of double bonds in the native hydrocarbon.

GC analysis was performed using a Varian 3800 gas chromatograph fitted with an HP5 ultra 2 capillary column ($50\text{ m} \times 0.32\text{ mm i.d.}$; $0.17\text{ }\mu\text{m}$ film thickness), flame ionisation (FID) detector, and septum–programmable injector (SPI). Samples were injected at an oven temperature of $45\text{ }^{\circ}\text{C}$; after 1 min, the oven temperature was raised by $30\text{ }^{\circ}\text{C min}^{-1}$ to $140\text{ }^{\circ}\text{C}$ and then by $3\text{ }^{\circ}\text{C min}^{-1}$ to $310\text{ }^{\circ}\text{C}$ where it was held for ten minutes. Peak areas were quantified using Galaxie chromatography software. The identity of individual compounds was confirmed by GC–MS analyses on a Thermoquest/Finnigan Trace DSQ benchtop quadrupole mass spectrometer fitted with a direct capillary inlet and on-column injector. Data were acquired in scan acquisition or selective ion monitoring and processed using Xcalibur software supplied with the instrument. The non-polar column (HP5) and operating conditions were similar to that described above for GC analyses. Compound identifications were based on comparisons with published mass spectra [2,3,14,15].

3. Results

Establishing healthy and culturable *B. braunii* cultures from single colony isolates was a difficult process due to the slow growth of this species and the need to remove other eukaryotic contamination such as fungi. This study reports the six strains that were successfully established to survive and prosper from isolation and long-term culturing. Figure 1 shows the colony counts over time (days) in the different cultures. The dip in the colony concentration at day 52 was due to all cultures being transferred from the original 3 mL multi-well culture into 30 mL of medium in tissue culture flasks, which resulted in a 1:10 dilution.

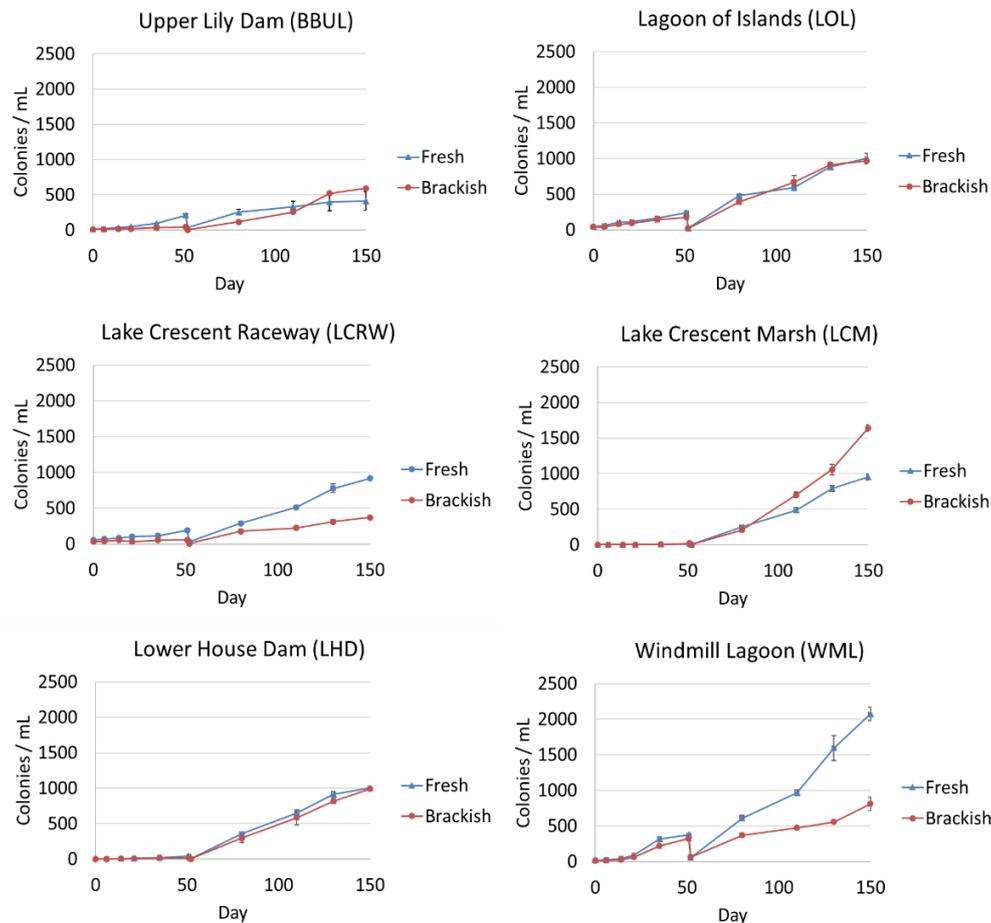


Figure 1. Colony counts for Australian *B. braunii* strains over time (days), blue line indicates freshwater media, red line indicates brackish (BW) media. The cultures were maintained in 3 mL multi-well plates from day 0 to 50, after which cultures were each transplanted into 75 mL tissue culture flasks containing 30 mL of either fresh AF-6 or brackish AF-6 media at day 51. The dip in colony concentration at day 52 throughout was due to dilution (1:10), as cultures were transferred from the original 3 mL culture growing in the multi-well plate into 30 mL medium. Strains LCRW and WML grew significantly better in freshwater medium, while LCM grew better in brackish growth medium. No significant differences in growth were observed for the other strains.

The WML strain originating from a coastal lagoon (<300 m from coastline) grew to higher densities at day 50 in the 3 mL 24-well plate in brackish medium than strains from the freshwater locations (Figure 1). However, growth rates of $<0.23 \text{ div day}^{-1}$ were similar in both fresh and brackish media.

Maximum colony growth rates (between days 52 and 130) of $0.23 \text{ div day}^{-1}$ were reached in freshwater grown LCRW and WML, while $0.22 \text{ div day}^{-1}$ were reached in brackish BBUL, LCM, and LHD. No significant differences occurred in growth rate between the freshwater and brackish

growth medium. All 36 cultures showed slower growth after 80 days, and most reached the stationary growth phase.

Figure 2 demonstrates the change of colony diameter and biovolume (size of sphere) in the growth of the six *B. braunii* strains in brackish water with a salinity of 4. All strains except BBUL showed significantly increased biovolume of colonies at a salinity of 4 at day 150. The possibility of cell division occurring in strain BBUL was dismissed due to the slow growth of this strain, and the culture was observed to be in stationary growth phase.

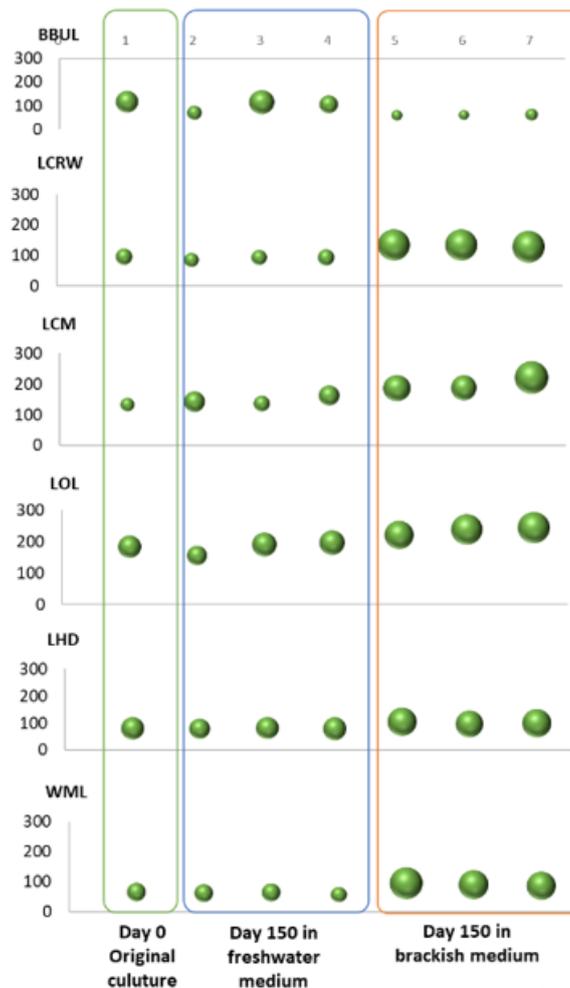


Figure 2. Change of colony diameter and biovolume (size of sphere) for the six Australian *B. braunii* strains, both in the original culture and after culturing for 150 days in either fresh or brackish growth media. The size of the sphere gives an indication of the increase or decrease in biovolume occurring as an average of 30 measured colonies.

Figure 3 shows the strain colony morphology of *Botryococcus* WML strains in this study. Within each strain, the colony morphology of *Botryococcus* strains in this study only altered slightly with culture conditions or age. Morphological variations have been found within and between different strains of the same and different races in normal freshwater media in the ANACC strains, and additional variation was not observed during growth in brackish media.

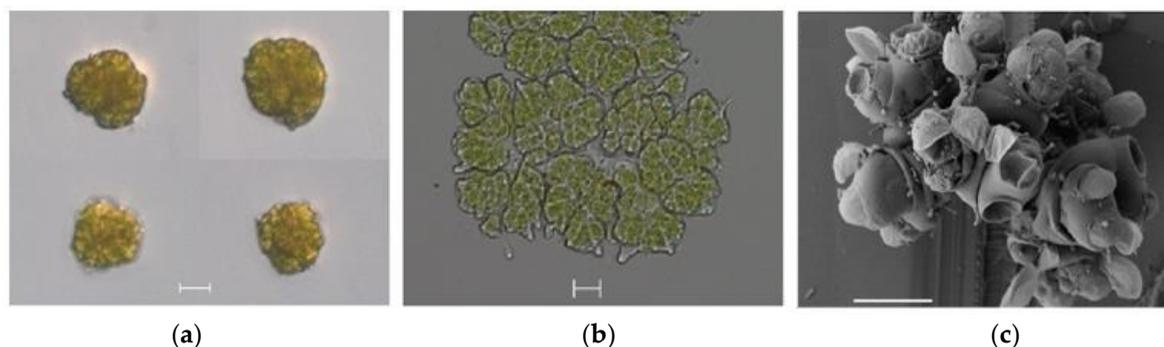


Figure 3. The Australian *B. braunii* strain CS-1046 WML, showing 30-day-old compact colonies; (a) cavity slide, a composite of four colonies from four separate frames, (b) coverslip mount, (c) 147-day-old scanning electron microscope; Scale bars = 10 μ m.

3.1. Pigment Analysis

Figure 4 shows the Chlorophyll a (Chl a) content (μ g/L) of the 6 Australian *B. braunii* strains. Higher Chl a values correlate with significantly better growth occurring for cultures (post inoculation to 30 mL) in fresh water medium than brackish medium (Figure 4). Total Chl a concentrations varied significantly between the fresh and brackish cultures as seen in strain LCRW, LHD, and WML (Figure 4). Figure 5 demonstrates the carotenoid pigment compositions of the six Australian *B. braunii* strains in this study. The Echinenone in both race B LCRW (11.4 μ g/L) and LHD (7.3 μ g/L) have generally higher values in the freshwater cultures compared to that of brackish water (6.9 and 3 μ g/L, respectively).

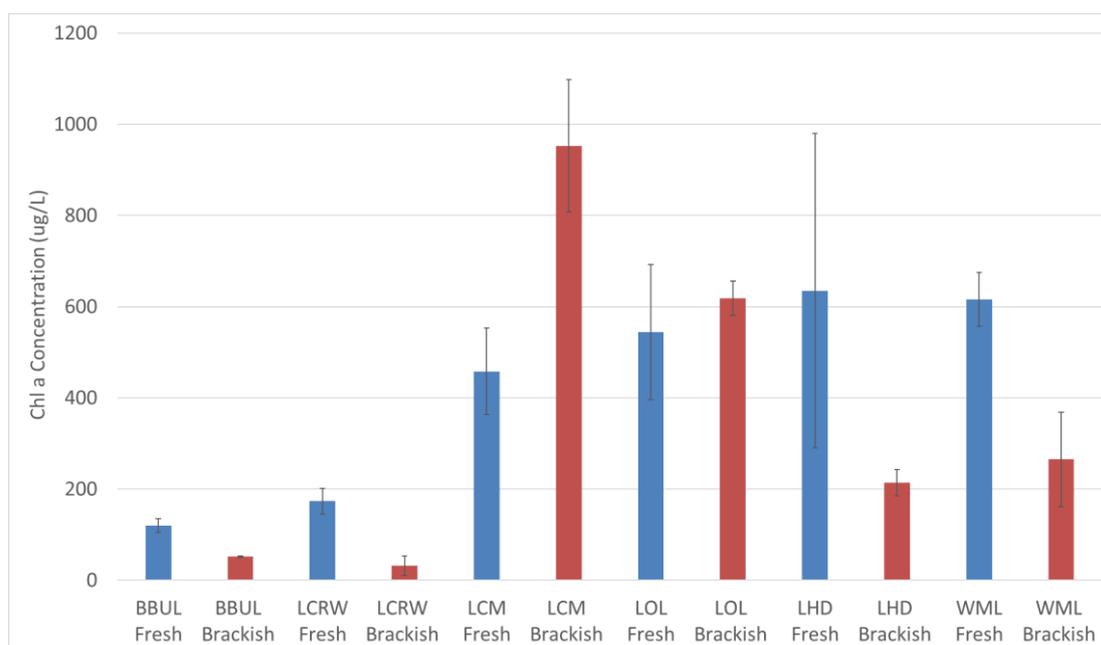


Figure 4. Chlorophyll a content (μ g/L) of the six Australian *B. braunii* strains. Freshwater cultures (blue bar) and brackish cultures (red bar) were grown over a period of 150 days.

3.2. Hydrocarbon Analysis

The hydrocarbon profiles of the Australian strains could be categorised into 2 types: Race A dominated by straight odd-chain hydrocarbons with 2 (alkadiene) or 3 (alkatriene) double bonds (C25–C33). Those characteristic of race B had predominantly botryococenes and methylated squalenes (C30–C37 isoprenoids). Table 2 summarises the percentage of total hydrocarbon and number of isomers

identified in the study. Twenty-two different straight odd-chain alkenes were identified in the race A strains; and 37 different botryococcenes and methylated squalenes were identified in the race B strains.

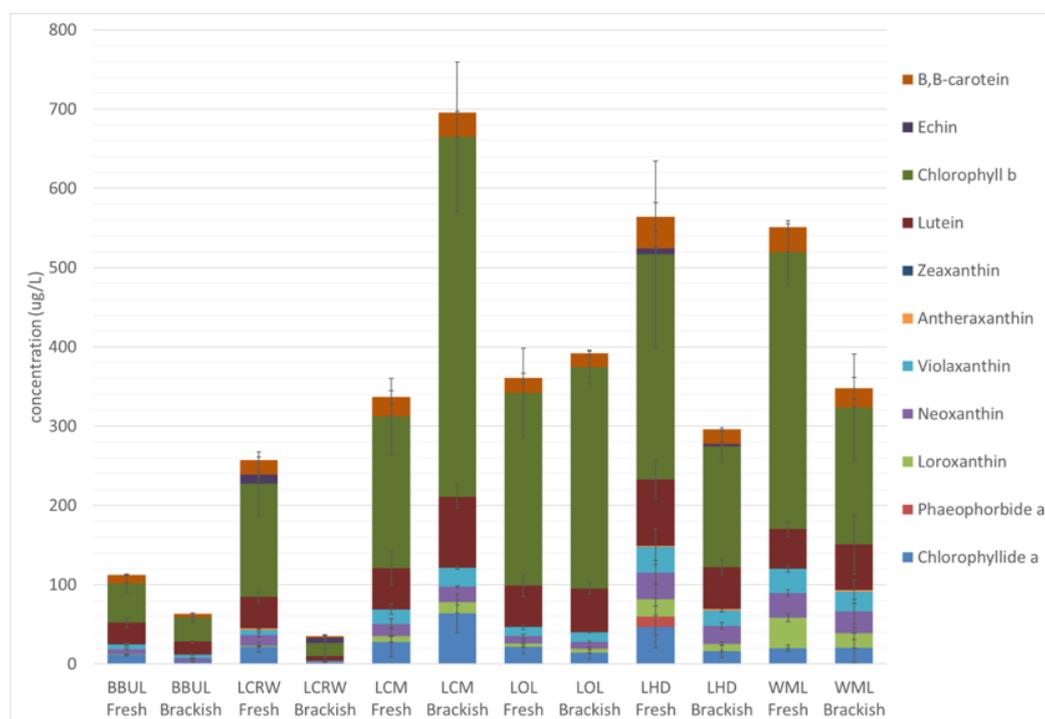


Figure 5. Pigment composition ($\mu\text{g/L}$) of 6 Australian *B. braunii* strains. The profile was typical of green algae with Chlorophyll b and lutein the dominant pigments. Each bar graph is the average result of the analysis of triplicate samples except in BBUL where duplicate samples were analysed due to undescribed contamination in replicate BBUL1.

Table 2. The average percentage (percentage total hydrocarbons \pm standard deviation) of *Botryococcus braunii* strains from the races A and B strains identified in the study.

	Number of Isomers	Average A	stdev	Average B	stdev
% of total hydrocarbons					
C25–C33 alkadienes	8	73.5	15.6		
C27–C33 alkatrienes	8	24.4	14.7		
C27–C31 alkatetraenes	6	2.0	0.9		
C31 botryococcenes	3			0.3	1.1
C32 botryococcenes	2			19.0	30.6
C33 botryococcenes	6			12.1	18.5
C34 botryococcenes	22			23.9	11.2
C35 botryococcenes	2			0.3	0.5
C36 botryococcenes	1			8.0	24.6
C40 lycopadiene	1				
Total hydrocarbon IN MEDIA (mg/L) excl contam		20.3	25.9	48.6	31.1
Hydrocarbon productivity (mg/L/d)		0.32	0.41	0.48	0.32

Figures 6 and 7 are the partial gas chromatograms showing the major hydrocarbons identified in the 6 Australian *B. braunii* strains with both the freshwater and brackish medium treatments. Each race A strain had between 14 and 21 of the 22 different straight odd-chain alkenes identified; each race B strain had between 7 and 26 of the 37 different botryococcenes and methylated squalenes identified. Strains BBUL, LCRW, and LHD showed the characteristics of race B strains, which have predominantly botryococcenes and methylated squalenes (C_{30} – C_{37} isoprenoids). Both BBUL and LCRW strains had no significant difference between the freshwater and brackish medium treatments (Figure 6), and strain

LHD had a more dominant peak (C_{29}) in the brackish treatment. Figure 7 shows the strains that have exhibited differences in their hydrocarbon profile due to the effect of the brackish treatment. Strain LCM is an unknown race, while C_{27} botryococcenes in the freshwater treatment of LCM are enhanced relative to the brackish treatment (Figure 5).

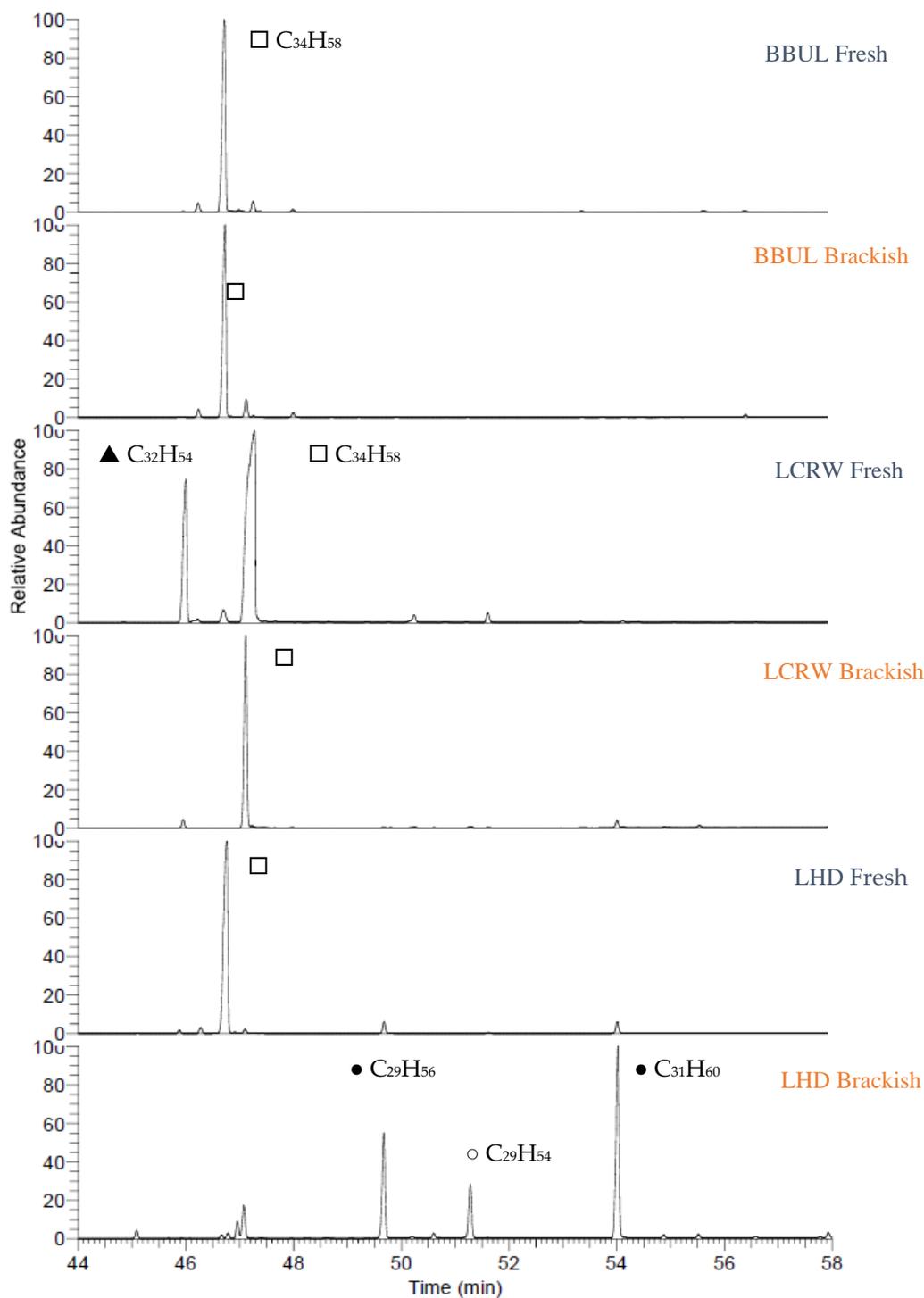


Figure 6. Partial gas chromatograms showing the major hydrocarbons identified in examples of the three races B of new Australian *B. braunii* strains analysed in this study: \square = monocyclic botryococcenes; \bullet = n-alkadienes; \circ = n-alkatrienes; \blacktriangle = botryococcenes.

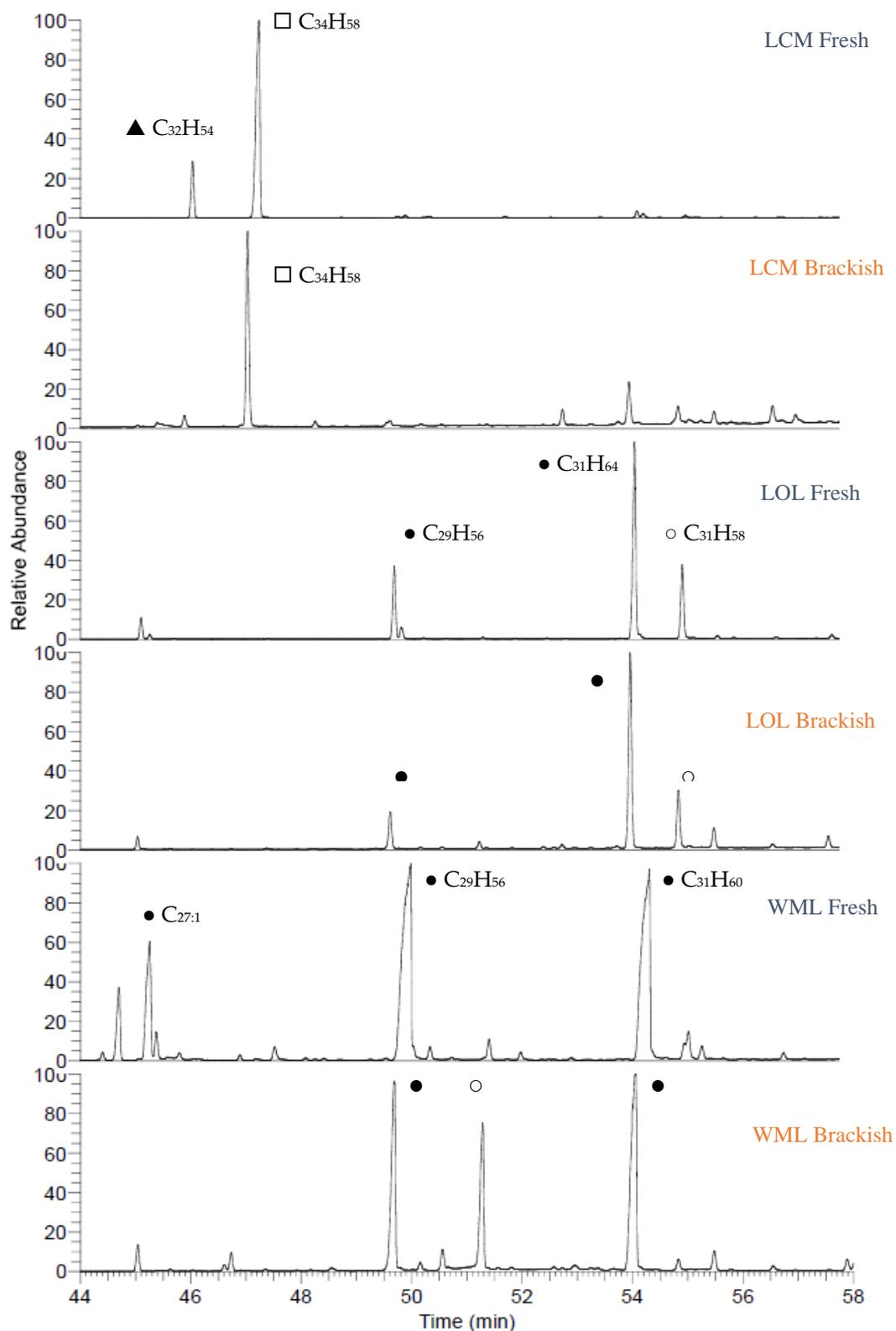


Figure 7. Partial gas chromatograms showing the major hydrocarbons identified in examples of the two races A of new Australian *B. braunii* strains analysed in this study \square = monocyclic botryococcenes \bullet = n-alkadienes; \circ = n-alkatrienes; \blacktriangle = botryococcenes.

Strains LOL and WML have the hydrocarbon profiles representative of race A with the characteristic C_{27} – C_{31} alkadienes and alkatrienes (Banerjee et al., 2002), and they are dominated by straight odd-chain

hydrocarbons with 2 double bonds (alkadiene) (Figure 6). The WML strain has dominant C₂₇ alkenes in freshwater treatment, while C₂₉ alkenes become more pronounced in the saline treatment.

The lipid content of the *B. braunii* BBUL strain was selected as a representative for the fatty acid composition of other *B. braunii* strains (Table 3). The sum of polyunsaturated fatty acids (PUFA) is 70.5% and the sum of saturated and monosaturated fatty acids is 29.5% of total fatty acids. The fatty acid profile is characteristic of typical Chlorophyceae microalgae with 15.9% palmitic acid and 13.6% of oleic acid. However, the linolenic acid is the dominant PUFA, at 60% of total fatty acids, and about 3% of docosahexaenoic acid is detected.

Table 3. Fatty acid composition (% of total fatty acids) of *B. braunii* strain BBUL.

Saturated	% of Total Fatty Acids
16:0	15.9
monoenoic	
18:1 ω 9	13.6
polyenoic	
18:2 ω 6	1.3
18:3 ω 3	58.9
20:4 ω 6	1.3
20:5 ω 3	3.1
22:5 ω 6	0.0
22:6 ω 3	2.9
SUM of Polyunsaturated fatty acids (PUFA)	70.5
ω 3/ ω 6 ratio	26.2
Biomass yield (DW g/L)	6.52
Total FAME (g/100g DW)	1.2

4. Discussion

Growth was overall slower in the 3 mL multi-well cultures in comparison to that of the 30 mL cultures. This was expected, as we have observed generally in *Botryococcus* maintenance that, in contrast to most other Chlorophyta microalgae, there can be a prolonged lag phase in growth of both old dense cultures and single colony isolates when transferred to fresh media. This may likely have more to do with the age and physiological condition of the colony(s) than attribution to lack of nutrient or light availability, as the latter is not readily plausible in instances such as this where the inoculum concentration was effectively so low at only 10s of colonies per mL. Some strains were more tolerant of brackish conditions than others, with BBUL, LCM, LHD, and LoI growing better, or as well as, their freshwater equivalents. In contrast, LCRW and WML grew significantly better in freshwater medium compared to brackish medium. The relationship between salinity tolerance and original location was inconclusive.

All 36 cultures reached stationary growth phase and showed slower growth after 80 days, which could be due to nutrients being limited in the culture medium or inadequate control of physical culture conditions such as temperatures and light intensities. Morphologies do not appear to match with either sample location or for hydrocarbon race, noting the sample size of some races.

The carotenoid composition of Australian *B. braunii* is similar to that of published studies [16–20], with lutein and β,β -carotene as the major carotenoids [21]. Other carotenoids identified in the 6 Australian *B. braunii* strains include linoxanthin, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, and echinenone. Matsuura et al. reported an echinenone-accumulating *B. braunii* BOT-20 strain that produced a small amount of hydrocarbons but high amounts of echinenone, 332 mg g⁻¹ dry biomass weight [22].

Grung et al. 1994 reported that some strains undergo color changes due to the production of secondary carotenoid pigments in the matrix under stress conditions. No color change was observed during cultivation in this study. Most strains have higher pigment concentrations in freshwater medium compared to that of the brackish water medium. In contrast, LCM strain has significantly

higher concentrations of all pigments in the brackish water treatment compared to that of the freshwater treatment (Figure 5). For example, higher linoxanthin and lutein concentrations in brackish grown LCM (14 ug/L and 89 ug/L) occurred than in the freshwater counterparts (7.6 ug/L and 52 ug/L). The carotenoid pigment accumulation can be directly related to higher cell growth, as the LCM strain has higher counts and colony count sizes in the brackish medium compared to that of the freshwater treatment (Figures 1 and 2).

The fatty acid profile of the BBUL strain is similar to other *B. braunii* strains with oleic, palmitic, and linolenic acids as the major fatty acids [1,3]. The intracellular lipids including triacylglycerols that are generally stored in the cytosolic lipid droplets of *B. braunii* strains were not suited for biodiesel production due to high levels of polyunsaturated fatty acids (70.5% of total fatty acids). The shorter chain fatty acids (number of carbons $<C_{20}$) with high levels of saturated and monounsaturated fatty acids (fewer double bonds) are more desirable for biodiesel production due to the increased oxidative and thermal stability of biodiesel. The study focused mainly on the extracellular hydrocarbons to demonstrate the potential of the non-destructive “milking” approach that can harvest the extracellular hydrocarbons to produce drop-in biofuels.

The characteristic C_{27} – C_{31} alkadienes and alkatrienes of the race A strains were identified. Minor amounts of atypical C_{27} – C_{31} alkatetraenes, C_{33} alkadienes, and alkatrienes were also observed. The latter were probably two carbon elongation products of the respective C_{31} alkenes. In contrast to the C_{33} alkadienes and alkatrienes, the corresponding alkatetraenes have possibly not been identified in race A strains due to the low levels present. The Race B strains (BBUL, LCRW, and LHD) dominated by botryococenes and methylated squalenes (C_{30} – C_{37} isoprenoids) were dominated by C_{34} compounds. Other *B. braunii* Race L with the characteristic C_{40} lycopadiene—a single tetraterpenoid hydrocarbon [1] and Race S that produce short-chained epoxy-*n*-alkanes and saturated C_{18} – C_{20} *n*-alkanes [9]—were not identified in this study.

The salinity had a minimal effect on lipid composition, with only strains LHD and WML showing a different hydrocarbon profile in comparison with the other strains. This could be explained by the culture ages as suggested by Okada et al. that botryococenes accumulate in the later stage of the culture growth, and the synthesis of botryococenes is shifted from the original C_{30} botryococcene toward the higher molecular weight C_{31} – C_{34} botryococenes [23]. As a result, C_{30} botryococcene is usually a minor component in the population of accumulated botryococenes in the later stages of a culture cycle. While the biovolume of LHD strains was generally larger than those in the freshwater medium, the cell growth stages in both treatments might not be the same with culture age. We would assume that a longer lag phase occurs in the brackish medium, and therefore, the LHD cells would be in late-stationary phase growth in the freshwater medium, and hence, the hydrocarbon profiles is dominated by C_{34} and C_{36} compounds at that culture age. The hydrocarbon and carotenoid pigment accumulation can be directly related to the cell growth where energy is diverted to a secondary metabolite production instead of reproduction, and they are due to the stress conditions that the strains are experiencing. Factors and conditions that influence the growth and biomass production of *B. braunii* will need to be further optimised. The potential of growing *B. braunii* in a brackish environment that utilises non-agricultural products is of great importance in the field of biofuel. The slow growth *B. braunii* in a brackish environment could make it a candidate for hydrocarbon-rich oils milking, where a large biomass is maintained at the stationary phase in brackish ponds, and only the lipids are removed on a semi-continuous basis, leaving the living cells to continue lipid production and become processed to biofuels. Future studies should also focus on the bioactivity of the *B. braunii* derived compounds such as exopolysaccharides and carotenoid pigments, which can be used as nutraceuticals in food and show other biotechnological potential.

5. Conclusions

The environmental impacts of fossil fuels have driven the rising interest of algae-derived biofuel for the sustainability bioenergy production. The slow growth and the difficulty in establishing healthy

cultures of *Botryococcus braunii*, as well as national biosecurity restrictions, have contributed to the limited data available for endemic Australian strains. The availability of endemic *B. braunii* strains from culture collections and obtaining key information for the optimisation of growth conditions will be required for any future commercial production of this sought-after group, including for biotechnology and bioactivity applications. This study is part of an effort to characterise six new endemic Australian *B. braunii* strains obtained from different locations and to investigate the effects of salinity on their growth, hydrocarbon, and pigment compositions. Some strains were more tolerable of brackish conditions than others; however, there are no recognisable links between salinity tolerance and original location, with maximum growth rates of $<0.23 \text{ div day}^{-1}$ observed, which were similar in both fresh and saline media. Salinity had a minimal effect on lipid composition, with only two of the six strains showing a different hydrocarbon profile in comparison with the other strains. While pigment composition showed only minor variations between fresh and brackish cultures, the concentrations varied significantly, with the freshwater cultures showing higher levels of pigment concentrations in comparison to the brackish cultures. Chl *a* concentration correlated well with the growth of the cultures, with higher values corresponding to enhanced culture growth. Chl *a* concentration varied significantly, with differences seen in LCRW, LHD, LCM, and WML strains. While pigment composition showed only minor variations between freshwater and brackish cultures, the composition was typical of green algae, with chlorophyll *b* and lutein the dominant pigments.

To achieve commercial viability, the yield of biomass and resultant hydrocarbon and carotenoid pigment would need to be much higher than was found in this preliminary study. Increasing the growth rate and biomass yield has occurred through culture manipulation for omega-3 oil containing heterotrophic microalgae and also for tailoring their fatty acid composition. In the case of *B. braunii*, culture conditions such as salinity could be similarly manipulated and optimised for carotenoid pigment production. The co-production of high-value bioproducts such as pigments during hydrocarbon production is also desirable, as it potentially adds greater value to the production process and improves process economics. This study also highlights the potential of using carotenoid pigment and hydrocarbon profiles obtained for six new endemic Australian *B. braunii* strains as biochemical makers for potential use in environmental research. Future research is also required on the potential production of exopolysaccharides and other bioactive compounds from these new Australian *B. braunii* strains to determine their suitability for novel applications and possible commercialisation.

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References

1. Banerjee, A.; Sharma, R.; Chisti, Y.; Banerjee, U. *Botryococcus braunii*: A renewable source of hydrocarbons and other chemicals. *Crit. Rev. Biotechnol.* **2002**, *22*, 245–279. [[CrossRef](#)] [[PubMed](#)]
2. Metzger, P.; Casadevall, E.; Pouet, M.; Pouet, Y. Structures of some botryococcenes: Branched hydrocarbons from the B-race of the green alga *Botryococcus braunii*. *Phytochemistry* **1985**, *24*, 2995–3002. [[CrossRef](#)]
3. Metzger, P.; Largeau, C. *Botryococcus braunii*: A rich source for hydrocarbons and related ether lipids. *Appl. Microbiol. Biotechnol.* **2005**, *66*, 486–496. [[CrossRef](#)] [[PubMed](#)]
4. Borowitzka, M.A. Microalgae in medicine and human health: A historical perspective. In *Microalgae in Health and Disease Prevention*; Elsevier: Amsterdam, The Netherlands, 2018; pp. 195–210.

5. Yamamoto, S.; Mandokoro, Y.; Nagano, S.; Nagakubo, M.; Atsumi, K.; Watanabe, M.M. Catalytic conversion of *Botryococcus braunii* oil to diesel fuel under mild reaction conditions. *J. Appl. Phycol.* **2014**, *26*, 55–64. [[CrossRef](#)]
6. Demura, M.; Ioki, M.; Kawachi, M.; Nakajima, N.; Watanabe, M.M. Desiccation tolerance of *Botryococcus braunii* (Trebouxiophyceae, Chlorophyta) and extreme temperature tolerance of dehydrated cells. *J. Appl. Phycol.* **2014**, *26*, 49–53. [[CrossRef](#)] [[PubMed](#)]
7. Senousy, H.H.; Beakes, G.W.; Hack, E. Phylogenetic placement of *botryococcus braunii* (trebouxiophyceae) and *botryococcus sudeticus* isolate utex 2629 (chlorophyceae) 1. *J. Phycol.* **2004**, *40*, 412–423. [[CrossRef](#)]
8. Kawachi, M.; Tanoi, T.; Demura, M.; Kaya, K.; Watanabe, M.M. Relationship between hydrocarbons and molecular phylogeny of *Botryococcus braunii*. *Algal Res.* **2012**, *1*, 114–119. [[CrossRef](#)]
9. Moheimani, N.R.; Cord-Ruwisch, R.; Raes, E.; Borowitzka, M.A. Non-destructive oil extraction from *Botryococcus braunii* (Chlorophyta). *J. Appl. Phycol.* **2013**, *25*, 1653–1661. [[CrossRef](#)]
10. Cheng, P.; Okada, S.; Zhou, C.; Chen, P.; Huo, S.; Li, K.; Addy, M.; Yan, X.; Ruan, R.R. High-value chemicals from *Botryococcus braunii* and their current applications—A review. *Bioresour. Technol.* **2019**, *291*, 121911. [[CrossRef](#)]
11. Furuhashi, K.; Saga, K.; Okada, S.; Imou, K. Seawater-cultured *Botryococcus braunii* for efficient hydrocarbon extraction. *PLoS ONE* **2013**, *8*, e66483. [[CrossRef](#)]
12. Rao, A.R.; Dayananda, C.; Sarada, R.; Shamala, T.; Ravishankar, G. Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents. *Bioresour. Technol.* **2007**, *98*, 560–564. [[CrossRef](#)] [[PubMed](#)]
13. Hooker, S.B.; VanHeukelem, L.; Thomas, C.S.; Claustre, H.; Ras, J.; Schluter, L.; Clementson, L.; vanderLinde, D.; Eker-Develi, E.; Berthon, J.-F. *The Third Seawifs HPLC Analysis Round-Robin Experiment (Seaharre-3)*; Goddard Space Flight Center: Greenbelt, MD, USA, 2009.
14. De Mesmay, R.; Metzger, P.; Grossi, V.; Derenne, S. Mono- and dicyclic unsaturated triterpenoid hydrocarbons in sediments from Lake Masoko (Tanzania) widely extend the botryococcene family. *Org. Geochem.* **2008**, *39*, 879–893. [[CrossRef](#)]
15. Gao, M.; Simoneit, B.R.; Gantar, M.; Jaffé, R. Occurrence and distribution of novel botryococcene hydrocarbons in freshwater wetlands of the Florida Everglades. *Chemosphere* **2007**, *70*, 224–236. [[CrossRef](#)] [[PubMed](#)]
16. Dayananda, C.; Sarada, R.; Kumar, V.; Ravishankar, G.A. Isolation and characterization of hydrocarbon producing green alga *Botryococcus braunii* from Indian freshwater bodies. *Electron. J. Biotechnol.* **2007**, *10*, 78–91. [[CrossRef](#)]
17. Grung, M.; Metzger, P. Algal carotenoids 53; secondary carotenoids of algae 4; secondary carotenoids in the green alga *Botryococcus braunii*, race L, new strain. *Biochem. Syst. Ecol.* **1994**, *22*, 25–29. [[CrossRef](#)]
18. Muntean, E.; Muntean, N.; Dragoş, N.; Bercea, V. Carotenoids as biomarkers in *Botryococcus braunii* algae. *Res. J. Agric. Sci.* **2008**, *40*, 49–54.
19. Tonegawa, I.; Okada, S.; Murakami, M.; Yamaguchi, K. Pigment composition of the green microalga *Botryococcus braunii* Kawaguchi-1. *Fish. Sci.* **1998**, *64*, 305–308. [[CrossRef](#)]
20. Van Den Berg, T.E.; Chukhutsina, V.U.; Van Amerongen, H.; Croce, R.; Van Oort, B. Light acclimation of the colonial green alga *Botryococcus braunii* strain Showa. *Plant Physiol.* **2019**, *179*, 1132–1143. [[CrossRef](#)]
21. Ambati, R.R.; Ravi, S.; Aswathanarayana, R.G. Enhancement of carotenoids in green alga-*Botryococcus braunii* in various autotrophic media under stress conditions. *Int. J. Biomed. Pharm. Sci.* **2010**, *4*, 87–92.
22. Matsuura, H.; Watanabe, M.M.; Kaya, K. Echinenone production of a dark red-coloured strain of *Botryococcus braunii*. *J. Appl. Phycol.* **2012**, *24*, 973–977. [[CrossRef](#)]
23. Okada, S.; Devarenne, T.P.; Murakami, M.; Abe, H.; Chappell, J. Characterization of botryococcene synthase enzyme activity, a squalene synthase-like activity from the green microalga *Botryococcus braunii*, Race B. *Arch. Biochem. Biophys.* **2004**, *422*, 110–118. [[CrossRef](#)] [[PubMed](#)]

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