

The background of the slide is a dark, almost black, space filled with numerous clusters of glowing spheres. The spheres are primarily green and blue, with some appearing as larger, more prominent clusters and others as smaller, scattered dots. Interspersed among these are several small, bright orange or red spheres. The overall effect is that of a microscopic or molecular scene, possibly representing the biological or chemical processes mentioned in the title. The text is contained within two white, rounded rectangular boxes with a slight green gradient on their borders.

*Botryococcus braunii* for the production of  
hydrocarbons and exopolysaccharides  
and the role of associated bacteria

João Diogo Guimarães Gouveia



*Botryococcus braunii* for the  
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*Botryococcus braunii* for the production of  
hydrocarbons and exopolysaccharides and the role  
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João Diogo Guimarães Gouveia

Thesis

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in the presence of the

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Dedicated to Francisco Gouveia (Grandad)

*A vida é de quem luta!!!*

\*\*\*\*\*

A path that is lighted  
Shall not hold for eternity  
As solid as it can be  
Its foundations have wings

A path that is dark  
Is a bless to the explorer  
With small steps I can sense and see  
The structure that supports even the tiniest bee

A true heart searches in darkness  
With that direction for intention  
Knowing that such is the path  
The darkness ahead turns into light behind me

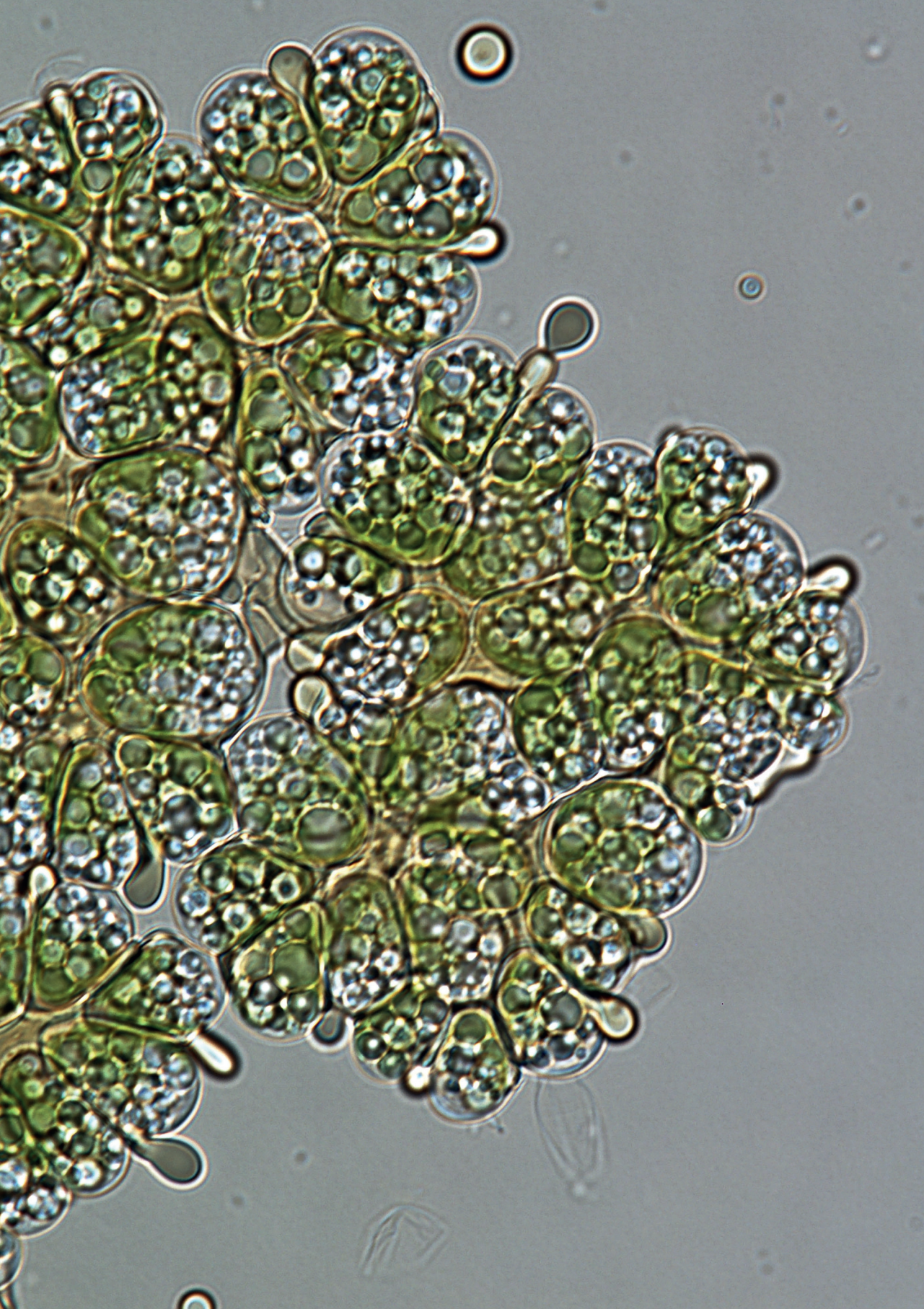
João Gouveia (a moment in Time)





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# Introduction and thesis outline

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## Chapter 1

## 1 Introduction

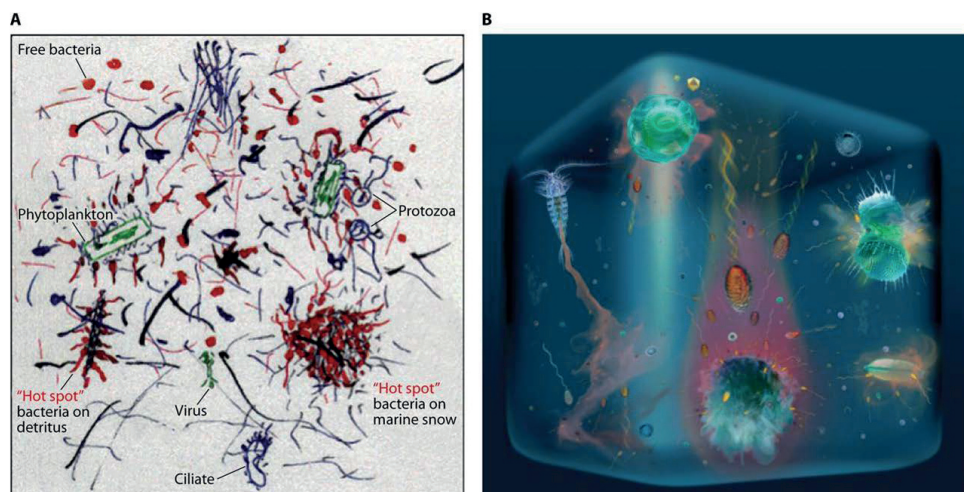
Petroleum has been an abundant resource in the last century and the main source of energy and polymers. However, this will change in the future because petroleum reservoirs are finite and it contributes to global warming (Dale 2007). New production platforms of biopolymers from sustainable sources are needed. A new promising alternative are microalgae. Microalgae are microscopic photosynthetic organisms that can be found all around the world in most aquatic environments. Microalgae obtain their energy from photosynthesis by absorbing radiation from the sun and converting CO<sub>2</sub> and water into sugar molecules and further into biomass. Microalgae are an attractive alternative over other agricultural crops because they do not need arable land for cultivation and they can grow in different aquatic systems such as marine and waste water (Wijffels and Barbosa 2010).

### 1.1 Microalgae and bacteria

Microalgae secrete extracellular substances such as simple amino acids, proteins and sugars around the algal cells or colonies which form the phycosphere (Bell and Mitchell 1972). The region of the phycosphere permit bacteria to grow in close proximity with the microalgae and facilitate interactions that can be beneficial or antagonistic in nature (Cole 1982). Usually one finds microalgae and bacteria as part of a larger ecosystem containing other elements such as exudates from other organisms and cell detritus. These larger ecosystems are termed “hot spots” where bacteria can thrive and many physicochemical process take place such as carbon, nitrogen and sulphur cycling (Azam 1998, Stocker and Seymour 2012). Fig 1.1 illustrates very well the complexity of interactions that occur in the marine environments, which also include the presence of microalgae (also known as phytoplankton).

Bacteria have been shown to be essential for growth of some microalgae via the production of Vitamin B<sub>12</sub> (cobalamin). Croft et al (2005) shows that in the absence of bacteria, *Porphyridium purpureum* is not able to grow, and if a bacterial species which can produce Vitamin B<sub>12</sub> is added, *Porphyridium purpureum* resumes growth. In the natural environment, microalgae usually associate with specific bacteria which provide advantages such as nutrient exchange (Eigemann *et al.* 2013, Ramanan *et al.* 2015). In other associations bacteria can contribute to cell division in diatoms and provide long term stability of these cultures (Windler *et al.* 2014). In laboratory conditions, a *Rhizobium* sp. bacterium was shown to promote growth of several microalgae including *Chlamydomonas reinhardtii* and *Chlorella*

*vulgaris* (*C. vulgaris*) although the mechanisms were not elucidated (Kim *et al.* 2014). A study suggests that *Rhizobium* spp. could promote health of microalgae by nitrogen fixation but only in the absence of other type of nitrogen sources in the medium (Hernandez *et al.* 2009).



**Fig. 1.1 Representation of the aquatic microcosms.** A) Illustration of the possible microbial community structures in the marine environment. Reprinted from reference Azam (1998) with permission from AAAS. B) Illustration of organic substances diffusion from many microbial organisms including microalgae exudes and lysis. *Science* Vol. 327, no.5966, 5 February 2010. Reprinted from reference by Roman Stocker, Justin Seymour and Glynn Gorick (2010) with permission from AAAS. Image A and B Reprinted from reference Stocker and Seymour (2012) with permission AAAS.

For large scale cultivation microalgae-bacteria associations can also be beneficial such as inducing higher degrees of flocculation because it allows for harvesting of biomass in a more cost effective process. One study at laboratory scale compared the flocculation activity between axenic and non-axenic cultures of *C. vulgaris*. It was found that the non-axenic *C. vulgaris* cultures had a flocculation activity that reached 92 % of total cell numbers compared to the axenic culture which only reached 2 % flocculation activity of total cell number (Lee *et al.* 2013). Although bacteria can be important and beneficial to microalgae, they can also be antagonistic (Wang *et al.* 2010, Liu *et al.* 2012, van Tol *et al.* 2017). One study showed bacteria can compete for essential nutrients in the presence of excess organic carbon resulting in reduced chlorophyll concentrations (Liu *et al.* 2012). Another study showed that the flavobacterium *Croceibacter atlanticus* inhibited cell division of the diatom *Thalassiosira*

*pseudonana* (van Tol *et al.* 2017). In other cases, some bacteria can release proteases causing the cells to lyse (Wang *et al.* 2010). It is clear from all the above examples that it is important to understand the microalgae and bacteria interactions in order to optimize cultivation of microalgae both for upstream and downstream processes.

## 1.2 *Botryococcus braunii*

*Botryococcus braunii* or *B. braunii* (Chlorophyta), shown in Fig. 1.2, is an interesting microalga because it can produce long chain hydrocarbons and extracellular carbohydrates (termed here exopolysaccharides) depending on the strain used (Wolf 1983, Fernandes *et al.* 1989, Metzger and Largeau 2005, Gouveia *et al.* 2017). *B. braunii* produces different types of hydrocarbons and for this reason it has been subclassified into four different chemical races, designated A, B, L and S (Metzger and Largeau 2005, Kawachi *et al.* 2012). Race A strains produce odd-numbered alkadienes and trienes ( $C_{25}$  to  $C_{31}$ ) (Metzger *et al.* 1986, Hilton *et al.* 1988, Metzger *et al.* 1989, Dayananda *et al.* 2007, Eroglu and Melis 2010), race B strains produce a class of isoprenoid derived compounds termed botryococcenes ( $C_{30}$  to  $C_{37}$ ) and methylated squalenes ( $C_{31}$  to  $C_{34}$ ) (Metzger and Casadevall 1983, Metzger *et al.* 1985b, Metzger *et al.* 1987, Nanamura 1988), race L strains synthesize lycopadiene ( $C_{40}$ ) and race S strains synthesize  $C_{18}$  epoxy-*n*-alkanes and  $C_{20}$  saturated *n*-alkanes (Metzger and Casadevall 1987).



**Fig. 1.2** *Botryococcus braunii* light microscope images. On the left *B. braunii* showing the typical colony morphology. On the right the hydrocarbon matrix (white arrow) in which the colony is embedded.

Some strains of *B. braunii* belonging to race A, can also produce large amounts of exopolysaccharides (EPS) which was first observed by an increase of viscosity in the medium (Casadevall *et al.* 1985). So far the largest amount of EPS produced by a *B. braunii* strain is reported as 4.0-4.5 g L<sup>-1</sup> (Fernandes *et al.* 1989). One drawback of *B. braunii* is the slow growth with productivities ranging between 0.1 and 0.2 g L<sup>-1</sup> d<sup>-1</sup> (Eroglu *et al.* 2011, Cabanelas *et al.* 2015) where other green microalgae such as *Chlorella* sp. can achieve 0.5 g L<sup>-1</sup> d<sup>-1</sup> (Hempel *et al.* 2012).

### 1.3 *Botryococcus braunii* and bacteria

Several studies have shown that *B. braunii* harbours several bacterial species. Earlier investigations report the presence of *Pseudomonas* sp. and *Flavobacterium* sp. among other bacteria (Chirac *et al.* 1985). Chirac and colleagues concluded that the presence of certain bacteria can affect the biomass productivity of *B. braunii* and could affect hydrocarbon production. Later investigations using 16S rRNA gene sequencing identified *Pseudomonas* sp. and *Rhizobium* sp. with the strain *B. braunii* UTEX and a significant increase in growth was observed when *Rhizobium* sp. was isolated and added to *B. braunii* UTEX culture (Rivas *et al.* 2010). Two recent studies using the strain *B. braunii* Ba10 showed the presence of *Hyphomonadaceae* spp. and they were proposed as a growth promoting bacteria (Tanabe *et al.* 2015). Few studies have looked at the role of bacteria on the EPS producing strains (Fernandes *et al.* 1989, Lupi *et al.* 1991) but no conclusions were drawn from these studies. So far all the above *B. braunii* and bacterial studies have focused on a few strains which makes it difficult to determine 1) if *B. braunii* needs bacteria to survive and 2) if bacteria have a synergistic or antagonistic effect on the EPS produced by *B. braunii*. To have a deeper understanding of the bacterial community that could be important for the growth of *B. braunii* and EPS accumulation, a descriptive community analysis is first needed in a larger sample of strains. In this thesis we looked at 16 *B. braunii* strains available from culture collections that range in type of hydrocarbons produced and EPS accumulation. Through identification of bacteria species, is then possible to infer or hypothesise their possible role. By applying changes in the bacterial community using antibiotics or Ultraviolet light treatment, we deduced which bacteria could be beneficial and which bacteria could be antagonistic through their presence. The investigation can lead to improved strategies for cultivation of *B. braunii* strains which can enable higher biomass productivities, hydrocarbon and EPS accumulation.

#### **1.4 Use of antibiotics to study microalgae and bacteria interactions**

Antibiotics are small molecules made by microbes that antagonize other microbes (Clardy *et al.* 2009). So in other words, antibiotics produced by microbes provides them with competitive environmental advantages. Antibiotics are also used by microbes as signalling molecules when present in low concentration, contributing to the maintenance of the microbial communities in the environment (Yim *et al.* 2007). Antibiotics can either be bactericidal, which will kill bacteria, or can be bacteriostatic, which will suppress growth. The mode of action of the antibiotic fall mainly in three categories: 1) inhibition of DNA replication; 2) inhibition of protein synthesis; 3) inhibition of cell wall turnover (Kohanski *et al.* 2007). Antibiotics have been used successfully in removing bacteria from microalgae cultures either by single use (one antibiotic) or in concoction (mix of two or more antibiotic) cocktails (Joo and Lee 2007, Han *et al.* 2016, Molina-Cardenas *et al.* 2016). In this thesis a similar approach was used primarily to change the bacterial community present with *B. braunii* to investigate the effects of bacterial community changes on the growth and EPS accumulation. And secondly, we tried simultaneously to obtain or create an axenic strain which would open new possibilities to study this microalgae and bacteria interactions.

#### **1.5 UV treatment to remove bacteria**

Ultraviolet (UV) irradiance is electromagnetic irradiance which falls between 100-400 nm. Further subdivisions of UV are used regarding the wavelength range. UV is a preferred method of sterilization for bacteria and viruses because of its mode of action being non-thermal and non-adulterating (Yen *et al.* 2014). It is widely used in pharmaceutical industry and water purification systems (Abshire 1988, Schmidt and Kauling 2007). UVC (200-280 nm) is the UV range used to inactivate microorganisms by damaging the genetic material inside the nucleus (Dai *et al.* 2012). UVC is widely used in medical interventions because it primarily effects prokaryotes while keeping eukaryotic cells viable. In this thesis, UVC treatment was applied for removing the bacteria from *B. braunii* CCALA778 strain and physiological responses were investigated.

#### **1.6 16S rRNA gene profiling for studying bacterial communities**

16S rRNA gene phylogenetic analysis has become an important tool to study bacteria and archaea microorganism evolution. The 16S rRNA gene is highly conserved as it is vital for cell function. The 16S rRNA gene length is in the range of 1600 base pairs with both conserved and variable regions, which provide the highly conserved attributes and at the same



time the differences that make it possible to distinguish different species and carry out phylogenetic analysis (Clarridge 2004). To process the sequence data, special pipelines are needed. Recently the NG-Tax pipeline has been developed that benchmarks against other highly used pipelines such as Qiime (Ramiro-Garcia *et al.* 2016). In this thesis the NG-Tax pipeline (Fig. 1.3) was used to treat the vast data collected before conducting bacterial community analysis.

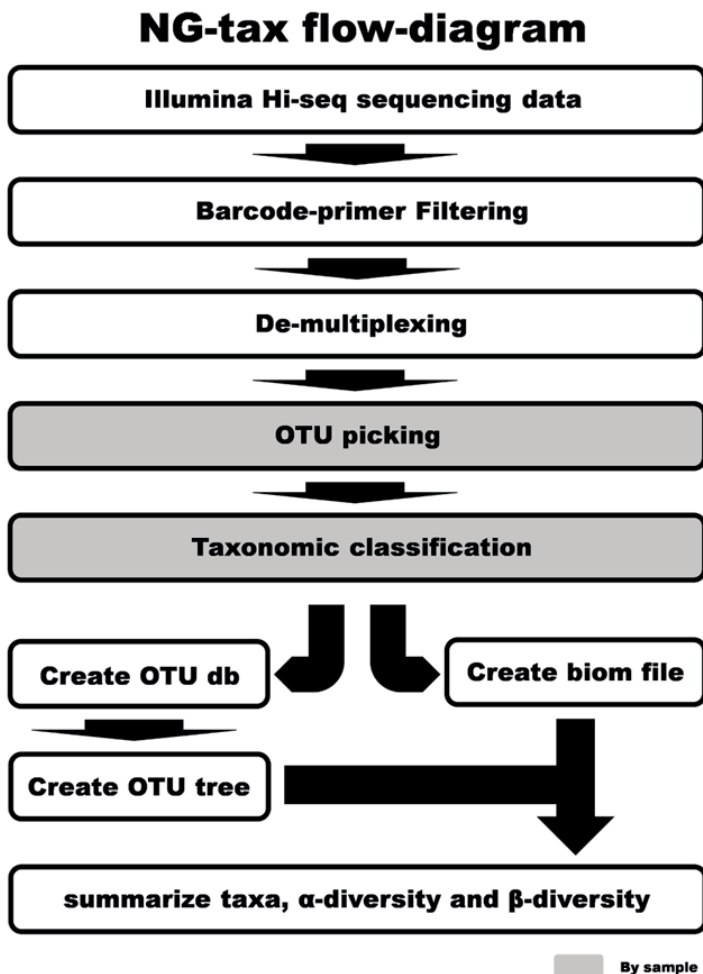


Fig. 1.3 NG-Tax flow-diagram. From reference Ramiro-Garcia et al (2016)

## 1.7 Thesis outline

The aim of this thesis is to primarily determine if *B. braunii* needs bacteria to survive and secondly to determine if bacteria have an effect on the biomass growth and EPS accumulation in CCALA778 strain which is known to produce high amounts of EPS. In the first two chapters, the investigation was aimed at comparing several strains of *B. braunii* for biomass productivity and hydrocarbon and total carbohydrate (intracellular and extracellular carbohydrate) content, followed by an investigation on the diversity of the associated bacterial community. In the following chapters, *B. braunii* CCALA778 strain with a known high EPS accumulation, was used to investigate the effects of bacteria on the production of EPS by changing the bacterial community via the use of antibiotics and treatment of CCALA778 culture with UVC.

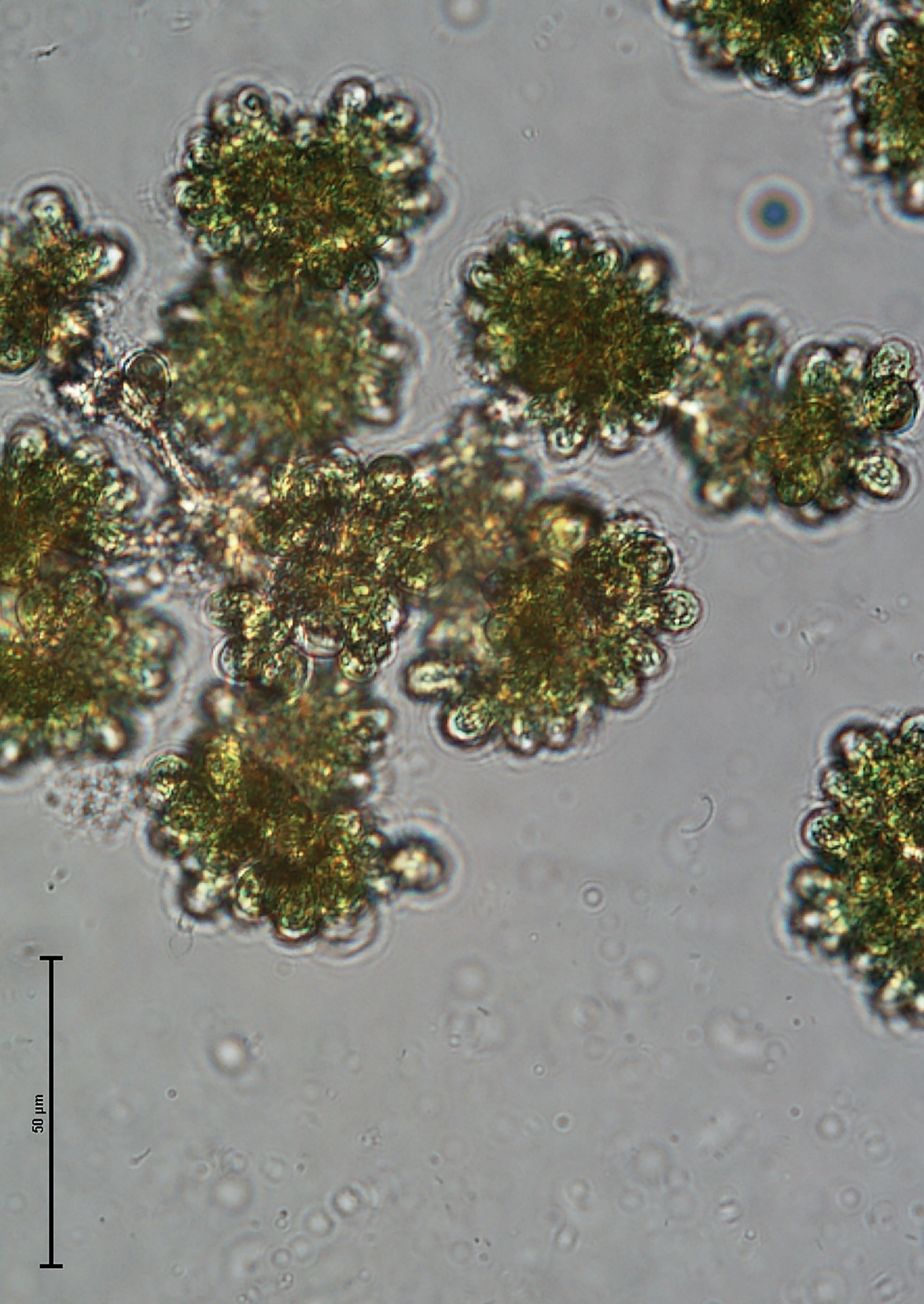
Chapter two: 16 strains of *B. braunii* from several culture collections were compared for biomass productivity and hydrocarbon and total carbohydrate content. The aim of this study was to find strains that have the highest biomass productivity and the highest hydrocarbon and total carbohydrate content. Conclusions are drawn in possible applications of *B. braunii* strains for both the hydrocarbon and EPS producers. From the 16 strains, CCALA778 is regarded as a good candidate for the production of EPS as well as to investigate the role of bacteria on biomass growth and EPS accumulation. AC761 is regarded as a good candidate for the production of botryococenes.

Chapter three: The bacterial community of 12 strains of *B. braunii* screened in chapter two was analysed. The aim of this study was to find possible symbionts and search for common or shared bacterial species by analysing the bacterial community. The possible role of the most abundant families and bacterial species identified are described and conclusions are drawn regarding correlations between specific bacteria communities and the different races of *B. braunii*.

Chapter four: Antibiotics were used to change the bacterial community of CCALA778 strain identified from chapter two as a high EPS producer. The aim was to study the effects of different bacterial community composition on the biomass productivity and EPS accumulation. Conclusions are drawn regarding the suitability of specific antibiotics to investigate *B. braunii* and bacteria interactions and the first effects of different bacterial community in EPS accumulation in CCALA778 were observed.

Chapter five: Strain CCALA778 was exposed to UVC which led to a reduction of the bacterial diversity as well as abundance. By comparing the biomass productivity and EPS accumulation and composition between the UVC treated and the untreated culture the role of bacteria found with *B. braunii* could be strongly inferred.

Chapter six: General discussion on the results from previous chapters. The role of bacteria with *B. braunii* is considered and conclusions are drawn in whether it needs bacteria for growth and EPS accumulation. The ability to cultivate *B. braunii* and other EPS producing microalgae in large scale reactors is considered. The importance of having axenic cultures for the study of bacterial interactions are addressed.



50 µm

# *Botryococcus braunii* strains compared for biomass productivity, hydrocarbon and carbohydrate content

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## Chapter 2

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**Abstract**

*Botryococcus braunii* can produce both long-chain hydrocarbons as well as carbohydrates in large quantities, and is therefore a promising industrial organism for the production of biopolymer building blocks. Many studies describe the use of different strains of *Botryococcus braunii* but differences in handling and cultivation conditions make the comparison between strains difficult. In this study, 16 *B. braunii* strains obtained from six culture collections were compared for their biomass productivity and hydrocarbon and carbohydrate content. Biomass productivity was highest for AC768 strain with  $1.8 \text{ g L}^{-1} \text{ day}^{-1}$ , while hydrocarbon production ranged from none to up to 42 % per gram biomass dry weight, with Showa showing the highest hydrocarbon content followed by AC761. The total carbohydrate content varied from 20 % to 76 % per gram of the biomass dry weight, with CCALA777 as the highest producer. Glucose and galactose are the main monosaccharides in most strains and fucose content reached  $463 \text{ mg L}^{-1}$  in CCALA778.

**Keywords:** *Botryococcus braunii*, hydrocarbons, carbohydrate, galactose, fucose, microalgae

## 2 Introduction

Human activities greatly depend on petroleum as both an energy source and industrial raw material (Dale 2007). Petroleum usage in the long term is both unsustainable due to depleting economically relevant sources and by rapid release of carbon dioxide in the environment. One potential source of biofuels and other biobased raw material compounds are microalgae. These photoautotrophic organisms are able to transform inorganic carbon into lipids such as triacylglycerols (TAGs) at a faster rate than agricultural oleaginous crops, and do not compete for arable land (Wijffels and Barbosa 2010). Besides lipids, other products of interest that microalgae may produce in large quantities are hydrocarbons and carbohydrate. Hydrocarbons are natural occurring compounds consisting entirely of hydrogen and carbon, and are one of the most important energy resources (Timmis and Qin 2010). Hydrocarbons derived from microalgae can be hydrocracked and transformed into aviation turbine fuel (Hillen *et al.* 1982). Carbohydrate have a range of industrial uses, including as thickeners, stabilisers and gelling agents in food products (Donot *et al.* 2012), as well as in the pharmaceutical and cosmeceutical industries (Buono *et al.* 2012, Borowitzka 2013).

One promising production host of biofuels and biobased materials is *Botryococcus braunii*. This eukaryotic microalga specie can be found across the world as a variety of strains with different physiological characteristics. Some strains of *B. braunii* can produce to up to 86 % hydrocarbons on cell dry weight basis (Brown *et al.* 1969), whereas other strains can produce to up to 4.5 g L<sup>-1</sup> carbohydrates into the medium (Fernandes *et al.* 1989). One advantage of *B. braunii* is that it secretes extracellular hydrocarbons and carbohydrates (Wolf 1983, Lupi *et al.* 1994, Volova *et al.* 1998, Kalacheva *et al.* 2002, Weiss *et al.* 2012) which allows the development of strategies for *in situ* extraction such as “milking” (Moheimani *et al.* 2013).

*B. braunii* can produce hydrocarbons with different chemical structures. These hydrocarbons play a role in the natural growth cycle of *B. braunii* (Khatri *et al.* 2014). Depending on what type of hydrocarbons are produced, *B. braunii* is subclassified into four chemical races, designated A, B and L (Metzger and Largeau 2005), and S, a recent assignment (Kawachi *et al.* 2012). Race A strains synthesize odd-numbered alkadienes and trienes (C<sub>25</sub> to C<sub>31</sub>) (Metzger *et al.* 1986, Hilton *et al.* 1988, Metzger *et al.* 1989, Dayananda *et al.* 2007, Eroglu and Melis 2010), race B strains synthesize a class of isoprenoid derived compounds termed botryococcenes (C<sub>30</sub> to C<sub>37</sub>) and methylated squalenes (C<sub>31</sub> to C<sub>34</sub>) (Metzger and Casadevall 1983, Metzger *et al.* 1985b, Metzger *et al.* 1987, Nanamura 1988), race L strains synthesize

lycopadiene (C<sub>40</sub>) and race S strains synthesize C<sub>18</sub> epoxy-*n*-alkanes and C<sub>20</sub> saturated *n*-alkanes (Metzger and Casadevall 1987).

In addition to hydrocarbons, *B. braunii* strains can produce large amounts of carbohydrates with the highest amounts so far reported as 4.0-4.5 g L<sup>-1</sup> (Fernandes *et al.* 1989). Extensive carbohydrate production was first observed by an increase of broth viscosity during growth (Casadevall *et al.* 1985). Since this initial report, other strains were found to produce carbohydrates with yields of 250 mg L<sup>-1</sup> for race A and B strains, and 1 g L<sup>-1</sup> for a race L strain (Allard and Casadevall 1990). Later, 1.6 g L<sup>-1</sup> for *B. braunii* LB572 and 0.7 g L<sup>-1</sup> for SAG30.81 (Dayananda *et al.* 2007) were reported. Galactose was identified as the main monomeric sugar constituent of all carbohydrates examined, with fucose and rhamnose as accompanying monomers. Glucose was detected in the L strain only (Allard and Casadevall 1990).

One drawback of using *B. braunii* as an industrial host is its slow growth compared to other photoautotrophic microorganisms. *B. braunii* biomass productivities range between 0.1 and 0.2 g L<sup>-1</sup> d<sup>-1</sup> (Eroglu *et al.* 2011, Cabanelas *et al.* 2015) where other green microalgae such as *Chlorella sp.* can achieve 0.5 g L<sup>-1</sup> d<sup>-1</sup> (Hempel *et al.* 2012). One commonly reported hypothesis for the slow growth is due to the synthesis of energetically expensive hydrocarbons (Banerjee *et al.* 2002).

There is an extensive body of work in last few decades describing different strains of *B. braunii* and it is clear that there is a high degree of morphological plasticity and physiological diversity amount the genus. It is probably due to this high diversity in the genus that *B. braunii* is not an easy organism to maintain and grow under laboratory conditions. Many methods of cultivation, different types of growth medium or culture conditions have been reported in the literature to study more in depth *B. braunii* individual strains as well as for comparing strains diversity (Metzger *et al.* 1989, Allard and Casadevall 1990, Dayananda *et al.* 2007, Eroglu *et al.* 2011, Cabanelas *et al.* 2015, Hegedüs *et al.* 2016, Moutel *et al.* 2016). While this has led to several reports on hydrocarbon and carbohydrate production for various *B. braunii* strains, it can be argued that it is still difficult to compare and assess physiological characteristics. This study attempts to establish reference conditions for future investigations of this interesting photosynthetic organism alongside its production of long chain hydrocarbons and/or carbohydrates.



The aim was to compare various readily available strains of *B. braunii* under the same culture conditions for biomass productivity and total hydrocarbon and carbohydrate content. Sixteen strains were tested in Erlenmeyer flasks, of which seven promising strains were additionally investigated in bubble columns. Comparison of the sixteen strains regarding biomass productivity, carbohydrate and hydrocarbon content is reported and major differences highlighted. Two strains are put forward as candidates for industrial applications.

## 2.1 Materials and Methods

### 2.1.1 Strains and media

Sixteen non-axenic *B. braunii* strains were obtained from culture collections (Table 1). Upon arrival, each strain was washed with sterile distilled water and revegetated in modified Chu 13 medium (Largeau *et al.* 1980) without citric acid or vitamins, with the following composition: 400 mg L<sup>-1</sup> KNO<sub>3</sub>, 200 mg L<sup>-1</sup> MgSO<sub>4</sub>·2H<sub>2</sub>O, 108 mg L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 104.8 mg L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 20 mg L<sup>-1</sup> Fe-Na<sub>2</sub>EDTA, 9.4 µg L<sup>-1</sup> Na<sub>2</sub>O<sub>4</sub>Se, 2.86 mg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 1.8 mg L<sup>-1</sup> MnSO<sub>4</sub>·4H<sub>2</sub>O, 220 µg L<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O, 90 µg L<sup>-1</sup> CoSO<sub>4</sub>·7H<sub>2</sub>O, 80 µg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 60 µg L<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 10 µl L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>. Final pH was adjusted to pH 7.2 with NaOH. The 16 strains were kept in an incubation room with the initial environment parameters: light intensity of 60 µmol photon m<sup>-2</sup> sec<sup>-1</sup>, light:dark photoperiod 18:6 h, ambient air CO<sub>2</sub>, temperature of 23°C and mechanical shaking at 60 rpm. Growth conditions - Prior to the experiment, the 16 strains inoculum were cultivated for two batch cycles under experimental conditions to minimize effects of changing environmental parameters.

Shake flask cultivation: For comparison of biomass productivity, hydrocarbon and carbohydrate content, the 16 strains were grown in Infors HT Multitron incubators in 250 mL conical flasks and a volume of 150 mL medium. Temperature was set at 23°C, with 2.5 % CO<sub>2</sub> enriched air and shaking at 90 rpm. Illumination was provided by Phillips lamps FL-Tube L 36W/77, with 150 µmol photon m<sup>-2</sup> sec<sup>-1</sup>, and a light:dark photoperiod of 18:6 h (photoperiod of 18:6h chosen based on earlier work done as described in Gouveia (2010)).

**Table 2.1** Origin of *Botryococcus braunii* strains. Information regarding the individual strains and the culture collections from where they were purchased. \* strains used for growth in bubble columns photobioreactors

| Culture Collection                                            | Botryococcus braunii Strain | Race       | Location                                              | Isolation, date of Isolation | Reference                         |
|---------------------------------------------------------------|-----------------------------|------------|-------------------------------------------------------|------------------------------|-----------------------------------|
| Berkeley                                                      | Showa                       | * Race B   | culturing tanks, Berkeley                             | By unknown, 1980             | Nonomura, A., 1988                |
| Scandinavian Culture Collection of Algae and Protozoa (SCCAP) | SCCAP K-1489                | not know   | Belgium, Nieuwvoort                                   | By G. Hansen, 2008           | No reference                      |
| Culture Collection of Algae at Goettingen University (SAG)    | SAG 30.81                   | Race A     | Peru, Dpto. Cuzco, Laguna Huaypo                      | By E. Hegewald, 1977         | Danyananda C., et al, 2007        |
| Culture Collection of Autotrophic Organisms (CCALA)           | CCALA-777                   | * not know | Porto da Castanheira (Poço dos Basílios) Portugal     | By Santos, 1975              | Fernandes H. L, et al, 1989       |
|                                                               | CCALA-778                   | * not know | Serra da Estrela ( Barragem da Erva da Fome) Portugal | By Santos, 1997              | No reference                      |
|                                                               | CCALA-835                   | Race A     | Peru, Dpto. Cuzco, Laguna Huaypo                      | By E. Hegewald, 1977         | Danyananda C., et al, 2007        |
| Culture Collection of Algae and Protozoa (CCAP)               | CCAP-807-2                  | * Race A   | Grasmere, Cumbria, England                            | By Jaworski, 1984            | Hilton J. et al, 1989             |
| UTEX Culture Collection of Algae                              | UTEX Bb 572                 | Race A     | Madingley, Cambridge, England                         | By M.I.R. Droop, 1950        | Eroglu E. Mellis A., 2010         |
|                                                               | UTEX Bb LB572               | Race A     | Cambridge, England                                    | By M.I.R. Droop, 1950        | Eroglu E., et al, 2011            |
| ALGOBANK-CANE                                                 | AC755                       | * Race A   | Lingault-Morvan, France                               | By Pierre Metzger, 1981      | Metzger, P., et al, 1985          |
|                                                               | AC759                       | * Race B   | Ayame, Ivory Coast                                    | By Pierre Metzger, 1984      | Metzger, P., et al, 1988          |
|                                                               | AC760                       | Race B     | Kossou, Ivory Coast                                   | By Pierre Metzger, 1984      | Metzger, P., et al, 1988          |
|                                                               | AC761                       | * Race B   | Paquemar, Martinique, France                          | By Pierre Metzger, 1983      | Metzger, P., et al, 1985          |
|                                                               | AC765                       | Race L     | Kossou, Ivory Coast                                   | By Pierre Metzger, 1984      | Metzger, P., et al, 1988          |
|                                                               | AC767                       | Race L     | Songkla Nakarin, Thailand                             | By Pierre Metzger, 1985      | Metzger, P., Casadevall, E., 1987 |
|                                                               | AC768                       | Race L     | Yamoussoukro, Ivory Coast                             | By Pierre Metzger, 1984      | Metzger, P., Casadevall, E., 1987 |

Flasks were inoculated with algae from a 10-day old, actively growing culture, such that the initial absorbance at 680 nm was 0.2. The Erlenmeyer flasks were capped with aseptically sterile film (Alphalabs). The experiment was conducted in triplicate and samples were taken at day 0, 6, 12 and 18 after inoculation. The cultivation period of 18 days for this experiment, was chosen based on similar cultivation times found in other studies (Kojima and Zhang 1999, Eroglu and Melis 2010).

**Bubble columns cultivation:** For determining cultivation viability at larger scale of selected strains from shake flask experiment, seven strains (marked with a star in Table 1) were cultivated in batch mode in a bubble column with a working volume of 400 mL. These custom-made glass tubes (Glass Instrumentmakerij, Wageningen University) are 400 mm in height, with an internal and external diameter of 40 and 60 mm, respectively. A water bath was used to pump water through an external water jacket, keeping the temperature of the cultures at 23°C. The bubble columns were kept vertical between two large light panels (bench panels: Mazda 5L KN RB ECO 118 I, fluorescent tubes: Philips Master TL-D 18W/865) which provided 150  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$  from two sides with a light:dark cycle of 18:6 h. Air enriched with 2 %  $\text{CO}_2$  was continuously injected at the bottom of the reactor at a rate of 0.5 vvm (volume air volume reactor<sup>-1</sup> min<sup>-1</sup>, 200 mL min<sup>-1</sup>). Air flow was controlled by a mass flow controller (Brooks 0254, Brooks instruments), and filter sterilized through a 0.2  $\mu\text{m}$  air filter (Acro 50, Pall Corporation) before it passed through a holed plate in the tube to create small bubbles. The enriched air provided inorganic carbon for growth, kept the reactor mixed and stabilized pH around 7.2. To compensate for evaporation, Milli Q water was added to the aeration tubes through a 0.2  $\mu\text{m}$  filter (Minisart, Sartorius stedim). An overflow bottle equipped with 0.2  $\mu\text{m}$  air filter (Acro 50, Pall Corporation) was connected to the top of the column, to enable air to escape and possible overflow of the culture to be kept free of contamination. The reactors were inoculated at biomass concentration of 0.1-0.3 g L<sup>-1</sup>, and sterile medium was added to 400 mL. Twice a week samples were taken through a port at the side of the reactor to determine pH and dry weight.

### 2.1.2 Biomass dry weight

Five mL aliquots of culture broth were filtered onto pre-weighed GF/D glass-fibre membranes (Whatman). The GF/D filters were dried at 100°C for 24 hours and weighted, and biomass amount was determined by subtraction. From the dry weight, biomass productivity was calculated using the following equation:  $\text{Productivity} = \frac{Cx_2 - Cx_1}{t_2 - t_1}$ , where Cx1 and Cx2 represent

the biomass concentration at the beginning ( $t_1$ ) and at the end of the experiment ( $t_2$ ), respectively. An univariate analysis of variance (one-way ANOVA) was carried out to compare the biomass productivity among different strains. As post-hoc test the Tukey's test was used. All statistical analyses were done at a significance level of 0.05. The software used was Genstat 64-bit Release 18.1.

### 2.1.3 Hydrocarbon extraction

Our method is adapted from the methodology developed by Folch and Dyer (Folch *et al.* 1957, Bligh and Dyer 1959). One mL of culture was transferred to a glass vial and 2.5 mL methanol and 1.25 mL dichloromethane were added and mixed for 6 hours. After the mixing step, 1.25 mL dichloromethane was added and mixed for one minute followed by addition of 1.25 mL 0.9 % (w/v) NaCl and mixed for another minute. Hereafter, samples were centrifuged for 5 minutes at 1500 g and the bottom phase was removed to a new glass vial using a glass Pasteur pipette and dried under nitrogen gas. The residue was resuspended in 300  $\mu$ L dichloromethane:methanol (v:v) and stored at  $-20^\circ\text{C}$ . For the hydrocarbon extraction of the strains cultivated in the bubble columns, the residue was resuspended in 1000  $\mu$ L hexane. Hydrocarbon extraction was performed using the last data point samples for both the Erlenmeyer flasks and bubble column cultures.

### 2.1.4 Hydrocarbon analysis

Hydrocarbons extracted from the strains cultivated in the Erlenmeyer flasks were measured by gas chromatography combined with mass spectrometry (GC-MS). The instrument used was a 7890A/5975C from Agilent Technologies, using a DB-Petro (100 m  $\times$  0.25 mm  $\times$  0.50  $\mu$ m) J&W Column using helium as the carrier gas, splitless injector, an operation temperature of  $280^\circ\text{C}$  and an injection volume of 1  $\mu$ L. The oven program was set at  $40^\circ\text{C}$  for 0.5 minutes, then ramped up by  $30^\circ\text{C}$  per minutes to  $250^\circ\text{C}$  followed by a  $5^\circ\text{C}$  per minute ramp to  $300^\circ\text{C}$  in 37.5 minutes, with a total run time of 55 minutes. Samples were diluted in dichloromethane:methanol 1:1 (v:v) Squalene ( $\text{C}_{30}\text{H}_{50}$ ,  $M = 410 \text{ g mol}^{-1}$ ) was the reference standard used. The concentration of the calibration standards were 50, 100, 250 and 500  $\text{mg L}^{-1}$ .

Hydrocarbon analysis for the strains cultivated in the bubble columns was carried out using GC-FID. The instrument used was an Agilent Technologies HP6890 series equipped with auto sampler, a using Restek Rxi-5ms (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) column. Helium was used as the carrier gas, and an hydrogen/air mixture detection, gas splitless injectors at  $350^\circ\text{C}$  oven

temperature and injection volume of 1  $\mu\text{L}$ . The oven program was 50°C for 1 minute, then 15°C per minute to 180°C, then 7°C per minute to 230°C, then 30°C per minute to 350°C and hold for 15 minutes with a total running time of 35 minutes. Samples were diluted in hexane, and several dilutions of standards using squalene were used. The dilutions were 1, 0.1, 0.01, 0.005 and 0.001 % (v/v).

### 2.1.5 Carbohydrate extraction

10 mL of culture from the last time point taken, was freeze dried using Zirbus technology Sublimator 2x3x3 and the pellet weight was determined. A pre-hydrolysis step was performed as follows: 72 % (w/w)  $\text{H}_2\text{SO}_4$  was added to the freeze dried sample (w/v) and hydrolyzed for 1 hour at 30°C, and stirred with glass pipette every 15 minutes. After the pre-hydrolysis water was added in 11.6 v/v (final concentration 1 M  $\text{H}_2\text{SO}_4$ ) to the mixture and placed at 100° C for 3 hours and shaken every 30 minutes. Hereafter the solution was allowed to cool on ice for 15 minutes and subsequently centrifuged for 15 minutes at 3000 g. Before analysis samples were diluted in water and 2.5  $\mu\text{L mL}^{-1}$  0.1 % (w/v) bromophenol blue in ethanol was added to the samples. For neutralization the pH, solid barium carbonate powder was added until a magenta colour was seen. Subsequently the solution was filtered through a 0.45  $\mu\text{m}$  pore size PTFE filter.

### 2.1.6 Carbohydrate analysis

Monomeric sugar composition was determined by High Performance Anion Exchange Chromatography (HPAEC) using an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (2 $\times$ 250 mm) in combination with a CarboPac PA guard column (2 $\times$ 25 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex). A flow rate of 0.3  $\text{mL min}^{-1}$  was used and the column was equilibrated with 16 mM NaOH. The following gradient was used: 0–26 min, 16 mM NaOH; 26–33 min, 16–100 mM NaOH; 33–78 min 0–1 M sodium acetate in 100 mM NaOH; 78–83 min 1M sodium acetate in 100 mM NaOH. L-Rhamnose, L-fucose, D-mannose, L-arabinose, D-glucose, D-xylose, D-galactose, sucrose, D-glucuronic acid and D-galacturonic acid (Sigma Aldrich) were used as standards for identification. For the bubble column growth, the total sugar content was measured using the Dubois Method (DuBois *et al.* 1956).

### 2.1.7 18S rRNA analysis

The genomic DNA was extracted after a grinding procedure using lysing matrix E tubes (6914–500, MP Biomedicals Europe) using a DNeasy Plant Mini kit (Qiagen GmbH,

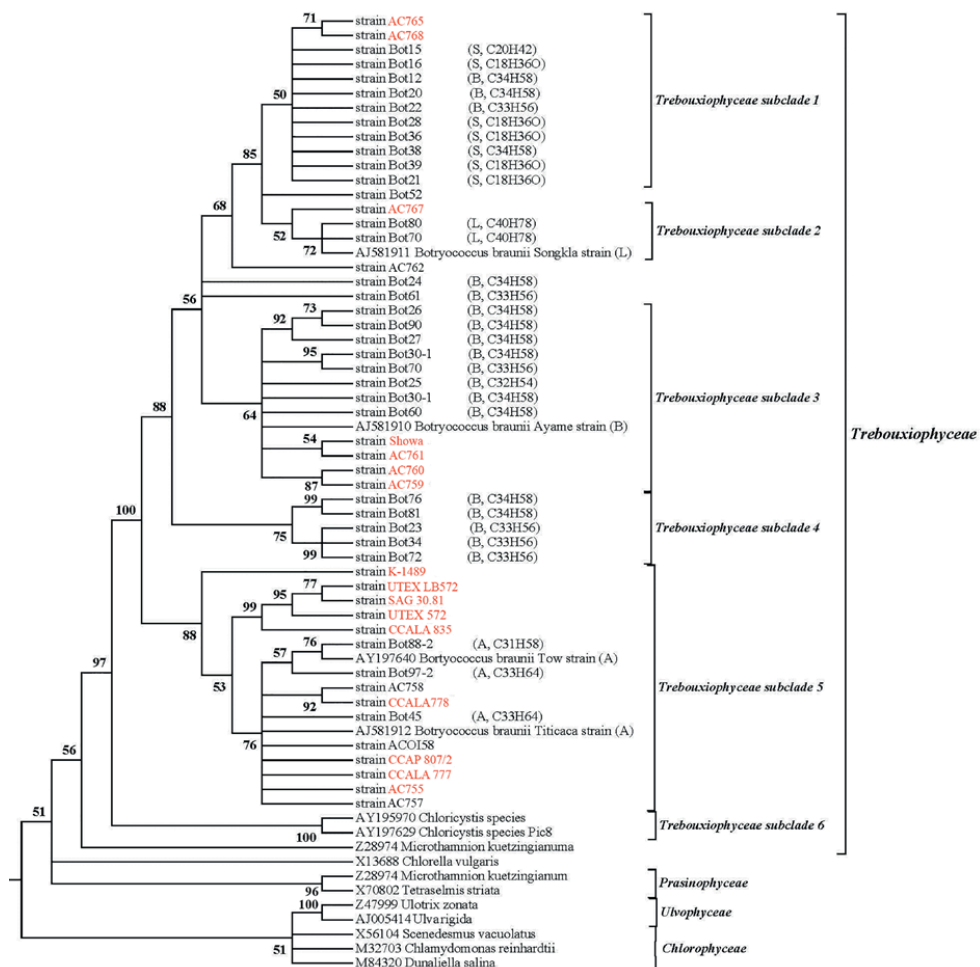
Germany) according to the specifications of the manufacturer. PCR amplification of fragments was done following Kawachi et al (Kawachi *et al.* 2012). Forward and reversed primer combination consisted of CV1 x CV2 and CV3 x CV4 primers according to Senousy *et al.* (Senousy *et al.* 2004).

After multiplication, the PCR templates were sequenced with CV1, CV2, CV3 and CV4 sequence primers. Using the PREGAP4 interface of the Staden package 2004 (Staden *et al.* 2003), raw trace data was processed into assembly ready sequences and sequences were base called by the PHRED base caller (Ewing and Green 1998). DNA sequence analysis and maximum divergence of 18S rRNA sequences were performed according to the method used by Kawachi et al (Kawachi *et al.* 2012).

## 2.2 Results

### 2.2.1 18S rRNA Analysis

To assess the relationships between our 16 culture collection strains we used 18S rRNA sequence analysis following the method that was used to characterize 31 BOT strains by Kawachi and co-workers (Kawachi *et al.* 2012), who strongly linked the *B. braunii* chemical races with their phylogenetic placement. Our strains fit well with Kawachi's results (Fig. 2.1).



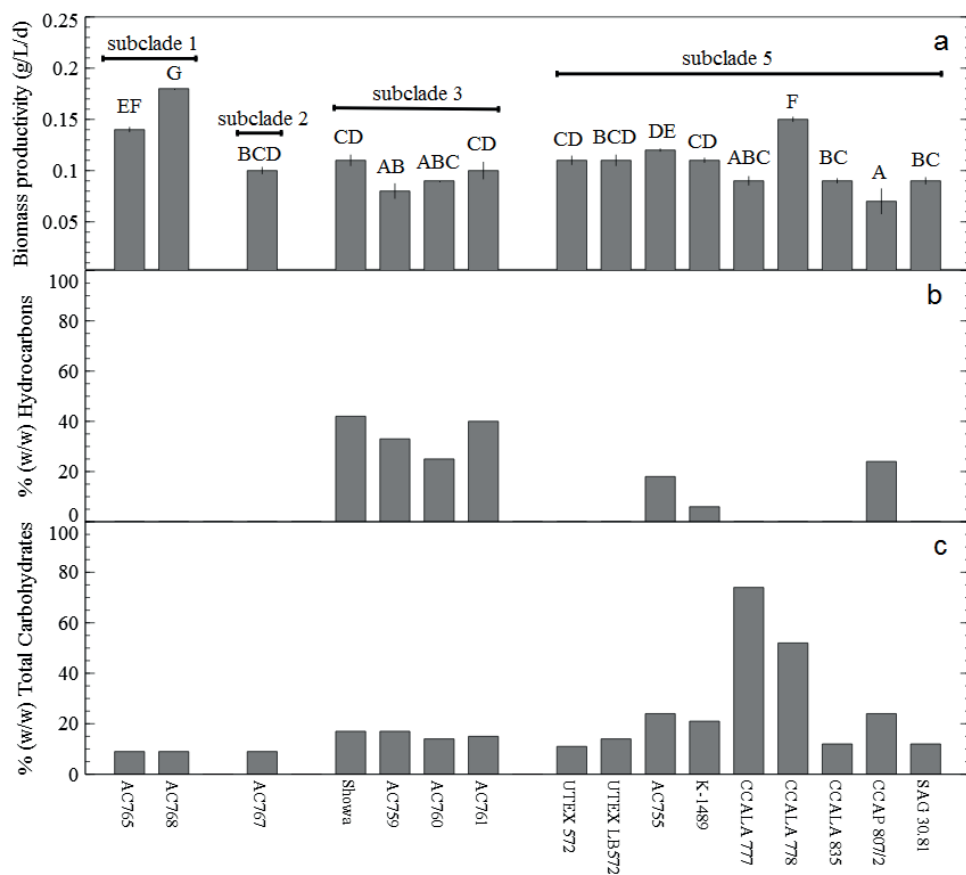
**Fig. 2.1** Phylogeny tree placement of 16 *Botryococcus braunii* strains. Strict consensus tree based with overlaid bootstrap values and consensus threshold of 50 % obtained by maximum-likelihood analysis on 18-rRNA gene sequences from 19 *Botryococcus* culture collection strains, 4 reference strains AJ581910 Ayame (B), AJ581911 Songkla (L), AJ581912 (A) and AY197640 Tow (A), 31 Bot strains from Kawachi *et al.*, 2012 and species from other chlorophytes that are used as an outgroup.

Strains UTEX 572, UTEX LB572, AC755, CCALA835, CCALA777, CCALA778, K-1489, CCAP807/2 and SAG30.81 are in subclade 5 which refers to race A. Strains AC759, AC760, AC761 and Showa are in subclade 3 which refers to race B, and strains AC765, AC767 and AC768 in subclade 1 and 2 which refers to race L. In Fig. 2.2, the assigned subclades 1, 2, 3 and 5 are used to group the strains and therefore it is also included in the Table 2.2 and Table 2.3.

### 2.2.2 Shake flask cultivation

The biomass productivity of the 16 strains was determined by assessing the total biomass increase over the cultivation period between time of inoculation and end of experiment (day 18). The biomass productivities in the shake flasks varied up to 2-fold between strains and showed statistically significant differences ( $P < 0.05$ ) in biomass productivity between strains (Fig. 2.2a). The three highest biomass producers were AC768, CCALA778 and AC765 with 0.18, 0.15 and 0.14 g L<sup>-1</sup> day<sup>-1</sup>, respectively. With 0.08 and 0.07 g L<sup>-1</sup> day<sup>-1</sup>, strains AC759 and CCAP807/2 respectively showed the lowest biomass productivity. The total hydrocarbon content in both the culture medium and biomass was determined at the end of experiment. Long-chain hydrocarbons were detected in seven strains (Fig. 2.2b). Of these, the highest hydrocarbon producing strains were Showa and AC761, which yielded a hydrocarbon percentage of around 40 % per gram biomass dry weight. Strain K-1489 was the lowest producer of the hydrocarbon producing strains, followed by AC755. The strains Showa, AC759, AC760 and AC761 produced C<sub>33</sub> to C<sub>37</sub> molecules, whereas K-1984, AC755 and CCAP807/2 produced C<sub>25</sub> to C<sub>31</sub> chain hydrocarbons (Table 2.2). No correlation was found between biomass productivity and hydrocarbon content.





**Fig. 2.2** Comparison of physiological traits for 16 *Botryococcus braunii* strains. a) biomass productivity; b) total hydrocarbon content; c) total carbohydrate content. The bars in plot A represent the standard error mean, with 3 replicates for all except AC760 and AC768 which 1 replicate was used, and AC755, AC761, AC767, CCAP807/2, SAG30.81, CCALA778, UTEX LB572 where 2 were used. Each letters above bars in graph a, represent statistical difference ( $P < 0.05$ ) by ANOVA

**Table 2.2** Hydrocarbon profile of seven strains of *Botryococcus braunii* grown in shake flasks. Strains are grouped into subclade 3 and 5 as a result of the phylogenetic placement results. Superscript letter *a* and *b* next to chemical formulas stand for different molecular structures. C? refers to unidentified compound

| Chemical formula                                    | Subclade 3 |       |       |       | Subclade 5 |       |           |
|-----------------------------------------------------|------------|-------|-------|-------|------------|-------|-----------|
|                                                     | Showa      | AC759 | AC760 | AC761 | K-1489     | AC755 | CCAP807/2 |
| <b>C<sub>23</sub>H<sub>44</sub>_320</b>             | -          | -     | -     | -     | -          | -     | 73        |
| <b>C<sub>25</sub>H<sub>48</sub>_348</b>             | -          | -     | -     | -     | -          | 48    | 93        |
| <b>C<sub>27</sub>H<sub>52</sub>_376</b>             | -          | -     | -     | -     | -          | 58    | 171       |
| <b>C<sub>29</sub>H<sub>56</sub>_404</b>             | -          | -     | -     | -     | 97         | 283   | 187       |
| <b>C<sub>31</sub>H<sub>60</sub>_432</b>             | -          | -     | -     | -     | 65         | 94    | -         |
| <b>C<sub>33</sub>H<sub>56</sub>_452<sup>a</sup></b> | -          | 55    | 58    | -     | -          | -     | -         |
| <b>C<sub>33</sub>H<sub>56</sub>_452<sup>a</sup></b> | 91         | -     | -     | 109   | -          | -     | -         |
| <b>C<sub>34</sub>H<sub>58</sub>_466<sup>b</sup></b> | 108        | -     | -     | 57    | -          | -     | -         |
| <b>C<sub>34</sub>H<sub>58</sub>_466<sup>b</sup></b> | 599        | -     | 57    | 509   | -          | -     | -         |
| <b>C<sub>34</sub>H<sub>58</sub>_466<sup>b</sup></b> | 138        | 195   | 334   | 186   | -          | -     | -         |
| <b>C<sub>34</sub>H<sub>58</sub>_466<sup>b</sup></b> | 84         | 270   | 240   | 77    | -          | -     | -         |
| <b>C?</b>                                           | 133        | -     | -     | -     | -          | -     | -         |
| <b>total hydrocarbons (mg L<sup>-1</sup>)</b>       | 1153       | 520   | 689   | 938   | 162        | 483   | 524       |

Total carbohydrate content in the culture medium and biomass, based on their monomeric sugar composition, were determined at the end of the growth experiment. Total carbohydrate content varied between 9 % and 74 % per gram biomass dry weight (Fig. 2.2c). Two A race strains showed highest amount of total carbohydrates with 74 % per biomass dry weight for CCALA777 which is the highest measured, followed by CCALA778 with 52 %. The monomeric carbohydrate composition was examined and the main constituent monosugars for the 16 strains are galactose, glucose, fucose, and arabinose (Table 2.3). Rhamnose was also detected in low but consistent amounts in all strains with values ranging from 2 to 9 mg L<sup>-1</sup>. The main sugar monomer was galactose for 10 strains, whereas glucose was the main monomer for the remaining six strains studied. Fucose is found to vary significantly between strains, ranging from no detection in AC759, AC760 and AC761 to 463 mg L<sup>-1</sup> in

CCALA778. Other *B. braunii* strains showing high amounts of fucose are AC755, CCALA777 and CCAP807/2 with 172 mg L<sup>-1</sup>, 279 mg L<sup>-1</sup> and 136 mg L<sup>-1</sup> respectively. Of the four race B strains, only Showa showed a small amount of fucose detected with 6 mg L<sup>-1</sup>.

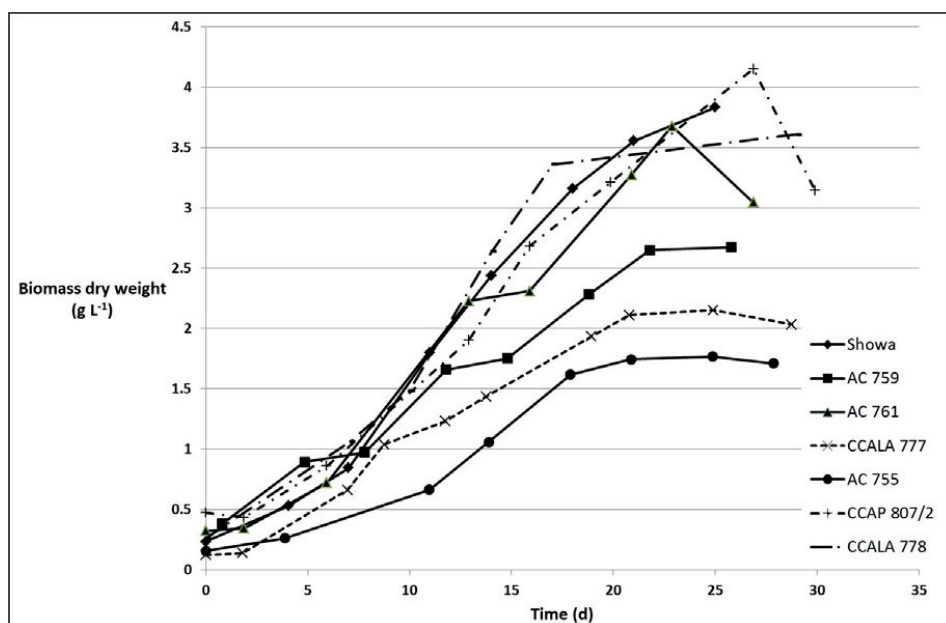
**Table 2.3** Monosaccharide composition. Monomeric sugar content (mg L<sup>-1</sup>) of the 16 strains of *Botryococcus braunii* grouped by subclades from the phylogenetic placement results. The entry n.d stands for not detected

|                   | <b>Fucose</b> | <b>Rhamnose</b> | <b>Arabinose</b> | <b>Galactose</b> | <b>Glucose</b> | <b>Total</b> |
|-------------------|---------------|-----------------|------------------|------------------|----------------|--------------|
| <b>Subclade 1</b> |               |                 |                  |                  |                |              |
| <b>AC765</b>      | 37            | 3               | 27               | 99               | 118            | 284          |
| <b>AC768</b>      | 70            | 3               | 12               | 131              | 113            | 328          |
| <b>Subclade 2</b> |               |                 |                  |                  |                |              |
| <b>AC767</b>      | 24            | 2               | 17               | 144              | 21             | 208          |
| <b>Subclade 3</b> |               |                 |                  |                  |                |              |
| <b>Showa</b>      | 6             | 3               | 102              | 199              | 163            | 473          |
| <b>AC759</b>      | n.d           | 5               | 18               | 210              | 82             | 316          |
| <b>AC760</b>      | n.d           | 4               | 18               | 165              | 99             | 286          |
| <b>AC761</b>      | n.d           | 3               | 17               | 230              | 93             | 343          |
| <b>Subclade 5</b> |               |                 |                  |                  |                |              |
| <b>UTEX 572</b>   | 4             | 3               | 10               | 107              | 158            | 282          |
| <b>UTEX</b>       | 7             | 3               | 9                | 86               | 213            | 319          |
| <b>LB572</b>      |               |                 |                  |                  |                |              |
| <b>AC755</b>      | 172           | 3               | 27               | 307              | 151            | 660          |
| <b>K-1489</b>     | 80            | 3               | 9                | 321              | 163            | 576          |
| <b>CCALA 777</b>  | 279           | 4               | 9                | 1115             | 374            | 1781         |
| <b>CCALA 778</b>  | 463           | 9               | 21               | 908              | 288            | 1688         |
| <b>CCALA 835</b>  | 29            | 3               | 14               | 88               | 143            | 277          |
| <b>SAG 30.81</b>  | 18            | 9               | 15               | 72               | 123            | 237          |
| <b>CCAP 807/2</b> | 136           | 5               | 28               | 134              | 178            | 480          |

Arabinose varies for most strains between  $9 \text{ mg L}^{-1}$  and  $28 \text{ mg L}^{-1}$ , but for Showa it is present in high amounts with  $102 \text{ mg L}^{-1}$ . Galactose is the largest portion of the total carbohydrate produce in the strains CCALA777 and CCALA778 with  $1115 \text{ mg L}^{-1}$  and  $908 \text{ mg L}^{-1}$  respectively. Similarly to hydrocarbon content, no correlation was found between biomass productivity and total carbohydrate.

### 2.2.3 Bubble columns cultivation

Based on highest biomass productivity, hydrocarbons and carbohydrate content from the shakeflask experiment, seven strains were scaled-up to bubble columns to assess the cultivation viability. Fig. 2.3 shows the biomass evolution for these strains grown for up to 30 days in the bubble columns.



**Fig. 2.3** Growth curves of *Botryococcus braunii* strains in bubble columns photobioreactors. Cultivation in batch mode in the range of 25 to 30 days. n=1.

In Table 2.4 the volumetric productivities (PV) and maximum specific growth rates ( $\mu_{\max}$ ) are given. Growth curves, volumetric productivities and maximum specific growth rates highlight Showa, CCALA778, CCAP807/2 and AC761 as the best performing strains in the bubble columns, with productivities ranging between  $0.12$  and  $0.15 \text{ g L}^{-1} \text{ day}^{-1}$ .

**Table 2.4** Volumetric productivity (PV) and maximum specific growth rate ( $\mu_{\max}$ ) of *Botryococcus braunii* grown in bubble columns. PV expressed in grams per litre per day and  $\mu_{\max}$  is the amount biomass growth per day. n=1.

| Strain    | PV (g l <sup>-1</sup> d <sup>-1</sup> ) | $\mu_{\max}$ (d <sup>-1</sup> ) |
|-----------|-----------------------------------------|---------------------------------|
| AC755     | 0.06                                    | 0.05                            |
| AC759     | 0.09                                    | 0.07                            |
| AC761     | 0.15                                    | 0.11                            |
| CCALA777  | 0.08                                    | 0.06                            |
| CCALA778  | 0.12                                    | 0.17                            |
| CCAP807/2 | 0.14                                    | 0.11                            |
| Showa     | 0.14                                    | 0.17                            |

The other strains show slower growth with lower biomass productivity. The hydrocarbon and carbohydrate content are shown in Table 2.5. Long chain hydrocarbons were detected in all seven strains with exception of CCALA778. AC761 shows the highest hydrocarbon content with 45 % per gram of biomass dry weight followed by Showa with 25 %. The lowest amount of hydrocarbon was found in the strain CCAP807/2 with 7 % of its biomass dry weight. Carbohydrate content in the seven strains varied between 4 % and 55 % for Showa and CCALA777 respectively.

**Table 2.5** Percentage of hydrocarbon and total carbohydrate content per biomass dry weight of *Botryococcus braunii* biomass cultured in bubble columns. The measurements were carried out on samples from the last day of growth for each strain. n=1.

| Strain    | % hydrocarbon | % carbohydrates |
|-----------|---------------|-----------------|
| AC755     | 16            | 25              |
| AC759     | 21            | 10              |
| AC761     | 45            | 10              |
| CCALA777  | 10            | 55              |
| CCALA778  | 0             | 9               |
| CCAP807/2 | 7             | 5               |
| Showa     | 25            | 4               |

### 2.3 Discussion

16 strains of *B. braunii* were compared under identical growth conditions. The results show similar strain variation as observed in the other studies with different growth conditions. And it also shows differences between different studies when comparing the same strains.

In the shakeflask cultivation, biomass productivity varies statistically significantly ( $P < 0.05$ ) among strains in the shake flask cultures, and align with reports from identical strains in similar experimental studies (Eroglu *et al.* 2011 ). The strain with the highest productivity ( $0.18 \text{ g L}^{-1} \text{ day}^{-1}$ ) AC768, produced no detectable hydrocarbons and had a relatively low level of carbohydrates (10 % w/w). However, this inverse correlation is not observed for all the strains. For example AC767 and SAG30.81 also show low carbohydrates and no hydrocarbon production, while biomass productivity remained at a low range with  $0.10$  and  $0.07 \text{ g L}^{-1} \text{ day}^{-1}$  respectively. On the other hand strains that produce high amounts of hydrocarbons such as Showa showed much higher productivity ( $0.11 \text{ g L}^{-1} \text{ day}^{-1}$ ). Similar results in the bubble columns were obtained, which suggests that the low biomass productivity of *B. braunii* compared to other algal species is specific and is independent of *B. braunii* producing hydrocarbons or carbohydrates. Low biomass productivity of *B. braunii* at this stage has to be accepted when utilized in large scale production for the extraction of hydrocarbons and carbohydrates until a strain with high productivity can be found that also can produce high amounts of product of interest.

Clear differences are also visible between strains when comparing hydrocarbon content under the same conditions. Seven strains out of sixteen produced hydrocarbons ranging from 6 % per gram of biomass dry weight in K-1489 to 42 % in the Showa strain, and these values are also found in literature (Metzger and Largeau 2005). Four strains (Showa, AC759, AC760 and AC761) produce  $C_{33} - C_{37}$  hydrocarbons and the remaining three strains for which hydrocarbons were detected (K-1489, AC755, CCAP807/2) produce  $C_{25} - C_{31}$  hydrocarbons, in accordance with previous reports (Metzger *et al.* 1985a, Metzger *et al.* 1988). For K-1489 strain, this is the first time that the hydrocarbon content is reported. The strain SAG30.81 is reported to contain up to 30 % of hydrocarbons (Dayananda *et al.* 2007) yet in our study no hydrocarbons were detected. Also, for a majority of other strains tested, no hydrocarbons were detected. Media composition between the various previous studies and this work do not vary significantly, therefore the absence of hydrocarbons in these strains are probably due to other factors. Other cultivation conditions, such as length of cultivation period and light regime might play a role. For example, the growth conditions for AC765, AC767 and AC768

strains were cultivated in cylindrical tubes, aerated with 1 % CO<sub>2</sub> under continuous illumination as described (Metzger *et al.* 1985b, Metzger *et al.* 1988). For SAG30.81 and UTEX LB572, light conditions were varied with different intensities in continuous and photo-period illumination parameters as described by Dayananda *et al.* (Dayananda *et al.* 2007). For CCALA777, the light intensity was of 250  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$  in continuous illumination, with 1 % CO<sub>2</sub> aeration in cylindrical tubes as described by Fernandes *et al.* (Fernandes *et al.* 1991). These differences in the growth conditions could have changed the hydrocarbon synthesis rate and explain why it was not detected in some strains of *B. braunii* in our study. Another possible explanation for these different results might be due to the different methods of hydrocarbon extraction used. In this study we used dichloromethane:methanol as solvent compared to hexane extraction applied by the other studies previously mentioned. But this reason does not stand so well as we have obtained hydrocarbons in the range of 6 % to 42 % for K-1489 and Showa respectively as well as hydrocarbon chains below and higher than C<sub>30</sub>.

Differences in total carbohydrate content are also evident ranging from 4 % of biomass dry weight in the Showa strain to 74 % in the CCALA777 strain. The high viscosity only appears in the CCALA777 and CCALA778. This is not surprising as there are only a few reports in the literature on the ability of *B. braunii* to produce large amounts of carbohydrates (Fernandes *et al.* 1989, Allard and Casadevall 1990, Fernandes *et al.* 1991, Lupi *et al.* 1994, Dayananda *et al.* 2007). The highest amount of total carbohydrates extracted from the culture broth in CCALA777 and CCALA778 was 1.78 g L<sup>-1</sup> and 1.68 g L<sup>-1</sup> respectively. Similar results are described in literature for the CCALA777 strain, which is also known by its identifier Acoi58 (Fernandes *et al.* 1989). It is well known the application of carbohydrates in the pharmaceutical industry (Moscovici 2015) due to the diverse biological active agents present. Carbohydrates from CCALA778 contains 27 % of fucose from the total carbohydrates, which translates to 140 g of fucose per Kg of biomass dry weight. Allard and Casadevall (1990) also show similar fucose content in a race A and L strain with 32 % of total carbohydrate content. Fucose is considered of high industrial value (Wijesinghe and Jeon 2012) and it has potential medicinal applications, namely anti-cancer properties (Liao *et al.* 2013). The carbohydrates of CCALA777 and CCALA778 strains, should be analysed in depth for its potential applications in industry and as well as more studies to investigate the mechanisms behind the high content of carbohydrates and fucose. Other monomeric composition of the total carbohydrates shows galactose and glucose as the main monomers across all strains. For CCALA777 and CCALA778 galactose is by far the largest fraction of

the carbohydrates moieties and is presumably the reason why viscosity increases (Fernandes *et al.* 1989, Allard and Casadevall 1990, Díaz Bayona and Garcés 2014). Recent work shows that galactose can be used for the production of bioethanol via fermentation (Lee *et al.* 2011, Park *et al.* 2014), therefore with the high amounts of galactose produced by CCALA777 and CCALA778, these strains can be considered as potential candidates as raw material for the production of bioethanol. Future research in *in situ* extraction of the carbohydrates from these two strains in a continuous cultivation system would further increase the feasibility for commercial scale application.

One point to bear in mind when comparing the strains is the fact that we have non-axenic (bacteria present) strains. Microalgae and bacteria interactions are of major interest as it has been shown that bacteria can have influence on the cultivation of microalgae (Cole 1982). Therefore we consider that attempts in making *B. braunii* axenic is of advantage as it can improve culture productivities and in this case increase the accuracy of the comparison between strains. Also is of interest to study the possible symbiosis existing with *B. braunii* and bacteria as a recent study using *B. braunii* Ba10 strain shows enhancement of biomass productivities with the presence of bacteria (Tanabe *et al.* 2015). In this case for example, CCALA778 strain produces high amounts of carbohydrates and if the culture contains high amounts of bacteria, in theory could be that these are degrading the sugars for carbon source and ultimately decreasing the amount of product accumulated and available for harvest.

From the bubble column cultivation, we can only speculate on the results as the batch mode cultivation was run only once for the selected strains. Strains CCALA778, AC761, Showa and CCAP807/2 seem viable for scale up cultivation as volumetric productivities show values in similar range or higher than the shakeflask cultivation. There are differences observed in biomass productivities, such as for AC755 and Showa, and also changes in the carbohydrate and hydrocarbon content. These differences may be explained by physical properties of the cultivation systems used. Biomass productivities can increase in systems such as bubble column reactors, for example because of improved light distribution and availability per cell (Kojima and Zhang 1999, Ugwu *et al.* 2005), or better gas mass transfer rates (Posten 2009). On the other hand, biomass productivities can decrease in these same systems because of shear stress induced by gas purge causing damage to cells (Barbosa *et al.* 2003) and in case of *B. braunii*, possibly damage to the colonies. Further work with strains such as CCALA778 and AC761 should be done at larger scale to optimize and characterize their potential as biofuel and bio-based raw materials.



Two aspects for future research and comparison of strains are 1) the characterization of the extracellular and intracellular hydrocarbons. Because *in situ* (“milking”) extraction is a viable option as shown by Moheimani and colleagues (Moheimani *et al.* 2013), a wide comparison would inform also which strains are best for hydrocarbon production facility. 2) the characterization of colony formation and structure as it could affect the extractability of products, growth rates related to light absorption and possibly downstream processes.

What also has been shown and is again evident, is the variability of the physiological characteristics of *B. braunii* strains relative to different literature studies. Therefore there would be a benefit of improving the species reference or taxonomy catalogue when dealing with the cosmopolitan *B. braunii*, for example, a similar approach taken by Dorienko and coworkers (Dariencko *et al.* 2015), who described the closely related species *Coccomyxa* using integrative taxonomy and DNA barcoding. This type of approach is suitable for microalgae that have high morphological and physiological similarities. A recent publication by Hegedus and colleagues follows similar approach with *B. braunii* A races (Hegedüs *et al.* 2016).

## 2.4 Conclusion

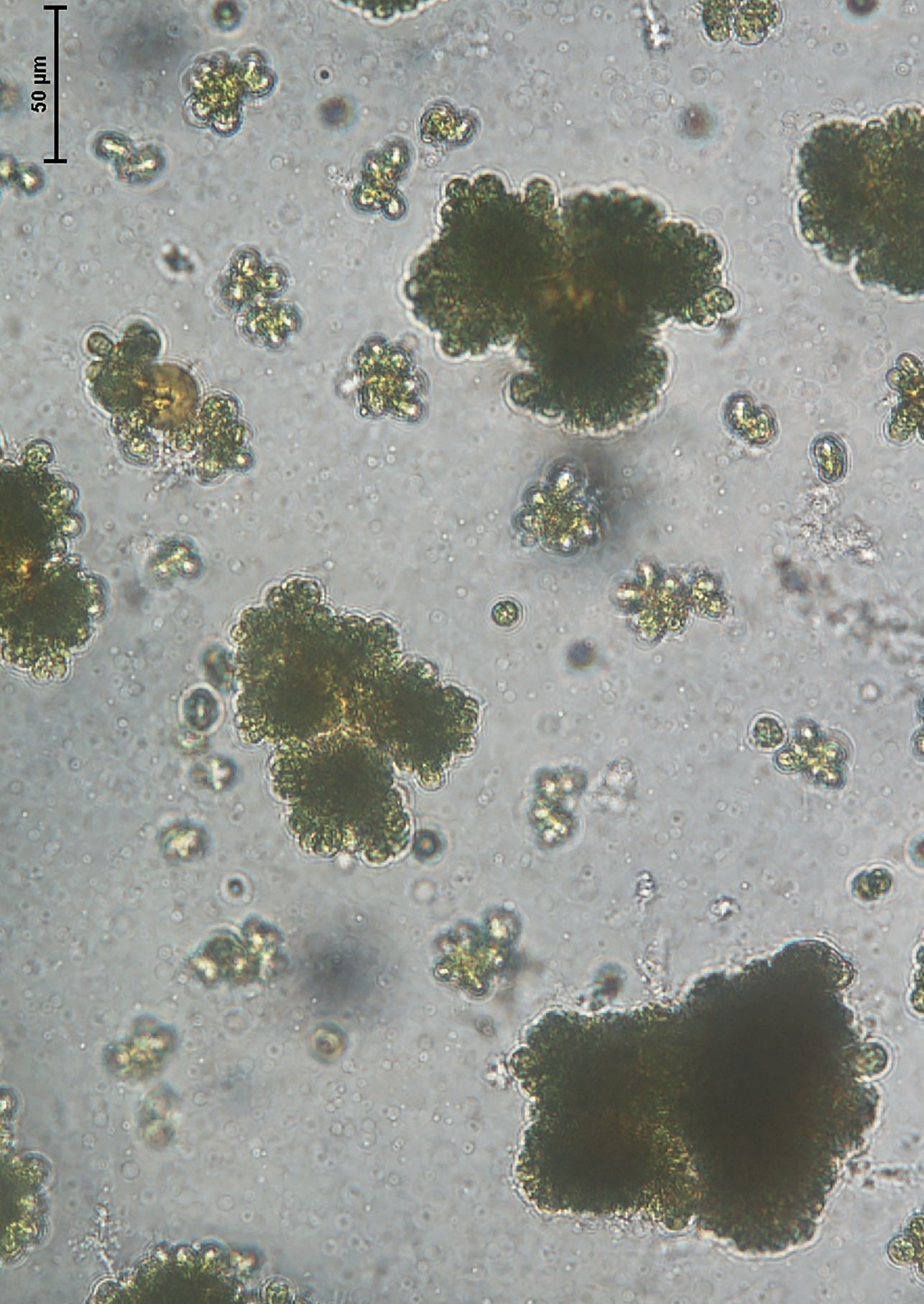
This study presents the physiological diversity of commercially available *B. braunii* strains grown in similar conditions with respect to biomass productivity, and hydrocarbon and carbohydrate content. The physiological characteristics of *B. braunii* from this study complemented with the literature can be as diverse as the number of strains compared. This variability keeps scientists challenged in the characterization and understanding of *B. braunii* and at the same time positive that the exploration of this specie can in the future yield a strain that will have the minimum required characteristics for industrial application. CCALA778 strains show potential as carbohydrate producers with feasible applications in different industries. For example, galactose in fermentation processes for the production of bioethanol and fucose for the application in cancer treatment. For hydrocarbons production the strain AC761 could be exploited. The slow growth of *B. braunii* is not correlated to the hydrocarbon content or total carbohydrate, but it remains the main obstacle to overcome for large scale production, this could also be overcome if applications with high value can emerge. For future work more can be done on the characterization of extracellular and intracellular hydrocarbon in the different strains, as well colony structure, as these will have an impact on the extractability of hydrocarbons and carbohydrates.

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# Associated bacteria of *Botryococcus braunii* (Chlorophyta)

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## Chapter 3

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**Abstract**

*Botryococcus braunii* (Chlorophyta) is a green microalga known for producing hydrocarbons and exopolysaccharides. Microalgae usually cohabit with bacteria and can form species-specific communities with environmental and biological advantages. Some bacteria though, can impair the growth of microalgae. In order to maximize biomass productivities and hence the desired hydrocarbon and exopolysaccharide compounds, more understanding of the bacterial community and interactions between *B. braunii* and the bacteria present in the cultures is required. Bacteria have been found and identified with a few *B. braunii* strains, but little is known about the bacterial community across the different strains. Knowledge of the bacterial community of *B. braunii* can help to optimize culture conditions to enhance the biomass productivity, hydrocarbon and carbohydrate accumulation. To understand better the bacterial community diversity of *B. braunii* we screened different strains from culture collections. Using 16S rRNA gene analysis by MiSeq we describe the bacterial diversity across twelve *B. braunii* strains and identify possible shared communities. We found three bacterial families common to all strains: *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae*. The results also suggest that additionally, each strain has its own specific bacteria that are not shared between strains.

**Keywords:** *Botryococcus braunii*, bacterial community, hydrocarbons, exopolysaccharides, microalgae

### 3 Introduction

In recent years the physiology and cultivation process of several microalgae with potential for large scale production was studied (Grima *et al.* 1999, Ugwu *et al.* 2005, Posten 2009, Blanken *et al.* 2016, Cabanelas *et al.* 2016). One microalga of interest for large scale cultivation is *B. braunii* because it can produce and secrete long chain hydrocarbons and exopolysaccharides (Fernandes *et al.* 1989, Metzger and Largeau 2005, Dayananda *et al.* 2007). Hydrocarbons are natural occurring compounds consisting entirely of hydrogen and carbon, and are one of the most important energy resources (Timmis and Qin 2010). *B. braunii* are characterized into different races (race A, B, L and S) depending on the type of hydrocarbons secreted (Metzger and Largeau 2005, Kawachi *et al.* 2012). Race A strains synthesize odd-numbered alkadienes and trienes (C<sub>25</sub> to C<sub>31</sub>), race B strains synthesize isoprenoid type compounds termed botryococcenes (C<sub>30</sub> to C<sub>37</sub>) and methylated squalenes (C<sub>31</sub> to C<sub>34</sub>), race L strains synthesize lycopadiene (C<sub>40</sub>) and race S strains synthesize C<sub>18</sub> epoxy-*n*-alkanes and C<sub>20</sub> saturated *n*-alkanes (Metzger and Largeau 2005, Dayananda *et al.* 2007, Eroglu *et al.* 2011, Kawachi *et al.* 2012). Exopolysaccharides have a range of industrial uses, such as thickeners, stabilisers and gelling agents in food products, as well as in the pharmaceutical and cosmeceutical industries (Buono *et al.* 2012, Donot *et al.* 2012, Borowitzka 2013). *B. braunii* comprises of a variety of strains from diverse parts of the world, which can differ in the hydrocarbon and exopolysaccharide content (Wolf 1983, Metzger *et al.* 1988, Allard and Casadevall 1990, Volova *et al.* 1998, Dayananda *et al.* 2007, Eroglu *et al.* 2011, Moutel *et al.* 2016).

Bacteria which can grow in close proximity of the algal cells or colonies due to the presence of exopolysaccharide substances surrounding the microalgae (Bell and Mitchell 1972), allow opportunities for mutually beneficial symbiotic interactions as well as opportunities for bacteria that can have antagonistic effects such as inhibition of microalgal growth, either causing cell lysis, or directly competing for nutrients (Cole 1982, Cooper and Smith 2015, Segev *et al.* 2016). Studies investigating interactions of other microalgae with bacteria show how important these interactions can be for the cultivation process (Guerrini *et al.* 1998, Kazamia *et al.* 2012, Kim *et al.* 2014, Windler *et al.* 2014). The microalgae tend to associate with specific bacteria providing environmental advantage such as nutrient exchange (Jasti *et al.* 2005, Eigemann *et al.* 2013, Ramanan *et al.* 2015). These species-specific associations could make the community more resilient to invasion by other microorganisms providing long term stability (Eigemann *et al.* 2013, Hays *et al.* 2015). Understanding the interactions of

microalgae and bacteria and how it can enhance the cultivation for industrial process, could lead to increase biomass productivity.

So far the bacterial community of *B. braunii* species is described in only a few studies. The earliest work is from Chirac and colleagues, who reported the presence of *Pseudomonas* sp. and *Flavobacterium* sp. in two strains of *B. braunii* (Chirac *et al.* 1982). Rivas and colleagues identified in *B. braunii* UTEX strain the presence of *Pseudomonas* sp. and *Rhizobium* sp. (Rivas *et al.* 2010). One more recent studies using *B. braunii* Ba10 strain showed the presence of rod shaped bacteria in the rim of the colony aggregations and proposed it is as growth promoting bacteria closely related to *Hyphomonadaceae* spp. (Tanabe *et al.* 2015). One important finding is that *B. braunii* is a Vitamin B<sub>12</sub> autotroph so it does not depend on bacteria for the synthesis of this important metabolite. But at the same time, other important type of mutualistic interactions that enhance biomass productivity may be taking place and therefore potentially important for the growth of *B. braunii* (Tanabe *et al.* 2014). So far, all studies investigating the bacteria present with *B. braunii*, have focused in only a few strains making it difficult to have a good overview of what bacterial community dominates *B. braunii* cultures.

In this study we looked at twelve strains of *B. braunii* obtained from several culture collections to investigate the bacterial community composition that is associated with *B. braunii*.

### 3.1 Materials and Methods

#### 3.1.1 Strain collections and media preparation

Twelve *B. braunii* strains were obtained from culture collections (Table 3.1) and transferred to Erlenmeyer flasks with modified Chu 13 medium (Largeau *et al.* 1980) without citric acid or vitamins, with the following composition: 1200 mg L<sup>-1</sup> KNO<sub>3</sub>, 200 mg L<sup>-1</sup> MgSO<sub>4</sub>·2H<sub>2</sub>O, 108 mg L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 104.8 mg L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 20 mg L<sup>-1</sup> Fe-Na<sub>2</sub>EDTA, 9.4 µg L<sup>-1</sup> Na<sub>2</sub>O<sub>4</sub>Se, 2.86 mg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 1.8 mg L<sup>-1</sup> MnSO<sub>4</sub>·4H<sub>2</sub>O, 220 µg L<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O, 90 µg L<sup>-1</sup> CoSO<sub>4</sub>·7H<sub>2</sub>O, 80 µg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 60 µg L<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 10 µl L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>. Final pH was adjusted to pH 7.2 with NaOH and NaHCO<sub>3</sub> was added to a final concentration of 5 mM. The 12 strains were grown in Infors HT Multitron incubators in 250 mL conical flasks and a volume of 150 mL. Temperature was set at 23°C, with 2.5 % CO<sub>2</sub> enriched air and shaking at 90 rpm. Illumination was provided by Phillips lamps FL-Tube L 36W/77, with 150 µmol



photon  $\text{m}^{-2} \text{sec}^{-1}$ , and a light:dark photoperiod of 18:6 h. Flasks were inoculated with *B. braunii* growing in the active growing phase, such that the initial absorbance at 680 nm was 0.2. The Erlenmeyer flasks were capped with aeresal sterile film (Alphalabs). Samples were taken at day 1, 4, 8 and 11 for 16S rRNA gene analyses.

### 3.1.2 DNA extraction

On sampling days, 5 mL of fresh culture were harvested with sterilized membrane filters (0.2  $\mu\text{m}$ , Millipore) using a vacuum apparatus. The filters were cryopreserved in  $-80^\circ\text{C}$  until further processing. DNA was extracted from the cryopreserved filters that were cut into small pieces with a sterile scissor. Filter pieces were transferred to a 2 mL sterilized tube with zirconia/silica beads (Biospecs), and 1 mL S.T.A.R buffer (Roche, USA) was added. Cells were homogenized for two rounds of 45 seconds, at the speed of 5500 rpm with Precellys (Bertin Technologies). Then DNA was extracted using the Maxwell 16 Tissue LEV Total RNA purification kit (Promega, USA) with aid of the Maxwell 16 instrument (Promega, USA). The purity and quantity of DNA was examined by electrophoresis on a 1% agarose gel and measured with a Nanodrop (ND1000, Thermo Fisher Scientific Inc., Wilmington). The extracted DNA was stored at  $-20^\circ\text{C}$  until further use.

### 3.1.3 16S rRNA gene amplification and Miseq sequencing

Amplicons of 350 base pairs from the V1-V2 region of 16S rRNA genes were generated by a two-step PCR strategy. Forward primer (27F-DegS = 5'GTTYGATYMTGGCTCAG 3' where M = A or C; R = A or G; W = A or T; Y = C or T) and an equimolar mixture of reverse primers (338R I = 5'GCWGCCTCCCGTAGGAGT 3' and II = 5'GCWGCCACCCGTAGGTGT 3' where M = A or C; R = A or G; W = A or T; Y = C or T). Eighteen bp Universal Tags 1 and 2 (Unitag1= GAGCCGTAGCCAGTCTGC; Unitag2= GCCGTGACCGTGACATCG) were appended at the 5' end of the forward and reverse primer, respectively (Daims *et al.* 1999, van den Bogert *et al.* 2011, Tian *et al.* 2016). The first PCR mix (50  $\mu\text{L}$ ) contained 10  $\mu\text{L}$  5 $\times$  HF buffer (Thermo Scientific<sup>TM</sup>, the Netherlands), 1  $\mu\text{L}$  dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion<sup>®</sup> Hot Start II High-Fidelity DNA polymerase (Thermo Scientific<sup>TM</sup>), 1  $\mu\text{M}$  of 27F-DegS forward primer, 1  $\mu\text{M}$  of 338R I and II reverse primers, 1  $\mu\text{L}$  template DNA and 32.5  $\mu\text{L}$  nuclease free water. Amplification included an initial denaturation at  $98^\circ\text{C}$  for 30 sec; 25 cycles of denaturation at  $98^\circ\text{C}$  for 10 sec; annealing at  $56^\circ\text{C}$  for 20 sec and elongation at  $72^\circ\text{C}$  for 20 sec; and a final extension at  $72^\circ\text{C}$  for 10 min. The PCR product size was examined by 1

% gel electrophoresis. The second PCR mix (100  $\mu\text{L}$ ) contained 62  $\mu\text{L}$  nuclease free water, 5  $\mu\text{L}$  of PCR1 product, 20  $\mu\text{L}$  5 $\times$  HF buffer, 2  $\mu\text{L}$  dNTP Mix, 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward and reverse primer equivalent to the Unitag1 and Unitag2 sequences respectively, each appended with an 8 nt sample specific barcode. Amplification included an initial denaturation at 98°C for 30 sec; 5 cycles of denaturation at 98°C for 10 sec, annealing at 52°C for 20 sec and elongation at 72°C for 20 sec; and a final extension at 72°C for 10 min. The concentration of PCR products was quantified with a Qubit Fluorometer (Life Technologies, Darmstadt, Germany) in combination with the dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). Purified products were then pooled in equimolar amounts of 100 ng  $\mu\text{L}^{-1}$  and sequenced on a MiSeq platform (GATC-Biotech, Konstanz, Germany).

#### 3.1.4 Processing MiSeq data

Illumina sequencing data were analyzed using NG-Tax pipeline (Ramiro-Garcia *et al.* 2016) yielding a total of 1460845 sequences. Briefly, in a first step, paired-end reads of 2 x 100 nucleotides were combined and only read pairs with perfectly matching primers and barcodes were retained. Demultiplexing, Operational Taxonomic Unit (OTU) picking, chimera removal and taxonomic assignment were performed within one single step using the *OTU\_picking\_pair\_end\_read* script in NG-Tax. Sequences were clustered to OTUs at the 99% identity level, and an OTU table was created with the most abundant sequences at a threshold of 0.1% minimum relative abundance of each sample. Taxonomic assignment of the OTUs was executed using the UCLUST algorithm (Edgar 2010) and the Silva\_126\_SSU Ref database, containing 1,675,819 unique full length 16S rRNA gene sequences. Seven samples from day 4 were removed from the results due to contamination during the PCR steps: AC755, AC759, AC760, AC767, AC768, CCAP and UTEX572. The 16S rRNA gene dataset obtained in this study is deposited in the Sequence Read Archive (SRA), NCBI with accession number SRP102970.

### 3.1.5 Microbial community analysis

For the interpretation of the microbial community data on family level, the Operational Taxonomic Unit (OTU) abundance table was converted to relative abundance and visualized as heatmaps using JColorGrid (Joachimiak *et al.* 2006). Ordination analyses to estimate the relationship of the *B. braunii* strains based on dissimilarity of the microbial community compositions among the individual samples was performed for all strains and for only the strains from CAEN culture collection. For both analyses it was used a standardized 97% OTU table (*decostand* function, *method = hellinger*) and the nMDS function *metaMDS* (*distance = Bray-Curtis*) from the *vegan* package in R (v.3.0.2) (R Core Team 2014, Oksanen *et al.* 2016). Betadispersion and permutation test were performed to test homogeneity dispersion within group of samples. *Adonis* from the *vegan* package in R (v.3.0.2) was used to test significant differences in bacterial community between strains. Hierarchical clustering analysis was performed using *hclust* function in R using *method = average*.

**Table 3.1** Information of the culture collections providers of *Botryococcus braunii* strains and location of origin.

| <i>Botryococcus braunii</i>                                   |                           |         |                                                      |                              |                                   |  |
|---------------------------------------------------------------|---------------------------|---------|------------------------------------------------------|------------------------------|-----------------------------------|--|
| Culture collection                                            | Strain (our abbreviation) | Race    | Location                                             | Isolation, date of isolation | Reference                         |  |
| Berkeley                                                      | Showa                     | Race B  | culturing tanks, Berkeley                            | by unknown, 1980             | Nonomura, A., 1988                |  |
| Scandinavian Culture Collection of Algae and Protozoa (SCCAP) | K1489                     | Race A  | Belgium, Nieuwoort                                   | by G. Hansen, 2008           | No reference                      |  |
| UTEX Culture Collection of Algae                              | UTEX LB572 (UTEX)         | Race A  | Cambridge, England                                   | by M. R. Droop, 1950         | Eroglu E., et al, 2011            |  |
| Culture Collection of Autotrophic Organisms (CCALA) check     | CCALA778 (CCALA)          | unknown | Serra da Estrela (Barragem da Erva da Fome) Portugal | by Santos, 1997              | No reference found                |  |
| Culture Collection of Algae and Protozoa (CCAP)               | CCAP807/2 (CCAP)          | Race A  | Grasmere, Cumbria, England                           | by Jaworski, 1984            | Hilton J. et al, 1989             |  |
|                                                               | AC755                     | Race A  | Lingoult-Morvan, France                              | by Pierre Metzger, 1981      | Metzger, P., et al, 1985          |  |
|                                                               | AC759                     | Race B  | Ayame, Ivory Coast                                   | by Pierre Metzger, 1984      | Metzger, P., et al, 1988          |  |
|                                                               | AC760                     | Race B  | Kossou, Ivory Coast                                  | by Pierre Metzger, 1984      | Metzger, P., et al, 1988          |  |
|                                                               | AC761                     | Race B  | Paquemar, Martinique, France                         | by Pierre Metzger, 1983      | Metzger, P., et al, 1985          |  |
| ALGOBANK-CAEN                                                 | AC765                     | Race L  | Kossou, Ivory Coast                                  | by Pierre Metzger, 1984      | Metzger, P., et al, 1988          |  |
|                                                               | AC767                     | Race L  | Songkla Nakarin, Thailand                            | by Pierre Metzger, 1985      | Metzger, P., Casadevall, E., 1987 |  |
|                                                               | AC768                     | Race L  | Yamoussoukro, Ivory Coast                            | by Pierre Metzger, 1984      | Metzger, P., Casadevall, E., 1987 |  |

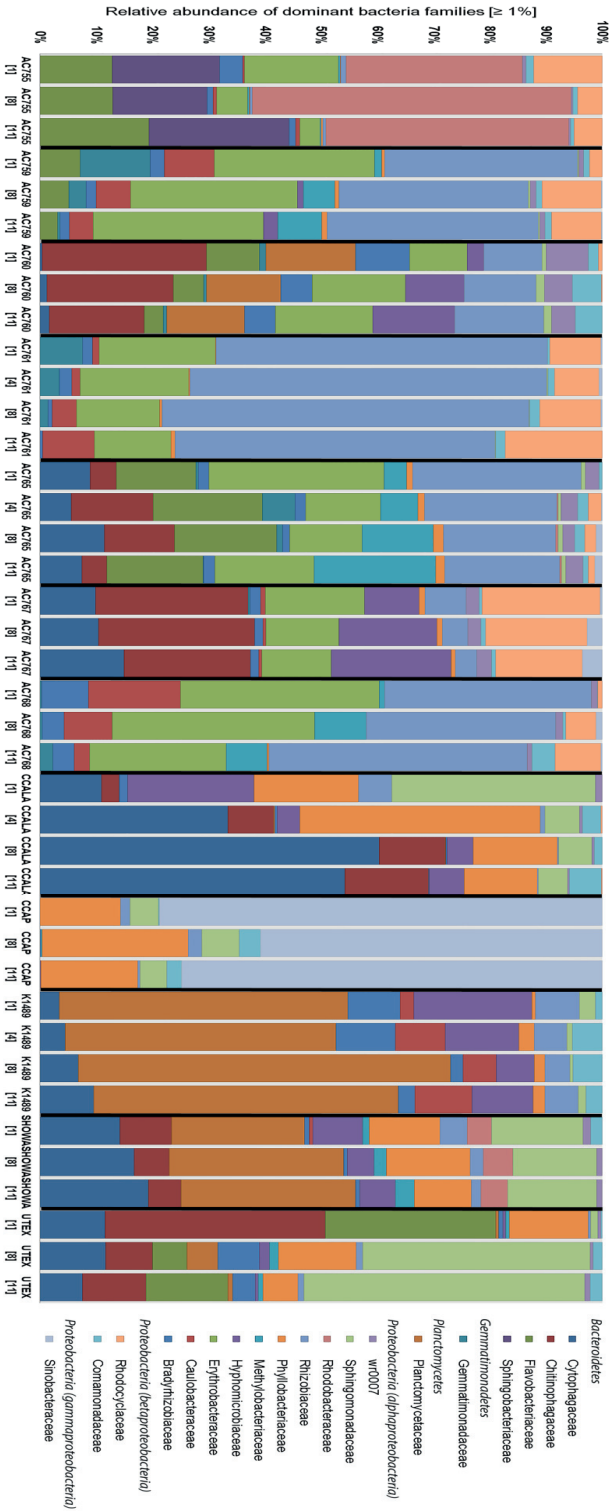
### 3.2 Results

Fig. 3.1 shows the bacterial families with relative abundance above 1 % and a total of four bacterial phyla associated with *B. braunii* strains. The four phyla found associated with *B. braunii* were the *Bacteroidetes*, *Gemmatimonadetes*, *Planctomycetes* and *Proteobacteria*. *Proteobacteria* was the predominant bacterial phylum and representatives of this taxon were found in all 12 strains. *Bacteroidetes* was found in all strains with exception to strains AC761, AC768 and CCAP. *Gemmatimonadetes* was found only in the CAEN culture (with AC prefix) strains with exception to AC755. *Planctomycetes* was found in AC760, CCALA, K1489, Showa and UTEX strains. Three families were found across all 12 *B. braunii* strains and all were *Proteobacteria*. These were the *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae*. *Rhizobiaceae* was represented 1 to 59 % of the bacterial reads. *Bradyrhizobiaceae* was found within the 1 to 8 % range. *Comamonadaceae* was found between 1 and 5 %. Two families of bacteria were only found in the strains obtained from the CAEN culture collection: *Erythrobacteraceae* with bacterial reads ranging 1 to 29 % and *Rhodocyclaceae* with 1 to 18 %.

Some families of bacteria were particularly dominant in specific strains. *Sinobacteraceae* was dominant in CCAP with relative abundances ranging from 59 to 78 %. *Planctomycetaceae* was dominant in K1489 strain with relative abundances between 46 and 51 %. *Rhizobiaceae* was dominant in AC761 with relative abundances between 55 and 64 %. Other families of bacteria became dominant as the cultures became older. *Rhodobacteraceae* was present in AC755 strain with relative abundances ranging from 28 % at day 1 to 40 % at day 11. *Sphingomonadaceae* was present in UTEX with 10 % at day 1 and increased its presence to 47 % at day 11. *Chytophagaceae* was dominant in CCALA strain with relative abundance ranging from 10 % at day 1 to 52 % at day 11.

Because we found three common families across all strains, we wanted to investigate in more detail the bacterial composition in these selected families and see if we could identify a unique microorganism present in all strains. Therefore we zoomed in and looked at the Operational Taxonomy Units (OTUs) distribution belonging to the three families (*Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae*). In addition we picked the OTUs found only in the strains obtained from the CAEN culture collection which belong to two families (*Erythrobacteraceae* and *Rhodocyclaceae*). The most abundant OTUs were selected and a total of 28 OTUs were investigated. From Fig. 3.2 it was clear that there was not an OTU that was found across all strains but rather each family comprised of several different

OTUs. The second important observation was that CCAP strain had no representative OTUs for *Bradyrhizobiaceae* and *Rhizobiaceae* in the most abundant OTUs. The most represented family taxon was *Rhizobiaceae* with 12 OTUs. From the three families found in the 12 strains, OTU 233 assigned to the genus *Rhizobium* had the highest OTU frequency abundance with 10 % and it was present in 7 out of 12 strains. The OTUs 143, 88 and 131 assigned to the genus *Shinella* were present in 9 out of 12 strains. The OTUs 477, 475 and 484 assigned to the genus *Bosea* covered 11 out of 12 strains. From the two families found only in the cultures originating from the CAEN culture collection, OTUs 333 and 539 were found in all seven CAEN strains with the assigned genus *Porphyrobacter* and *Methyloversatilis* respectively.



**Fig. 3.1 Relative abundance of bacterial families in 12 *B. braunii* strains.** Strain abbreviations are used as explained in Table 1. Each bar displays the bacterial family relative abundance above 1%. Strains are labelled below with sample day within square brackets. Bacterial Families are organized according to the phyla (in italics) they belong to.



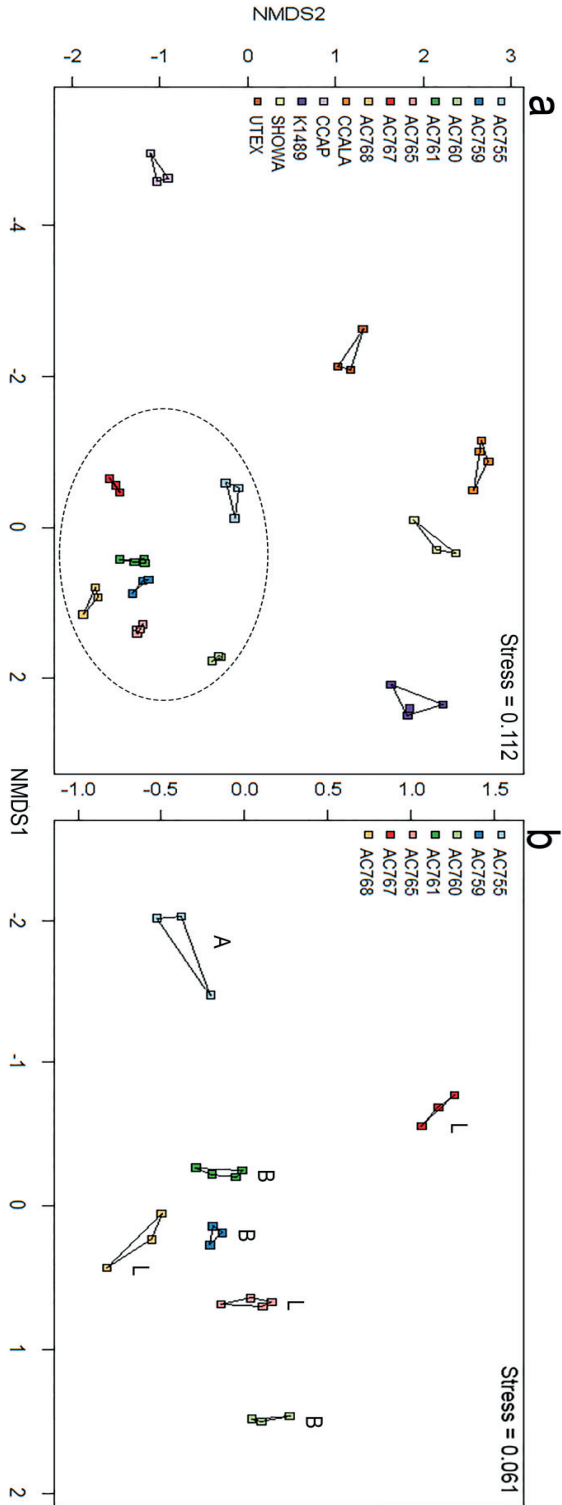


**Table 3.2 NCBI database blast of OTUs for selected families.** Closest first three neighbours with highest identity match and with a minimum of 85 % coverage for each OTU. NCBI blast on the 1<sup>st</sup> February 2016, except the OTU 662 which the blast search from 30<sup>th</sup> August 2016 and OTU 63 and 415 on February 2017.

| OTU | nearest neighbour1                       | Genbank acc. | nearest neighbour2                         | Genbank acc. | nearest neighbour3                            | Genbank acc. |
|-----|------------------------------------------|--------------|--------------------------------------------|--------------|-----------------------------------------------|--------------|
| 475 | <i>Hypomicrobium nitratovorans</i> (100) | NR_121713.1  | <i>Hypomicrobium nitratovorans</i> (100)   | NR_118448.1  | <i>Bosea latipri</i> (100)                    | NR_108515.1  |
| 477 | <i>Bradyrhizobium lupini</i> (100)       | NR_134836.1  | <i>Bradyrhizobium lupini</i> (100)         | NR_044869.2  | <i>Rhodopseudomonas palustris</i> (100)       | NR_103926.1  |
| 484 | <i>Bosea robiniae</i> (100)              | NR_108516.1  | <i>Bradyrhizobium lupini</i> (99)          | NR_134836.1  | <i>Bradyrhizobium ottawaense</i> (99)         | NR_133988.1  |
| 502 | <i>Bradyrhizobium daqingense</i> (100)   | NR_118648.1  | <i>Bradyrhizobium labiabi</i> (100)        | NR_117513.1  | <i>Beijerinckia doebereineri</i> (100)        | NR_116304.1  |
| 88  | <i>Rhizobium rhizoryzae</i> (100)        | NR_133844.1  | <i>Rhizobium flavum</i> (100)              | NR_133843.1  | <i>Rhizobium azibense</i> (100)               | NR_133841.1  |
| 115 | <i>Rhizobium rhizoryzae</i> (100)        | NR_133844.1  | <i>Rhizobium flavum</i> (100)              | NR_133843.1  | <i>Rhizobium azibense</i> (100)               | NR_133841.1  |
| 143 | <i>Rhizobium rhizoryzae</i> (100)        | NR_133844.1  | <i>Rhizobium flavum</i> (100)              | NR_133843.1  | <i>Rhizobium azibense</i> (100)               | NR_133841.1  |
| 233 | <i>Rhizobium paranaense</i> (100)        | NR_134152.1  | <i>Rhizobium rhizoryzae</i> (100)          | NR_133844.1  | <i>Rhizobium flavum</i> (100)                 | NR_133843.1  |
| 555 | <i>Variovorax guangxiensis</i> (100)     | NR_134828.1  | <i>Variovorax paradoxus</i> (100)          | NR_074654.1  | <i>Variovorax boronicumulus</i> (100)         | NR_114214.1  |
| 566 | <i>Hydrogenophaga flava</i> (100)        | NR_114133.1  | <i>Hydrogenophaga bisnensis</i> (100)      | NR_044268.1  | <i>Hydrogenophaga defluvi</i> (100)           | NR_029024.1  |
| 567 | <i>Hydrogenophaga flava</i> (100)        | NR_114133.1  | <i>Hydrogenophaga bisnensis</i> (100)      | NR_044268.1  | <i>Hydrogenophaga defluvi</i> (100)           | NR_029024.1  |
| 333 | <i>Sphingomonas gae</i> (100)            | NR_134812.1  | <i>Sphingomonas ginsengisoli</i> (100)     | NR_133664.1  | <i>Porphyrobacter colymbi</i> (100)           | NR_114328.1  |
| 539 | Uncultured bacterium (100)               | KY606782.1   | <i>Methyloversatilis discipulorum</i> (71) | KY284088.1   | <i>Methyloversatilis discipulorum</i> (71)    | KY284080.1   |
| 63  | <i>Thioclava</i> sp. (100)               | CP019437.1   | <i>Rhodobacter</i> sp. (100)               | KY608089.1   | Uncultured <i>Rhodobacter</i> sp. (100)       | KY606875.1   |
| 819 | <i>Dyadobacter jiangsuensis</i> (100)    | NR_134721.1  | <i>Dyadobacter fermentans</i> (100)        | NR_074368.1  | <i>Dyadobacter tibetensis</i> (88)            | NR_109648.1  |
| 832 | <i>Dyadobacter jiangsuensis</i> (100)    | NR_134721.1  | <i>Dyadobacter fermentans</i> (100)        | NR_074368.1  | <i>Dyadobacter tibetensis</i> (88)            | NR_109648.1  |
| 415 | Uncultured bacterium (100)               | KI769749.1   | Uncultured bacterium (91)                  | KI724695.1   | Uncultured <i>Pilancomyces</i> sp. (87)       | JX576019.1   |
| 45  | <i>Frigidibacter albus</i> (100)         | NR_134731.1  | <i>Paracoccus sediminis</i> (96)           | NR_134122.1  | <i>Nioella nitratrieducens</i> (94)           | NR_134776.1  |
| 69  | <i>Frigidibacter albus</i> (100)         | NR_134731.1  | <i>Paracoccus sediminis</i> (100)          | NR_134122.1  | <i>Nioella nitratrieducens</i> (97)           | NR_134776.1  |
| 302 | <i>Sphingorhabdus arenillioris</i> (100) | NR_134184.1  | <i>Sphingopyxis italcia</i> (100)          | NR_108877.1  | <i>Parasphingopyxis lamellibrachiae</i> (100) | NR_113006.1  |
| 310 | <i>Sphingomonas yuntinensis</i> (100)    | NR_133866.1  | <i>Sphingomonas canadensis</i> (100)       | NR_108892.1  | <i>Blastomonas natatoria</i> (100)            | NR_113794.1  |
| 355 | <i>Blastomonas natatoria</i> (100)       | NR_113794.1  | <i>Sphingomonas wu-sincola</i> (100)       | NR_040825.1  | <i>Blastomonas natatoria</i> (100)            | NR_040824.1  |

The most abundant OTUs (as listed in Fig. 3.2) were subjected to a Blast search against the NCBI database to infer their nearest neighbours (Table 3.2). OTUs 88, 115, 143 and 233 were similar in their nearest neighbours with four different *Rhizobium* spp. as candidates. Similar blast results were seen also for OTUs 566 and 567 with nearest neighbours *Hydrogenophaga* spp.. The OTUs 819 and 832 with *Dyadobacter* spp. as nearest neighbour dominated CCALA bacterial community. Some OTUs showed different species as closest neighbours such as OTUs 45 and 69 with *Frigidibacter albus*, *Paracoccus sediminis* and *Nioella nitratreducens*. The OTU 415 with high abundance in K1489 belonging to *Planctomycetaceae*, had as closest neighbours uncultured bacterium and third closest neighbour uncultured *Planctomyces* spp. with the latter showing 87 % identity. The OTU 333 present only in the strains from CAEN culture collection, had 100 % identity with *Sphingomonas* as closest two neighbours, and third neighbour also with 100 % identity as *Porphyrobacter*.

Non-metric multidimensional scaling ordination was performed for the 12 strains to determine the bacterial community dissimilarities (Fig. 3.3a). *B. braunii* strains from the CAEN culture collection clustered together when compared to the other strains indicating these strains were similar to each other in bacterial community composition. This was supported by hierarchical cluster analysis showing CAEN strains in their own cluster (Supplementary Fig. 3.1). The strains K1489, UTEX, CCAP, CCALA and Showa represented separate clusters. The homogeneity of dispersion within each strains with 1000 permutations showed no significant difference ( $F=0.323$ ). Using adonis to test for bacterial community similarities between all strains, the results showed that the bacterial communities were significantly different ( $DF = 11$ , Residuals = 28,  $R^2 = 0.921$ ,  $P = 0.001$ ). Fig 3.3b zooms in to the CAEN culture collection strains. Races A, B and L are subdivisions of *B. braunii* according to the type of hydrocarbons produced. No clustering by type of hydrocarbons produced was seen by the distribution of the race B and race L strains which were found mixed, namely race B AC759 and AC761 with race L AC765 and AC768. Similarly, the bacterial community between CAEN strains were significantly different ( $DF = 6$ , Residuals = 16,  $R^2 = 0.904$ ,  $P = 0.001$ ).



**Fig. 3.3 Non-metric multidimensional scaling (NMDS) ordination (based on Bray-Curtis distance matrix) of 16S rRNA gene sequences of 12 *B. braunii* strains.** a) ordination of all strains with CAEN cultures clustering together (within the ellipse dotted line); b) ordination of the CAEN culture collection strains only. Capital letters in plot b refer to the race subclassification based on the type of hydrocarbons produced.

### 3.3 Discussion

It is evident that *B. braunii* possesses a highly diverse bacterial community as seen by the range of bacterial phyla and families present in all the strains used in this study (Fig. 3.1 (for a more comprehensive list see Supplementary Fig. 3.2)).

From the bacterial community analysis (Fig. 3.3a,b), each *B. braunii* strain showed a specific bacterial community. Another feature from the community analysis, is that the *B. braunii* strains from the CAEN culture collection clustered near each other while *B. braunii* strains from other culture collections appeared as separate groups. This implies that the culture collection from which the strain was obtained correlates stronger to the bacterial profile of the strain than the location from where it was isolated (Table 3.1). OTUs 539 (*Methyloversatilis*) and 333 (*Porphyrobacter*) were only found with the CAEN cultures and contributed towards these strains clustering in close proximity. OTU 333 (*Porphyrobacter*) was specially high in relative abundance and contributed to the distinctive clustering of the CAEN culture collection strains. The remaining strains also contained their specific OTUs that contributed towards their own clustering: OTU 819 (*Dyadobacter*) and 832 (*Dyadobacter*) with CCALA, OTU 310 (*Sphingomonas*) with UTEX and K1489 with OTU 415 (*Planctomyces*). Bacterial community between three race B and three race L were mixed together (Fig. 3.3b) therefore no correlation was found between bacterial community and the type of hydrocarbons produced between the two races. Similar observations were made in another study using six strains of *B. braunii* in which authors did not find correlation between the bacteria and type of hydrocarbon produced (Chirac *et al.* 1985).

Three bacterial families were found to be present with all twelve strains of *B. braunii*: *Bradyrhizobiaceae*, *Rhizobiaceae*, and *Comamonadaceae*. Two families were found abundantly only in the strains from the CAEN culture collection: *Erythrobacteraceae* and *Rhodocyclaceae*. The OTUs 88, 115, 143 and 233 blast hits showed that these bacteria species were related to *Rhizobium* spp. (Table 3.2). *Rhizobium* spp. are known to form nodules in the roots of several plants within the family of legumes and are best known for nitrogen fixation. Bacteria fixing nitrogen was investigated in association with microalgae and it has been shown that they can enhance microalgae growth (Hernandez *et al.* 2009). *Rhizobium* spp. found associated with *B. braunii* could have a similar role. Rivas *et al.* (2010) also found a *Rhizobium* sp. associated with *B. braunii* in particular UTEX LB572 strain, and Kim *et al.* (2014) showed the presence of *Rhizobium* sp. with *B. braunii* 572. Recent studies also showed *Rhizobium* spp. present with *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and

*Scenedesmus* spp. (Kim *et al.* 2014). *Rhizobium* spp. seem important to *B. braunii* strains as it appeared in all 12 strains with more prominence in the CAEN cultures and K1489 with three to four OTUs (Fig. 3.2). For the remaining strains CCALA, CCAP, Showa and UTEX, *Rhizobium* spp. is represented only with one OTU.

OTU 475 from *Bradyrhizobiaceae* family showed 100 % similarity with the species *Hyphomicrobium nitrativorans* as the two closest neighbours and was present in 10 out of 12 *B. braunii* strains. *H. nitrativorans* is a known denitrifier isolated from a seawater treatment facility (Martineau *et al.* 2013). Denitrification is the process of reducing nitrate into a variety of gaseous with the final being dinitrogen. Because denitrification mainly occurs in the absence of oxygen it is unlikely that this is happening within our cultures that are well oxygenated. The 3<sup>rd</sup> closest neighbour for OTU 475 was *Bosea lathyri* and it is associated with root nodules legumes (De Meyer and Willems 2012). The fact this OTU 475 is abundant with K1489 strain, makes it interesting to further investigate its role.

OTUs 555, 566 and 567 from *Comamonadaceae* family appeared in seven out of twelve strains. The three closest neighbours of OTU 555 were *Variovorax* spp. and for OTUs 566 and 567 were *Hydrogenophaga* spp.. *Variovorax* and *Hydrogenophaga* spp. are not known for being symbionts but may be able to support ecosystems by their ability to degrade toxic compounds and assist in nutrient recycling, therefore potentially producing benefits to other microorganisms (Yoon *et al.* 2008, Satola *et al.* 2013). *Comamonadaceae* appeared also as one of the main bacteria families associated with cultivation of microalgae in bioreactors using a mix of fresh water and municipal water as part of water treatment strategy (Krustok *et al.* 2015).

*Erythrobacteraceae* and *Rhodocyclaceae* were only found in the strains from CAEN culture collection. OTU 333 (*Erythrobacteraceae*) first two closest neighbours were from *Sphingomonas* spp., and third closest neighbour was *Porphyrobacter* spp. isolated from water in a swimming pool. Most *Porphyrobacter* spp. isolated originate from aquatic environments (Tonon *et al.* 2014) and are associated with fresh water sediments (Fang *et al.* 2015). *Porphyrobacter* spp. have also been associated with other microalgae such as *Chlorella* spp. (Biondi *et al.* 2016). OTU 539 (*Rhodocyclaceae*) second and third closest neighbour was *Methyloversatilis discipulorum* which is a bacteria found in biofilms formation in engineered freshwater installations (van der Kooij *et al.* 2017). It is not clear why OTU 333 and 539 were specifically found only in the strains originating from the CAEN culture collection, but it

could be an introduced species during handling. None the less, these two OTUs were present in high relative abundance namely OTU 333 (Fig. 3.2), and would be interesting to know if they have a positive or negative influence on the growth of the CAEN strains. It would be interesting to confirm such statement by attempting the removal of these OTUs and investigate the biomass growth.

*Sinobacteraceae* was dominant in CCAP (Fig. 3.1). This family was proposed in 2008 with the characterization of a bacteria from a polluted soil in china (Zhou *et al.* 2008). A recent bacteria related to hydrocarbon degradation showed similarities with *Sinobacteraceae* (Gutierrez *et al.* 2013). OTU 63 was highly abundant in CCAP and could have a negative impact in the cultivation of CCAP strain by reducing its hydrocarbon content.

The *Bacteroidetes* family *Cytophagaceae* dominated the culture CCALA at later stages of growth (Fig. 3.1). *Cytophagaceae* has also been found present on laboratory scale photobioreactor cultivation using wastewater for production of microalgae biomass (Krustok *et al.* 2015). The two OTUs that dominate the bacterial community in CCALA were OTU 819 and OTU 832. The Blast search on NCBI database approximates these two OTUs as *Dyadobacter* spp. which have also been found co-habiting with *Chlorella* spp. (Otsuka *et al.* 2008).

*Planctomycetaceae* dominated the bacterial community in K1489 strain (Fig. 3.1) with OTU 415. This family can be found in freshwater biofilms and also strongly associated with macroalga (Abed *et al.* 2014, Lage and Bondoso 2014). Species in this family could possibly be involved in metallic-oxide formation and be co-players in sulphate-reduction with the later involving also a sulphur-reducing bacteria (Shu *et al.* 2011).

*Rhodobacteraceae* was present up to 55 % of bacterial relative abundance in AC755. Members of this family have been also isolated from other microalgae, namely *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Schwenk *et al.* 2014). The OTUs 45 and 69 blast searches in NCBI database showed the closest neighbours to be *Frigidibacter albus*, *Paracoccus sediminis* and *Nioella nitratireducens* (Table 3.2). All three neighbours were isolated from water environments (Pan *et al.* 2014, Li and Zhou 2015, Rajasabapathy *et al.* 2015).

*Sphingomonadaceae* is mostly found in freshwater and marine sediments (Newton *et al.* 2011). OTUs 302, 310 and 355 from this family were found in six out of 12 strains above 1 %

relative abundance. OTU 310 was only found in the UTEX strain with *Sphingomonas* spp. as the two closest neighbours. *Sphingomonas* spp. are shown to co-habit with other microalgae such as *C. sorokiniana* and *C. vulgaris* (Watanabe *et al.* 2005, Ramanan *et al.* 2015). *Sphingomonas* spp. have been shown to be able to degrade polycyclic aromatic hydrocarbons (Tang *et al.* 2010a) and could possibly be degrading the hydrocarbons secreted by *B. braunii* as its carbon source.

Another characteristic of many bacteria, is the ability to produce exopolysaccharides such as species from the *Rhizobiaceae* and *Bradyrhizobiaceae* family (Bomfeti *et al.* 2011, Freitas *et al.* 2011, Carareto Alves *et al.* 2014). This characteristic could play a role on the colony aggregation of *B. braunii* as exopolysaccharides are known to be essential for biofilm formation (Flemming *et al.* 2007). Therefore it would be interesting in the future to study this possible relationship as *B. braunii* is a colony forming organism. Such studies could involve the introduction of bacteria associated with colony formation such as *Terramonas ferruginea* as it has been associated with inducing flocculation in *C. vulgaris* cultures (Lee *et al.* 2013).

With the present high microbial diversity, *B. braunii* showed qualities in resilience towards microbial activity, probably due to its colonial morphology and protective phycosphere made of hydrocarbons and exopolysaccharides (Weiss *et al.* 2012). A number of microbes are potentially beneficial such as *Rhizobium* spp. which have been shown to have a positive effect on the biomass productivities of *B. braunii* UTEX (Rivas *et al.* 2010), and *Hydrogenophaga* with the ability to degrade toxic compounds (Yoon *et al.* 2008). There are also microbes that may cause detrimental effects on hydrocarbon productivities of *B. braunii* such as *Sphingomonas* spp. (OTU 310) with ability to degrade hydrocarbons (Tang *et al.* 2010a). The removal of such detrimental microbes could enhance cultivation allowing more nitrogen available for biomass production and increase hydrocarbon accumulation of *B. braunii* at larger industrial scale.

### 3.4 Conclusion

*B. braunii* can host a diverse microbial community and it is likely that it forms symbiotic relationships with the members from the *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae* family, which all belong to the phylum *Proteobacteria*. There is not a specific bacterial community correlated to the different types of hydrocarbons produced by race A and B and mostly likely also not race L and S. *B. braunii* has many strains and each

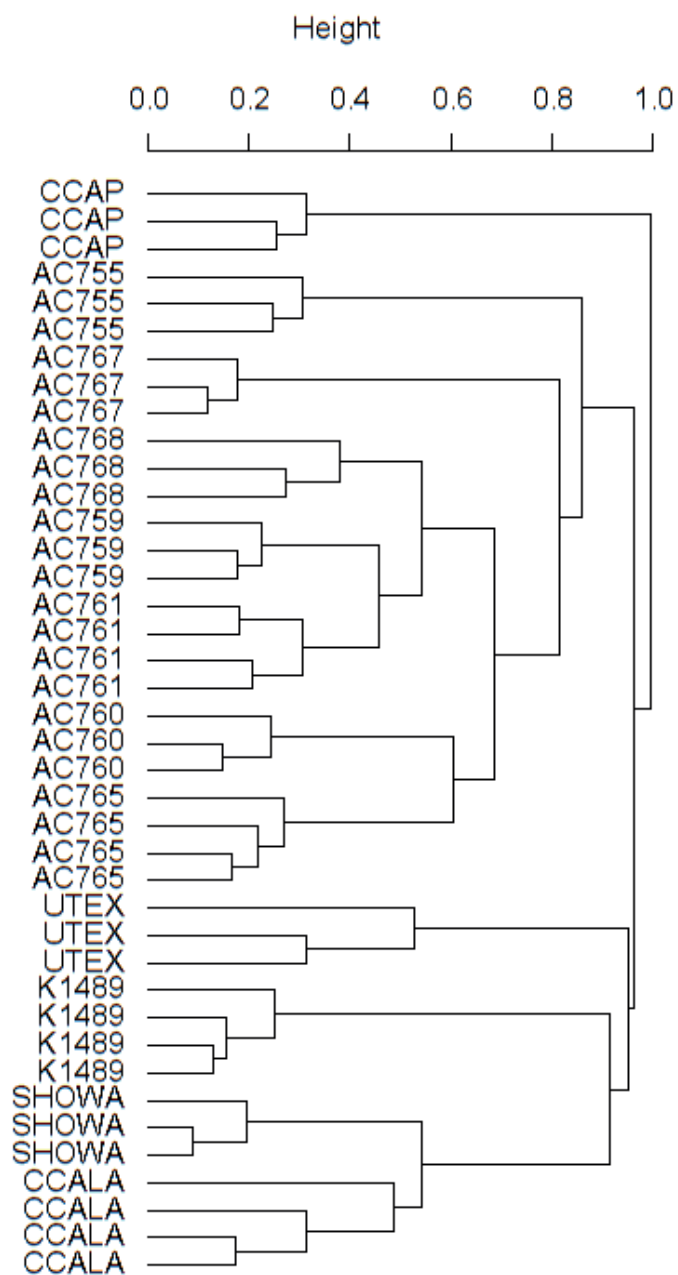
seems to have their own species-specific bacterial community. With a diverse microbial community present, it is also likely that some bacteria are having antagonistic effects on *B. braunii* such as competition with nutrients and degradation of hydrocarbons. The knowledge of bacterial communities present with *B. braunii* allows better understanding of what types of interactions might be occurring and therefore open new opportunities for cultivation strategies to enhance biomass growth.

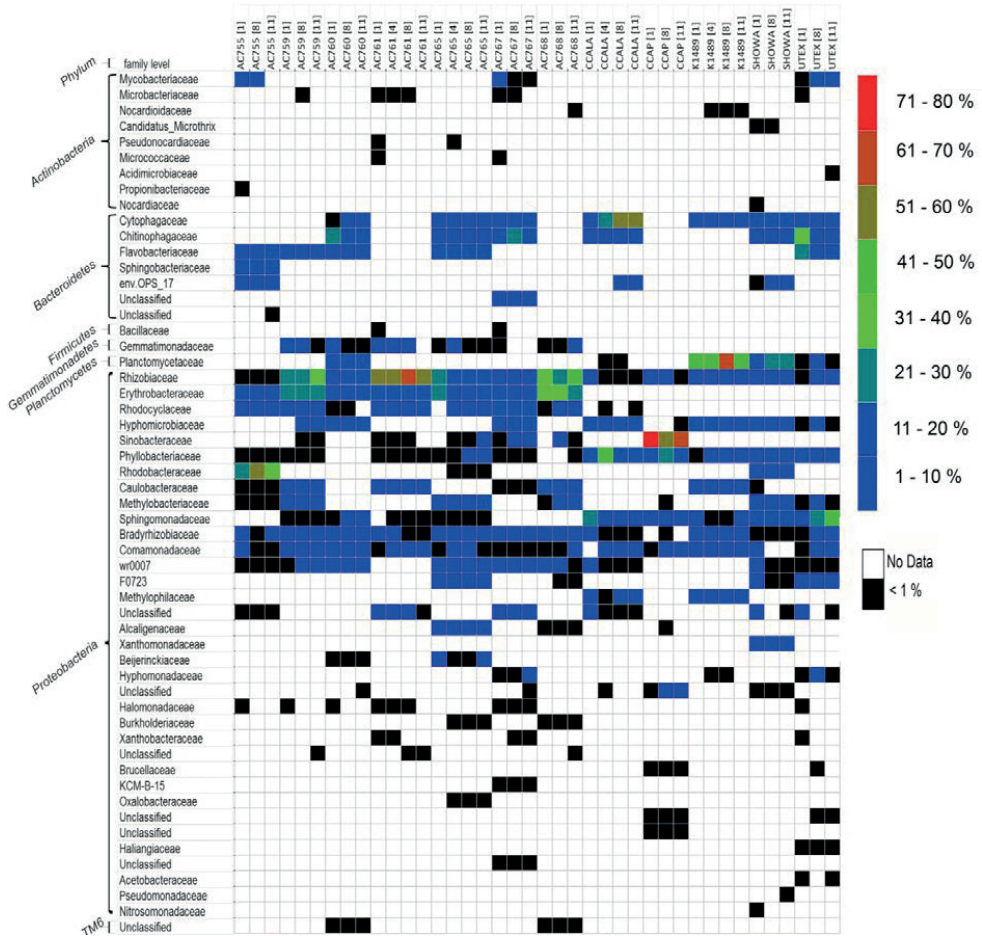
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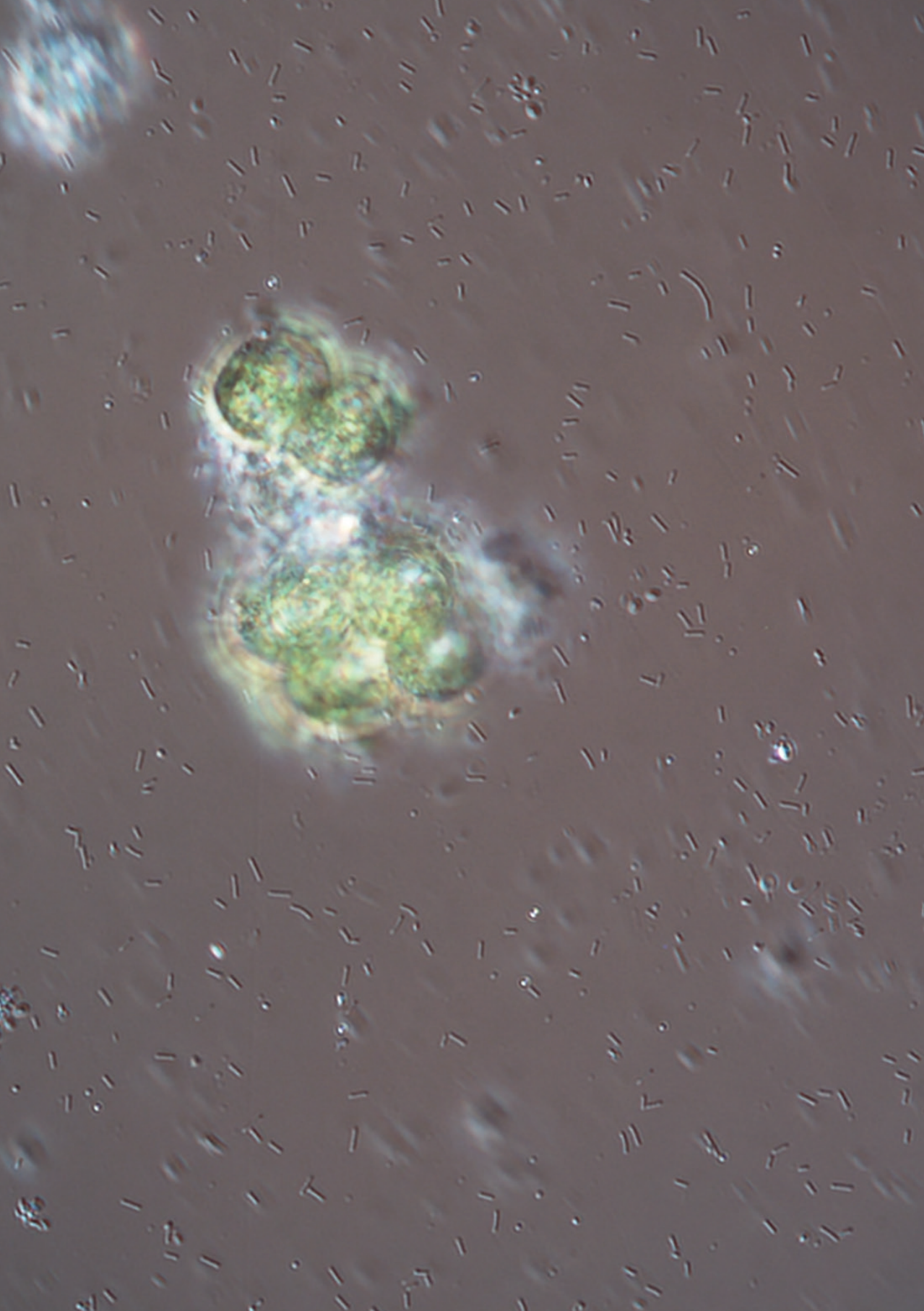
Supplementary Fig. 3.1 Hierarchical clustering of *B. braunii* strains. Strains from CAEN culture collection (AC prefix) cluster together in comparison to the other strains.





**Supplementary Fig. 3.2 Family taxa relative abundance heatmap of 12 *B. braunii* strains.** On the left, is the family taxa classification. On the right the colour coded label is the relative abundance in percentage.





# The impact of antibiotics on *Botryococcus braunii* growth, exopolysaccharide production and associated bacteria

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## Chapter 4

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**Abstract**

Microalgae are a potential host organism for industry to produce biobased polymers. Improving cultivation techniques and strategies can lead to enhanced biomass productivities and product accumulation. Bacterial communities associated to microalgae can have a beneficial or antagonistic effect on algae growth and product formation. Understanding the potential to modify or even eradicate these associations can lead to improved cultivation techniques and production strategies. *B. braunii* is a commercially interesting microalga because it can produce long-chain hydrocarbons and high amounts of exopolysaccharides. In this study we investigated the effects of applying several antibiotics and concoctions, on the growth and extracellular carbohydrate accumulation in *B. braunii* CCALA778 and on the associated bacterial community composition. Most antibiotics and concoctions suppressed growth of CCALA778, except Penicillin. After the release of antibiotic pressure, CCALA778 was able to resume growth, except for the cultures treated with Gentamycin, Chloramphenicol and Linezolid. Exopolysaccharide production was dependent on biomass growth but a decrease was observed in the Penicillin-treated culture. Gram-negative bacteria with the two most abundant belonging to the genera *Terrimonas* and *Blastomonas*, dominated the bacterial communities. Application of single antibiotics and mixes had significant impact on the bacterial communities composition.

**Keywords:** *Botryococcus braunii*, exopolysaccharides, antibiotics, bacterial community, microalgae

## 4 Introduction

One emerging area of investigation, which is important to consider for making microalgae production into an economically feasible industrial production platform, is the understanding of the algae-associated bacterial community composition and their interactions with the algal hosts (Wang *et al.* 2014). In natural aquatic environments bacteria and microalgae coexist due to the presence of exopolysaccharide substances surrounding the microalgae, which has been termed the phycosphere (Bell and Mitchell 1972, Ramanan *et al.* 2015). The interaction between microalgae and bacteria can be mutually beneficial as well as antagonistic in nature, such as growth inhibition of microalgae, either by causing cell lysis, or directly by competing for nutrients (Cole 1982, Cooper and Smith 2015, Segev *et al.* 2016). In some cases bacteria are essential to microalgae that depend on them for the synthesis of vitamin B<sub>12</sub> (Croft *et al.* 2005). Generally, microalgae tend to associate with specific bacteria that provide a growth advantage such as nutrient exchange (Jasti *et al.* 2005, Eigemann *et al.* 2013, Ramanan *et al.* 2015). The associations with specific species, could make the community more resilient to invasion by other microorganisms and provide long term stability (Eigemann *et al.* 2013, Hays *et al.* 2015).

One way to investigate the interactions between microalgae and bacteria, is to enforce changes in the bacterial community and study the impact on the alga or the community as a whole. For instance, this can be achieved by using antibiotics (Joo and Lee 2007, Han *et al.* 2016, Molina-Cardenas *et al.* 2016). Antibiotics are small molecules made by microbes that serve as antagonistic agents to provide them a competitive advantage over other microbes (Clardy *et al.* 2009). Antibiotics can either be bactericidal, which will kill bacteria, or can be bacteriostatic, which suppress growth (Kalghatgi *et al.* 2013).

A microalga of interest for industrial application is *Botryococcus braunii* CCALA778 as it produces high amounts of exopolysaccharides (EPS) (Fernandes *et al.* 1989, Lupi *et al.* 1991). EPS from *B. braunii* contains fucose and uronic acids, which have potential applications such as cancer treatment or reduction of metal-associated toxicity in wastewater, respectively (Wijesinghe and Jeon 2012, Díaz Bayona and Garcés 2014). As many other *B. braunii* strains, CCALA778 shows the presence of a diverse range of bacteria cohabiting with the microalgae but their role or function are unknown (Fernandes *et al.* 1989). As no further studies have

been conducted with the CCALA778 strain, it is not currently known if this strain needs these bacteria for its survival and whether the bacteria have any role in EPS production.

In this study we aimed to investigate the effects of antibiotics on the bacterial community composition of *B. braunii* CCALA778 and the effects on algal biomass growth and EPS accumulation. The impact of antibiotics was monitored during a period of nine days after which antibiotics pressure was released and the algae cultures were monitored for an additional six days.



## 4.1 Materials and Methods

### 4.1.1 Strain and media preparation

*B. braunii* CCALA778 strain was obtained from the Culture Collection of Autotrophic Organisms (Trebou, Czech Republic) and transferred to Erlenmeyer flasks with modified CHU 13 medium (Largeau *et al.* 1980) without citric acid and with the following composition: 1200 mg L<sup>-1</sup> KNO<sub>3</sub>, 200 mg L<sup>-1</sup> MgSO<sub>4</sub>·2H<sub>2</sub>O, 108 mg L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 104.8 mg L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 20 mg L<sup>-1</sup> Fe-Na<sub>2</sub>EDTA, 9.4 µg L<sup>-1</sup> Na<sub>2</sub>O<sub>4</sub>Se, 2.86 mg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 1.8 mg L<sup>-1</sup> MnSO<sub>4</sub>·4H<sub>2</sub>O, 220 µg L<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O, 90 µg L<sup>-1</sup> CoSO<sub>4</sub>·7H<sub>2</sub>O, 80 µg L<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 60 µg L<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 10 µl L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>. Final pH was adjusted to pH 7.2 with NaOH and NaCO<sub>3</sub> was added to a final concentration of 5 mM and subsequently filter sterilized prior to the start of the experiment.

### 4.1.2 Biomass cultivation parameters

CCALA778 was grown in Infors HT Multitron incubators in 250 mL conical flasks and a volume of 120 mL medium. Temperature was set at 23°C, the atmosphere was 2.5 % CO<sub>2</sub> enriched air and cultures were shaken at 90 rpm. Illumination was provided by Phillips lamps FL-Tube L 36W/77, with 150 µmol photon m<sup>-2</sup> sec<sup>-1</sup>, and a light:dark photoperiod of 18:6 h.

### 4.1.3 Experimental design

Culture flasks were inoculated with actively growing *B. braunii*, such that the initial absorbance at 680 nm was 0.2. The Erlenmeyer flasks were capped with aeraseal sterile film (Alphalabs) to allow gas exchange and maintain the flask sterility. The experiment was conducted in triplicate for a period of 16 days. Antibiotics were added at 3 days interval starting at day 0 up to day 9 to maintain antibiotic pressure. Samples were taken at day 0, 2, 4, 8 and 10. To determine culture response post antibiotic treatment, cultures were left to recover for seven days between day 9 and 16, under the same experimental conditions before taking the last samples for analyses.

### 4.1.4 Antibiotic treatment

Six different antibiotics were used as sole antibiotic additions to cultures with CCALA778. In addition, four combinations of some of the six antibiotics were tested (Table 4.1). The four mixes of antibiotics were prepared to combine mode of actions against both Gram-positive and Gram-negative bacteria with the purpose to increase toxicity of the cocktail to the bacterial community. Individual antibiotic stocks were prepared at 8.0 mg mL<sup>-1</sup> for Metranidazole, Penicillin G sodium salt, and Gentamycin sulphate whilst Rifampicin,

Linazolid and Chloramphenicol stocks had a concentration of  $2.7 \text{ mg mL}^{-1}$ . Antibiotics were added before sampling at day 0 and after sampling at day 6. Antibiotic addition was performed always at the same time of the day. Sampling and antibiotics addition were done in a flow cabinet under sterile conditions. Antibiotics were added using sterile serological pipette with filter from the individual stocks directly to the Erlenmeyer flasks. For the treatments containing the mixes, the antibiotics were added in succession. The volume of antibiotic stocks added to the Erlenmeyer flasks was based on the final concentrations required for the individual antibiotics (Table 4.1). As the experiment progressed in time and volume in the Erlenmeyer decreased, the volume of antibiotics added was adjusted to give the same final concentration. A triplicate of Erlenmeyer flasks containing the same medium, but without addition of antibiotic was used as control.

**Table 4.1. Antibiotics treatment used and respective final concentrations.** Antibiotics were added at day 0, 3, 6 and day 9.

| <b>Antibiotic treatment</b>             | <b>Antibiotic conc (mg/mL)</b> | <b>Supplier</b> |
|-----------------------------------------|--------------------------------|-----------------|
| Chloramphenicol ( <i>Chlora</i> )       | 0.1                            | Sigma           |
| Gentamycin Sulphate ( <i>Gen</i> )      | 0.1                            | Sigma           |
| Linezolid ( <i>Lin</i> )                | 0.1                            | Sigma           |
| Metronidazole ( <i>Metro</i> )          | 0.1                            | Sigma           |
| Penicillin G Sodium salt ( <i>Pen</i> ) | 0.1                            | Sigma           |
| Rifampicin ( <i>Rif</i> )               | 0.05                           | Duchefa         |
| <b>Combined AB treatment</b>            | <b>AB conc (mg/mL)</b>         |                 |
| <i>Metro + Pen</i>                      | 0.1 + 0.1                      |                 |
| <i>Pen + Rif</i>                        | 0.1 + 0.05                     |                 |
| <i>Metro + Rif</i>                      | 0.1 + 0.05                     |                 |
| <i>Metro + Rif + Pen</i>                | 0.1 + 0.05 + 0.1               |                 |

#### 4.1.5 Cell dry weight

Five mL aliquots of culture broth were filtered onto pre-weighed GF/D glass-fibre membranes (Whatman). The GF/D filters were dried at 100°C for 24 hours and weighted again. Biomass was determined by subtracting the initial filter weight from the weight of the filtered sample.

#### 4.1.6 Total chlorophyll content

Total chlorophyll content was determined by preparing a methanol extract. In brief, 500 µL -20 °C methanol was added to 500 µL culture broth, and centrifuged for 3 min at 21,000 x g. After centrifugation, supernatant was discarded and the pellet was dissolved in 1 mL cold methanol, vortexed for 1 min, and subsequently heated to 60 °C for 30 minutes. After incubation, the sample was vortexed for 10 seconds and allowed to cool to room temperature before it was again centrifuged for 3 min at 21,000 x g. 200 µL of the supernatant was diluted in 800 µL of methanol and absorbance was measured at 470, 652, 665 and 666 nm. Total chlorophyll was calculated using the following equation:  $Chl\ a = (16.72 * Abs_{665}) - (9.16 * Abs_{652})$ ;  $Chl\ b = (34.09 * Abs_{652} - 15.28 * Abs_{665})$ ;  $Total\ chlorophyll = Chl\ a + Chl\ b$  (Porra *et al.* 1989).

#### 4.1.7 Exopolysaccharide (carbohydrate) content

Carbohydrate content was determined using the method first reported by Dubois (DuBois *et al.* 1956). The carbohydrate content was measured only in the supernatant fraction. In brief, 1000 µL culture broth was centrifuged (except for day 16 samples which only 500 µL culture broth was used) for 3 min at 21,000 x g to pellet cells, and the supernatant was carefully transferred to an Eppendorf tube. The supernatant was lyophilized using a Sublimator 2x3x3 (ZIRBUS Technology) during 28 hours and stored at -20 °C until further analysis. The lyophilized sample was subsequently hydrolyzed by adding 500 µL 2.5 M HCl and incubated for 3 h while vortexing hourly, and neutralized thereafter by adding 500 µL 2.5 M NaOH solution. A centrifugation step of 1 min. at 21,000 x g was done to pellet potential bacterial cell debris and the hydrolyzed sample was stored at -20 °C. Thereafter, 50 µL of the stored hydrolyzed sample was added to 450 µL demineralized water. Gently, 500 µL 5% phenol in water solution was pipetted into the tube followed by 2.5 mL 96 % sulphuric acid added directly onto the liquid surface, and the mixture was incubated at room temperature for 10 min. Hereafter, the tube was placed in a 35 °C water bath for 30 min, while vortexing every 5 min. Subsequently, the sample was left at room temperature for 5 min. prior to absorbance measurements. A dilution series of a standard stock solution, which contained a total of 1 g L<sup>-1</sup> monosaccharides, with a ratio of 8/15 D-galactose, 4/15 D-Fucose and 3/15 D-glucose was

prepared as these monosugars are the main constituents of *B. braunii* carbohydrates (Gouveia *et al.* 2017). The absorbance of the standards and samples was measured at 486 nm and used to calculate the carbohydrate content in the samples. Differences in exopolysaccharide concentrations between treatments was tested based on the means using ANOVA. ANOVA was tested at day 10 and 16 using the function ‘aov’ in R (version 3.3.2).

#### 4.1.8 Bacteria colony morphology

Cultivable bacterial colonies present in the different antibiotics treatments were incubated on agar plates and photographed at day 10. Lysogeny Broth agar (LB) and Plate Count Agar (PCA) were used and prepared according to manufacturer’s specifications. From each Erlenmeyer flask, 100 µL of 10<sup>4</sup> times diluted culture broth was pipetted onto LB and PCA agar in Petridish under sterile conditions inside a flow cabinet. Plates were incubated at room temperature for three days before imaging.

#### 4.1.9 DNA extraction and 16S rRNA gene amplification

On day 0 and day 10, 5 mL liquid culture broth was sampled from all Erlenmeyer flasks and stored at -80 °C. Samples were then lyophilized for 28 h and stored at -20 °C for later analysis. Lyophilized culture broth was resuspended and washed in 5 mL dH<sub>2</sub>O before centrifugation at 4816 x g for 5 min and discarding the supernatant to get rid of the EPS that obstructed DNA extraction. The washed culture broth samples were resuspended in 1 mL S.T.A.R buffer (Roche,USA) and transferred to a 2 ml sterilized tube with 0.1 mm zirconia/silica beads (Biospecs) and 5 glass beads of 3 mm. Cells were subjected to three rounds of homogenization for 45 seconds at a speed of 5500 rpm with Precellys Beadbeater (Bertin Technologies). Then DNA was extracted using the Maxwell 16 Tissue LEV Total RNA purification kit (Promega, USA) with aid of a Maxwell 16 instrument (Promega, USA). The purity and quantity of DNA were examined by electrophoresis on a 1% agarose gel and measured with a Nanodrop (ND1000, Thermo Fisher Scientific Inc.). The extracted DNA was stored at -20 °C until further use.

Amplicons of size 350 base pairs from the V1-V2 region of 16S rRNA genes were generated by a two-step PCR strategy. For the first step, forward primer 27F-DegS (5’GTTYGATYMTGGCTCAG 3’ where M = A or C; R = A or G; W = A or T; Y = C or T) and an equimolar mixture of reverse primers 338R I (5’GCWGCCTCCCGTAGGAGT 3’) and 338R II (5’ GCWGCCACCCGTAGGTGT 3’ where M = A or C; R = A or G; W = A or T; Y = C or T) were used (Daims *et al.* 1999). Eighteen base pairs Universal Tags 1 and 2

(Unitag1= GAGCCGTAGCCAGTCTGC; Unitag2= GCCGTGACCGTGACATCG) were appended at the 5' end of the forward and reverse primers, respectively (Daims *et al.* 1999, van den Bogert *et al.* 2011, Tian *et al.* 2016). The first PCR mix (50  $\mu$ L) contained 10  $\mu$ L 5 $\times$  HF buffer (Thermo Scientific<sup>TM</sup>, the Netherlands), 1  $\mu$ L dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion<sup>®</sup> Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 1  $\mu$ M of Unitag1-27F-DegS forward primer, 1  $\mu$ M of Unitag2-338R I and II reverse primers, 10  $\mu$ L template DNA and 22.5  $\mu$ L nuclease free water. Amplification included an initial denaturation at 98°C for 30 sec; 25 cycles of denaturation at 98°C for 10 sec; annealing at 56°C for 20 sec and elongation at 72°C for 20 sec; and a final extension at 72°C for 10 min. The PCR product size was examined by 1 % gel electrophoresis. The second PCR mix (100  $\mu$ L) contained 62  $\mu$ L nuclease free water, 5  $\mu$ L of PCR1 product, 20  $\mu$ L 5 $\times$  HF buffer, 2  $\mu$ L dNTP Mix, 2 U of Phusion<sup>®</sup> Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward primer (Unitag1) and reverse primer (Unitag2) appended with an 8 nt sample specific barcode for each sample. Amplification included an initial denaturation at 98°C for 30 sec; 5 cycles of denaturation at 98°C for 10 sec, annealing at 52°C for 20 sec and elongation at 72°C for 20 sec; and a final extension at 72°C for 10 min. The concentration of PCR products was quantified with a Qubit Fluorometer (Life Technologies, Darmstadt, Germany) in combination with the dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). Purified products were then pooled in equimolar amounts of 100 ng  $\mu$ L<sup>-1</sup> and sequenced on a MiSeq platform (GATC-Biotech, Konstanz, Germany).

#### 4.1.10 Processing of MiSeq data

Illumina sequencing data were analyzed using NG-Tax pipeline (Ramiro-Garcia *et al.* 2016) yielding a total of 9080216 sequences. Briefly, in a first step, paired-end reads of 2 x 100 nucleotides were combined and only read pairs with perfectly matching primers and barcodes were retained. Demultiplexing, Operational Taxonomic Unit (OTU) picking, chimera removal and taxonomic assignment were performed within one single step using the *OTU\_picking\_pair\_end\_read* script in NG-Tax. Sequences were clustered to OTUs at the 98.5% identity level, and an OTU table was created with the most abundant sequences at a threshold of 0.1% minimum relative abundance of each sample. Taxonomic assignment of the OTUs was executed using the UCLUST algorithm (Edgar 2010) and the Silva\_126\_SSSU Ref database, containing 1,675,819 unique full length 16S rRNA gene sequences. Raw data is deposited at NCBI Genbank with study accession number SRP107367.

#### 4.1.11 Microbial community analyses

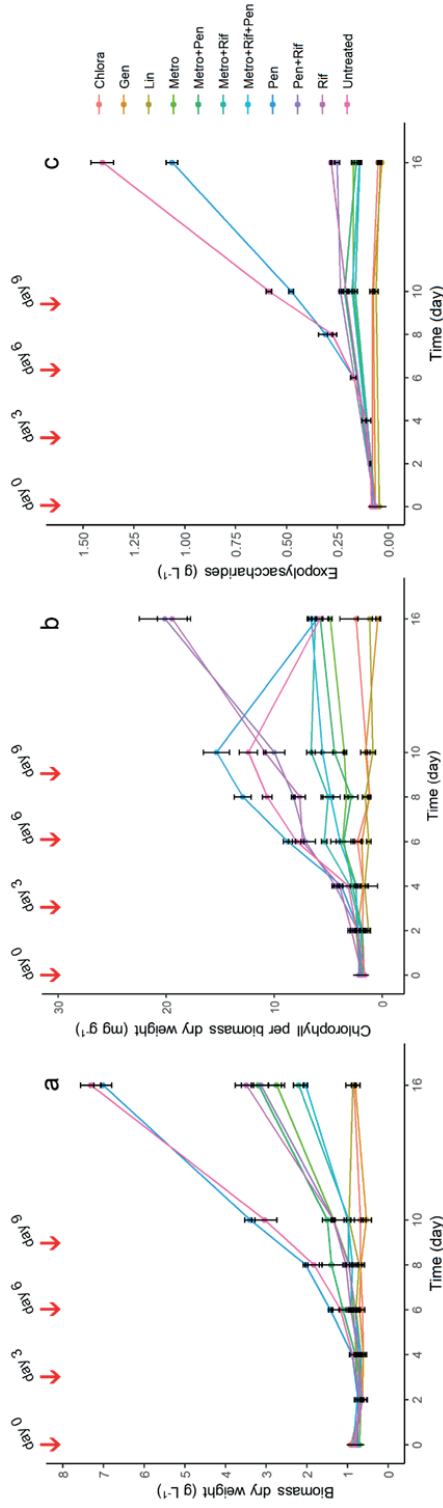
The OTU abundance table was converted to relative abundance and visualized as heatmap using Microsoft Office Excel 2010. Ordination analyses to estimate the similarities of the *B. braunii* CCALA778 based on the microbial community compositions from the different antibiotics treatments was performed using a standardized OTU table (*decostand* function, *method = hellinger*) and the nMDS function *metaMDS* (*distance = Bray-Curtis*) from the *vegan* package in R (v.3.3.2) (R Core Team 2014, Oksanen *et al.* 2016). Diversity indexes within samples were calculated using Richness and Shannon index for day 0 and day 10 using the functions *specnumber* and *diversity* respectively from *vegan* package. The treatments *Chlora*, *Gen* and *Lin* were not tested for significance because of sequence data was poor in quality or was missing due to low DNA concentrations. T-test paired comparison was conducted between day 0 and 10 to check for significant changes in the bacterial community over time. T-test Two-Samples comparison was conducted at day 10 relative to the untreated culture. P-value of 0.05 was used. T-test calculations were done using Microsoft Office Excel 2010. Betadiversity analysis was performed using the multivariate analysis of variance test *adonis* from the *vegan* package to test for significant differences in the bacterial community between treatments at day 10. Prior to betadiversity analysis, homogeneity dispersion within group of samples were determined using *betadisper* and *permutest* from the *vegan* package.

## 4.2 Results

After 10 days of cultivation under antibiotic pressure, clear differences in *B. braunii* cell dry weight can be observed between the treatments and three groups can be distinguished on day 10 (Fig. 4.1a). For one of the groups, no growth was observed during the 10 days period and these treatments included the cultures treated with *Chlora*, *Gen*, *Lin*, *Metro+Rif*, and *Metro+Rif+Pen*. The second group, with suppressed growth to approximately 1.5 g L<sup>-1</sup> cell dry weight, included the cultures treated with *Metro*, *Rif*, *Metro+Pen* and *Pen+Rif*. The third group with normal growth were the untreated and the *Pen*-treated cultures that accumulated 3.0 and 3.4 g L<sup>-1</sup> cell dry weight at day 10, respectively. After day 9 no further antibiotics were added and the growth measured between day 10 and day 16 showed the algae recovery potential after the antibiotic treatment. For all antibiotics treatments, cells were still viable with the exception of the treatments with *Gen*, *Chlora* and *Lin* where growth was not resumed. For two treatments, *Metro+Rif* and *Metro+Rif+Pen*, for which no growth was observed during the initial 10 days, cell dry weight increased to 2.2 and 2.0 g L<sup>-1</sup>, respectively

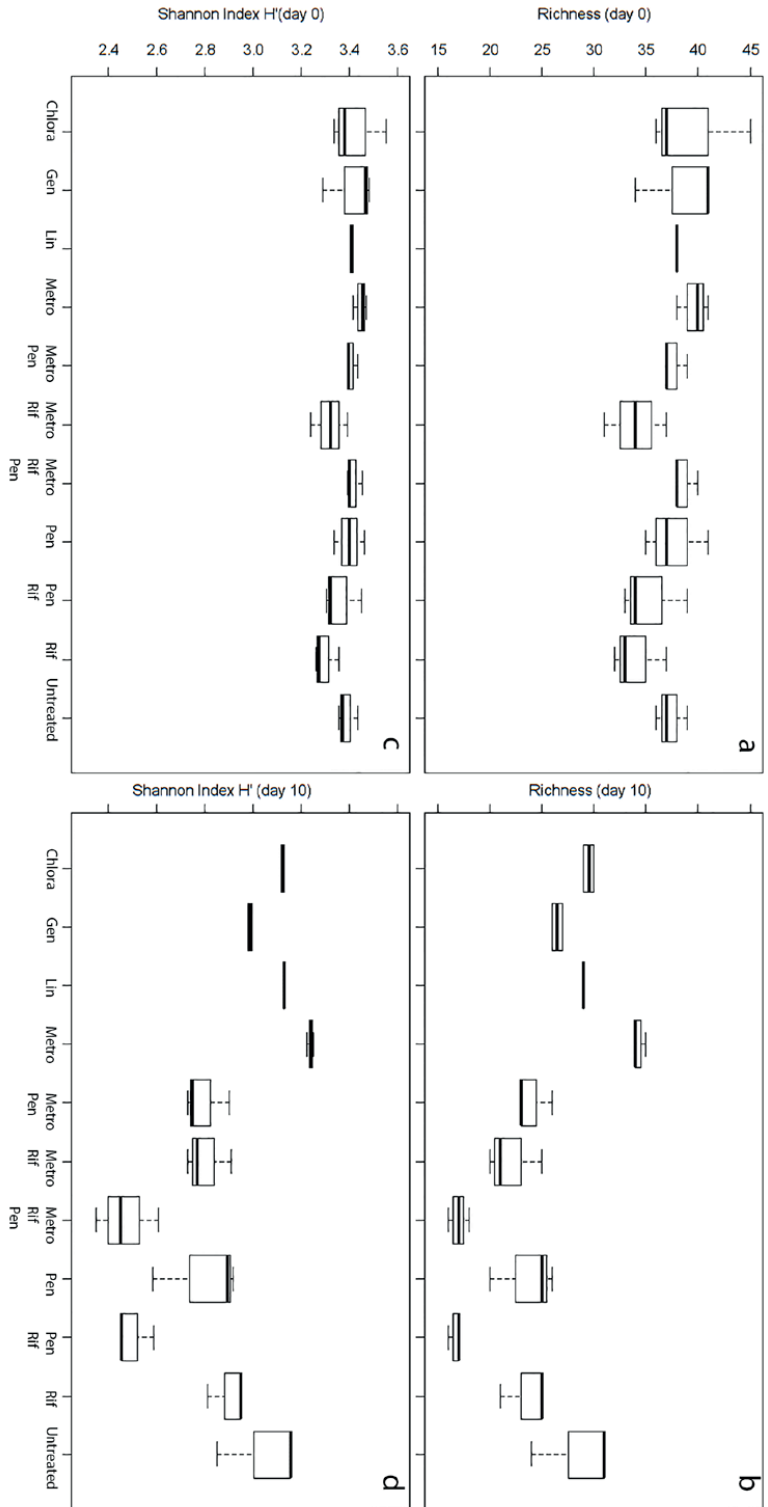
when antibiotic pressure was lifted. *Pen*-treated and untreated cultures continued to grow to 7.0 and 7.3 g L<sup>-1</sup> cell dry weight, respectively.

Based on the chlorophyll per biomass dry weight at day 10, four groups can be distinguished for the different antibiotics treatments (Fig. 4.1b). The group with a decrease in chlorophyll per biomass dry weight comprised of the *Chlora*, *Lin* and *Gen* treated cultures, the same cultures for which growth did not recover after day 10. A group with a very low increase in chlorophyll per biomass dry weight of approximately 3 to 7 mg g<sup>-1</sup> included the treatments with *Metro*, *Metro+Pen*, *Metro+Rif* and *Metro+Rif+Pen*. Two treatments with moderate chlorophyll per biomass dry weight between 9 and 11 mg g<sup>-1</sup> were *Rif* and *Pen+Rif*. For the *Pen*-treated and untreated cultures for which normal growth was observed, chlorophyll per biomass dry weight was the highest during the first 10 days with 15 mg g<sup>-1</sup> and 12 mg g<sup>-1</sup> respectively. From day 10 to day 16, when the antibiotics supplementation was stopped, a sharp bend of the chlorophyll per biomass dry weight concentration for the untreated and *Pen*-treated cultures was observed. In contrast, the two treatments that showed a moderate chlorophyll content until day 10 recovered and the chlorophyll per biomass dry weight increased substantially for this group reaching concentrations of approximately 20 mg g<sup>-1</sup>. The group of treatments with only a low chlorophyll per biomass dry weight increase till day 10 had a marginal increase to day 16 with exception of *Metro+Rif* which had a slight decline in the chlorophyll per biomass dry weight. The treatments that showed a decrease in chlorophyll per biomass dry weight at day 10 did not recover after the antibiotics pressure was lifted after day 10.



**Fig. 4.1 – Effects of antibiotics on *B. braunii* CCALA778:** a) Biomass dry weight; b) Chlorophyll per biomass dry weight; and c) Exopolysaccharides content. Error bars represent standard deviations of three replicates. Red arrows pointing down mark the days of antibiotic shots at day 0, 3, 6 and 9.





**Fig. 4.2** Boxplot of distribution of species diversity for the different antibiotics treatments. a) Richness at day 0; b) Richness at day 10; c) Shannon Index at day 0; d) Shannon Index at day 10. n=3 except for Gen and Chlora where n=2 and Lin n=1. Abbreviations for treatment names are listed in Table 1.

The secreted carbohydrate fraction, termed exopolysaccharide (EPS), was measured for all treatments (Fig. 4.1c). Three groups could be distinguished during the first 10 days: halted EPS formation, impaired EPS formation and normal EPS formation, which was a reflection of the growth pattern. At day 10 EPS accumulated up to  $0.48 \text{ g L}^{-1}$  for the *Pen*-treated cultures and  $0.59 \text{ g L}^{-1}$  for the untreated cultures, followed by further increase up to  $1.1 \text{ g L}^{-1}$  and  $1.4 \text{ g L}^{-1}$  for the *Pen*-treated and untreated cultures, respectively by day 16. Both *Pen*-treated and untreated cultures, which had similar biomass growth, showed differences in their EPS accumulation at day 10 and 16 with  $P=0.005$  and  $P=0.006$  respectively ( $DF=1$ , Residuals=4) with the untreated cultures producing significantly higher amounts of EPS. For the treatments for which growth was resumed after day 10 the EPS concentration was approximately between  $0.25 - 0.28 \text{ g L}^{-1}$ , while the cultures for which no growth was obtained the EPS concentration seemed to slightly decrease over time.

After cultivation of *B. braunii* CCALA778 under antibiotic pressure, changes in the associated bacterial community were observed. Alpha diversity analyses (Fig. 4.2a,b,c and d) indicated that changes in the bacterial community occurred for all conditions during the first 10 days after the start of the experiment (data values can be found in Supplementary Table 4.1). Richness and Shannon's Index ( $H'$ ) of the algae-associated bacterial community were affected in a similar way. Generally, a decrease in richness and diversity was observed for all antibiotics treatments including the untreated control culture. However, this decrease in diversity was not significant for the untreated-culture between day 0 and 10, whilst all the cultures treated with antibiotics showed significant differences between day 0 and 10 for both Richness and Shannon Index, with exception of *Metro+Rif*, which only showed a significance difference in diversity for the Shannon Index and not for Richness (Table 4.2, for full t-test details see Supplementary Table 4.2). The *Metro*-treated cultures showed the least changes by day 10 and the largest changes in diversity were observed for the *Metro+Rif+Pen* and *Pen+Rif* treatments. When day 10 antibiotics-treated cultures were each compared to the untreated culture at day 10, alpha diversity indices were significantly lower for treatments *Metro+Rif+Pen* and *Pen+Rif* (Table 4.3, for full t-test details see Supplementary Table 4.2). Also for the other antibiotics treatments richness and diversity indices were lower than the untreated control (except for *Metro*), but these differences were not significant.

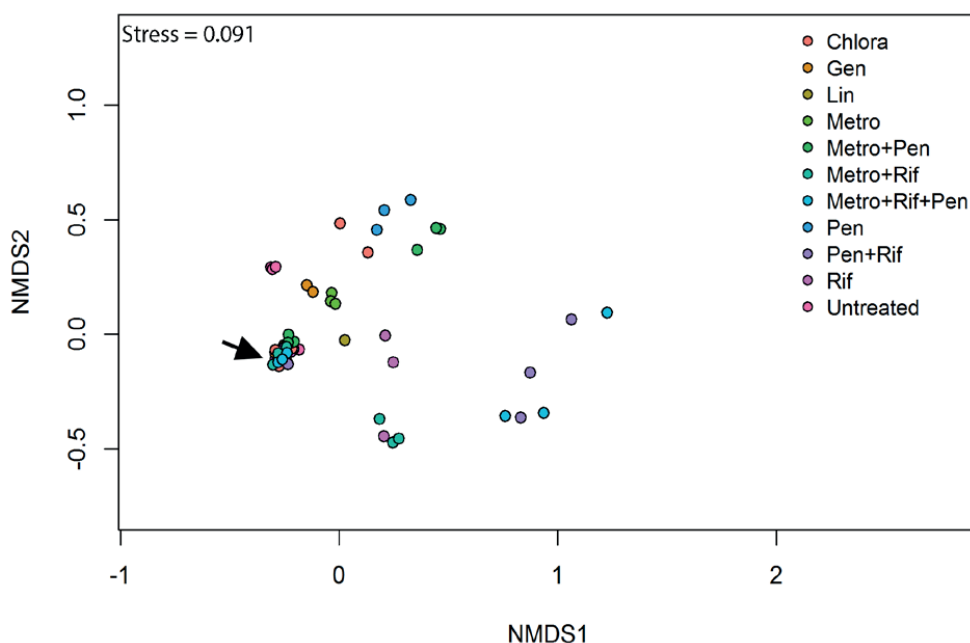
**Table 4.2. Analysis of significance by t-test paired for Richness and Shannon index for each treatment between day 0 and day 10.** Values with an \* mean significantly different in bacterial diversity. Table with full results are presented in Supplementary Table 4.2.

| Treatment | Metro d0  | Metro+Pen d0  | Metro+Rif d0  | Metro+Rif+Pen d0  | Pen d0  | Pen d0      | Pen+Rif d0 | Rif d0        | untreated d0 |
|-----------|-----------|---------------|---------------|-------------------|---------|-------------|------------|---------------|--------------|
| nt        | vs        | vs            | vs            | vs                | vs      | vs          | vs         | vs            | vs           |
|           | Metro d10 | Metro+Pen d10 | Metro+Rif d10 | Metro+Rif+Pen d10 | Pen d10 | Pen+Rif d10 | Rif d10    | untreated d10 |              |
| Richness  | 0.0472*   | 0.0006*       | 0.0649        | 0.0017*           | 0.0117  | 0.0079*     | 0.0250     | 0.1124        |              |
| P-value   |           |               |               |                   | *       | *           | *          |               |              |
| Shannon   | 0.0072*   | 0.0043*       | 0.0339*       | 0.0097*           | 0.0173  | 0.0001*     | 0.0119     | 0.1176        |              |
| P-value   |           |               |               |                   | *       | *           | *          |               |              |

**Table 4.3. Analysis of significance by t-test two-sample for Richness and Shannon index at day 10.** Values with an \* mean significantly different in bacterial diversity. Table with full results are presented in Supplementary Table 4.2.

| Treatment | untreated | untreated | untreated | untreated     | untreated | untreated | untreated | untreated |
|-----------|-----------|-----------|-----------|---------------|-----------|-----------|-----------|-----------|
|           | vs        | vs        | vs        | vs            | vs        | vs        | vs        | vs        |
|           | Metro     | Metro+Pen | Metro+Rif | Metro+Rif+Pen | Pen       | Pen+Rif   | Rif       |           |
| Richness  | 0.0740    | 0.1399    | 0.0751    | 0.0083*       | 0.1688    | 0.0070*   | 0.1363    |           |
| P-value   |           |           |           |               |           |           |           |           |
| Shannon   | 0.1451    | 0.0844    | 0.0925    | 0.0099*       | 0.1571    | 0.0074*   | 0.2469    |           |
| P-value   |           |           |           |               |           |           |           |           |

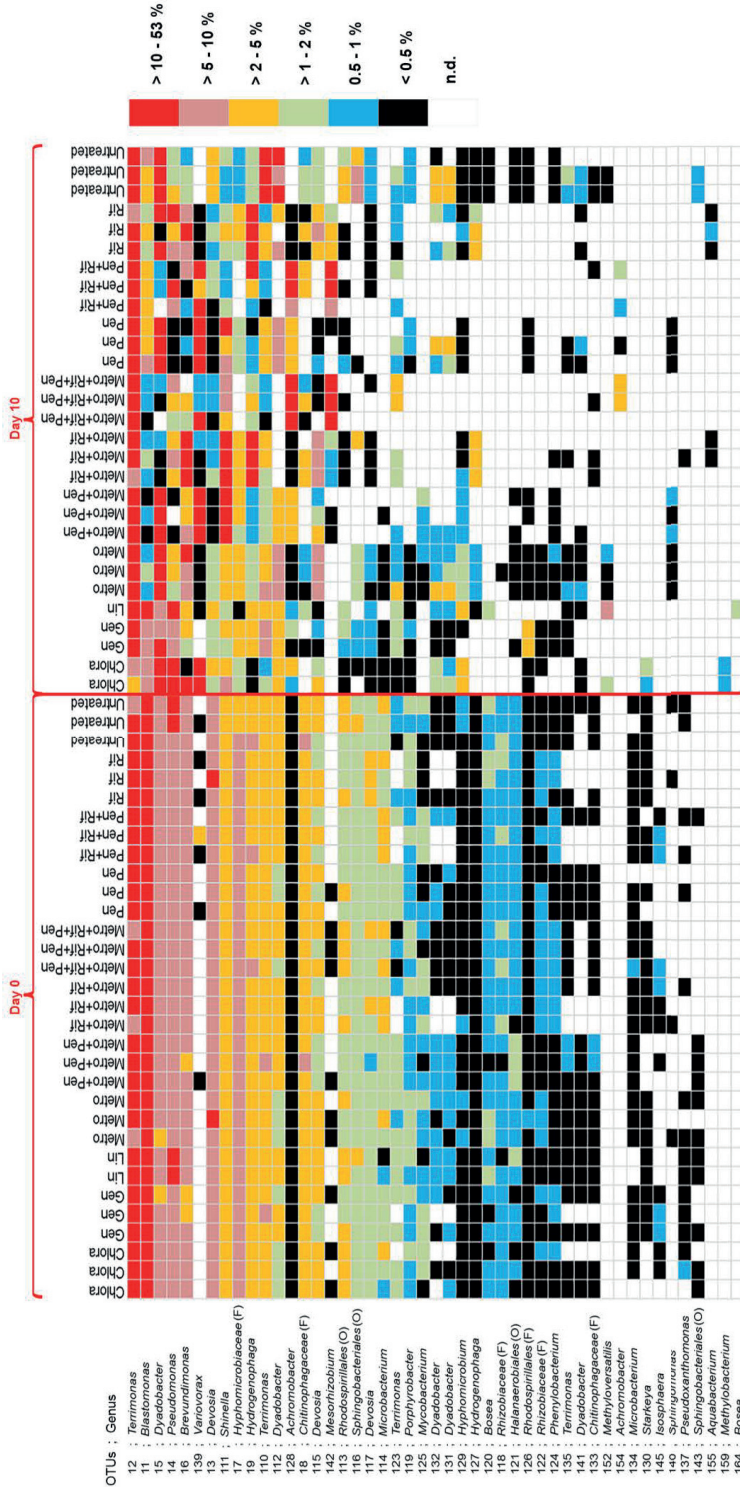
Changes in bacterial community composition were also measured in terms of betadiversity. As with alpha diversity, it was observed that the bacterial communities changed during the 10 day experiment for all treatments including the untreated culture (Fig. 4.3). The bacterial compositions of all cultures at day 0 were clustered together and radiated out after 10 days and mostly clustered by treatment. At day 10 bacterial communities between treatments were significantly different  $P=0.001$  ( $DF=10$ ,  $R^2=0.89$ ). The smallest dissimilarities in the bacterial community composition compared to the untreated control were observed for the *Gen*, *Metro*, *Chlora* and *Lin* treatments, whilst the largest dissimilarities were observed for the *Metro+Rif+Pen* and *Pen+Rif* treatments. Some treatments showed a tendency to cause similar changes in the bacterial community, namely the group with the treatments *Chlora*, *Pen* and *Metro+Pen* and a second group with the treatments *Metro+Rif+Pen* and *Pen+Rif*. The treatments *Rif*, *Metro+Rif+Pen* and *Pen+Rif* showed a larger within treatment variation compared to the other treatments (Supplementary Table 4.3).



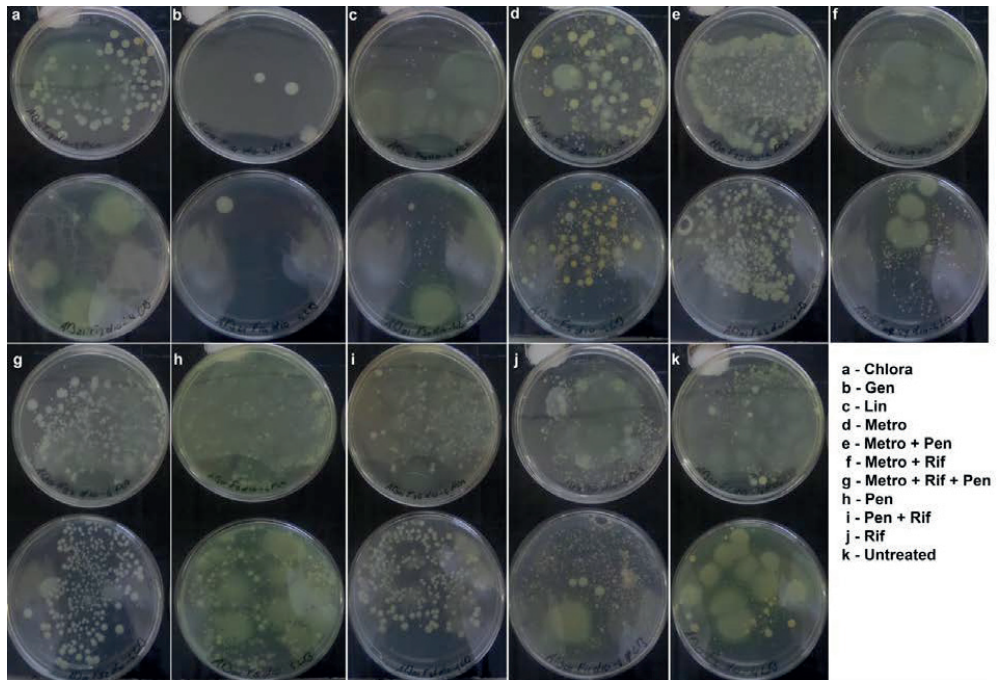
**Fig. 4.3** Non-metric multidimensional scaling (nMDS) ordination (based on Bray-Curtis distance matrix) of 16S rRNA gene sequences from antibiotic treated and untreated *B. braunii* CCALA778. All samples from day 0 are present in the cluster indicated with the black arrow.

Relative abundance changes for individual bacterial species during the antibiotics treatments can be seen in the OTU (Operational Taxonomic Unit) heatmap (Fig. 4.4). Generally, OTUs disappeared during the 10 days antibiotics treatments. However, also for the untreated control changes in the bacterial compositions between day 0 and day 10 could be seen with the disappearance of OTUs (from the top most abundant OTUs to the bottom less abundant OTUs) 128 (*Achromobacter*), 114 (*Microbacterium*), 125 (*Mycobacterium*), 118 (*Rhizobiaceae*) and 122 (*Rhizobiaceae*) from all replicates, but these OTUs did not disappear from all other treatments. Several OTUs i.e. OTU 11 (*Blastomonas*), OTU 15 (*Dyadobacter*), OTU 14 (*Pseudomonas*), OTU 19 (*Hydrogenophaga*), OTU 111 (*Shinella*), OTU 12 (*Terrimonas*) and OTU 110 (*Terrimonas*) could not be eradicated by any of the antibiotics used. However, most OTUs were eradicated by one or more antibiotics. OTUs from day 0 that were consistently eradicated by the use of antibiotics included OTU 17 (*Hyphomicrobiaceae*), OTU 112 (*Dyadobacter*), OTU 18 (*Chitinophagaceae*), OTU 115 (*Devosia*), OTU 113 (*Rhodospirillales*), OTU 116 (*Sphingobacteriales*), OTU 117 (*Devosia*), OTU 119 (*Porphyrobacter*), OTU 129 (*Hyphomicrobium*), OTU 127 (*Hydrogenophaga*), OTU 120 (*Bosea*), OTU 126 (*Rhodospirillales*) and OTU 124 (*Phenylobacterium*) (Supplementary Fig. 4.1 as subset of Fig. 4.4). The most effective antibiotics for removing these OTUs were *Metro+Rif+Pen* and *Pen+Rif*, which removed 9 and 10 out of these 13 OTUs respectively, followed by *Metro+Pen* and *Rif*, which removed 7 and 4 out of 13 OTUs respectively. The least effective antibiotics for removing bacteria were *Metro* and *Gen* which removed only 2 out of 13 OTUs.

The effects of antibiotic treatment on the bacterial community were also observed through bacteria isolated on LB and PCA agar plates (Fig. 4.5a-k). The different treatments showed different colony morphologies. For *Gen* and *Lin*, a low number of bacterial colonies was evident and only two morphologies were visible. In contrast, the untreated CCALA778 showed a high number of colonies and a more diverse number of colony morphologies both on the LB and PCA agar plates. The use of *Pen* led to a different community of cultured bacteria that were characterized by the yellow colouration of a bacterium, which spread like a lawn on the agar plate covering the whole surface (Fig. 4.5h), in contrast to the circular colony forming bacteria most seen on the untreated LB and PCA agar plates (Fig. 4.5k). No quantitative effect of the antibiotics was seen on the bacterial community, except for the treatments where the algae did not grow (*Chlora*, *Lin*, *Gen*) where fewer bacteria were found (Supplementary Fig. 4.2).



**Fig. 4.4 Heatmap of OTU relative abundance at day 0 and day 10 for the different antibiotic treatments.** The heatmap displays only the OTUs of which at least one sample had a relative abundance above 0.5%. OTUs are ordered from higher to lower abundance. Colour code legend on the right represents the relative abundance in percentage. n.d. means no reads detected. Taxonomy classification on the left includes families and orders as the closest classification labelled with (F) and (O) respectively when genus is not assigned.



**Fig. 4.5 Bacterial colony formation on LB and PCA agar plates.** Images taken at day 10 for each treatment using one representative agar plate per treatment. In each figure, top plate is PCA agar and bottom plate is LB agar.

### 4.3 Discussion

The aim of this study was to evaluate the effects of antibiotics on the bacterial community and performance of *B. braunii* CCALA778. In general, both the bacteria richness and diversity of the bacteria decreased for cultures treated with antibiotics, but none of the antibiotics or antibiotic cocktails could entirely eradicate the diverse bacterial community of *B. braunii* (Fig. 4.4). First it is important to note changes in the bacterial community composition that occurred independently of antibiotic treatment. Changes in the bacterial community composition for the untreated samples could be expected as during batch growth, physico chemical properties of the medium change over time, such as availability of nutrients, changes in pH and increase of EPS or other polymeric substances (Ramanan *et al.* 2016). Generally different antibiotics (or antibiotics cocktails) affected the bacterial community composition in a different but reproducible way (Fig. 4.3 and Fig. 4.4) and the mixes of antibiotics appeared to have a larger effect in changing the bacterial community composition compared to antibiotics used individually. With the *Metro+Rif+Pen* and *Pen+Rif* mixes, the largest

changes in BCC were observed. Similar studies investigating the removal of bacteria from microalgae cultures also obtained higher efficiency in removal of bacteria when mixing antibiotics (Lee *et al.* 2015, Molina-Cardenas *et al.* 2016). Antibiotics are known to create synergies when mixed together and therefore it is an active field of research for finding novel concoctions for medicinal applications (Bollenbach 2015). Antibiotic mixes are more important when dealing with Gram-negative bacteria due to their extra outer membrane which is very effective in protecting against antimicrobial agents. Synergies between antibiotics happen when one antibiotic can induce disruptions to the outer membrane facilitating the diffusion of another antibiotic to enter the cell (Miller 2016). The bacteria that were consistently eradicated by the use of antibiotics (Fig. 4.4) were all Gram-negative and most antibiotics used in this study can target both Gram-negative and Gram-positive bacteria.  $\beta$ -Lactams such as penicillin usually create synergies with other antibiotics which can disrupt the outer membrane of Gram-negative bacteria. Once the outer-membrane is disrupted,  $\beta$ -Lactams can diffuse through specific channels called porin and disrupt cell wall synthesis (Delcour 2009, Miller 2016). This is likely the mechanism of bacterial community alterations that occurred with the mixes *Metro+Pen*, *Metro+Rif+Pen* and *Pen+Rif*. Antibiotics that were applied individually were also successful in removing bacteria. For example, the addition of *Pen* led to complete removal of OTUs 18 (*Chitinophagaceae*), 117 (*Devosia*) and 127 (*Hydrogenophaga*), while *Gen* and *Chlora* also removed bacteria such as OTUs 18 (*Chitinophagaceae*), 132 (*Dyadobacter*), 131 (*Dyadobacter*) and OTUs 19 (*Hydrogenophaga*), 18 (*Chitinophagaceae*), 113 (*Rhodospirillales*), 116 (*Sphingobacteriales*), respectively. Other studies also showed similar effects of *Pen*, *Chlora*, and *Gen* for the removal of bacteria from several microalgae cultures or aquatic environments (Lai *et al.* 2009, D'Costa and Anil 2014, Lee *et al.* 2015, Han *et al.* 2016).

Some bacteria, such as OTU 12 (*Terrimonas*) and OTU 11 (*Blastomonas*), OTU 13 (*Devosia*), OTU 111 (*Shinella*) and OTU 19 (*Hydrogenophaga*) were not affected by antibiotics. The reason could be that these bacteria possess genes conferring resistance to antibiotics (Delcour 2009, Yang *et al.* 2013).

Although all antibiotics and concoctions led to changes in the bacterial community composition, not all antibiotics were equally suitable for creating bacterial community changes. One important aspect is the viability of the algae culture after antibiotic treatment. In a normal batch culture, biomass increases over time with an increase of chlorophyll per biomass dry weight as the cultures become light limited, followed by a decline in the



chlorophyll per biomass dry weight as some nutrients like nitrogen become depleted (Li *et al.* 2008, Benavente-Valdés *et al.* 2016). We observed a similar pattern and values for the untreated and Pen-treated cultures (Fig. 4.1b). For *Gen*, *Chlora* and *Lin*, the treatments showed high toxicity to CCALA778 with the concentrations used and with the repeated administration every 3 days. It is unlikely that the removal of certain bacteria due to the antibiotics, were the cause for the lack of growth as other antibiotics also removed the same bacteria as with the *Gen*, *Chlora* and *Lin* treatments. Other studies using *Chlora* also show similar results above concentrations of 50  $\mu\text{g Chlora mL}^{-1}$  for different microalgae species (Lai *et al.* 2009, Molina-Cardenas *et al.* 2016). The toxicity of *Gen* and *Chlora* and *Lin* is probably due to their primary mode of action by inhibiting bacterial protein synthesis through irreversible binding to the 30S ribosomal subunit. These antibiotics may inhibit microalgae as mitochondria and chloroplasts also harbor 30S ribosomal subunits (Manuell *et al.* 2007). The slow decrease of the chlorophyll concentration in the presence of these antibiotics (Fig. 4.1b) suggest this may also be the case for CCALA778.

*Pen* was the only antibiotic that did not hinder growth of CCALA778 showing similar trend in growth as the untreated cultures. *Pen* belongs to the class of  $\beta$ -Lactam antibiotics which specifically inhibit cell wall synthesis by stopping the formation of peptidoglycan cross-links, which is found only in bacteria (Kohanski *et al.* 2010). In addition, penicillin is known as a growth promoter for higher plants such as *Bouvardia ternifolia* (Robert *et al.* 1989), but no significant growth increase was found in our study using *B. braunii*. Another  $\beta$ -Lactam antibiotic is ampicillin (not used in our study) and it has also been shown to promote and not hinder growth of microalgae (Kvíděrová and Henley 2005) suggesting that  $\beta$ -Lactam antibiotics are most suitable for mediating bacterial community changes in microalgae without disruption of algae growth. Also, related to the *Pen*-treatment, was the reduced EPS accumulation when compared to the untreated culture. This results may indicate that changes to the bacterial community of *B. braunii* CCALA778 could have an effect on the EPS accumulation. It is not clear to us at this stage how the changes in the bacterial community changed the EPS accumulation but efforts are underway to look at this question.

The mechanisms behind the effects of *Metro* on (eukaryotic) microalgae cells are still poorly understood, although growth inhibition has been reported (Kolodziejska *et al.* 2013). In anaerobic prokaryotes and protozoa this antibiotic is partially reduced to its active form. In this form it can covalently bind DNA, blocking the transcription (Lanzky and Halting-

Sørensen 1997, Kolodziejaska *et al.* 2013). This could explain why we see growth suppression in the CCALA778 cultures treated with *Metro*.

*Rif* inhibits prokaryotic DNA-dependent RNA polymerase and mitochondria and chloroplasts also use this type of polymerase. However, research on rat liver cells, suggested that in vivo mitochondria are normally not permeable for *Rif* to penetrate and thus transcription of mitochondrial DNA is not effected (Gadaleta *et al.* 1970). Surzycki (1969) reported that chloroplasts from *Chlamydomonas reinhardi* are affected by *Rif*. This might indicate the chloroplast membrane is permeable enough for *Rif* to block the RNA polymerase inside chloroplasts. Transcription and translation of most genes and proteins involved in biosynthesis of the photosynthetic machinery (including chlorophyll) would not be affected, because these genes are encoded on the nuclear genome of the alga (Kobayashi *et al.* 2014). Some core photosynthetic proteins, however, are located on the plastid-genome. If biosynthesis of these proteins is stopped by *Rif*, it may disrupt photosynthesis. This causes *B. braunii* cells to only grow heterotrophically during which the non-functioning chloroplast is still replicated and thus no changes in chlorophyll levels were noticed (Tanoi *et al.* 2011, Im *et al.* 2012).

Although in this study we were not able to make axenic *B. braunii* cultures by using antibiotics, its application may still be important in further attempts to create an axenic *B. braunii* CCALA778 culture, due to the availability of yet other antibiotics and different combinations of antibiotics. *Chlamydomonas reinhardtii* was successfully treated with an antibiotic cocktail making it bacteria-free (Kan and Pan 2010). It is likely that the bacteria removed successfully by antibiotic treatment in this study can equally be removed from other microalgae cultures although the antibiotic effects on the microalgae growth and culture viability could be different, also because different microalgal species harbor different bacterial communities (Ramanan *et al.* 2015). Other methods for creating axenic microalgae cultures include treatments such as, differential centrifugation, filtration, UV treatment, the use of detergents and repeated plating, all of which have previously been successfully applied (Shishlyannikov *et al.* 2011, Lee *et al.* 2015). In addition, a combination of the different methods thereof offer substantial opportunities to modify bacterial communities associated to microalgae in a desired direction for improving microalgae biomass cultivation and or product formation.

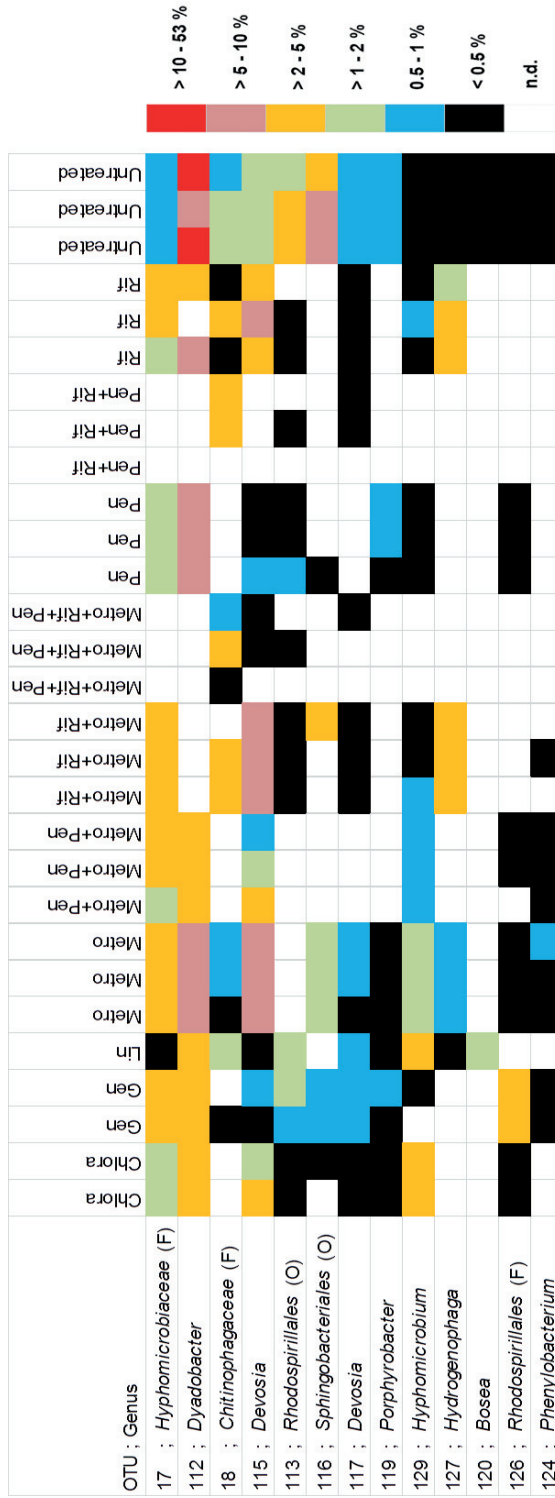
#### 4.4 Conclusion

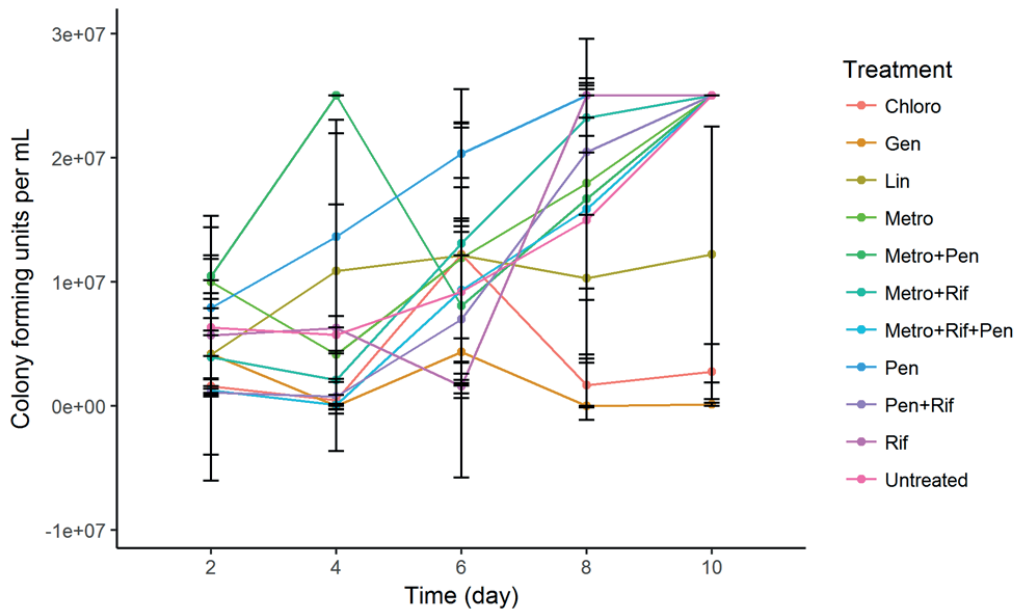
Antibiotic treatments have antibiotic-specific effects on the bacterial community of *B. braunii* CCALA778, which was dominated by Gram-negative bacteria, but they are unlikely to lead to axenic microalgae cultures when used as the only treatment when the bacterial diversity and abundance is high. Most antibiotics used in this study led to inhibition of *B. braunii* CCALA778 growth, but in most cases growth was resumed after the supplementation with antibiotics was stopped. Only *Gen*, *Lin* and *Chlora* showed a strong toxicity towards CCALA778 and cultures lost viability. The most effective antibiotic concoctions for the removal of bacteria associated to CCALA778 were *Metro+Rif+Pen* and *Pen+Rif*.

#### Acknowledgments

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Supplementary Fig. 4.1. Subset of heatmap Fig. 4.4 illustrating the OTUs consistently eradicated by one or more antibiotics





**Supplementary Fig. 4.2. Bacterial colony counts on agar plates.** LB and PCA agar triplicate plates were incubated at 25 degrees Celsius for three days for the treatment samples taken between day 2 and day 10. Numbers shown are averages of the six agar plates and error bars indicate standard deviation. Only *Gen*, *Chlora* and *Lin* Colony Forming Units (CFUs) at day 10, can be reliably determined with the dilution used. The remaining treatments CFU count was over the maximum CFU count and therefore cannot be accurately determined.

**Supplementary Table 4.1.** Diversity index values for Richness and Shannon index for all treatments at day 0 and 10.

| Treatment | Day | Richness | Shannon | Treatment     | Day | Richness | Shannon |
|-----------|-----|----------|---------|---------------|-----|----------|---------|
| Untreated | 0   | 36       | 3.3587  | Chlora        | 0   | 37       | 3.38    |
| Untreated | 0   | 37       | 3.3735  | Chlora        | 0   | 45       | 3.5533  |
| Untreated | 0   | 39       | 3.4364  | Chlora        | 0   | 36       | 3.3382  |
| Untreated | 10  | 24       | 2.8529  | Chlora        | 10  | 29       | 3.1321  |
| Untreated | 10  | 31       | 3.1602  | Chlora        | 10  | 30       | 3.1195  |
| Untreated | 10  | 31       | 3.1572  | Lin           | 0   | 38       | 3.4166  |
| Metro     | 0   | 41       | 3.4723  | Lin           | 0   | 38       | 3.4035  |
| Metro     | 0   | 38       | 3.4185  | Lin           | 10  | 29       | 3.1301  |
| Metro     | 0   | 40       | 3.4578  | Metro+Pen     | 0   | 39       | 3.4357  |
| Metro     | 10  | 34       | 3.2534  | Metro+Pen     | 0   | 37       | 3.3918  |
| Metro     | 10  | 35       | 3.2452  | Metro+Pen     | 0   | 37       | 3.399   |
| Metro     | 10  | 34       | 3.226   | Metro+Pen     | 10  | 26       | 2.9006  |
| Pen       | 0   | 37       | 3.3999  | Metro+Pen     | 10  | 23       | 2.7305  |
| Pen       | 0   | 41       | 3.4641  | Metro+Pen     | 10  | 23       | 2.749   |
| Pen       | 0   | 35       | 3.3383  | Pen+Rif       | 0   | 34       | 3.3052  |
| Pen       | 10  | 26       | 2.9197  | Pen+Rif       | 0   | 33       | 3.3237  |
| Pen       | 10  | 25       | 2.8932  | Pen+Rif       | 0   | 39       | 3.4526  |
| Pen       | 10  | 20       | 2.5853  | Pen+Rif       | 10  | 17       | 2.4548  |
| Rif       | 0   | 37       | 3.3578  | Pen+Rif       | 10  | 16       | 2.4543  |
| Rif       | 0   | 33       | 3.2742  | Pen+Rif       | 10  | 17       | 2.5867  |
| Rif       | 0   | 32       | 3.2645  | Metro+Rif     | 0   | 34       | 3.3222  |
| Rif       | 10  | 25       | 2.9507  | Metro+Rif     | 0   | 31       | 3.2412  |
| Rif       | 10  | 21       | 2.8134  | Metro+Rif     | 0   | 37       | 3.3949  |
| Rif       | 10  | 25       | 2.9517  | Metro+Rif     | 10  | 21       | 2.7674  |
| Gen       | 0   | 41       | 3.4835  | Metro+Rif     | 10  | 25       | 2.9091  |
| Gen       | 0   | 34       | 3.2915  | Metro+Rif     | 10  | 20       | 2.7308  |
| Gen       | 0   | 41       | 3.4695  | Metro+Rif+Pen | 0   | 40       | 3.4553  |
| Gen       | 10  | 27       | 2.9814  | Metro+Rif+Pen | 0   | 38       | 3.3917  |
| Gen       | 10  | 26       | 2.9957  | Metro+Rif+Pen | 0   | 38       | 3.4008  |
|           |     |          |         | Metro+Rif+Pen | 10  | 17       | 2.3479  |
|           |     |          |         | Metro+Rif+Pen | 10  | 18       | 2.6093  |
|           |     |          |         | Metro+Rif+Pen | 10  | 16       | 2.4521  |

**Supplementary Table 4.2.** T-test results.

| <b>Student t-test Paired-Samples between day 0 and 10</b> |                 |               |                       |                      |               |                       |
|-----------------------------------------------------------|-----------------|---------------|-----------------------|----------------------|---------------|-----------------------|
| <b>Treatment Pairs</b>                                    | <b>Richness</b> |               |                       | <b>Shannon Index</b> |               |                       |
|                                                           | <b>df</b>       | <b>t stat</b> | <b>p value (0.05)</b> | <b>df</b>            | <b>t stat</b> | <b>p value (0.05)</b> |
| untreated 0 - untreated 10                                | 2               | 2.726         | 0.11237               | 2                    | 2.652         | 0.11760               |
| Metro 0 - Metro 10                                        | 2               | 4.438         | 0.04721               | 2                    | 11.733        | 0.00719               |
| Pen 0 - Pen 10                                            | 2               | 9.165         | 0.01170               | 2                    | 7.497         | 0.01733               |
| Rif 0 - Rif 10                                            | 2               | 6.200         | 0.02504               | 2                    | 9.095         | 0.01188               |
| Metro+Pen 0 - Metro+Pen 10                                | 2               | 41.000        | 0.00059               | 2                    | 15.271        | 0.00426               |
| Pen+Rif 0 - Pen+Rif 10                                    | 2               | 11.200        | 0.00788               | 2                    | 147.432       | 0.00005               |
| Metro+Rif 0 - Metro+Rif 10                                | 2               | 3.733         | 0.06486               | 2                    | 5.290         | 0.03392               |
| Metro+Rif+Pen 0 - Metro+Rif+Pen 10                        | 2               | 24.568        | 0.00165               | 2                    | 10.082        | 0.00970               |

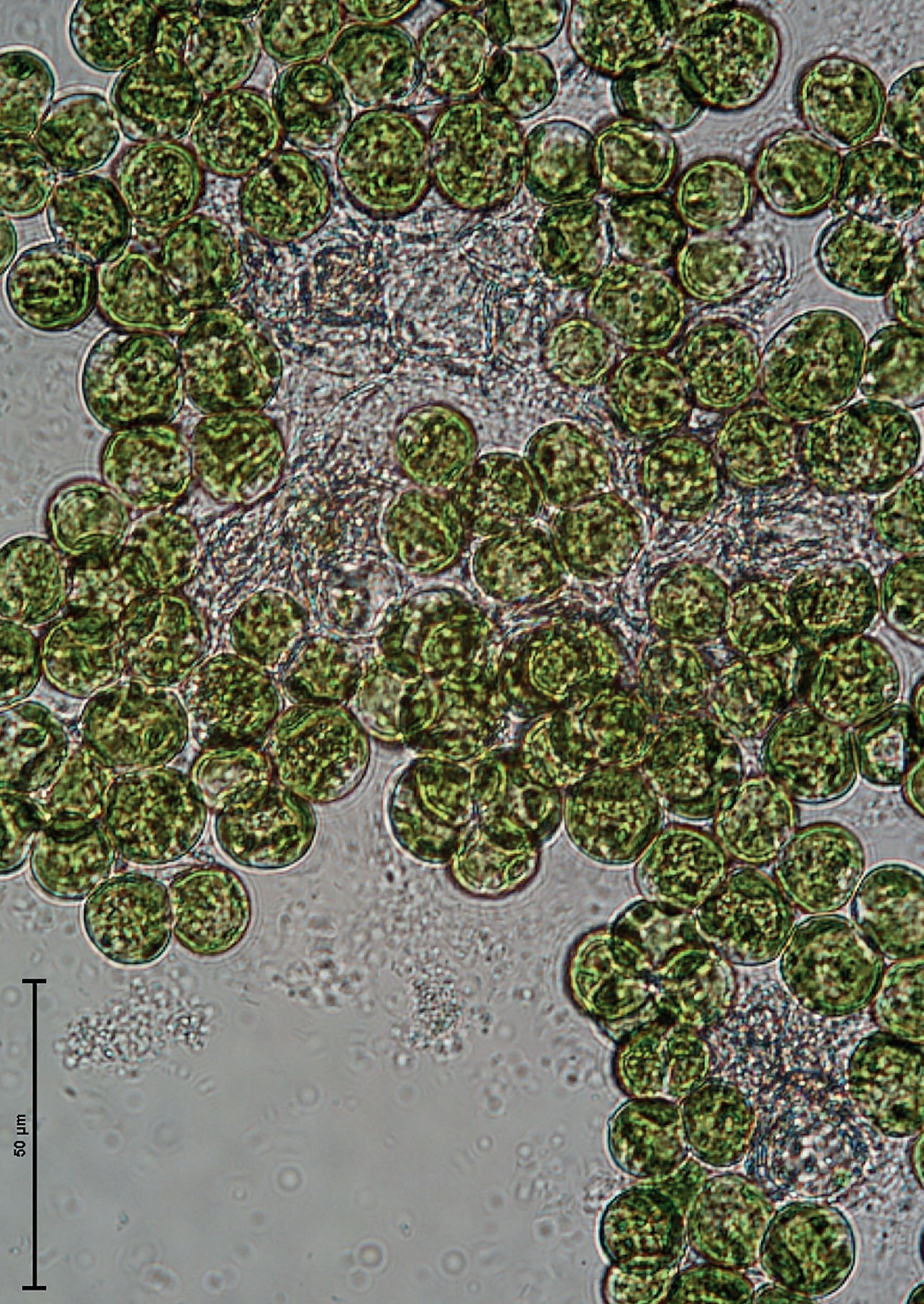
| <b>Student t-Test Two-Samples between untreated and antibiotic treatments at day 10</b> |                 |               |                       |                      |               |                       |
|-----------------------------------------------------------------------------------------|-----------------|---------------|-----------------------|----------------------|---------------|-----------------------|
| <b>Treatment Samples</b>                                                                | <b>Richness</b> |               |                       | <b>Shannon Index</b> |               |                       |
|                                                                                         | <b>df</b>       | <b>t stat</b> | <b>p value (0.05)</b> | <b>df</b>            | <b>t stat</b> | <b>p value (0.05)</b> |
| untreated 10 - Metro 10                                                                 | 2               | -2.404        | 0.07402               | 2                    | -1.807        | 0.14508               |
| untreated 10 - Pen 10                                                                   | 2               | 1.677         | 0.16884               | 2                    | 1.738         | 0.15711               |
| untreated 10 - Rif 10                                                                   | 2               | 1.861         | 0.13632               | 2                    | 1.355         | 0.24689               |
| untreated 10 - Metro+Pen 10                                                             | 2               | 1.838         | 0.13988               | 2                    | 2.284         | 0.08438               |
| untreated 10 - Pen+Rif 10                                                               | 2               | 5.091         | 0.00703               | 2                    | 5.027         | 0.00735               |
| untreated 10 - Metro+Rif 10                                                             | 2               | 2.390         | 0.07513               | 2                    | 2.201         | 0.09255               |
| untreated 10 - Metro+Rif+Pen 10                                                         | 2               | 4.854         | 0.00832               | 2                    | 4.617         | 0.00990               |

**Supplementary Table 4.3. Homogeneity of multivariate dispersions for each treatment at day 0 and 10.** Average distance mean calculated with spatial mean using function "betadisper" from vegan package. nd means not determined.

| Treatment - Day      | Average distance to the mean | Replicate # |
|----------------------|------------------------------|-------------|
| Untreated - 0        | 0.050                        | 3           |
| Metronidazole - 0    | 0.031                        | 3           |
| Penicillin - 0       | 0.025                        | 3           |
| Rifampicin - 0       | 0.037                        | 3           |
| Gentamycin - 0       | 0.052                        | 3           |
| Chloramphenicol - 0  | 0.060                        | 3           |
| Linezolid - 0        | 0.016                        | 2           |
| Metro+Pen - 0        | 0.031                        | 3           |
| Pen+Rif - 0          | 0.046                        | 3           |
| Metro+Rif - 0        | 0.045                        | 3           |
| Metro+Rif+Pen - 0    | 0.026                        | 3           |
| Untreated - 10       | 0.056                        | 3           |
| Metronidazole - 10   | 0.045                        | 3           |
| Penicillin - 10      | 0.069                        | 3           |
| Rifampicin - 10      | 0.131                        | 3           |
| Gentamycin - 10      | 0.031                        | 2           |
| Chloramphenicol - 10 | 0.070                        | 2           |
| Linezolid - 10       | nd                           | 1           |
| Metro+Pen - 10       | 0.061                        | 3           |
| Pen+Rif - 10         | 0.165                        | 3           |
| Metro+Rif - 10       | 0.071                        | 3           |
| Metro+Rif+Pen - 10   | 0.149                        | 3           |







50  $\mu\text{m}$

# Effect of removal of bacteria by UVC treatment on the productivity of *Botryococcus braunii* capable of producing exopolysaccharides

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## Chapter 5

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Submitted to Algal Research

**Abstract**

*B. braunii* CCALA778 is a green microalga that can produce large amounts of extracellular carbohydrates and therefore is a potential host for industrial applications such as materials, food and pharmaceutical products. The downside of *B. braunii* is its slow growth and therefore, improvements on the biomass productivity or carbohydrate production will make this microalgae more attractive for industrial exploitation. During cultivation microalgae are normally in the presence of bacteria and these can be beneficial or antagonistic. The role or effects of bacteria present in *B. braunii* CCALA778 are not yet fully elucidated. We used UV-C treatment to reduce bacterial abundance present in *B. braunii* CCALA778 and 16S rRNA gene amplicon MiSeq sequencing for bacterial community analysis. The effect of reduced bacteria on the biomass growth and production of carbohydrates was analysed. It is shown that by UV-C treatment the bacterial population could be removed without harming *B. braunii*. *B. braunii* CCALA778 does not need bacteria present to grow or to produce exopolysaccharides (EPS). Bacteria were antagonistic to *B. braunii* CCALA778 as it reduced its biomass growth and extracellular carbohydrates significantly. CCALA778 with less than 1 % relative abundance of bacteria accumulated  $826 \pm 61 \text{ mgL}^{-1}$  of EPS by day 15 compared to  $422 \pm 135 \text{ mgL}^{-1}$  accumulated EPS in the untreated culture.

**Keywords:** *Botryococcus braunii*, Ultra Violet-C light, bacteria, exopolysaccharides, microalgae

## 5 Introduction

*Botryococcus braunii* (*B. braunii*) is a green microalga that can produce long chain hydrocarbons or extracellular carbohydrates (termed here exopolysaccharides) depending on the strain used (Fernandes *et al.* 1989, Allard and Casadevall 1990, Banerjee *et al.* 2002, Metzger and Largeau 2005, Gouveia *et al.* 2017). *B. braunii* is considered a potential candidate for industrial applications and production of biofuels. Hydrocarbons derived from microalgae can be hydrocracked and transformed into aviation turbine fuel (Hillen *et al.* 1982). Exopolysaccharides (EPS) have a range of industrial uses, including thickeners, stabilisers and gelling agents in food products, as well as in the pharmaceutical and cosmeceutical industries (Buono *et al.* 2012, Donot *et al.* 2012, Borowitzka 2013).

*B. braunii* is a colony forming microalga found in fresh and brackish water across the world. Depending on what type of hydrocarbons are produced, *B. braunii* is subclassified into four chemical races, designated A, B, L (Metzger and Largeau 2005), and S (Kawachi *et al.* 2012). Some strains of *B. braunii* can produce up to 86 % hydrocarbons on biomass dry weight basis (Brown *et al.* 1969), whereas the carbohydrate producing strains can produce up to 4.5 g L<sup>-1</sup> of EPS into the medium (Fernandes *et al.* 1989, Lupi *et al.* 1994, Dayananda *et al.* 2007, Díaz Bayona and Garcés 2014). The EPS from *B. braunii* is mainly composed of galactose and minor quantities of other sugars as fucose, rhamnose, glucose and uronic acids residues (Fernandes *et al.* 1989, Allard and Casadevall 1990, Díaz Bayona and Garcés 2014). The drawback of *B. braunii* is that it is a slow growing microorganisms and therefore it is not currently attractive for industrial applications (Cabanelas *et al.* 2015).

Bacteria which are present with the hydrocarbon producing strains of *B. braunii*, have been shown to both enhance or antagonize the biomass productivity and hydrocarbon production (Chirac *et al.* 1985, Tanabe *et al.* 2012). The early work from Chirac and colleagues in which they described the presence of *Pseudomonas* sp. and *Flavobacterium* sp. in two strains of *B. braunii* concluded that bacteria were not essential for the production of hydrocarbons although it was shown that numerous bacteria could exert both beneficial or antagonistic influence on the growth yields and hydrocarbon production (Chirac *et al.* 1982). A more recent study using *B. braunii* Ba10 strain revealed the presence of rod shaped bacteria in the rim of the colony aggregations and it was proposed as a growth promoting bacterium closely related to *Hyphomonadaceae* spp. (Tanabe *et al.* 2015). Few studies have looked at the role of bacteria on the exopolysaccharide producing strains and although bacteria were observed growing in association with the growth of *B. braunii* ACOI58, no conclusions were drawn

from this study (Fernandes *et al.* 1989). Lupi and colleagues (1991) suggested that EPS is produced by *B. braunii* based on similar sugar composition of the strain used in their study compared to several other strains of *B. braunii*, in which galactose and fucose were detected as main components. It is therefore not clear what is the role of bacteria with the *B. braunii* strains which produce exopolysaccharides. Questions such as, are the bacteria important for the growth of *B. braunii*, or do the bacteria exert any effects on the EPS produced by *B. braunii* still need to be elucidated. The objective of this study is to understand the role of bacteria on the growth and on the production of EPS of *B. braunii* CCALA778 strain.

To study the role or effects of bacteria present in microalgae cultures, it is necessary to remove them. Several approaches are commonly used such as, centrifugation, washing and sieving methods, and the use of antibiotics (Bruckner and Kroth 2009, Lee *et al.* 2015, Han *et al.* 2016). Another method of sterilization commonly used in environmental technology, medical practices and pharmaceutical industry is the use of UV (Schmidt and Kauling 2007, Carney and Lane 2014, Yen *et al.* 2014). Ultra-violet light C (UVC) which is in the range of 200 – 280 nm, damages the genetic material in the nucleus especially the range between 250 – 270 nm which is strongly absorbed by nucleic acids (Dai *et al.* 2012). Studies in progress to test the treatment of infectious diseases with UVC, show promising results in killing prokaryotic microorganisms while keeping eukaryotic mammalian cells viable. It was found that the UVC doses necessary to damage mammalian cells were ten times higher than the doses necessary to inactivate *E. coli* (which is considered to be one of the most resistant species to UV). UV exposure has also been successfully used to control bacterial abundance with microalgae such *Tetraselmis* sp. (Doud *et al.* 2014, Sharma *et al.* 2014).

In this study we used batch cultures of *B. braunii* CCALA778 and investigated the effects of bacteria on biomass productivity and on the production of total carbohydrates and the EPS fraction. UVC treatment was used to reduce the bacterial abundance of CCALA778 and comparisons were made to the untreated CCALA778. 16S rRNA gene analysis was used to determine the bacterial relative abundance, as well as to assess the efficiency of UVC method in reducing the bacterial present in CCALA778. Biomass dry weight, colony size and total carbohydrates were measured as well as carbohydrate composition.

## 5.1 Materials and Methods

### 5.1.1 Strain and media preparation

*B. braunii* CCALA778 strain was obtained from the Culture Collection of Autotrophic Organisms (Třebon, Czech Republic) and transferred to Erlenmeyer flasks with modified CHU 13 medium as described previously (Gouveia *et al.* 2017). NaHCO<sub>3</sub> was added to a final concentration of 5 mM.

### 5.1.2 UVC treatment

#### *UV treated*

*B. braunii* CCALA778 cells were plated on square modified CHU 13 agar plates after dilution to  $4.4 \cdot 10^3$  -  $4.4 \cdot 10^5$  cells/plate. Cells were exposed to UVC dose of 82,500  $\mu\text{J cm}^{-2}$  (15-20 % survival) using the CL1000 Ultraviolet Crosslinker. After a 24 h incubation in dark, the plates were transferred in a 25 °C incubator with 50  $\mu\text{mol photon m}^{-2} \text{sec}^{-1}$  light. After 3 – 4 weeks colonies without a visible bacterial aura around, were resuspended in 5  $\mu\text{L}$  modified CHU 13 medium and spotted on modified CHU 13 agar plates. After 3 weeks colonies without visible bacterial contamination were transferred to a new modified CHU 13 agar plate. Last step was repeated one more time before selected colonies were resuspended in 200  $\mu\text{L}$  liquid modified CHU 13 medium and used as an inoculum for growth. Hereafter, the UV treated CCALA778 was kept for six months with identical cultivation parameters as described in the experimental design and subcultured every 2 to 4 weeks before being used in this experiment.

#### *UV-treated with added bacteria (UV+Bac)*

Preparation of the UV+Bac treated CCALA778 was prepared in the following way: First bacteria were isolated from the untreated CCALA778 onto Lysogeny Broth agar (LB). Briefly, 100 $\mu\text{L}$  of  $10^4$  times diluted culture volume was pipetted onto LB petridish in sterile conditions inside a flow cabinet. Plates were incubated at room temperature for three days before storing at 4 °C. At the start of the experiment (day 0), 3 mL of Modified CHU 13 medium was pipetted onto the petri dish containing the bacteria and mixed thoroughly with a sterile rod. Next, approximately 2 mL were pipetted and added to an Erlenmeyer flask containing approximately 120 mL of UV treated CCALA778.

### 5.1.3 Experimental design

UV treated, UV+Bac and untreated CCALA778 culture flasks were prepared with actively growing cells, such that the initial absorbance at 680 nm was 0.2. The Erlenmeyer flasks were

capped with aeraseal sterile film (Alphalabs) to allow gas exchange at the same time it maintained the flask sterility. The experiment was conducted in triplicate and samples taken at day 0, 3, 6, 9, 12 and 15. Cultivation parameters were set as previously described (Gouveia *et al.* 2017).

#### 5.1.4 Biomass dry weight

Five mL aliquots of culture broth were filtered onto pre-weighed GF/D glass-fibre membranes (Whatman). The GF/D filters were dried at 100 °C for 24 hours and weighted, and biomass amount was determined by subtraction.

#### 5.1.5 Extracellular carbohydrate (exopolysaccharide content)

The extracellular carbohydrate content, termed here as exopolysaccharide, was determined using the method first reported by Dubois (DuBois *et al.* 1956). In brief, 1000 µL culture broth was centrifuged for 3 min at 21,000 x g to pellet cells, and 500 µL of the supernatant was carefully transferred to an Eppendorf tube. The supernatant was lyophilized using a Sublimator 2x3x3 from ZIRBUS technology during 28 hours and stored at -20 °C until further analysis. The lyophilized sample were subsequently hydrolyzed by adding 500 µL of 2.5M HCl and incubated for 3 h while mixing hourly, and neutralized thereafter by adding 500 µL of 2.5M NaOH solution. A centrifugation step of 1 min at 21,000 x g was done to pellet potential bacterial cell debris and the hydrolyzed sample was stored at -20 °C. For analysing the exopolysaccharide content, 50 µL of the stored hydrolyzed sample was added to 450 µL demineralized water. Gently, 500 µL 5% phenol in water solution was pipetted into the tube followed by 2.5 mL of 96 % concentrated sulphuric acid added directly onto the liquid surface, and the mixture was incubated at room temperature for 10 minutes. Hereafter, the tube was placed in a 35 °C water bath for 30 min, while mixing every 5 min. Subsequently, sample was left at room temperature for 5 min prior to absorbance measurement. A dilution series of a standard stock solution, which contained a total of 1 g L<sup>-1</sup> monosaccharides, with a ratio of 8/15 D-galactose, 4/15 D-fucose and 3/15 D-glucose was prepared as these monosugars are the main constituents of *B. braunii* CCALA778 carbohydrates (Gouveia *et al.* 2017). The absorbance of the standards and samples was measured at 486 nm and the exopolysaccharides content determined.

#### 5.1.6 Total carbohydrate content and composition

Hydrolysis of algae samples was performed as described previously by Gouveia *et al.* (2017). The sugar composition was determined by high performance anion exchange chromatography



(HPAEC) using an ICS-3000 Ion Chromatography HPLC system equipped with a CarboPac PA-1 column (250 × 2 mm) in combination with a CarboPac PA guard column (25 × 2 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, USA) at 20 °C as reported by Gilbert-López *et al.* (2017). Elution was also performed at 28 °C for separation of glucose and glucosamine.

### 5.1.7 Colony size measurements

3 mL of culture broth for all time points were harvested for colony size measurements using Mastersizer 2000 (Malvern Instruments) with the following settings: Particle Refractive Index of 1.5, Dispersant Refractive Index of 1.33 and Obscuration levels set for 1 to 2 %. Volume of culture broth used was dependent on biomass concentration at the different time points. Size of colonies are expressed in Volume Weighted Mean  $D[4,3] = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$ , where  $n_i$  is the number of particles with diameter  $d_i$ .

### 5.1.8 Confocal imaging

Cultures of algae growing in log phase were stained for DNA presence and analysed with confocal microscopy to visualize algal nuclei, but also checked for bacterial presence. A comparison was made for 9 days grown UV treated, UV+Bac and untreated CCALA778 cultures. DNA staining was performed by incubating cells in growth medium for > 5 minutes in a mixture with a final concentration of 2 µg/ml DAPI in fixative. Staining was achieved in the presence of 2 % para-formaldehyde and 0.025% glutardialdehyde as fixatives combined with 0.01% Triton X-100 to allow dye penetration through extracellular EPS and into cells. Thin coverslip-slide mountings were made of the cells. Confocal images were obtained with a Zeiss Confocal Laser Scanning Microscope 510 with a Axiovert 200M equipped with a 63x 1.4 NA Plan-Apo objective. DAPI was excited with an Ar diode laser of 405 nm (30 mW) at 10% and emission was collected at BP 420-490 nm. Corresponding bright field images were omitted, representative selected images were increased in image resolution and FL-brightness (+10-15%), and aligned in Photoshop 2015CC.

### 5.1.9 DNA extraction and 16S rRNA gene amplification

On day 0 and day 15, 5 mL of liquid culture broth was sampled from all Erlenmeyer flasks and stored at -80 °C. Sample was spun down at 4816 x g for 5 min and the supernatant was discarded. Next, 5 mL of sterile dH<sub>2</sub>O was added and centrifugation step repeated followed by the removal of the supernatant to get rid of the EPS that obstructed DNA extraction. The pellet was resuspended in 1 mL S.T.A.R buffer (Roche,USA) and transferred to a 2 ml

sterilized tube with 0.1 mm zirconia/silica beads (Biospecs) and 5 glass beads of 3 mm. Cells were subjected to three rounds of homogenization of 60 seconds at a speed of 5500 rpm with Precellys Beadbeater (Bertin Technologies). After, sample was heated for 5 min at 95 °C and 300 rpm to help break down any polymeric substances around the colony matrix of CCALA778. After the heating step, sample was centrifuged for 5 min at 21 000 x g at +4 °C. Then, DNA was extracted using the Maxwell 16 Tissue LEV Total RNA purification kit (Promega, USA) with aid of a Maxwell 16 instrument (Promega, USA). The purity and quantity of DNA were examined by electrophoresis on a 1% agarose gel and measured with a Nanodrop (ND1000, Thermo Fisher Scientific Inc.). The extracted DNA was stored at -20 °C until further use.

Amplicons of size 350 base pairs from the V1-V2 region of 16S rRNA genes were generated by a two-step PCR strategy. For the first step, forward primer 27F-DegS (5'GTTYGATYMTGGCTCAG 3' where M = A or C; R = A or G; W = A or T; Y = C or T) and an equimolar mixture of reverse primers 338R I (5'GCWGCCTCCCGTAGGAGT 3') and 338R II (5' GCWGCCACCCGTAGGTGT 3' where M = A or C; R = A or G; W = A or T; Y = C or T) were used (Daims *et al.* 1999). Eighteen bp Universal Tags 1 and 2 (Unitag1= GAGCCGTAGCCAGTCTGC; Unitag2= GCCGTGACCGTGACATCG) were appended at the 5' end of the forward and reverse primers, respectively (Daims *et al.* 1999, van den Bogert *et al.* 2011, Tian *et al.* 2016). The first PCR mix (50 µL) contained 10 µL 5× HF buffer (Thermo Scientific™, the Netherlands), 1 µL dNTP Mix (10 mM; Promega, Leiden, the Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific), 1 µM of Unitag1-27F-DegS forward primer, 1 µM of Unitag2-338R I and II reverse primers, 10 µL template DNA and 27.5 µL nuclease free water. Amplification included an initial denaturation at 98°C for 30 sec; 25 cycles of denaturation at 98°C for 10 sec; annealing at 56°C for 20 sec and elongation at 72°C for 20 sec; and a final extension at 72°C for 10 min. The PCR product size was examined by 1 % gel electrophoresis. The second PCR mix (100 µL) contained 62 µL nuclease free water, 5 µL of PCR1 product, 20 µL 5× HF buffer, 2 µL dNTP Mix, 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward primer (Unitag1) and reverse primer (Unitag2) appended with an 8 nt sample specific barcode for each sample. Amplification included an initial denaturation at 98°C for 30 sec; 5 cycles of denaturation at 98°C for 10 sec, annealing at 52°C for 20 sec and elongation at 72°C for 20 sec and a final extension at 72°C for 10 min. The concentration of PCR products was quantified with a Qubit Fluorometer (Life Technologies, Darmstadt,

Germany) in combination with the dsDNA BR Assay kit (Invitrogen, Carlsbad, CA, USA). Purified products were then pooled in equimolar amounts of 134 ng  $\mu\text{L}^{-1}$  and sequenced on a MiSeq platform (GATC-Biotech, Konstanz, Germany).

#### 5.1.10 Processing of MiSeq data

Illumina sequencing data were analyzed using NG-Tax pipeline (Ramiro-Garcia *et al.* 2016) yielding a total of 1768266 sequence. Briefly, in a first step, paired-end reads of 2 x 100 nucleotides were combined and only read pairs with perfectly matching primers and barcodes were retained. Demultiplexing, Operational Taxonomic Unit (OTU) picking, chimera removal and taxonomic assignment were performed within one single step using the *OTU\_picking\_pair\_end\_read* script in NG-Tax. Sequences were clustered to OTUs at the 98.5% identity level, and an OTU table was created with the most abundant sequences at a threshold of 0.1% minimum relative abundance of each sample. Taxonomic assignment of the OTUs was executed using the UCLUST algorithm (Edgar 2010) and the Silva\_126\_SSU Ref database, containing 1,675,819 unique full length 16S rRNA gene sequences. The 16S rRNA gene dataset obtained in this study is deposited in the Sequence Read Archive (SRA), NCBI with accession number SRP107368.

#### 5.1.11 Microbial community analyses

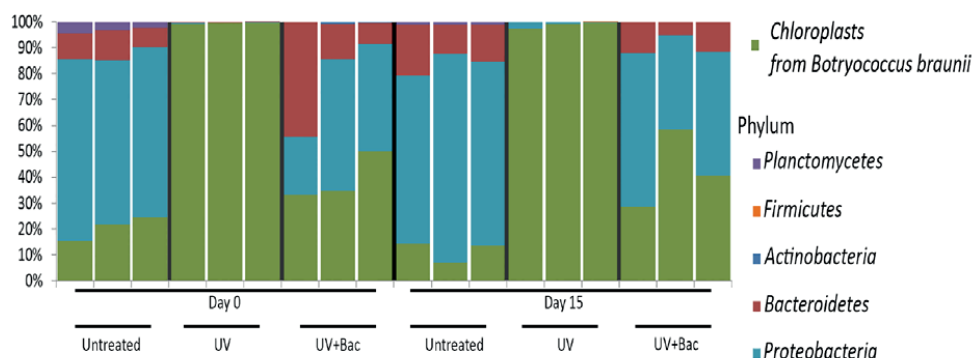
For the interpretation of the microbial community data on phylum level, the Operational Taxonomic Unit (OTU) abundance table was converted to relative abundance and visualized as a bar chart using Microsoft Office Excel 2010. OTUs assigned to chloroplasts are grouped together under chloroplasts from *Botryococcus braunii*.

#### 5.1.12 Statistical analysis

An univariate analysis of variance (one-way ANOVA) was carried out to compare the biomass dry weight at day 12 and day 15 and to compare the average colony size from day 0 to day 15 for the different treatments. ANOVA was carried out using R open source software (version 3.1.1) with the *aov* function and the post-hoc test the Tukey's test with the *TukeyHSD* function. All statistical analyses were done at a significance level of 0.05.

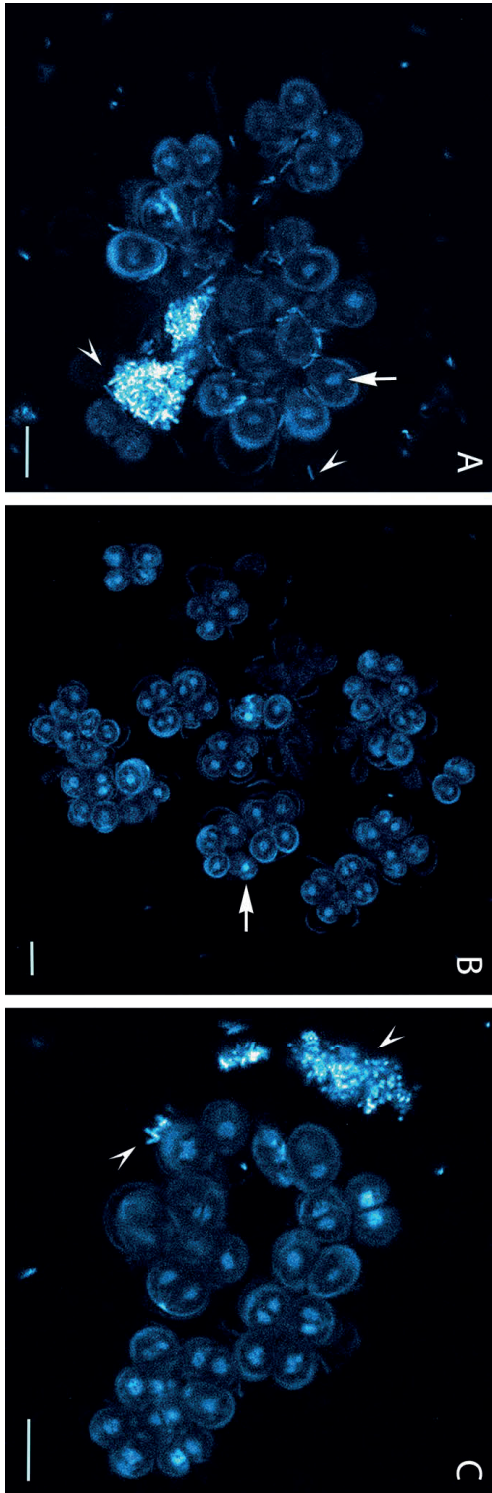
## 5.2 Results

The effects of UVC treatment on the abundance of bacteria in *B. braunii* strain CCALA778 are shown in Fig. 5.1. The UV treated CCALA778 showed almost 100 % of the 16S rRNA genes assigned to chloroplast both at day 0 and 15. The bacteria relative abundance in the UV treated CCALA778 was less than 1 % in most samples with the highest relative abundance being 3 % for one replicate at day 15. In contrast the untreated CCALA778 showed 70 % of the 16S rRNA genes assigned to bacterial origin for both day 0 and 15. The UV+Bac treated CCALA778 showed higher relative abundance of bacteria compared to the UV treated CCALA778 and less compared to the untreated CCALA778. In both untreated and UV+Bac treated CCALA778 at day 0 and 15, the dominant phylum was *Proteobacteria* followed by *Bacteroidetes*, with the exception of one replicate of UV+Bac treated CCALA778 at day 0. In lower relative abundance the phyla *Planctomycetes*, *Firmicutes* and *Actinobacteria* were detected for both UV+Bac and untreated cultures.



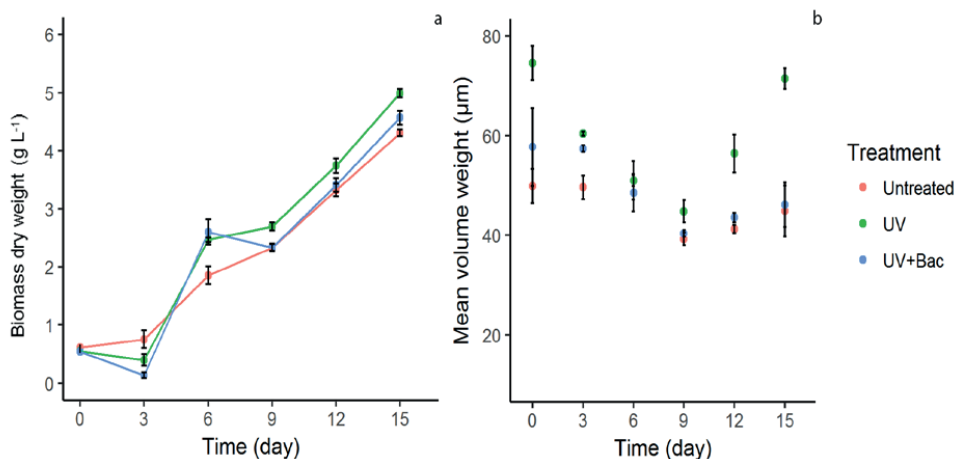
**Fig. 5.1 Relative abundance of bacteria compared to *Botryococcus braunii* strain CCALA778.** Green colour represents CCALA778 quantified by the chloroplast 16S rRNA gene. Remaining colours represents the different bacterial phyla present in each sample. Each bar represents an individual sample with n=3 per untreated, UV and UV+Bacteria (Bac) added treatment.

Confocal microscopy also confirmed the reduction of bacterial abundance after the UV treatment (Fig. 5.2). Bacteria clumps were observed in the untreated and UV+Bact CCALA778 (Fig. 5.2A and Fig. 5.2C respectively), whilst the UV treated CCALA778 showed no bacterial DNA staining (Fig. 5.2B).



**Fig. 5.2 DAPI staining of *Botryococcus braunii* CCALA778 and bacterial DNA.** DAPI accumulates in the DNA of the nuclei of algae (indicated with an arrow), and of the bacteria (indicated with an arrowhead) for A) untreated CCALA778, B) UV treated CCALA778, and C) UV+Bacteria (Bac) CCALA778. Scale bars are 10  $\mu\text{m}$ .

Fig. 5.3a shows the growth curve of all treatments for CCALA778 cultures. In the first 3 days a decrease was observed in the biomass dry weight of UV treated and UV+Bac treated CCALA778 followed by a fast growth between day 3 and 6. From day 9 onwards, the UV treated CCALA778 showed higher biomass compared to UV+Bac treated and untreated CCALA778. For both the untreated and UV+Bac treated CCALA778 biomass dry weight was similar between day 9 and 12, with UV+Bac treated CCALA778 showing a slight increase by day 15 over the untreated CCALA778. ANOVA analysis revealed that UV treated CCALA778 biomass accumulation was significantly higher than UV+Bac treated and untreated CCALA778 both at day 12 and day 15 (Table 5.1).



**Fig. 5.3 *Botryococcus braunii* strain CCALA778 growth and morphology after UVC treatment compared to untreated and UV+Bacteria (Bac) treated.** a) Growth was measured as biomass dry weight over time. b) Colony size measured as mean volume weight. Some treatment points are overlapping. Error bars represent standard deviation (n=3).

A larger average colony size was observed for the UV treated CCALA778 (Fig. 5.3b) compared to the untreated and UV+Bac treated CCALA778. At day 0, the average colony size measured for the UV treated CCALA778 was 75 μm with standard deviation  $\pm 3.5$  μm compared to  $57 \pm 7.8$  μm for the UV+Bac treated CCALA778 and  $50 \pm 3.4$  μm for the untreated CCALA778. From day 0 to day 9, all culture conditions showed a decrease in their

average colony size. From day 9 to 15 all culture conditions made a switch from decreasing to increasing average colony size. The UV treated CCALA778 showed a large increase of average colony size from day 9 to 15 whilst the UV+Bac and untreated CCALA778 had a small increase in colony size. ANOVA analysis revealed that UV treated CCALA778 colony size was significantly larger than untreated CCALA778 at all time points except for day 6 and significantly larger than UV+Bac treated CCALA778 at all time points except day 3 and day 6 (Table 5.2).

**Table 5.1. ANOVA of biomass growth.** Post-hoc performed with Tukey multiple comparison of means with 95 % family-wise confidence interval.

| Treatment Comparison | day 12 | day 15 |
|----------------------|--------|--------|
| UV - untreated       | Y      | Y      |
| UV - UV+Bac          | Y      | Y      |
| UV+Bac - untreated   | N      | N      |

<sup>†</sup> P < 0.05 Yes = Y, No = N

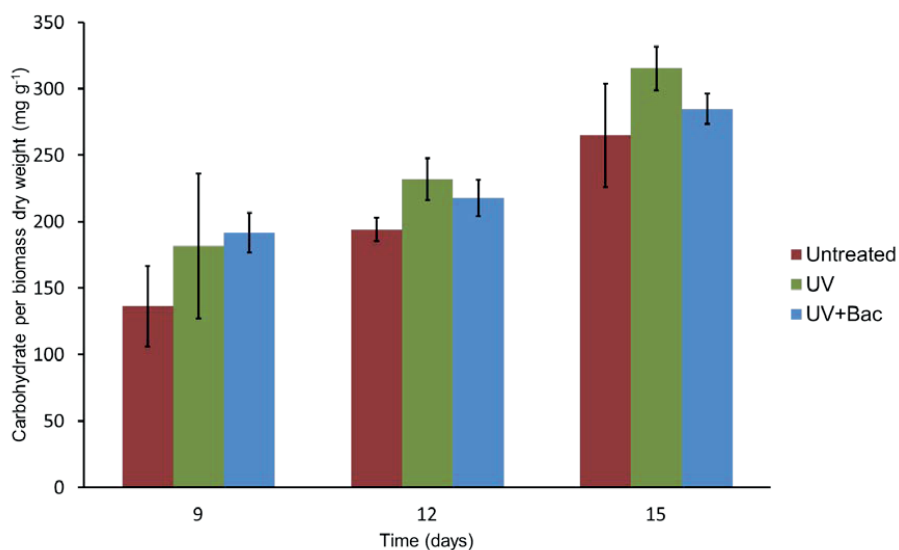
**Table 5.2. ANOVA of colony size.** Post-hoc performed with Tukey multiple comparison of means with 95 % family-wise confidence interval.

| Treatment Comparison | day 0 | day 3 | day 6 | day 9 | day 12 | day 15 |
|----------------------|-------|-------|-------|-------|--------|--------|
| UV - untreated       | Y     | Y     | N     | Y     | Y      | Y      |
| UV - UV+Bac          | Y     | N     | N     | Y     | Y      | Y      |
| UV+Bac - untreated   | N     | Y     | N     | N     | N      | N      |

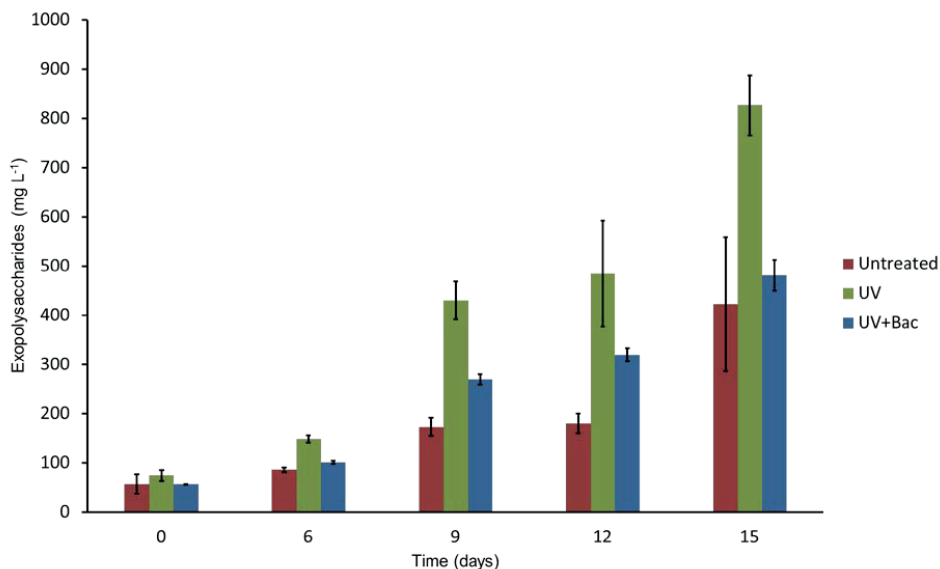
<sup>†</sup> P < 0.05 Yes = Y, No = N



Total carbohydrate was measured and the amount per dry weight biomass was determined for all conditions at day 9, day 12 and day 15 (Fig. 5.4). All conditions showed an increase of total carbohydrate per dry weight over time. Untreated CCALA778 had the lowest amounts of total carbohydrates per dry weight biomass at day 9, day 12 and day 15. On the other hand, UV treated CCALA778 showed higher total carbohydrate per dry weight biomass over the UV+Bac treated and untreated CCALA778, except in day 9 where UV+Bac treated CCALA778 had the highest ratio.



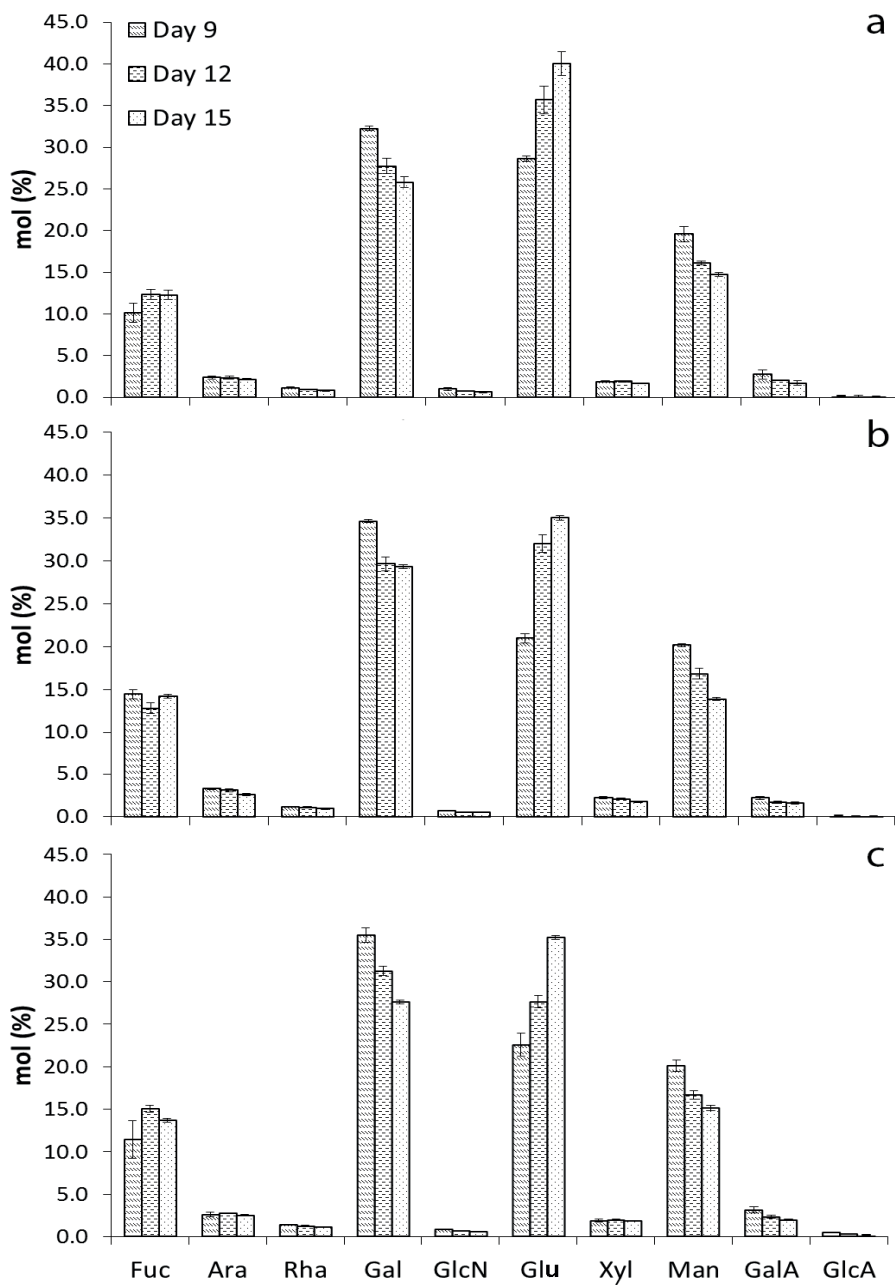
**Fig. 5.4** Total carbohydrate content of *Botryococcus braunii* strain CCALA778 per dry biomass between day 9 and day 15. Error bars represent standard deviation (n=3).



**Fig. 5.5 Accumulation of exopolysaccharides of *Botryococcus braunii* strain CCALA778 over time.** Error bars represent standard deviation (n=3).

The amount of exopolysaccharides measured for all culture conditions overtime, are shown in Fig. 5.5. All conditions showed an increase of exopolysaccharides over time with UV treated CCALA778 having by far the highest amount measured over the UV+Bac treated and untreated CCALA778. From day 6 till day 15 the accumulation of exopolysaccharides was higher in UV treated CCALA778 when compared to the UV+Bac and untreated CCALA778, reaching  $826 \pm 61 \text{ mgL}^{-1}$  at day 15. The highest exopolysaccharide production by UV+Bac treated and untreated CCALA778 was  $481 \pm 31 \text{ mgL}^{-1}$  and  $422 \pm 135 \text{ mgL}^{-1}$  respectively.

The carbohydrate composition of the biomass and the exopolysaccharide remained the same for all different conditions at day 9, 12 and 15 (Fig. 5.6). UV treated, UV+Bac treated and untreated CCALA778 all showed similar carbohydrate composition with D-galactose (Gal) and D-glucose (Glu) as the most abundant carbohydrate fractions, followed by D-mannose (Man) and D-fucose (Fuc). In lower quantities D-arabinose (Ara), D-rhamnose (Rha), D-glucosamine (GlcN), D-xylose (XyL), D-galacturonic acid (GlaA) and D-glucuronic acid (GlcA) were detected. In all treatments, both the relative amount of Gal and Man monomers decreased over time while Glu increased.



**Fig. 5.6** Total carbohydrate composition of *Botryococcus braunii* strain CCALA778. a) untreated culture, b) UV treated culture and c) UV+Bacteria (Bac) added culture. Error bars represent standard deviation (n=3).

### 5.3 Discussion

UVC treatment was successful in reducing the abundance of bacteria present in CCALA778 as seen from the 16S rRNA gene sequence analysis and the confocal imaging in Fig. 5.1 and Fig. 5.2 respectively. The results showed that UVC treatment is a feasible method to remove nearly all bacteria from CCALA778 strain. In recent years this method has also been shown to control and reduce bacteria from other microalgae such as *Tetraselmis* sp. (Sharma *et al.* 2014). Although it was not possible to create an UV treated CCALA778 100 % free of bacteria, it is likely that further UVC treatment upon UV treated CCALA778 could yield an axenic culture. Further treatments such as the use of  $\beta$ -Lactam antibiotics like Penicillin is also suggested as it has been shown that it can remove bacteria without harming *B. braunii* CCALA778 or other microalgae (D'Costa and Anil 2014). Two bacterial species which resisted UVC treatment were identified as *Dyadobacter* sp. and *Phenylobacterium* sp. *Dyadobacter* sp. has been isolated from glacial ice which is known to be an environment with high UV exposure, which could explain why the *Dyadobacter* sp in our culture resisted UVC treatment (Liu *et al.* 2014).

Reduction of bacterial abundance led to significant changes on the morphology and physiology of CCALA778. The results suggest that bacteria interfere with the growth of CCALA778 as seen from Fig. 5.3a in which the highly reduced bacteria abundance seen with the UV treated CCALA778 cultures, showed significantly higher biomass accumulation from day 9 onwards compared to the untreated CCALA778. The same trend was seen when bacteria were added to the UV treated CCALA778. UV+Bac biomass accumulation was reduced to similar densities as the untreated CCALA778. The reason why bacteria reduced the growth of CCALA778 is probably due to nutrient competition such as phosphate. One study investigating the nutrient-mediated interactions between algae and bacteria showed that phosphorus demand by bacteria can occur when organic carbon is present, proliferating bacteria growth (Liu *et al.* 2012). The high amounts of exopolysaccharide produced by *B. braunii* CCALA778, provides the organic carbon necessary for the bacteria to grow and hence, most likely inducing competition for nutrients.

Reduction of bacterial abundance also created significant changes in the average colony size of CCALA778. UV treated CCALA778 showed significant larger average colony size when compared to the untreated CCALA778 and the UV+Bac treated CCALA778. CCALA778 also displayed a colony size rhythmic cycle with decreasing and increasing of size overtime. It is not clear from our results the mechanisms behind this cycle, but it could be due to an

environmental stress such as light or nutrient limitation. It is hypothesised that higher exopolysaccharide content present in the UV treated CCALA778 was the reason why larger colony size were observed. Zhang and Kojima (1998) also related the size of colonies with the exopolysaccharide content, although different colony size trends were seen in their study. In Zhang and Kojima study, colony size was a function of light availability with a positive correlation where colony size decreased with decreasing light availability, while in our study we see a switch from decreasing to increasing colony size whilst the light availability continues to decrease. Also to note, Zhang and Kojima (1998) observed a decrease in exopolysaccharide content over time which is opposite of our findings and that could explain why they observed the size of the colony decreasing over time. In a recent study it was concluded that Iron (III) rich medium also influences the size and shape of colonies with *B. braunii* Bot-22 strain and that addition of glucose enhances the effect of Iron (III) (Tanoi *et al.* 2013).

UV treated CCALA778 showed higher total carbohydrate per dry biomass by day 15 while the untreated CCALA778 showed the lowest as seen in Fig. 5.4. It is likely that the bacteria are responsible for the lower amounts of total carbohydrates per dry biomass found in the untreated CCALA778 as it is readily available in the medium as a carbon source. This is probably best supported with the results seen in the accumulation of exopolysaccharides over time in Fig. 5.5. The untreated CCALA778 containing higher abundance of bacteria compared to the UV treated CCALA778 (Fig. 5.1) showed lower amounts of exopolysaccharide. Also when bacteria were added to UV treated culture, UV+Bac treated CCALA778 showed a reduction of exopolysaccharides, suggesting that the bacteria are feeding on the exopolysaccharide secreted and therefore reducing its content. In a previous study using the *B. braunii* strain ACOI58, the role of bacteria in the production or stimulation of EPS accumulation could not be determined, although similar attempts were also tried by introducing bacteria present in ACOI58 to *B. braunii* UTEX 572 (Fernandes *et al.* 1989). The composition of the carbohydrates produced by CCALA778 remained unchanged between treatments further indicating that the presence or absence of bacteria does not affect the composition of carbohydrates. Similar compositions of carbohydrates present with *B. braunii* are reported in five other strains (Allard and Casadevall 1990).

UVC treatment has proved to be effective and appropriate in reducing bacteria abundance in *B. braunii* CCALA778 strain as it does not affect its growth or exopolysaccharide accumulation. Based on these results, UVC treatment is recommended as a purification

method for *B. braunii* CCALA778 strain and most likely will be effective for other strains of *B. braunii*. Our results suggest that *B. braunii* is not in symbiotic relationship with bacteria as we did not observe any antagonistic effects with the reduction of the 16S rRNA genes assigned to bacteria below 1 % relative abundance. It is important to mention that the medium used in our study contained no vitamins or other co-factors that could substitute compounds produce by bacteria, such as Vitamin B<sub>12</sub>. We infer that *B. braunii* CCALA778 does not need bacteria to grow or to produce exopolysaccharides in the large quantities that it is known for, rather it is undesirable as it reduces exopolysaccharide accumulation. *B. braunii* is still a slow growing microalgae and attempts to improve its growth is needed if we are to use it in industrial applications. Our current UV treated CCALA778 strain can be used further to investigate enhance biomass productivity or to explore potential application for the EPS produced.

## 5.4 Conclusions

From this study we can conclude that bacteria are antagonistic to *B. braunii* CCALA778 cultures. We have shown that by reducing bacterial abundance, the biomass growth and EPS accumulation in *B. braunii* CCALA778 increases. *B. braunii* CCALA778 is probably not in a symbiotic relationship with bacteria and is the organism producing the exopolysaccharides. Moreover, it can do it without the presence of bacteria. UVC treatment is an effective method to reduce bacteria in *B. braunii* CCALA778 strain and possibly make axenic strains with further treatment.

## Acknowledgments

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50  $\mu\text{m}$



# General discussion

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## Chapter 6

## 6 Background

*B. braunii* are green microalgae that have the potential to be used as a source of sustainable polymers as they produce large amounts of long chain hydrocarbons and exopolysaccharides (EPS) (Metzger and Largeau 2005, Gouveia *et al.* 2017). The drawback of *B. braunii* is that growth is slow and therefore improvements on biomass growth or hydrocarbons and EPS production is essential to make use of these microalgae for large scale production (Banerjee *et al.* 2002, Tanabe *et al.* 2015). Like many other microalgae, *B. braunii* are associated with other microorganisms such as bacteria which can have a synergetic or antagonistic effect, but whether *B. braunii*'s associated bacteria, could lead to improvements of biomass growth, and hydrocarbons and EPS production was unknown (Chirac *et al.* 1985, Fernandes *et al.* 1989). The aim of this study was to investigate if *B. braunii* was dependent on bacteria for growth or not, and to determine if the associated bacteria present were beneficial or antagonistic towards the growth and production of hydrocarbons and EPS. In this thesis we have shown that we can improve the biomass growth and EPS accumulation of *B. braunii* CCALA778 by removing the associated bacteria by treating the cultures with Ultra Violet-C light (UVC).

### 6.1 Microalgae and bacteria associations

Microalgae and bacteria associations have in recent years received much attention as many studies are showing that bacteria have a beneficial role in many aspects of microalgae cultivation (Cole 1982, Kazamia *et al.* 2012, Lee *et al.* 2013, Wang *et al.* 2014, Amin *et al.* 2015, Cho *et al.* 2015, Ramanan *et al.* 2016). For example, *Sulfitobacter* spp. promote diatom cell division via secretion of hormone indole-3-acid, synthesized by the bacterium using both diatom-secreted and endogenous tryptophan (Amin *et al.* 2015). Another benefit of bacteria present with microalgae cultures includes the recycling of nutrients. For example, *Halomonas* sp. excretes siderophores that help to solubilize precipitated iron and therefore stimulating the growth of the red marine microalgae *Dunaliella bardawil* (Wang *et al.* 2014). Although we have seen that bacteria can be important and beneficial to microalgae, they can also be antagonistic (Wang *et al.* 2010, Liu *et al.* 2012, van Tol *et al.* 2017). One study looking at microalgae-bacteria interactions, showed bacteria competing for phosphate in the presence of excess organic carbon resulting in reduced chlorophyll concentrations (Liu *et al.* 2012). Another study showed how the flavobacterium *Croceibacter atlanticus* inhibits cell division of the diatom *Thalassiosira pseudonana* (van Tol *et al.* 2017). In other cases, some bacteria can release proteases when attached to the microalgae cell wall causing the cells to lyse

(Wang *et al.* 2010). It is clear from literature that the beneficial effects of bacteria in microalgae cultures depend very much on the microalgae specie and on the bacteria.

In this thesis we looked at the microalga *B. braunii* and its associated bacteria. In chapter three we showed how 12 strains of *B. braunii* are associated with wide diversity of bacteria with their own specific bacterial community. The reason we find a large bacterial diversity is probably because *B. braunii* can excrete large amounts of hydrocarbons and EPS which the bacteria can feed on. For example, among the various species of bacteria found we identified *Sphingomonas* spp. that are known to degrade hydrocarbons (Tang *et al.* 2010b). In chapter five with the use of UVC treatment with the EPS producing strain CCALA778, we reduced the bacteria diversity and the abundance to less than 1 % relative abundance. The results indicate that *B. braunii* CCALA778 probably does not need bacteria to survive. Furthermore, we showed how *B. braunii* CCALA778 associated bacteria were antagonistic by reducing the biomass concentration when present. The reason *B. braunii* CCALA778 biomass growth was reduced in the presence of bacteria was probably due to nutrient competition by the bacteria. Heterotrophic bacteria obtain their carbon source from reduced organic molecules, and since *B. braunii* excretes large amounts of EPS, the bacteria can use the available organic carbon for growth while also absorbing other essential compounds such as phosphate that would otherwise be available for the growth of *B. braunii* CCALA778. The EPS produced by *B. braunii* CCLA778 is composed mainly of galactose (Fernandes *et al.* 1989, Gouveia *et al.* 2017) which many bacteria can degrade and use for growth (Beisel and Afroz 2016). Not only the associated bacteria were antagonistic to *B. braunii* CCALA778 growth, but they also decreased the EPS content. The decrease in EPS content by the presence of bacteria is seen as detrimental to an industrial process because the EPS is the compound of interest for commercialization.

Our results indicate that *B. braunii* which secretes large amounts of organic carbon in the form of EPS and hydrocarbon, probably do not benefit from the presence of bacteria because bacteria will most likely degrade these compounds for their growth therefore reducing both biomass concentration, EPS and hydrocarbon productivity. We extend the conclusions from this thesis to hypothesize that any microalgae that excrete large amounts of organic carbons and which have value for industrial application are not compatible with the presence of bacteria. So it seems that it is redundant to add any bacteria to these microalgae with the intention to enhance their growth. Another microalgae known to produce large amounts of EPS is *Porphyridium cruentum* and it has received much scientific interest because of the EPS

potential medical applications (Xiao and Zheng 2016). In order to cultivate microalgae that secrete large amounts of organic carbons, such as *B. braunii* and *P. cruentum*, it is therefore essential to protect them from any bacterial contamination. Currently, industrial production of microalgae cannot prevent completely bacterial contamination both in open ponds or in a closed photobioreactor (PBR) (Lee 2001, Wang *et al.* 2013). Therefore efforts to develop large scale PBR which prevent bacterial contamination is crucial to the advancement of the microalgae industry. A promising solution is UV treatment in order to sterilize PBR units (Essam *et al.* 2006, Doud *et al.* 2014). Doud and colleagues showed that a UV treated PBR used to grow *Synechocystis* sp., was maintained sterile for over three weeks in contrast to the non-treated PBR (Doud *et al.* 2014).

## 6.2 Microalgae axenic cultures

Axenic cultures are defined as cultures containing one specie or strain. Axenic cultures are essential when conducting genomic, physiological or biochemical studies. To determine if a microalgae culture is axenic or not, simple agar plate monitoring is not enough because most of the bacteria that exist are actually uncultivable (Fredricks and Relman 1996). To detect the presence of bacteria, 16S rRNA gene analysis has become the standard protocol (Janda and Abbott 2007). 16S rRNA gene sequencing is a sensitive and robust method which is currently becoming more affordable as new technologies allow for more samples to be analysed at one given time (Clarridge 2004). 16S rRNA gene sequencing offers a robust and easy way to determine the axenic status of microalgae cultures and the data should be revealed in publications when possible. In chapter four and five we clearly see the benefits of using 16S rRNA gene analysis to determine bacterial diversity and its relative abundance present with *B. braunii* CCALA778. After the use of UVC to remove bacteria, we still detected a low relative abundance of bacteria below 1 % compared to the 70 % found in the untreated cultures. In order to investigate the genomics and transcriptomics attributes of *B. braunii* which are the hydrocarbons and EPS production, we need to have axenic strains, therefore efforts should be put in removing the last traces of bacteria from CCALA778 strain.

## 6.3 Antibiotic and UVC treatment

We know now that bacteria associated with *B. braunii* CCALA778 are antagonistic and therefore methods to remove bacteria are required. The antibiotics used in this thesis did remove some bacteria but overall a large diversity and abundance remained in the antibiotics-treated cultures that did not kill *B. braunii* CCALA778 (chapter four). We cannot extrapolate

the results of the effects of these antibiotics to the other *B. braunii* strains due to the fact that in chapter three we observed that different *B. braunii* strains have different bacterial community composition and therefore the same antibiotics and mixes would act differently. If we were to continue using antibiotics to remove the bacteria from *B. braunii* CCALA778 culture, the next step would be to find different combinations of antibiotics with synergy potential as we found that most associated bacteria with CCALA778 were Gram-negative (chapter four). Antibiotic combinations are important when dealing with Gram-negative bacteria because they possess an outer membrane which provides protection against antimicrobial agents (Delcour 2009). Synergies between antibiotics happen when one antibiotic can induce disruptions to the outer membrane facilitating the diffusion of another antibiotic to enter the bacterial cell (Miller 2016). The challenge is however, to find the correct antibiotics combination to remove the bacteria. The large number of possible antibiotic combinations makes this approach almost practically unfeasible (Bollenbach 2015). Therefore we recommend other type of treatment to remove bacteria from *B. braunii* cultures and similar microalgae species with a large bacterial diversity and abundance. Here we recommend the use of UVC treatment as an effective method for reducing bacteria abundance and diversity as well as to obtain bacteria free cultures. In chapter five, we successfully reduced the 16S rRNA genes assigned to bacteria to less than 1 % in the UVC treated culture compared to the 70 to 90 % of the 16S rRNA genes assigned to bacteria in the untreated culture. Since the UVC treatment was performed six months before the actual experiment, we can also conclude that UVC treatment did not damage the ability of *B. braunii* CCALA778 to grow or to produce EPS. UVC damages the genetic material in the nucleus of the bacteria specially between 250 – 270 nm as this is the wavelength that is most strongly absorbed by nucleic acids and therefore the most lethal range of wavelength to microorganisms and is known as the “germicidal spectrum” (Dai *et al.* 2012). In the pharmaceutical industry for example, laboratory scale UVC treatment is being applied to inactivate viruses and bacteria in cloudy liquid media (Schmidt and Kauling 2007).

#### **6.4 Recommendations for future research**

In light of what has been mentioned in the preceding sections future research should focus on:

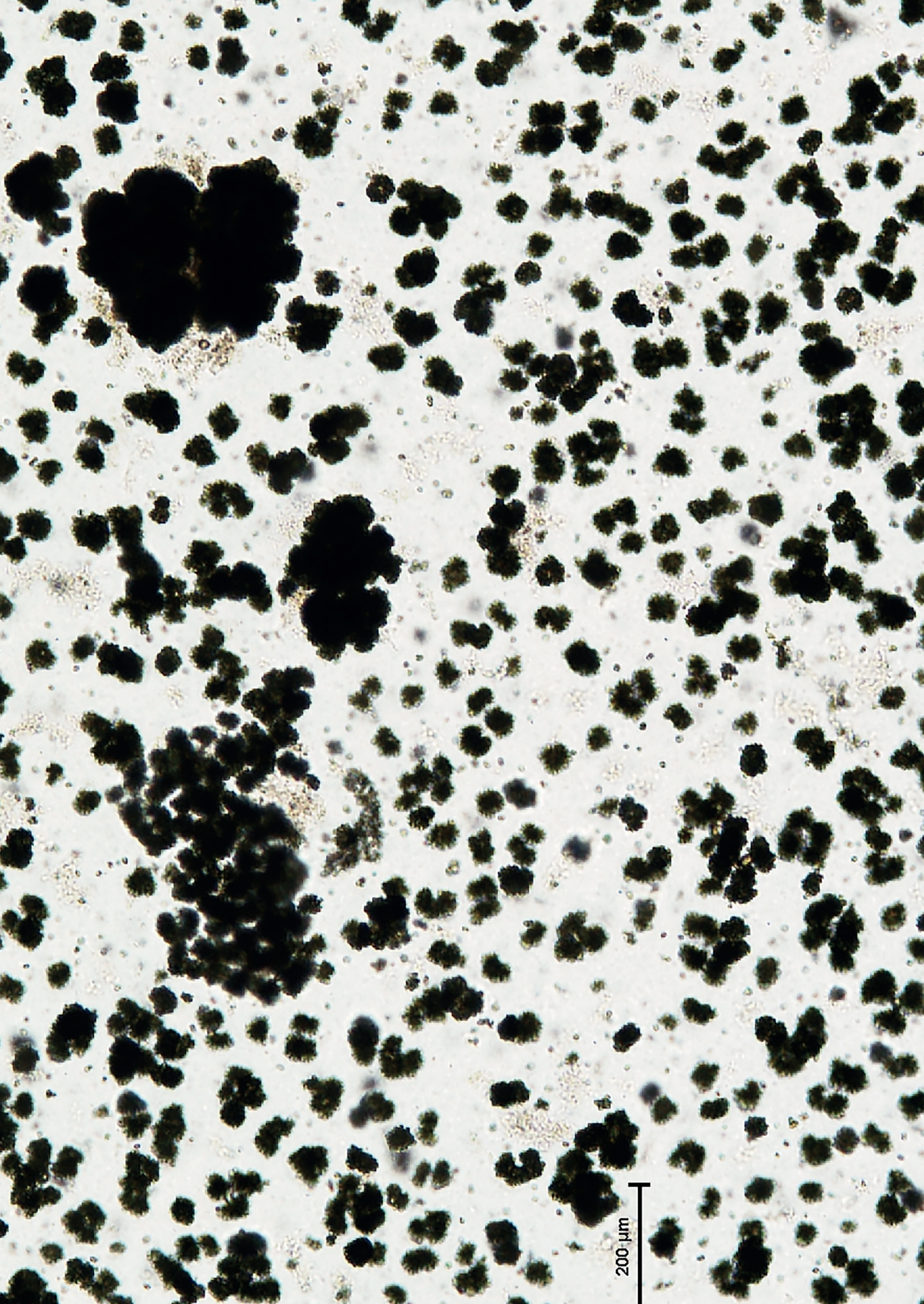
- 1) Understanding if microalgae species that secrete EPS are generally adversely affected by associated bacteria. Obtaining insights to this question will help the scientific community investigating microalgae and bacteria interactions.

2) Obtaining axenic strains as these are necessary in order to study genomics and transcriptomic of microalgae . UVC treatment for the removal of bacteria from *B. braunii* CCALA778 cultures shows promising results and with a few bacteria left, it is of value to further attempt the removal of the last traces of bacteria to obtain an axenic culture. This will allow physiological studies related to EPS formation which could lead to improved productivities.

## 6.5 Final remarks

Microalgae and bacteria are not always a good match. *B. braunii* CCALA778 most probably does not require the presence of bacteria for survival or the production of EPS and here we showed how some bacteria are antagonistic to *B. braunii* by decreasing the biomass accumulation and how they can be detrimental to industrial processes by reducing the concentration of EPS. Likewise, other microalgae probably do not need bacteria to survive and introduction of bacteria via handling or large scale cultivation, could end up decreasing the biomass productivity of the microalgae by nutrient competition. Microalgae such as *B. braunii* and others that secrete large amounts of organic carbon such as EPS, probably do not benefit with the addition of beneficial bacteria. Microalgae species that secrete large amounts of EPS are more susceptible to bacterial contamination and therefore large scale production in PBR units is considered a risk. Therefore the microalgae industry will benefit immensely with the development of PBR units that prevent bacterial contamination.





200  $\mu\text{m}$



# References

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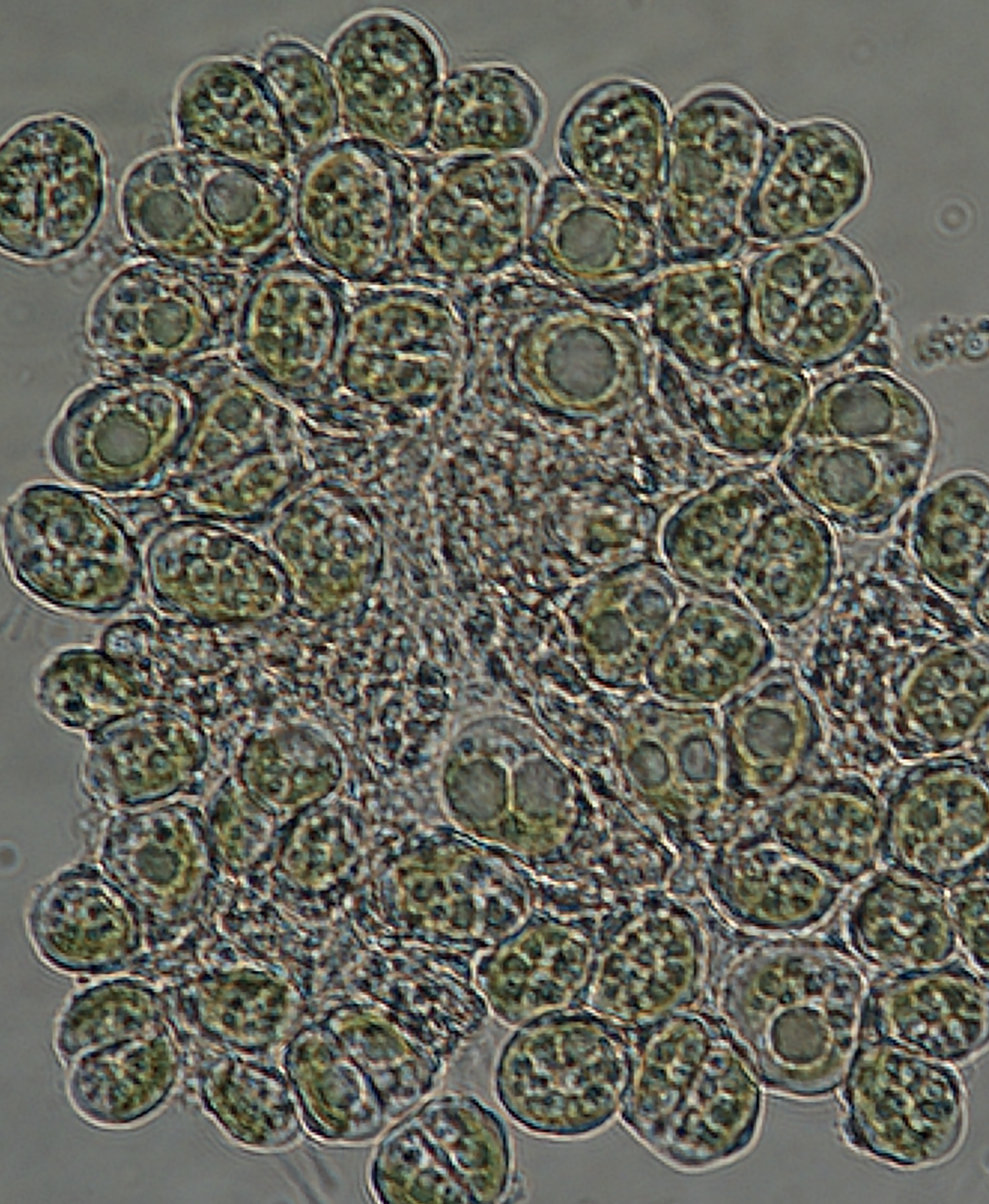
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# Summary

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Microalgae are photosynthetic organisms that are found worldwide in many different aquatic environments and therefore display an immense biological diversity. They are a promising source of many useful polymers that have industrial applications such as food, fuel, material and pharmaceutical. One microalga that has gathered quite a research community is *Botryococcus braunii*. The reason for its scientific club is the fact it can synthesize long chain hydrocarbons molecules from C<sub>20</sub> to C<sub>40</sub>. These hydrocarbons have been found in oil-shales and tests show that it can be used as aviation fuel. Besides producing hydrocarbons, some strains of *B. braunii* can produce exopolysaccharides (EPS) composed mainly of galactose and a small fraction of fucose. The EPS has interesting rheological properties for the food industry and potential active compounds that could be used in the pharmaceutical industry .

Like many other microorganisms, microalgae in the natural environment are usually in the presence of bacteria. The presence of bacteria with microalgae can either have a beneficial or an antagonistic effect. For *B. braunii* little is known about the bacteria community present especially for the EPS producing strain. For that reason, the aim of this thesis was to investigate *B. braunii*'s associated bacteria with the aim of improving *B. braunii*'s biomass growth and hydrocarbon and EPS content. In chapter one, we introduced the topic of microalgae as a potential source of sustainable polymers and we introduced the species *B. braunii*, describing its characteristics and scientific interest. The topic of microalgae and bacteria associations is also introduced by looking at other studies from literature.

In chapter two, 16 publically available strains of *B. braunii* were ordered from culture banks and screened for biomass productivity, hydrocarbon and total carbohydrate content. The aim of the study was to identify one or more good strains that displayed high biomass productivity as well as hydrocarbon or total carbohydrate content. In seven strains out of 16 cultivated in 250 mL volume Erlenmeyer flaks, we detected 5 to 42 % content of hydrocarbons of the dry biomass with four strains producing botryococcenes (C<sub>30</sub>-C<sub>34</sub>) and three strains producing alkanes (C<sub>20</sub>-C<sub>25</sub>). Two strains showed high amounts of EPS content above 50 % per dry biomass. Seven strains comprising of the strains with higher biomass productivity plus the highest hydrocarbons and EPS content, were tested for scalability using bench scale 800 mL volume bubble column reactors. Two strains, AC761 which produces botryococcenes and CCALA778 which produces EPS, were selected as the most promising *B. braunii* strains for industrial production of hydrocarbon and EPS.

In chapter three, we studied the bacterial community associated with *B. braunii*. We cultured 12 strains from the initial 16 from chapter two and extracted the DNA from samples taken over a time period of 12 days. It was clear from this study that *B. braunii* hosts a variety of bacterial species whilst still maintaining its growth. The bacteria families *Rhizobiaceae*, *Bradyrhizobiaceae* and *Comamonadaceae* were found in all 12 strains. These families which belong to the phylum *Proteobacteria* could have an important role regarding *B. braunii* growth. Each strain displayed a different bacterial community composition but all the strains from the CAEN culture collection clustered near each other suggesting that the algae culture collection could have an influence on the bacterial community composition. Bacteria genus identification based on 16S rRNA gene amplicon similarity showed several genera present including *Rhizobium* spp. and *Variovorax* spp.. Two genera were found that are possibly linked to hydrocarbon degradation: *Sphingomonas* spp. and *Rhodobacter* spp..

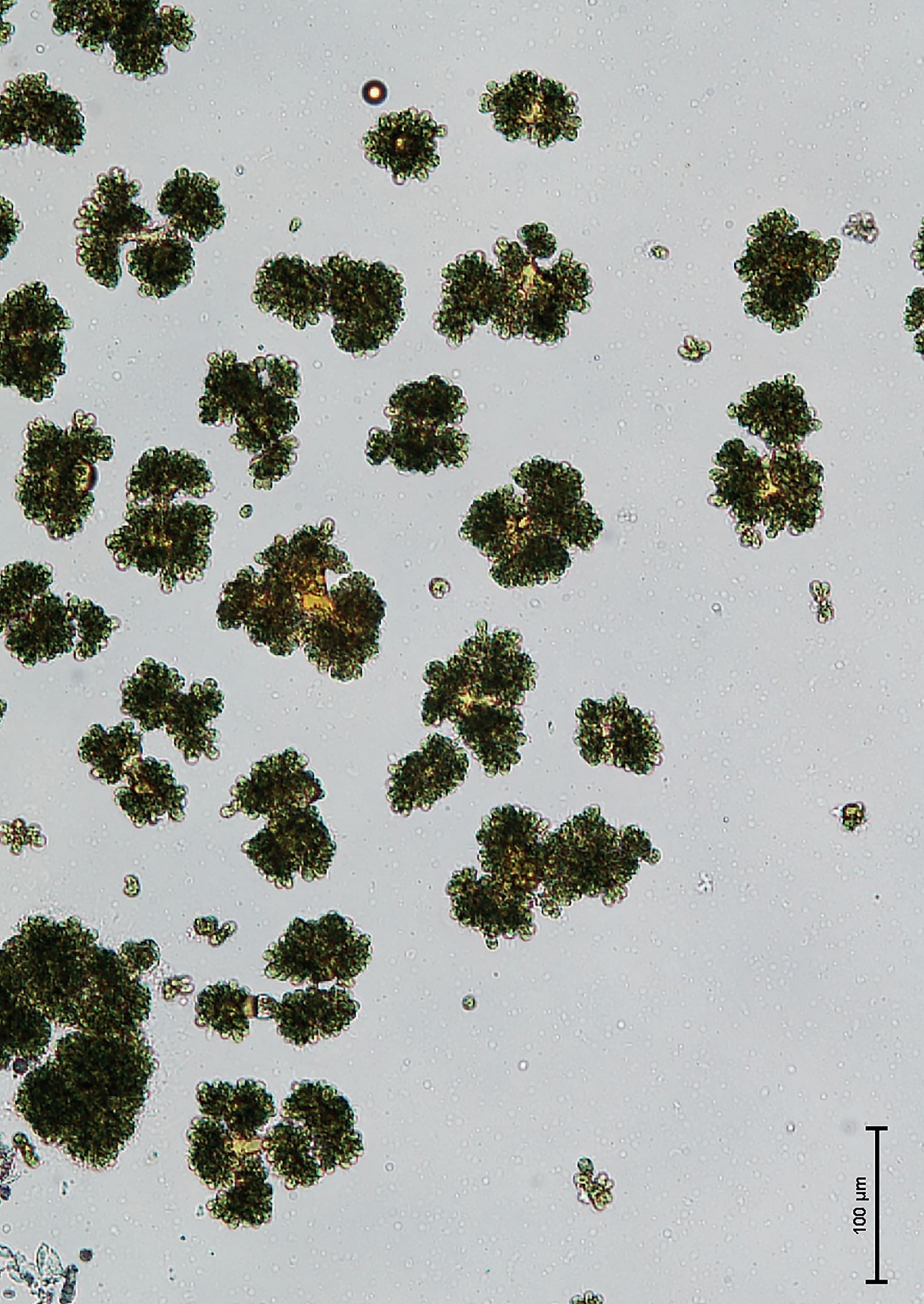
In chapter four, we investigated further *B. braunii* CCALA778 which was shown in chapter two to accumulate high amounts of EPS. We investigated the effects of antibiotics on algal growth, EPS accumulation and bacterial community composition of CCALA778. Taxonomical identification by 16S rRNA gene analysis indicated that most of the bacteria present with CCALA778 were Gram-negative. Of all antibiotics and antibiotic mixes, only the treatment with Penicillin did not affect the growth of *B. braunii*. The remaining antibiotics halted the growth of CCALA778 while they were active. The exceptions were with the antibiotics Chloramphenicol, Gentamycin and Linezolid which permanently ceased the growth of CCALA778. The accumulation of EPS seemed to be related to biomass growth, but we did also observe a reduction of EPS with the cultures treated with Penicillin suggesting that bacteria could have an effect on the EPS content. Antibiotics had specific effects on the bacterial community with all treatments showing significant changes over time. The most efficient treatment in removing bacteria were the mixes Metronidazole-Rifampicin-Penicillin and Penicillin-Rifampicin which were the only treatments to show significant changes in the bacterial community when compared to the untreated cultures after 10 days of cultivation. Antibiotics and antibiotic mixes can create changes in the bacterial community but it is unlikely that they alone can lead to axenic *B. braunii* cultures.

In chapter five, we used Ultra Violet-C light (UVC) to reduce bacteria diversity and abundance present in *B. braunii* CCALA778. UVC is highly effective in inactivating bacteria and for that reason is being investigated further in medicinal applications. After applying the UVC to *B. braunii* CCALA778, we were able to reduce the relative abundance of 16S rRNA

genes assigned to bacteria to less than 1 % compared to the 70% in the non-treated cultures. With the UVC treated CCALA778 we observed several physiological changes. The UV treated cultures with reduced bacterial load showed nearly double the EPS accumulation when compared to the untreated. To confirm that we did not see an artefact in our results due to the UVC treatment, UVC treated cultures were also inoculated with bacteria from the untreated and we observed a reduction of EPS similar to what we saw with the untreated cultures. There were no changes to the EPS composition after the removal of the bacteria. Other physiological changes were observed, namely that colony size of *B. braunii* CCALA778 significantly increased when compared to the untreated culture and the UV treated with bacteria. We hypothesise that the increase in colony size was probably due to the fact there was more EPS accumulated which helped with cell aggregation. We also observed an increase on the biomass growth in the UV-treated CCALA778 which we hypothesized being related to the fact that there was none or hardly any competition for essential micronutrients such as phosphate. From this study we concluded that the associated bacteria present with *B. braunii* CCALA778 were antagonistic. We believe the reason why the bacteria were antagonistic is because of the readily available EPS which is a rich source of organic compounds that bacteria could use for their own proliferation allowing them to compete with *B. braunii* for essential nutrients.

In chapter six, we discuss the implications from our previous 4 experimental chapters. The aim of the study was to improve the biomass productivity and hydrocarbon and EPS content of the microalgae *B. braunii*. In brief, *B. braunii* displayed a wide range of physiological traits regarding biomass productivity and hydrocarbon and total carbohydrate content. We showed that *B. braunii* can co-habit with a wide range of bacteria diversity and abundance and that the associated bacteria were antagonistic to CCALA778 by affecting its biomass growth. We also showed that by removing the associated bacteria we can increase the EPS accumulation. Currently most of the research on microalgae and bacteria interactions, focus on the positive side, but we must understand also how bacteria can be antagonistic to microalga growth. Bacteria can be antagonistic to microalgae by competing for nutrients and also being detrimental to industrial process by degrading the product of interest in the case of organic carbons such as EPS. Therefore it is unlikely we can use the benefits that bacteria can provide such as enhancing growth to improve the cultivation of *B. braunii* and other similar microalgae species that secrete EPS. Since bacteria can be antagonistic to microalgae that secrete large amounts of organic compounds such as EPS, it is imperative to minimize

contamination in large scale photobioreactors (PBR). It is important because in large scale PBR, contamination can occur leading to downtime of the reactors. If microalgae industry is to advance, it must develop PBR units that prevent contamination of bacteria from the surrounding environment.



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A PhD study is seldom a lonely affair. During my studies many colleagues and friends contributed to my success and I would like to acknowledge them on the journey to become a doctoral graduate.

First of all I would like to acknowledge my co-promoters and promoter. Thank you **Maria Barbosa** for believing in me and giving me the opportunity to start my PhD at Bioprocess Engineering. From our first conversation over the phone, there was a pleasant resonance which continued over the years. Perhaps because we both have “lusitano” blood, but also because we both saw *B. braunii* growing ever so green in the bioreactors. Thank you **Detmer Sijkema** for your guidance and mentoring over the last period of my PhD. Your critical thinking and analysis of our results are a wonderful example of first class scientific inquiry and for that I am fortunate and grateful to have been your student. Thank you **Rene Wijffels** for also accepting me into the Wageningen Bioprocess Engineering family. I enjoyed many of our discussions and your points of view when interpreting our results. It was important to me to learn to interpret the results in a pragmatic fashion and not venture in wild observations and predictions. I will always remember your relaxed and humour disposition balanced with the sharp critical thinking. I am also forever grateful for your support all the way to the final stages of my thesis. In the end, we did not investigate *B. braunii* cultivation using apples or fish, but instead, we have a good understanding of *B. braunii* cultivation in the presence of many bacteria. I must also acknowledge the role of **Douwe van der Veen** who supervised me during my first three years. Although we did not see things eye to eye there were lessons learned and those I carry and are the important things to remember.

In the department nothing happens without the support of the technical and secretarial team. Thank you **Fred van den End**, **Wendy Evers**, **Sebastiaan Haemers**, **Snezana Gagic**, **Miranda Berger** and **Marina van der Vegte** for making the life of a PhD candidate much easier and more graceful.

I am much thankful to the academic and research staff at BPE for the good times we had over the years. A special memory goes to a luxury vegan dinner at **Marian Vermue** family home that gave me a break from my life in the tent during the first weeks of my PhD. Being present

at several work anniversary celebrations from our long standing colleagues. One memory was the celebrations at **Dirk Martens** home with lovely crowd, tasty food and getting to exchange some football skills with **Marcel Janssen**. Thank you **Arjen Rinzema** for giving away the wonderful IKEA white table, as it decorated lightly my living room. **Hans Reigh** for some interesting discussions and trips to Impulse and Loburg to watch seminars on diverse scientific topics. Many thanks to the rest of the crew in BPE: **Douwe van der Veen, Packo Lamers, Ruud Weusthuis, Rouke Bosma, Corjan Berg, Mathieu Streefland** and **Michel Eppink**.

**BPE** is full of opinions, and it was delightful, insightful and other times foolish to engage in many discussions at the BPE coffee corner. But all had a role in creating the supporting environment within BPE. The times we all gathered at the coffee corner will forever be remembered and surely missed.

In October 2012, BPE was inoculated with a special half a dozen PhD candidates for a batch run of four years and a bit more for some of us. The great batch includes **Ilse Remmers, Xiao Pan, Richard Postma** and **Abdulaziz Al Sayyari**. I recall that first day with warm feelings. You can only have a good batch when the one before also sets the standards. For the ones present when I entered we count with the following heroes and heroines: **Lenny de Jaeger, Anne Klok, Marjon van Es, Sina Salim, Lenneke de Winter, Guido Breuer, Rupali Desai, Tim de Mooij** and **Ana dos Santos**.

From microbiology I had the pleasure of being part of the **Moleico** group with **Jie Lian, Johanna Gutleben** and **Georg Steinert**, among others. I learned much about sponges and bacteria and the statistical program R studio. I will always remember the diversions and expansions of rational thinking and discussions around Alpha & Beta diversity, multivariate analysis and Powerpoint presentations!

A big thank you to my student team that help me during my PhD. **Kaushik, Rhoda** and **Patrick**. I very much enjoyed to see our experiments flowing wild and delivering scratching head moments. Supervision was also a new experiment for me and I have learned valuable lessons from you.

I also had the chance to work with a wonderful international team as part of the European project that funded my PhD. The challenging saga of *B. braunii* towards a **SPLASH** “Sustainable **PoLymers** from **Algae Sugars** and **Hydrocarbons**”.



During my studies I also had lots of great moments with the **VLAG PhD council members**. The fun moments were a great complement to our achievements we got. Namely seeing our efforts to make a PhD student journey better by introducing a PhD guide book, by highlighting areas of improvement in what are our expected roles, and by representing and supporting our VLAG School at international peer review panel meetings. We count here with **Edita, Jonathan, Anne, Tjerk, Geraldine, Laura, Natalia and Anika**.

**Labuitje committee for 2013** was the best team ever with **Marjon van Es, Ward and Rupali**. It was great to organize this awesome day over our many meetings and having come up with the competition of “building a house out of cardboard”. Brilliant!

Half way through my PhD I was inspired to follow the footsteps of Lenny (FameLab 2014 finalist) and joined the **Famelab 2015** competition for the Netherlands. I met inspiring scientists that are not afraid of stepping up to the stage and passionately talk about their research and aspirations. It was wonderful to share the story and life of “*Little Green Shoes*” with this epic crew who pitch and talk their walk! Bravo to all of you.

The squash team was a satisfying endeavour with an almost elite bunch of athletes. Here we count with gang **Marcel de Haan** our almost champion if it wasn't for his shoes which somehow always were disintegrating, **Ward Blanken**, our almost champion that many times had something on his knee, but whose skills allowed him up the ranks without too much running, **Youri van Nolan**, our up rising star who started as a newbie and ended his season with some strikes to make any one up their game.

If you come and live in the Netherlands you soon learn that cycling is a must and one should make the most of it along the splendour landscape during summer times. All our trips were memorable as well as some of the speeds we achieved of course. The road riders were **Ilse Remmers, Lenny de Jaeger, Ward Blanken, Guido Breuer, Anne Klok, Edgar Suarez, Simon and Ben Schaap**.

Running was the second activity that I engaged in throughout my time in Wageningen. It was often a morning, evenings and lunch time activity for me and some of the other BPE runners. It was great to share many morning runs with **Gerard Lam** and **Lenneke de Winter**. It was always a good time to share some gossip and other news. I hope you guys are still running and will keep doing so for many more years to come. Also I have to recall my best half marathon time at 01:43 HH:MM with a special company of **Lenneke** and her dad.

Swimming was another sport that BPE PhD candidates seemed to love. There was initially a great momentum initiated with **Lenneke** and **Wendy Evers** for quite some time with swims before work. Other times, together with **Iago Teles**, we took the morning swimming sessions to the extreme using it to do some swimming but mainly to set some discussions on par and point to the pink elephant on centre of the room (laughing is the best medicine they say).

For many reasons one sometimes secretly wishes that we keep the life we had with our colleagues forever. Such bonding are these moments in life that they are worth more than **gold**. The evenings spend playing worms with **Ward**, **Youri** and **Agi**. I loved the foolishness, the seriousness and the beer. I look forward to the 10 year Worms reunion fellas! The many encounters with no other than a brother from Brazil **Iago**. I cannot recall all the countless times that we express in virtuosi lyrical manifestos all the many thoughts that crossed our minds about our lifes, from our original backgrounds (the great brazil e Lusitania) to cultural differences and PhD comedy in the presence of the “granda vizinha” who often joined us. Other times the Iberian team would line up a drink or two mixed in with a dinner to discuss endlessly other matters besides PhD and for that I thank **Rafael Cubero** for these evenings. And the many BBQs that kept appearing during the summer times either at **Carl Safi’s**, **Youri’s** or at **Jeroen’s** home. These were a treat to be present *meneers* so thank you for organizing these splendid get togethers. I am also thankful to **Ruchir Khandelwal** with whom I shared many discussions on isoteric legends from Indian history and well as the 8 day fasting spend together at his home. It was a special and rewarding challenge

To my office mates **Richard Postma** and **Agi Janoska**. It was a great pleasure to share most the full PhD time with you and to have the many meet ups outside the office. It was a pleasure to get to know you both. Also how amazing to see **Richard** during his PhD getting married, and becoming a father! **Agi**, you were always in good mood and it was great to have that happiness around and that I could borrow your car time to time travel to my ultra-marathons in France and Belgium...Agexegerê!!!

There were some wonderful international dinners also organized around many of our homes in Wageningen. It was great to have such a relaxing time and good food. Thank you for these nice evenings and afternoons dear colleagues: **Rupali**, **Mitsue Leon Saiki**, **Jorijn Janssen**, **Ilse**, **Gerard**, **Xiao**, **Yimin**, **Imma** and **Luci**.

Although I was not always there for Friday's drinks, the ones that I could join, it was always nice to be part of. Thank you **Camilo, Pauline, Youri, Jeroen, Edgar, Fabian** and **Enrico** for the great evenings shared around the many bars in Wageningen.

Not directly connected to the PhD there were other great forces manoeuvring and moving through space. In 2014, I joined the Wageningen based dance group **InMotion dance traces**. For more than three years we gathered every Friday after work to train together, to move and inspire each other, to break norms and habits and to discover new possibilities. The ripple effects of the work we did went unexpectedly beyond our dance studio. We shared much courage and vulnerabilities, much passion and frustrations and the group kept just getting more stronger and stronger. So beautiful to be part of this group. I loved it every moment, with trainings, dinners, rehearsals and performances. I am grateful that you could be part of this time of my life and I hope we stay connected for as long as possible! Much love to you **Marisa, Gonne, Femke, Petra, Veronika, Sandy, Lian, Elena, Rachel, Linda, Anouk, Shuhang, Amandine, Erikwim, Angela, Annabel, Tanya, Xander, Robin** and **Meng**. Also part of the dancing scene, was **Raul, Iris, Monique, Erik, Orestis, Eloi** and **Vital**.

In my final stretch of the PhD I had the amazing support of my dear beloved **Petra Vossenber**g. Thank you for your support, discussions and feedback during the final stretch of my PhD in our South East Asia adventure.

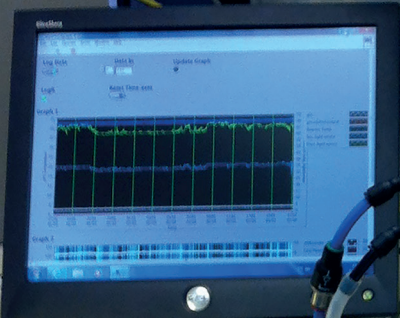
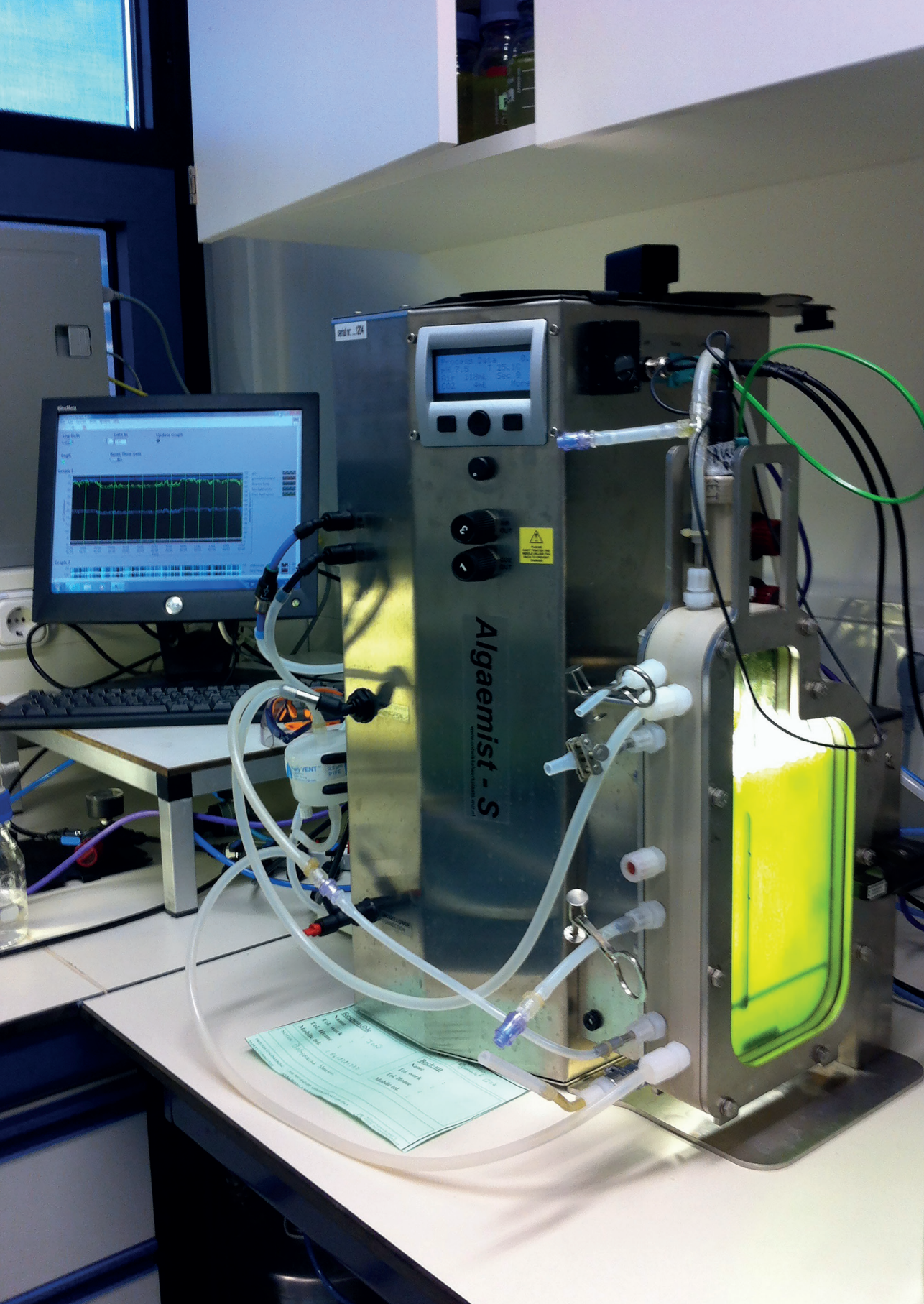
To my **family** who has always been there to support me whenever I returned to Portugal, offering always that endless partnership and loyalty that can crack fears and doubts the size of mountains. Although most were not physically present in Wageningen, they were always near spiritually and energetically. Thank you for being there with unconditional love.

A big thank you to **Irma Mandler** who shared with me the beginnings of my PhD journey and some challenging PhD moments. During this time we both ended a journey and initiated another one with much love. We also collaborated and prepared a workshop for public speaking and presentations that was a success. And you were at my side for the FameLab competition final in Leiden which made all the difference. Also thank you for the lovely times in Berlin with **Basti** and **Elio**. It was always a perfect time to recharge and regroup ones energy and focus. I look forward to more encounters.

**A dedication to Running:** Running was and still is a life endeavour with challenges always rising and demanding new limits. The PhD is a long effort and requires dedication and

persistence as with running. During my PhD, running was a perfect ally that permitted me to push and push limits which demanded *perseverance*, *will power* and a *braveheart*. Running kept me strong towards achieving my doctorate diploma. The little green shoes and many others were a fine support who saw the roads and trails which I passed and blazed. To run far is to dream with defiance. **As with any endeavour such as a PhD, we should dream and defy the limits within our minds.**





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Datei: 1000

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# List of publications

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## Published

Gouveia, J. D., Ruiz, J., van den Broek, L. A. M., Hesselink, T., Peters, S., Kleinegris, D. M. M., Smith, A. G., van der Veen, D., Barbosa, M. J. and Wijffels, R. H. (2017) '*Botryococcus braunii* strains compared for biomass productivity, hydrocarbon and carbohydrate content', Journal of Biotechnology.

Sambles, C., Moore, K., Lux, T. M., Jones, K., Littlejohn, G. R., Gouveia, J. D., Aves, S. J., Studholme, D. J., Lee, R. and Love, J. (2017) 'Metagenomic analysis of the complex microbial consortium associated with cultures of the oil-rich alga *Botryococcus braunii*', Microbiologyopen, 2017;e00482.

Littlejohn, G. R., Gouveia, J. D., Edner, C., Smirnov, N. and Love, J. (2010) 'Perfluorodecalin enhances in vivo confocal microscopy resolution of *Arabidopsis thaliana* mesophyll', New Phytologist, 186(4), 1018-25.

## Submitted for publication

Joao D. Gouveia\*, Jie Lian\*, Georg Steinert, Hauke Smidt, Detmer Sipkema, Rene H. Wijffels, Maria J. Barbosa. 'Associated bacteria of *Botryococcus braunii* (Chlorophyta)', submitted to Microbial Biotechnology.

\*Authors contributed equally

Joao D. Gouveia, Patrick Tijm, Georg Steinert, Rene H. Wijffels, Maria J. Barbosa, Detmer Sipkema. 'The impact of antibiotics on *Botryococcus braunii* growth, exopolysaccharide production and associated bacteria', submitted to MicrobiologyOpen.

Joao D. Gouveia, Antoine Moers, Yvonne Griekspoor, Lambertus van den Broek, Jan Springer, Lolke Sijtsma, Detmer Sipkema, Rene H. Wijffels, Maria J. Barbosa. 'Effect of removal of bacteria by UV-C treatment on the productivity of *Botryococcus braunii* capable of producing exopolysaccharides', submitted to Algal Research.

# ONE WAY ANOVA - TO COMPARE MEANS

①  $H_0: \mu_1 = \mu_2 = \mu_3$   
 $H_a$ : AT LEAST 1 difference among the means

② ANALISE DEGREES OF FREEDOM TO GET CRITICAL VALUE

$df_{\text{BETWEEN}} = k - 1 = 3 - 1 = 2$

$df_{\text{WITHIN}} = N - k = 9 - 3 = 6$

FROM TABLE  $t_{df, \text{between}}$   $t_{df, \text{within}}$

CRITICAL = 5.14

## ④ CALCULATE THE VARIANCE

$M_s \text{ BETWEEN} = \frac{SS_{\text{BETWEEN}}}{df_{\text{BETWEEN}}} = \frac{0.22}{2} = 0.12$

$M_s \text{ WITHIN} = \frac{SS_{\text{WITHIN}}}{df_{\text{WITHIN}}} = \frac{13.34}{6} = 2.22$

## ⑤ CALCULATE F VALUE

$F = \frac{M_s \text{ BETWEEN}}{M_s \text{ WITHIN}} = \frac{0.12}{2.22} = 0.05 < 5.14$   
 FAIL TO REJECT  $H_0$

INITIAL = 5.14  
 $F = \frac{0.12}{2.22} = 0.05$

- ①
- ②
- ③
- ④

③ SUM OF THE SQUARES  
 $\bar{X}_1 = 2.67$   
 $\bar{X}_2 = 2.67$   
 $\bar{X}_3 = 3.00$

$\frac{G}{N} = \frac{25}{9} = 2.78$

$SS_{\text{TOTAL}} = \sum (x - \bar{x})^2 = (-2.28)^2 + (2.28)^2 + \dots$

$SS_{\text{TOTAL}} = 13.6$

$SS_{\text{WITHIN}} = \sum (x_1 - \bar{x}_1)^2 + (x_2 - \bar{x}_2)^2 + (x_3 - \bar{x}_3)^2 + \dots$   
 $(-2.67)^2 + (2.67)^2 + \dots$

$SS_{\text{WITHIN}} = 13.34$

$SS_{\text{BETWEEN}} = 13.6 - 13.34 = 0.22$



# Educational courses and activities

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## **Courses**

Teaching and supervising MSc thesis  
Interpersonal communication  
Scientific writing  
Applied statistics  
Multivariate analysis for food/data scientists  
Lisbon Microalgae Biotechnology Advance Course  
Science communication Master class  
Voice matters

## **Symposium & Congress**

Microalgae process design: from cells to photobioreactors  
BFMM2013 Symposium  
YAS2014 Symposium (Poster)  
YAS2016 Symposium (Poster and pitch)  
NBV mini symposium  
2<sup>nd</sup> Wageningen PhD Symposium (pitch)  
European Phycology Congress 6  
European roadmap for an Algae-based industry (poster)

## **Social media communication**

FameLab The Netherlands 2015 (pitch your work competition)  
Radio interview with De Kennis van Nu



## About the author

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João was born in Lisbon, Portugal on the 16<sup>th</sup> of November 1979. He spent most of his youth in the region of Parede and Carcavelos which are well known for its beautiful coast line. Growing up near the Atlantic coast had a big influence in his life as he started to get involved with many sea explorations from a young age, namely spear hunting and later surfing. By the age of 14 it was clear he had a passion for the ocean and in his mind the seed to become a marine scientist was growing strong.

Most of his high school education was in Portugal, but in his final year he decided, as did his twin brother, to study in the USA as part of an exchange program. This move would change his life direction permanently. After finishing high school he moved to Wales UK to study marine and freshwater biology at the University of Wales, Aberystwyth where he graduated in 2003. The next two years were spend travelling in Europe in a Mercedes camper van with his first love. Travelling had become a life style since leaving Portugal at the age of 17. He travelled and visited different countries, and the last year were spend travelling and exploring Portugal. Mid 2005 there was a need for a new direction and so he moved back to the UK and focused on a research oriented career. During this period, he worked at a pharmaceutical company and at University of Derby as a laboratory technician. With other sights in his mind and heart, he decided to move to south Ireland where he worked as a marine scientist at Sherkin Island Marine Station, studying and monitoring the phytoplankton succession in the region. His time in Sherkin Island set a new momentum in his life direction as it was the beginning of the line that would take him to Wageningen University to do his PhD.

In 2008 João moves to Exeter as research assistant in Prof. John Love's group at the University of Exeter. Here he worked for the first time with the prodigious green microalga *Botryococcus braunii*, which produces cornucopias amounts of hydrocarbons. During his four and a half years at Exeter he also completed his Master by Research degree, graduating in 2010 with the thesis entitled "The effects of light quality in the morphology and hydrocarbon production of *Botryococcus braunii*". With his knowledge on and experience with *B. braunii*, João had a chance to follow a bioprocess engineering specialization after being offered a PhD position at Wageningen University at Bioprocess Engineering group lead by Prof. Rene

Wijffels. His studies were part of an European project named “SPLASH” initially lead by Dr. Maria Barbosa. During his five years of PhD he was also member of the VLAG PhD council supporting and representing the PhD candidates interests. In his final year he also joined the Centennial Committee lead by Prof. Louise Fresco in preparations of the Centennial Celebrations of Wageningen University. In 2017 he finished his PhD and his work is presented here in this thesis booklet.

The last five years of João’s life were also filled with other endeavours and achievements that have become part of his lifestyle shaping and directing his beliefs, his heart and motivations as well as his decisions. With his adventure companion and Exeter buddy Hugo Pinto, he started running and training as a ultra-runner and has now completed many ultra distances above 60 km in the Netherlands, Belgium, France, England and Hungary. His latest achievement in ultra running was completing the 100 Km CCC race as part of the UTMB event in 2016. He has also run several marathons in the Netherlands, Copenhagen, Berlin and Singapore. He stills keeps running and reaching new limits. The other big change in his life happened when he joined the dance group *InMotion dance traces* lead by Marisa Grande. The work of somatic training in body movement and improvisation has challenged his body and mind to new possibilities. His love for the art of movement has been important also in how he started to relate to science creating in him a strong view on the benefits of arts and science being inseparable. With InMotion dance traces he has also had the pleasure to perform in Wageningen, Utrecht and Amsterdam. It was also in the InMotion group that he met his lovely partner Petra.

João is currently living in Singapore with Petra. They both do their science research at their respective institutions, and both also dedicate time to their continuous dance exploration, having created a rigorous schedule with training and performances.





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