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Non-destructive oil extraction from Botryococcus braunii (Chlorophyta)

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Abstract

Some of the key reasons for why the production of biofuels from microalgae have not yet succeeded as a source of sustainable transport fuel are the costs involved and the amount of energy needed to obtain the oils compared to the energy contained in the final fuel. The key energy costs are in the dewatering of biomass followed by extraction of the oil, disposal of biomass, and the energy content of the nutrient fertiliser needed for regrowing the algae. In this study, we bypass all of these barriers by using a different approach towards cutting energy and fertiliser costs in the production of biofuels from microalgae—rather than growing the algae in the presence of fertilisers such as N and P, followed by harvesting the whole algae cells, and the energetically costly drying of cells and extraction of the fuel from the cells, this process makes use of the natural tendency of the green alga, *Botryococcus braunii* to release oils from the cell into the extracellular matrix during and after growth. Here, we non-destructively and repeatedly harvest the external oil (hydrocarbons) from *B. braunii* CCAP 807/2. Extraction with several solvents showed that hexane was not compatible with *B. braunii*, but that heptane in contact with *B. braunii* for less than 20 min did not negatively affect this alga. As an alternative, solvent-free method, we tested physical methods of extracting the extracellular oil. Light and temperature did not affect the extraction of the external oil from *Botryococcus*, but gentle pressure (i.e. 'blotting') was an effective method for external oil recovery. Less than 1 h of blotting also did not affect the physiology of *Botryococcus*. Both the heptane extraction and the non-destructive 'blotting' methods had no significant effect on growth and photosynthesis (F_v/F_m, ETR_{max}) of *B. braunii*. Our results indicate that over a period of 6 days, we can repeatedly extract over 35 % (using heptane) and 1 % (using 'blotting') of the total oil, mainly in the form of external hydrocarbon in stationary phase cells without damage to the cells.

Keywords: *Botryococcus;* Chlorophyta; Hydrocarbon; Extraction; Renewable;

Introduction

There is significant worldwide interest in sustainable renewable oil production from microalgae (Borowitzka and Moheimani 2013, Lundquist et al. 2010). The lower doubling time and, hence, higher growth rate of microalgae is the key to offering a potentially more attractive feedstock for bioenergy compared to terrestrial plants. Furthermore, microalgae can be grown on non-arable land using saline and hypersaline water sources (Fon Sing et al. 2013), thus not competing with food crops for water and land. However, like all plants, algae require fertilisers to grow and to achieve high productivities. In line with Redfield ratio (Redfield 1958, Stumm and Morgan 1996), the stoichiometric formula for algae is estimated to be $C_{106}H_{263}O_{110}N_{16}P$—hence about 64 g of nitrogen (=390 g of $NaNO_3$) and 9 g of phosphorus (=34 g of $NaHPO_4$) are required to produce 1 kg of microalgae biomass.

In a conventional process of algae-to-biofuel production, the main steps are to: (a) grow algae with a high concentration of oil, (b) harvest and dewater the algae biomass, and (c) extract the lipids and convert them to biofuel. A major drawback in the efforts to use algae, as well as any other oil-producing plants as sources of renewable energy, is that current harvesting and post harvesting techniques are expensive and require a high energy input (Moheimani et al. 2011). Microalgal triglycerides, the main source of algal oils for biofuels, are secondary metabolites and are mainly produced when microalgae growth is limited (Ratledge and Kristiansen 2001; Hejazi et al. 2004). Over the last few decades, there has been a major research and development effort worldwide on optimising this conventional algae-to-biofuel process (Sheehan et al. 1998; Borowitzka and Moheimani 2013), but so far there is no large-scale commercial algae biofuel production. Several species of algae are being cultured commercially for other high value products (Borowitzka and Moheimani 2013) as microalgae as a source of bioenergy are not yet economical and sustainable.

Almost all microalgae, like other oil-producing plants, produce and store their oil intracellularly. The green alga *Botryococcus braunii* is an exception as this alga releases some of the oil in the form of long-chain aliphatic hydrocarbons from the cells into the extracellular matrix (Blackburn 1936; Metzger and Largeau 2005). *B. braunii* has been widely studied, especially due to its involvement in the formation of current fossil fuel resources (Cane and Albion 1973, Wake and Hillen 1981, Metzger and Largeau 2005). Because of its high hydrocarbon content, *B. braunii* has been identified as an appropriate species for biofuel production. This alga can accumulate hydrocarbons to as high as 40–50 % of the ash free dry weight (Metzger and Largeau 2005) which can be converted to fuel. For example, Dayananda and colleagues (2006) showed that hydrocracking of botryococcenes of *B. braunii* resulted in 67 % gasoline, 15 % aviation turbine fuel, 15 % diesel fuel and 3 % residual bio-oil. However, the slow growth of this alga presents a significant problem for the development of this alga as a source of commercial biofuels.

A particular innovative suggestion that addresses a number of the cultivation and associated cost problems with algal biofuel production is the concept of 'milking' or the 'non-destructive' oil extraction of the external hydrocarbons or oil from microalgae (Frenz et al. 1989; Hejazi et al. 2004). The concept is very simple: rather than growing the algae in the presence of fertilisers (i.e. nutrients such as N and P) followed by harvesting the whole algal cells and the energetically costly drying of the cells and extraction of the fuel from the cells, the non-destructive oil extraction process intends to extract the algal oils into a solvent from live algae cells without killing the cells. This would mean that, rather than growing new algae cells after extraction, the cells can be re-used for further lipid production. This concept of harvesting the oil released while recycling rather than discarding the bio-catalytic algae cells is similar to 'milking'. Traditional algae production systems 'kill' the 'cow' (algae) to extract the 'milk' rather than re-using the cow for many batches of milk. To date, all of studies on the process of 'milking microalgae' have focused on using solvent extraction by brief contact between the cells and the solvent. Using hexane or any other possible biocompatible organic solvent enabled the in situ collection of a fraction of hydrophobic hydrocarbons (up to 30 %) of *B. braunii* that are embedded in the extracellular matrix of the colony (Frenz et al. 1989). Due to the short extraction time, the cells were able to recuperate and could be returned to the culture vessel for further growth. The most important advantage of such a process is that the production step in which biomass is generated does not need to be continuously repeated. When using solvent, the key factor is the selection of an inert hydrophobic chemical with a low inhibition of cell viability, being incapable of mixing with the algal culture medium and with a suitable density for phase separation for the extraction process with a favourable partitioning of the compound of interest (Sim and Chang 1993). The polarity of the solvent is also an important property that affects the recovery efficiency of the compounds of interest (Sim et al. 2001).

For the production of algal oil for fuel, there are additional concerns over using solvents for milking microalgae. One concern is the long-term toxicity of the solvent used. For instance, Kleinegris et al. (2011) showed that direct contact between *Dunaliella salina* cells and an organic solvent such as dodecane often resulted in cell death. The toxicity of long carbon chain solvents such as hexane, heptanes and dodecane may disrupt the plasma membrane of some algae such as *D. salina* and rupture the algae cells, causing lipids or other compounds such as carotenoids to leak into the medium. This could give a false sense of a 'milking' process, but it is really a solvent extraction as the cells are no longer viable after the solvent treatment. Therefore, if solvent is used, it is critical to test the viability of cells after the contact with the solvent. A second concern is the fact that the solvents themselves are made from fossil fuel. For example, if the solvent used represents a typical percentage of the final product of 80 %, then the fuel value of the final product is more due to the solvent rather than the algal oil. Finally, the separation of solvent from the product and its reuse is likely to be required, but this is likely to have a high energy cost.

The main question is whether the external oil of *B. braunii* can be harvested repeatedly without damaging the cells using a solvent-free procedure. In this study, we investigated repeated solvent extraction as well as a potential method for replacing solvents or minimising solvent use in repeatable non-destructive oil extraction of the extracellular oil from *B. braunii*.

Materials and methods

Culture maintenance

The non-axenic culture of *B. braunii* Kützing strain CCAP 807/2 was obtained from the Culture Collection of Algae and Protozoa, UK. The alga was maintained on modified 3N and 3P Chu13 medium (Largeau et al.. 1980). The culture was free of any other microalgae but contained a small load of bacteria. The 500 mL of cultures were maintained in 1 L non-aerated, unstirred Erlenmyer flasks at 25 °C under a 12:12 light–dark cycle at 120 ± 20 μmol photons m^{-2} s^{-1}.

Growth and photosynthesis measurements

Growth parameters (ash free dry weight, cell counts) were determined using the methods described in Moheimani and Borowitzka (2007). Total lipid (external and internal lipid) was by the method of Kates and Volcani (1966) as adapted by Mercz (1994) and as described in Moheimani and Borowitzka (2006). Qualitative and quantitative (see non-destructive oil extraction) measurements of extracted oil fractions was by thin layer chromatography (TLC) as previously described by Fried and Sherma (1982) and Ackman (1991). Standard TLC plates (MERK, TLC Silica Gel 60 F_{254}) were used with a solvent mixture of 25 % (*v*/*v*) diethylether, 75 % (*v*/*v*) hexane with 0.1 % acetic acid (*v*/*v*) added to facilitate phase separation. *B. braunii* total protein and carbohydrates were determined using the methods described in Moheimani et al. (2013).

A Water PAM (Walz GmbH) was used for fluorescence measurements. Samples were dark adapted for 20 min and rapid light curves (RLCs—Ralph and Gademann 2005), and F_v/F_mwere measured using the in-built protocols and the maximum electron transport rate (ETR_{max}) was calculated by fitting the data to a double exponential decay function (Platt et al. 1980). Gross photosynthesis and dark respiration rates were measured at constant cell densities of $9 \pm 0.5 \times 10^5$ cells mL^{-1} using a Clark-type oxygen electrode (Rank Brothers, Bottisham) as previously described by Moheimani et al. (2013). Photosynthetic rates were measured on 10–15 min dark adapted samples between 0 and 2 mg O_2 L^{-1} , to minimise any potential effect of O_2 inhibition on photosynthesis, at 25 °C and a saturating irradiance of 100 μmol photons m^{-2} s^{-1} (Kirk 1994) provided by a quartz halogen lamp. For calibration, the oxygen content in the air-saturated medium was determined using the tables of Carpenter (1966).

Using light, temperature and pressure for extracting external hydrocarbon

A drop of a stationary phase cell suspension was placed on a microscope slide with a concave well or a normal flat microscope and covered by cover slip, taking care to avoid air bubbles under the cover slip. The slides were exposed to irradiances of 0, 100, and 800 μmol photons m^{-2} s^{-1} and at 15, 25 and 35 °C in humidified air to avoid volume loss of the samples for 4, 8 and 12 min before observing oil release under a compound light microscope at 40× magnification. This experiment was conducted five times.

Solvent based non-destructive oil extraction

n-Hexane and *n*-heptane were used to non-destructively extract external hydrocarbons from cultured *B. braunii*. When the cultures had reached stationary phase at $9 \pm 0.5 \times 10^5$ cells mL^{-1}, the culture was divided into six 100 mL cultures in 250 mL Erlenmyer flasks. For external hydrocarbon extraction, 20 mL of solvent was added to the cultures (Sim et al. 2001; Frenz et al. 1989), mixed for 0 (control), 5, 10, 20, 40 and 60 min on a rotary shaker at 125 rpm providing a mixing rate of of 20 ± 3 s^{-1} determined by measuring the time required for India ink to mix completely in the Erlenmeyer flask (Moheimani et al.. 2011). Using a 10-mL pipette, the solvent (contained algal lipids) was then removed carefully, placed in a pre-weighed vial and evaporated with pure N_2 gas (lipids remain in the vial after evaporation). The hydrocarbon content was then measured gravimetrically using a 5-digit balance. The algae were then kept in the same growth conditions as previously and the extractability of the samples was re-tested after 2, 3 and 7 days. In the repeated solvent extraction experiments, the culture after removal of the solvent was incubated under the normal growth conditions described above.

Solvent-free non-destructive oil extraction

Blotting (physical pressure) was used to non- destructively extract external oils from *B. braunii*. As a simple means of applying physical pressure, floating *B. braunii* colonies were placed between stacks of filter paper with a defined pressure applied (blotting). *B. braunii* samples (12–15 mL) from the cultures were concentrated from 0.2 to 30 ± 2 g of ash free dry weight (AFDW) L^{-1} by gentle filtration of 10 kPa using a 25 mm GF/F Whatman glass-fibre filter. A 25-mm 1-μm pore Whatman polycarbonate (Nucleopore) filter was then placed on the algae side of the GF/F filter, and a 47 mm diameter 0.2 μm pore size mixed cellulose ester filter was placed on top of the Nucleopore filter. This 'algae pack' was then placed between two PVC plates and a pressure of 215–875 Pa applied, a process we have termed 'blotting'. After a certain time (1–4 h), the filters were separated, and the blotted oil fractions in the mixed cellulose ester filter were extacted with 3 mL chloroform in a 5-mL glass vial by vortexing for 2 min. The chloroform was then evaporated using a stream of pure N_2 gas in fume hood. The algal oil fraction in the glass vial then was redissolved in 120 μL chloroform and spotted on a pre-activated TLC plate as well as a known amount of olive oil and squalene (50 μL L^{-1}) as standards and chromatographed (Moheimani et al. 2013). The surface area of the separated oil fractions (triglycerides, hydrocarbons, phospholipids, etc.) on the TLC plate were then measured. To be able to quantify the amount of extracted hydrocarbons a standard curve was prepared from squalene stock solutions (between 10 and 100 μg dissolved in chloroform) on a separate TLC plate. In the repeated blotting experiment, the filter containing the algae was then resuspended in fresh medium so that the algae could continue to grow.

Results

Solvent based non-destructive oil extraction

Initially various solvents were used to test the possibility of non-destructive oil extraction from *B. braunii* CCAP 807/2. Experiments were carried out on suspended *B. braunii* colonies at stationary phase with a cell density of $9 \pm 0.5 \times 10^5$ cells mL^{-1} (=0.2 ± 0.04 g AFDW L^{-1}), with a proximate composition of 31 ± 3 % total oil, 37 ± 4 % total carbohydrate and 29 ± 5 % total protein (percent of AFDW). To be able to use a solvent for non-destructive oil extraction, the solvent must not be harmful to cell metabolism, and we therefore studied the effect of the solvents on photosynthesis. The results indicated that hexane is not a suitable solvent for *B. braunii* (Fig. 1). Any contact time with hexane longer than 2.5 min negatively affected photosynthesis, and 2.5 min contact time was not long enough to collect any external oil using either hexane or heptane (Fig. 1). On the other hand, heptane did not significantly affect the photosynthetic parameters determined for up to 20 min contact time, and F_v/F_m was only reduced significantly when the exposure period was longer than 20 min (repeated measures one-way ANOVA $P < 0.05$) (Fig. 1). The optimum contact time for non-destructive oil extraction using heptane was between 5 and 20 min (Fig. 1).

Thin layer chromatography showed that with less than 20 min contact time, the composition of the extracted oil was >90 % hydrocarbons, <7 % triglycerides, and <3 % phospholipids indicating little, if any cell damage. On the other hand, increase in contact time extracted more triglycerides and phospholipids indicative of damaged cells. A heptane extraction time of 20 min was therefore selected as the solvent for testing a repeatable solvent-based non-destructive oil extraction process.

The repeatable non-destructive extractability of *B. braunii* external oil was then tested over a period of six days using 5, 10 and 20 min contact time between heptane and the medium containing *B. braunii*. Repeatable non-destructive extraction of the external oil was achievable under these conditions (Fig. 2). Although oil could be extracted consistently using a 5-min contact time, the extracted amount was the same as 10 min contact time but 30 % less than that when a contact time of 20 min was used (Fig. 2a). An oil recovery of 3–7 mg oil g^{-1}*B. braunii* day^{-1} was found between day

1 and day 6 with a 20-min contact time. Increase in contact time between heptane and *B. braunii* from 5 to 20 min significantly reduced F_v/F_m(repeated measures one-way ANOVA $P < 0.05$, Fig. 2c). However, under all of these conditions, *B. braunii* F_v/F_m was not affected during the 6-day period of the experiment (RM one-way ANOVA $P < 0.05$, Fig. 2c). Results show that after 6 days of algal incubation, an almost similar amount of oil could be extracted again, while 1 or 2 days of incubation was insufficient for renewed production of extractable extracellular oil (Fig. 2a).

Solvent-free non-destructive oil extraction

Preliminary microscopic observations indicated that *B. braunii* CCAP 807/2 colonies can release their external oil when placed on a microscope slide under a cover slip (Fig. 3). This suggested an opportunity of oil recovery by physical means rather than solvent extraction. In order to determine whether it was the effect of light, temperature or pressure caused by the cover slip that caused the visible oil release from colonies of *B. braunii*, simple tests were carried out. These showed that that the *Botryococcus* colonies released oil only when pressure was applied and this oil release was not affected by light or increased temperature (Table 1).

Based on the outcome of the previous experiment, a series of experiments were carried out to investigate the use of pressure to non-destructively extract the external oils of *B. braunii*. These experiments were carried out on colony-forming and floating *B. braunii* cells in stationary phase with a cell density of $8.6 \pm 0.6 \times 10^5$ cells mL^{-1} (=0.19 ± 0.02 g AFDW L^{-1}), with a proximate composition of 29.5 ± 4 % total oil, 36 ± 3 % total carbohydrate and 25 ± 8 % total protein.

The blotting experiments showed that almost all of measured photosynthesis parameters were affected negatively by blotting and that an increase in blotting time increases this negative effect (Fig. 4). For instance, between the control (0 h) and 0.5 h of blotting, F_v/F_m was reduced by 11 % (Fig. 4). However, no significant differences were found between ETR_{max} and F_v/F_mbetween 0.5 and 1 h of blotting (RM one-way ANOVA, $P > 0.05$). On the other hand, both ETR_{max} and F_v/F_m were markedly reduced between 2 and 24 h of blotting, resulting in death of the culture after 10 and 24 h blotting time (Fig. 4). Based on the TLC results, when less than 3 h of blotting was applied, over 97 % of the blotted oil was hydrocarbons, suggesting that cell lysis had not occurred. Increasing the blotting time resulted in an increase in the phospholipid and triglyceride content of the blotted oil.

In order to test whether *B. braunii* colonies from which oil was extracted by blotting could continue to photosynthesise and the de novo secreted oil could be extracted again; the blotting procedure was repeated over a period of 6 days incubation in the light (Fig. 5). Repetitive non-destructive oil extraction by blotting was possible after 6 days of incubation (Fig. 5). Again, as shown in the heptane extraction experiment, no new oil was recoverable after 1 day of incubation (Fig. 5a), indicating that the algae needed more time to produce more extracellular oil. The composition of the recovered oil was >95 % hydrocarbons, <3 % triglycerides and <2 % phospholipids, also indicating little cell damage. While the original blotting on day 0 resulted in 43 ± 6 % reduction in F_v/F_m, the blotting did not significantly affect the F_v/F_m of the blotted cells between day 0 and day 6 (RM one-way ANOVA, $P > 0.05$, Fig. 5c), showing that the repeated blotting did not affect the physiology of the algae cells post the primary blotting.

Discussion

The main advantage of non-destructive oil extraction from microalgae is the avoidance of the need for addition of fertilisers to the culture. The conventional microalgae-to-biofuel production process requires a large amount of fertilisers (Borowitzka and Moheimani 2013). Nitrogen fertilisers are made from fossil fuel, and there is a likelihood of peak phosphorous (Cordell et al. 2009), which means that fertiliser will be an issue in renewable biofuels production. In the process of non-destructive oil extraction, the use of fertilisers will be limited compared to conventional algae culture.

This study demonstrates that in principle the external oil of *B. braunii* can be extracted non-destructively from colonies using either compatible solvents such as heptane or a suitable physical method such as applying pressure and capillary forces. After extraction, the harvested algae colonies were photosynthetically active and could, in the absence of nutrients, produce new oil which could be extracted again after 6 days. Although repeated solvent-based non-destructive oil extraction from other strains of *B. braunii* has been reported previously (for example, see Sim et al. 2001), this is the first description of a non-solvent-based method has been successful.

The idea of 'milking' algae cells repeatedly by non-destructive solvent extraction is not new and *Botryococcus* is not the only alga for which non-destructive oil extraction has been attempted. A number of algae species (i.e. *Spirulina*, *Dunaliella* and *Nannochloropsis*) and also the halophilic bacterium *Halomonas elongata* have been tested for the potential of non-destructive extraction of high value products or biofuel (Sauer and Galinski 1998; Hejazi et al. 2004; Eroglu and Melis 2010; Sim et al. 2001; An et al. 2004). For instance, milking yields for β-carotene from *D. salina* by (Hejazi et al. 2004) were up to 55 % of β-carotene with a productivity of 0.06 mg L^{-1} day^{-1}. However, Kleinegris et al. (2011) showed that direct contact between the *D. salina* cells and an organic solvent such as dodecane in a two-phase system often resulted in cell death and that the β-carotene recovery through this system can destroy the cells.

Frenz et al. (1989) showed that botryocococcene recovery yields from *B. braunii* depend on the physiological state of the algae and the total hydrocarbon content of the biomass. They attempted non-destructive oil extraction with hexane showing yields ranging from 7.4 to 14 % and up to 38 % of the total hydrocarbons. Evidence of cell survival was not provided. In our experiments, hexane was an effective solvent for extraction of oil but significantly inhibited photosynthesis, limiting the capacity of repeated oil harvesting from live cells. Dihexyl-ether rather than hexane in a two-phase reactor for continuous extraction of hydrocarbon from *B. braunii* resulted in a maximum hydrocarbon productivity of 28 mg L^{-1} day^{-1} (Sim et al. 2001). However, based on the findings of Kleinegris et al. (2011), it is not clear if the method of Sim et al. (2001) was non-destructive. An et al. (2004) showed a threefold increase in *B. braunii* hydrocarbon recovery when a two-stage cell-recycle extraction process was used compared to a direct contact with the solvent. They also noticed serious growth inhibition of *B. braunii* by *n*-octane (An et al. 2004). These studies show that while significant productivities of oil could be obtained, the survival of the algal cells was not necessarily guaranteed.

Nevertheless, the results of potential non-destructive oil extraction of *B. braunii* are very interesting. In general, the lipid productivities of the targeted strains for biofuel production [e.g. *Nanochloropsis* (Zittelli et al. 2003), *Tetraselmis* (Moheimani 2013), *Chlorella* (Kanazawa et al. 1958), *Pleurochrysis* (Moheimani and Borowitzka 2006)] using conventional methods are in the range of 4–120 mg L^{-1} d^{-1} depending on the type of cultivation system used. Although the overall productivity of non-destructively extracted external hydrocarbon of *Botryococcus* (0.3–7 mg oil g^{-1}*Botryococcus* day^{-1} = 0.06–1.4 mg L^{-1} day^{-1}) achieved in this study is far lower than what has been reported for other microalgae, this process certainly has not yet been optimised.

The fact that short extraction times of 20 min with heptane could extract a significant proportion of the algae oil without compromising algae viability as measured by F_v/F_mindicates that in principle the algae cells could be exposed again to the light to allow further extractable oil to be produced, without requiring regrowing of the algae cells. When using short extraction times of 5 min, only about 20 % of the oil that was found to be extractable over 20 min could be obtained (Fig. 2a). Similar amounts could be extracted after 1, 2 and 6 days. It is not clear whether this represents de novo excreted oil or residual oil not extracted from the previous attempts. It appears unlikely that solvent-based non-destructive process can be put to use for the extraction of bulk hydrocarbons as renewable fuel. Not only is the repeated generation of the solvent by thermal distillation costly, the likelihood of solvent loss within the algal biomass, followed by air stripping and/or bacterial degradation sheds doubts on the likely success of harvesting algal oil for fuel by using solvents. Solvent extraction however could be a feasible process when the target oil is of significantly higher commercial value than the solvent itself.

As yet, we have tested our method only for a period of 7 days, and it remains to be seen for how long such a method can be continued. For instance, the blotting experiment clearly indicated that solvent-free non-destructive repetitive extracellular oil extraction of *B. braunii* is possible when pressure is applied to recover the external oil. The repeated blotting method however recovered less hydrocarbon per day (maximum rate 0.3 mg oil g^{-1}*Botryococcus* day^{-1}) compared to the solvent-based method (maximum rate 7 mg oil g^{-1}*Botryococcus* day^{-1}). There also is a need to design a suitable reactor with integrated continual physical pressure extraction of the oil for this process and to study the long-term reliability of such an oil recovery process. The productivities shown in the current work are not yet at a commercially relevant level. However, the productivity (grams of oil per litre per day) depends directly on the yield of algal cells (grams per litre) and the rate of production of 'new' hydrocarbons released to the outside of the algae cells. For example, if after growth and first oil recovery the cells were suspended into a tenfold smaller volume, resulting in tenfold higher cell densities, then the photosynthetic oil production is expected to be also tenfold higher, provided that light, oxygen, and carbon dioxide can be kept at non-limiting conditions. Furthermore, the conditions for optimum external oil production have not yet been determined. Another possible future improvement of continued oil harvesting from *Botryococcus* cells could the use of immobilised biofilms of *Botryococcus*. It is well known that dewatering is a very cost and energy expensive part of the conventional algae-to-biofuel production (Moheimani et al. 2011). The algae needs to be grown, dewatered and then biomass can be converted to biofuel by several methods (de Boer et al. 2012). If non-destructive oil extraction from *B. braunii* can be scaled up when the alga is immobilised, this can potentially remove the dewatering stage. Obviously, this will only be viable while cells are still alive. In this case, the use of a physical, non-destructive hydrocarbon extraction method could also reduce costs by facilitating the dewatering process. Therefore, in authors view an immobilised culture of *B. braunii* seems to be the most realistic method for scaling up non-destructive oil extraction. However, the main limit for such a process would be the number of times that *B. braunii* oil can be non-destructively extracted and the oil recovery rate. This is yet to be tested. The question of either solvent-based or non-solvent-based non-destructive oil extraction would be used in the future scaled system is yet to be addressed. While, the non-solvent-based non-destructive oil extraction is more environmentally friendly, the solvent-based method showed to extract more oil. If the solvent-based method is to be scaled up, there will be a need for solvent recovery process.

In conclusion, there as yet exists no suitable system for long-term non-destructive oil extraction from microalgae, in particular for bulk extraction of low-value fuel oils. However, our results show that in principle such a process is possible, especially the non-solvent method for the extraction of external oil from *B. braunii*.

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Fig. 1 Extraction of external oils from *Botryococcus braunii* CCAP 807/2 with *n*-hexane and *n*-heptane: Effects if time of contact with solvent on oil extraction and photosynthesis of the algae cells. Data are: mean ± range, $n = 3$, for total oil in the cells and extracted oil measurements; mean ± SE, $n = 5$ for PAM fluorometery measurements (ETR_{max} & F_v/F_m); mean ± range, $n = 3$ for O_2 electrode measurements of photosynthesis and respiration rates; mean ± SE, $n = 15$ for controls

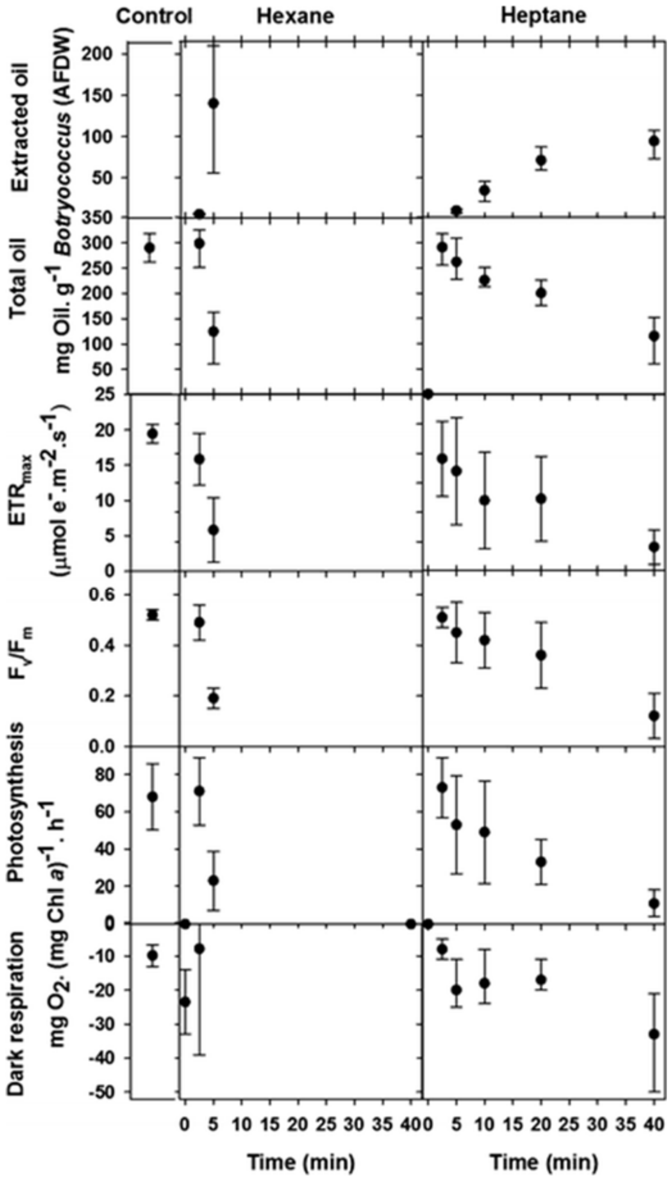

Fig. 2 Repetitive non-destructive extraction of external oils from *Botryococcus braunii* CCAP 807/2 using *n*-heptane. **a** Amount of oil extracted; **b** total oil remaining in cells, and **c** F_v/F_m . *Circles* = 5 min contact time, *squares* = 10 min contact time; *triangles* = 15 min contact time. Data are mean ± range for total oil and extracted oil measurements. $N = 3$; mean ± SE, $n = 6$ for F_v/F_m measurements

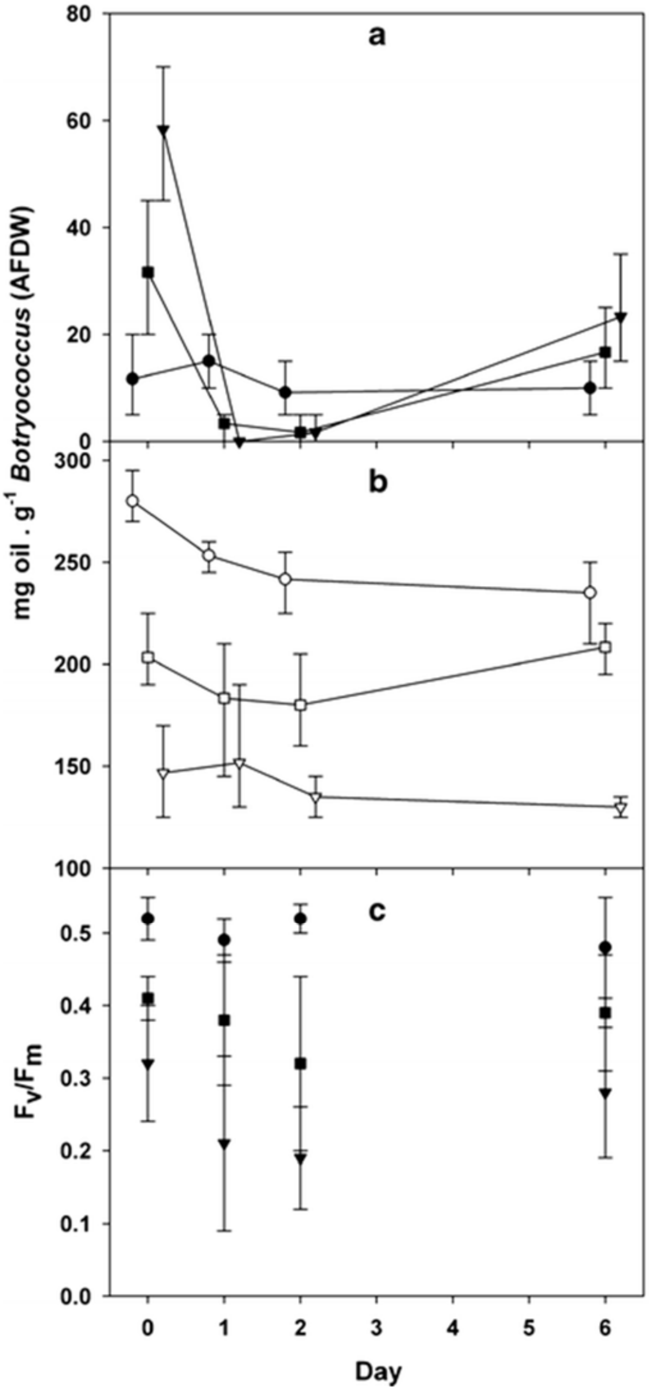

Fig. 3 *Botryococcus braunii* colony CCAP 807/2, **a** immediately after being placed on slide and **b** the same colony 5 min after being on the slide under compound microscope with cover slip (microscope light was left on)

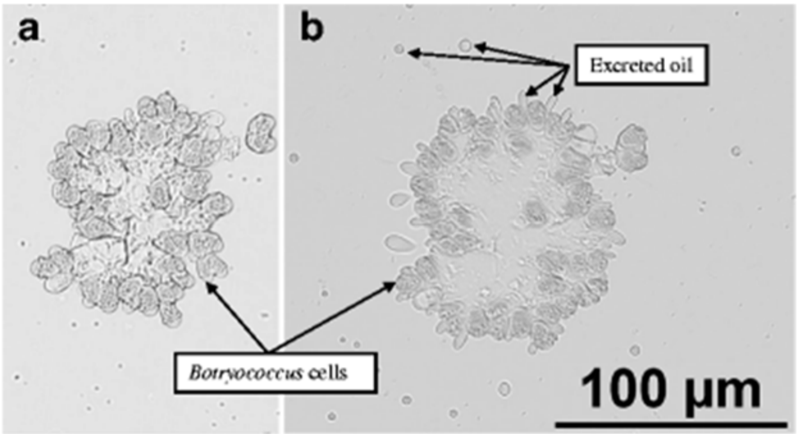

Fig. 4 Effect of blotting time on external oil extractability and the photophysiology of *Botryococcus braunii* 807/2. Data are mean ± range, n = 3 for total oil and extracted oil measurements; mean ± SE, n = 5, for PAM fluorometery measurements

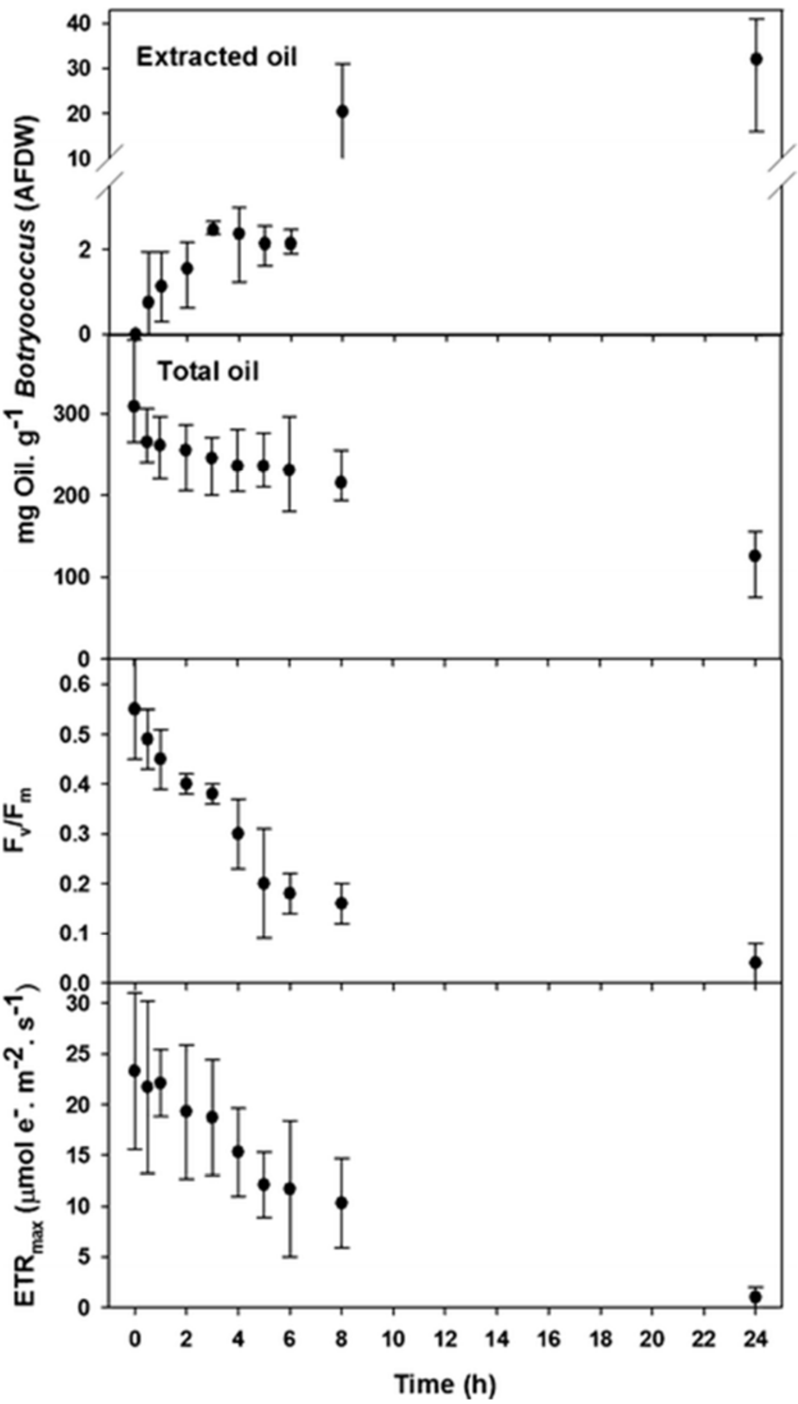

Fig. 5 Repetitive non-destructive extraction external oil from *Botryococcus braunii* CCAP 807/2 using 3 h blotting. a extracted oil, b total oil remaining after blotting, and cF_v/F_m. Data are mean ± range $n = 3$ for total oil and extracted oil measurements; mean ± SE $n = 6$ for F_v/F_m measurements

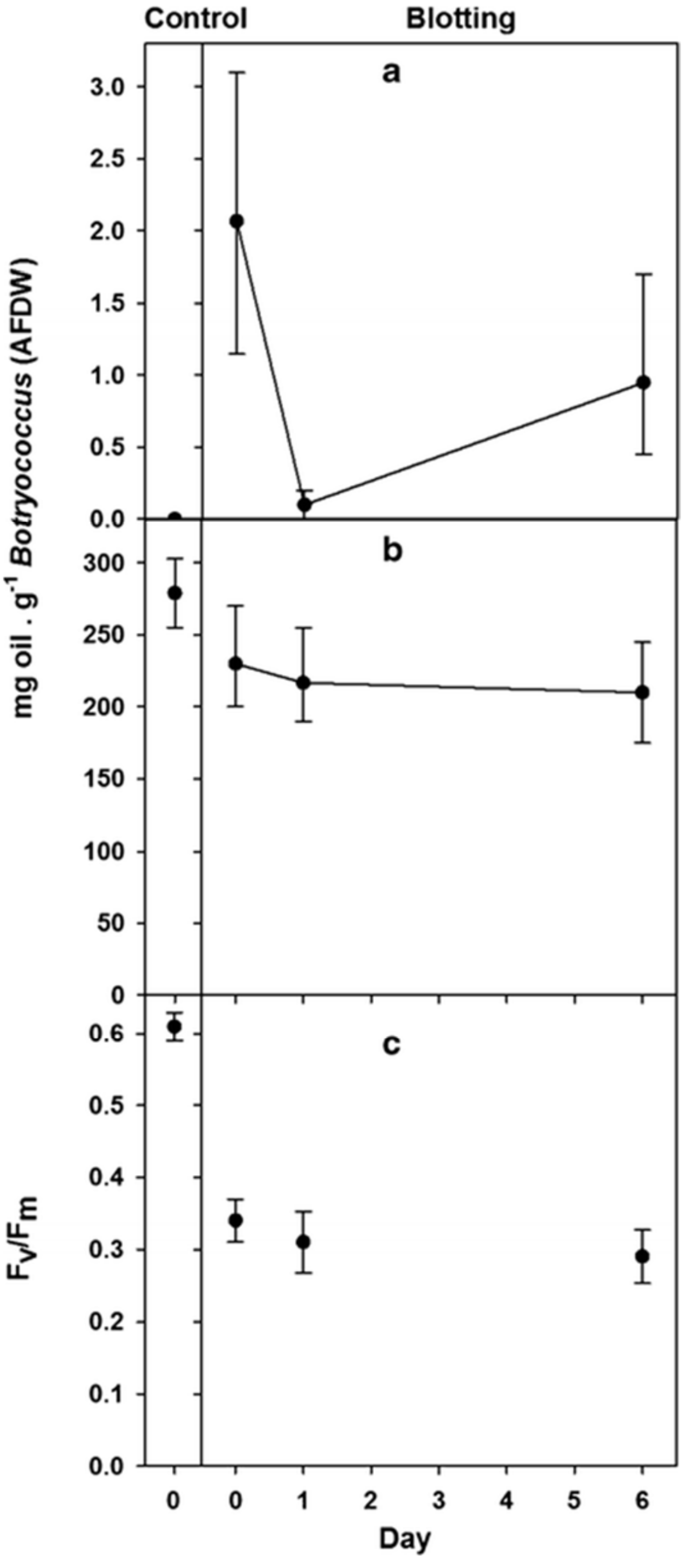

Table 1 Effect of light vs. temperature vs. pressure on the oil release from *B. braunii* CCAP 807/2 colonies

		0 µmol photons m−2 s−1			**100 µmol photons m−2 s−1**			**800 µmol photons m−2 s−1**		
		15 °C	25 °C	35 °C	15 °C	25 °C	35 °C	15 °C	25 °C	35 °C
Slide with concave well	4 min	−	−	−	−	−	−	−	−	−
	8 min	−	−	−	−	−	−	−	−	−
	12 min	−	−	−	−	−	−	−	−	−
Flat slide	4 min	−	−	−	−	−	−	−	−	−
	8 min	+	+	+	+	+	+	+	+	+
	12 min	+	+	+	+	+	+	+	+	+