Pure

Bangor University

DOCTOR OF PHILOSOPHY

Microalgal photobioreactors for power plant CO2 mitigation and bioenergy

Hulatt, Christopher J.

Award date:
2011

Awarding institution:
Bangor University

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 27. Apr. 2024

Microalgal Photobioreactors for Power Plant CO_2 Mitigation and Bioenergy

Christopher J. Hulatt

A Thesis in Partial Fulfillment of the Requirements of Bangor University
for the Degree of Doctor of Philosophy

LLYFRGELL PRIFYSGOL BANGOR UNIVERSITY LIBRARY

Acknowledgements

A massive thank-you to my supervisor Prof. David Thomas for all his help and support over the last few years. Despite the many problems, setbacks and general trouble during my research, he invariably helped and supported my studies in every single way possible. In short, I could not have asked for a better supervisor.

A huge thank-you to Louiza Norman, not least for her top-class analytical skills, but particularly for her support and fantastic attitude to life in general. Although I'm sure my sometimes laid-back approach caused her untold stress, she invariably carried it off with a smile and was a true pleasure to work with.

Thanks to Joe McDonald (Varicon Aqua Solutions Ltd, UK), Martin Pilbin, Richard Hotchkiss and Roger Bewley, Sam Pearshouse and Alan Pearshouse (RWE npower, UK) for organizing and co-ordinating the project and meetings, for insightful opportunities and for valuable discussions associated with the research. Thanks especially for their patience every time I started talking about DOM!

Thanks to Naomi Thomas for her analytical support and generally cheering me up in the lab.

Thanks to Sarah Carver and Aino-Maija Lakaneimi for their help and support during my time at Tampere University of Technology and, with the odd exception, for keeping me out of trouble! Thanks to Jaakko Puhakka and Olli Tuovinen for their valuable contributions.

Thanks to Ian Nichols for helping me with everything I built.

Thanks to Dr David Ellis and Rosemary Crichton for introducing me to algal cultivation and the use of laboratory equipment.

Thanks to Dr David Bowers, Ian Pritchard, Gwyn, Joan, John East, Paul Kennedy and Stathys Papadimitriou for supplying me with various pieces of equipment and information when I needed it.

Thanks to university PhD committee members Lewis LeVay & Hilary Kennedy.

Thanks to Dr. Colin Jago for the long-term loan of the PAM fluorometer and Dr. John Turner for the long-term loan of the light-meter.

Thanks to Raquel and Andy for coffee, cookies and company.

Thanks to the Engineering and Physical Sciences Research Council (EPSRC) and RWE npower for studentship funding.

Statement of Contributions to Results Chapters

Chapter 3 - Chris Hulatt designed and carried out the practical work under the supervision of David Thomas. He carried out the data analyses and was the lead author with input from David Thomas into manuscript preparation and revision. Louiza Norman and Naomi Thomas contributed nutrient and bacterial analysis to the data set. David Ellis contributed bacterial measurements to the preliminary data set (Chapter 3.1).

Chapter 4 - Chris Hulatt designed and carried out the practical work under the supervision of David Thomas. He carried out the data analyses and was the lead author with input from David Thomas into the manuscript preparation and revision. Louiza Norman contributed nutrient analyses to the data set.

Chapter 5 - Chris Hulatt and Aino-Maija Lakaniemi jointly designed and carried out the practical work under the supervision of David Thomas and Jaakko Puhakka. Chris Hulatt and Aino-Maija Lakaniemi both carried out the data analyses. Chris Hulatt was the lead author with input from Aino-Maija Lakaniemi, David Thomas and Jaakko Puhakka into the manuscript.

Chapter 6 - Chris Hulatt designed and carried out the practical work under the supervision of David Thomas. He carried out the data analyses was the lead author with input from David Thomas into the manuscript preparation and revision.

Chapter 7 - Sarah Carver conceived the experimental design together with Chris Hulatt. The first set of experiments and analyses were conducted by Chris Hulatt under the supervision of Sarah Carver at Tampere University of Technology, Finland. Sarah Carver conducted subsequent experiments, from which Chris Hulatt performed elemental and ammonium analysis at Bangor University, Wales. Sarah Carver was the lead author on the paper, but all co-authors had input into the manuscript preparation and revision.

Appendix 1 – Chris Hulatt was involved with production of algal biomass, preliminary enrichments and latter stages of manuscript preparation.

Summary.

The overall hypothesis tested in this research was that microalgae are an efficient method for power-plant flue-gas CO_2 mitigation and bio-energy production. Research examined the losses of organic matter from algal cells, finding that potentially significant quantities of organic carbon and energy may be lost in this manner. The work also examined the energy balance of laboratory-scale gas-sparged photobioreactors. Manipulation of the power input for gas-sparging influenced the productivity and net energy balance of photobioreactors, and it was concluded that optimization of power input to microalgal photobioreactors had significant implications for the environmental impacts and sustainability of technologies. The effect of nitrogen source (nitrate, urea, ammonium) on the productivity of microalgae was tested. It was found that the nitrogen source supplied did not impact growth under the test conditions. However, measurements of nitrogen dynamics in intensive bubble column photobioreactors showed that supply of fertilizer nitrogen was an important energy burden in the production of microalgal biomass. The long-term cultivation of *Scenedesmus obliquus* in an outdoor tubular photobioreactor was achieved in a cool-temperate climate, with productivity comparable to the literature. The evidence from this research showed that it is important to design microalgal cultivation systems in such a way that a significant positive energy return may be achieved for any given global location. The anaerobic co-digestion of microalgal biomass with cellulose yielded significant quantities of H_2, showing that a mixture of microalgal biomass and cellulose improved yields of gas. The overall conclusion from the work was that microalgal technologies have significant potential for CO_2 mitigation and bio-energy, but that it will require significant research aimed at reducing the energetic demands of production to improve net energy production from these systems.

Contents

Chapter 1.

General Introduction

1.1. Rationale

As a result of current economic issues, political pressures and public concerns relating to greenhouse gas emissions to the atmosphere, the power and transport fuel industries are under significant pressure to reduce the environmental impacts of their activities. The research in this work concerns the potential use of photosynthetic microalgae for industrial CO_2 mitigation, and for the production of bio-energy.

1.2. Carbon dioxide and renewable energy policy.

In Europe, two important developments in government policy are the EU emissions trading scheme (EU-ETS) (Georgopoulou et al., 2006; Kirat & Ahamada, 2011) and more recently the EU renewable energy directive (EU-RED) (Klessman et al., 2010). The EU-ETS is a 'cap and trade' system for CO_2 emissions permits (Kirat & Ahamada, 2011), affecting carbon dioxide emissions from industrial plant with an energy input of over 20 megawatts. In particular, the scheme affects the electricity generation sector (Kirat & Ahamada, 2011). The scheme operates by issuing CO_2 'emissions certificates' to companies at the start of a trading period. If more CO_2 is emitted than their allocated certificates allow, extra certificates must be obtained. If less than the allocated amount of CO_2 is emitted, the spare allowances may be traded (Kirat & Ahamada, 2011). The EU-ETS trading periods measure several years. The first trading period commenced in 2005 and ended in 2007. The current trading period commenced in 2008 and ends in 2012.

In the first trading period prices for CO_2 emission reached 30 euros/ T (Schiermeier, 2006), and the price was somewhat volatile (Egenhofer, 2007; Schiermeier, 2006), leading to concern over the future cost of CO_2 emissions. During the first trading period the market price of CO_2 fell when leaked data showed that the

total allowances were greater than the actual emissions from plant covered by the scheme (Schiermeier, 2006).

The EU-RED (Directive 2009/28/EC) was formally adopted in April 2009. In brief, it sets targets for its member states to achieve an overall 20% renewable energy contribution to gross energy consumption in the EU by 2020 (Klessman et al., 2010). National targets are allocated based on gross domestic product (GDP). The directive drives the use of renewable energy across various sectors including transport and electricity generation. The requirements may be met by different sources of renewable energy, including biomass energy.

1.3. Options for CO_2 mitigation, renewable fuels and algae.

As a result of current UK government policy, there is a need to develop processes which mitigate CO_2 emissions and provide renewable sources of energy. There are a variety of technology options under development for reducing CO_2 emissions from fossil-fuel fired power plant flue gas. Chemical engineering methods such as amine and oxyfuel-based CO_2 capture systems coupled to geological storage may provide a solution (Bouillon et al., 2009), and are receiving considerable research at present (Herzog, 2010; Gibbins & Chalmers, 2008). However, these processes have a significant negative impact on power plant efficiency and the price of electricity (Bouillon et al., 2009; Giovanni & Richards, 2010; Markewitz et al., 2009).

Power plant may combust alternative solid and liquid biomass, thereby avoiding the combustion of an equivalent amount of fossil fuel. Biomass may be combusted alone or co-fired with coal for example (Demirbas, 2003; De & Assadi, 2009). This is an excellent method for making use of existing power plant infrastructure, although plant may require certain modifications for effective

operation (Damstedt et al., 2007). Another option is to reduce dependence on conventional thermal power plant by using renewable technologies such as wind (Pinto et al., 2010) or photovoltaic (PV) systems (Ibrahim et al., 2010).

Alternatively, the use of photosynthetic microalgae has been suggested as a potential method for both removing the CO_2 from power plant flue gas whilst simultaneously producing bio-energy (Kadam, 1997; Vunjak-Novkovic et al., 2006). The overall hypothesis investigated in this work is that *cultivating microalgae is an efficient method for mitigating CO_2 emissions from power plant and producing bio-energy.*

1.4. Introduction to photoautotrophic microalgae

Microalgae are a diverse group of photoautotrophic organisms which, in terms of evolutionary history, pre-date terrestrial plants. In nature, they inhabit a wide range of terrestrial and aquatic habitats and may exist in single-celled forms or as part of larger colonies (Graham & Wilcox, 2000). In particular the phytoplankton– species living suspended in the upper reaches of the seas and oceans– play a major role in regulating global carbon cycles, their contribution equal in magnitude to terrestrial plants (Graham & Wilcox, 2000). Part of the reason for their significant contribution to the carbon cycle is their rapid growth rates- under optimal conditions they often form large blooms (Fernandez et al, 1993). However, microalgae are highly suited to intensive cultivation in engineered environments, where their high photosynthetic efficiency makes them an attractive means of fixing large amounts of carbon dioxide and producing renewable bio-energy (Ho et al., 2010; Zijffers et al., 2010). The concept of using microalgae to produce fuel was discussed 50 or more years ago (Wijffels & Barbosa, 2010), and various intensive programs of research have been

conducted in the USA (Department of Energy Aquatic Species Program, 1976-1996) and in Japan (Project by the Research Institute of Innovative Technology for the Earth (RITE) on biological CO_2 fixation and utilisation, 1990-1999). However, most research in these periods was driven by the price of petroleum fuel: When oil prices dropped, research ceased (Pienkos & Darzins, 2009; Wijffels & Barbosa, 2010).

The process for converting inorganic carbon to organic matter using light energy can be summarized simply by the overall equation for photosynthesis: (Eq. 1.1 from Falkowski & Raven, 2001).

$$CO_2 + H_2O + \geq 8 \text{ photons} = (^1/_6)\, C_6H_{12}O_6 + O_2 \qquad (1.1)$$

The mechanisms of photosynthesis are described extensively elsewhere, and the reader is referred to Falkowski & Raven (2001) and Blankenship (2005) for a complete background of photosynthetic biochemistry in aquatic systems. However, it is important to note that the complete process for biomass production extends far beyond equation 1, and incorporates all aspects of microalgal physiology.

1.5. Limits to production

The maximum efficiency of microalgal photosynthesis is in the order of 10% (Williams & Laurens 2010; Dismukes et al., 2008). This value corresponds to the conversion of solar energy to primary photosynthetic products, and is arrived at by examining the basic properties of sunlight and mechanisms of photosynthesis. However, this value is never reached in practice, with practical measurements of around 1-3% (Williams & Laurens, 2010; Weyer et al., 2009). Park et al., (2011) take additional factors into account and predicted a maximum solar-to-biomass energy

conversion efficiency of 1.3 to 2.4%. The following is a non-exhaustive description of the important properties of light and performance of microalgal photosynthesis, detailing some of the loss factors in microalgal solar energy conversion.

The limits to production are fundamentally determined by the available light energy. Some works have considered the use of artificial light (Chen et al., 2011), but this is unlikely to be feasible because: i) artificial light powered using energy from non-renewable sources is a highly unsustainable method of producing biomass and ii) artificial light powered by renewable sources introduces additional loss processes to the energy conversion system. It is thus essential to efficiently use natural sunlight for cultivation of microalgae.

The solar radiation spectrum (W m^{-2} nm^{-1}) reaching the surface of the Earth is shown in Figure 1.1. The extra-terrestrial and direct surface spectra are shown. Photosynthetic organisms can only utilize the photosynthetically active radiation (PAR) band, generally from 400 to 700 nm, which accounts for 45.7% of total solar energy (Nagaraja Rao, 1984). As a result, around half of sunlight energy is not actually utilized by microalgae.

The photosynthetic performance of microalgae can be described by the relationship between photosynthesis and irradiance (PI curve, Fig. 1.2). At low (sub-saturating) irradiances, the rate of photosynthesis is limited by the available light, so that the photosynthetic rate increases approximately linearly with increasing irradiance. At high irradiances such as in direct sunlight, the rate of photosynthesis is not limited by the rate of photon, capture but by downstream bottlenecks such as Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) activity, regeneration of ribulose 1,5 bisphosphate acceptor and the maximum turnover rate of Cytochrome b_6-*f* (MacIntyre et al., 2002; Melis 2009). Subsequent increases in irradiance beyond

the saturation point may result in a decrease in photosynthetic rate and damage to photosystems (photoinhibition). Microalgae have various mechanisms to redirect and dissipate excess absorbed light energy, e.g. the xanthophyll cycle (Serodio et al., 2008), and this energy is effectively wasted.

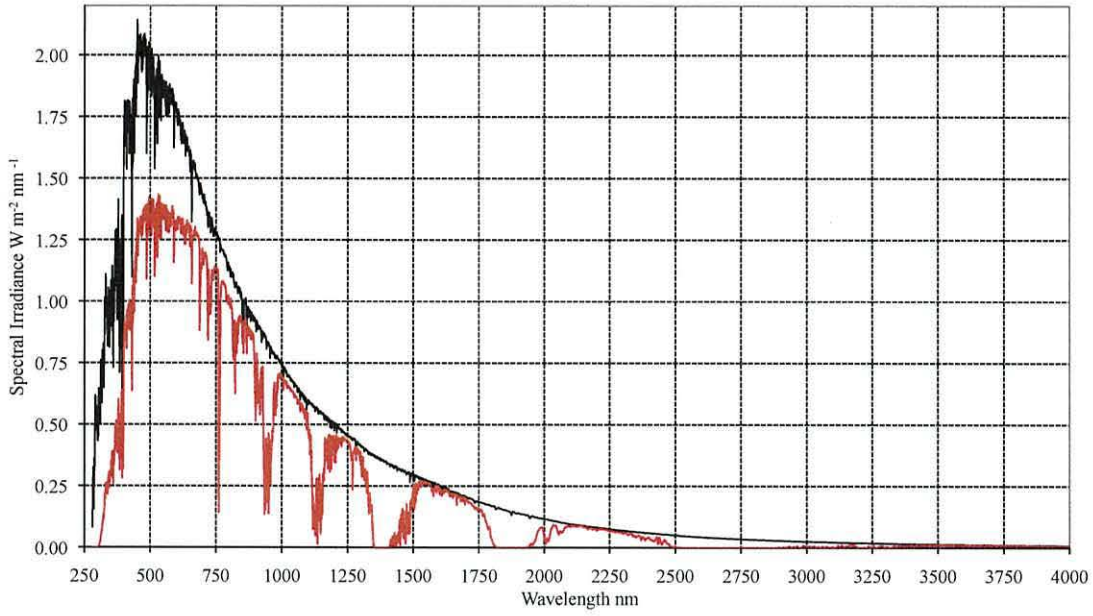


Figure 1.1. Extra terrestrial (black) and Earth surface irradiance (red) spectra of natural sunlight. Data from USA National Renewable Energy Laboratory for photovoltaic modelling (ISO 9845-1, 1992).

Due to mutual shading, irradiance inside a cultivation vessel of microalgae decreases with distance from the surface according to the Beer-Lambert law (Chen et al., 2011). The heterogenous light regime means that different cells may be both photoinhibited and photo-limited within the same cultivation vessel (Melis, 2009;

Molina Grima et al., 1996). Aside from the availability of light and physiological limits of microalgal photosynthesis, other factors also reduce the efficiency of energy conversion. These include reflection of light (liquid surface, bubbles, container surface); absorption of light (container materials, water, non-photosynthetic cell components); metabolic processes/ respiration (Chisti, 2007; Fernandez et al., 1997; Park et al., 2011).

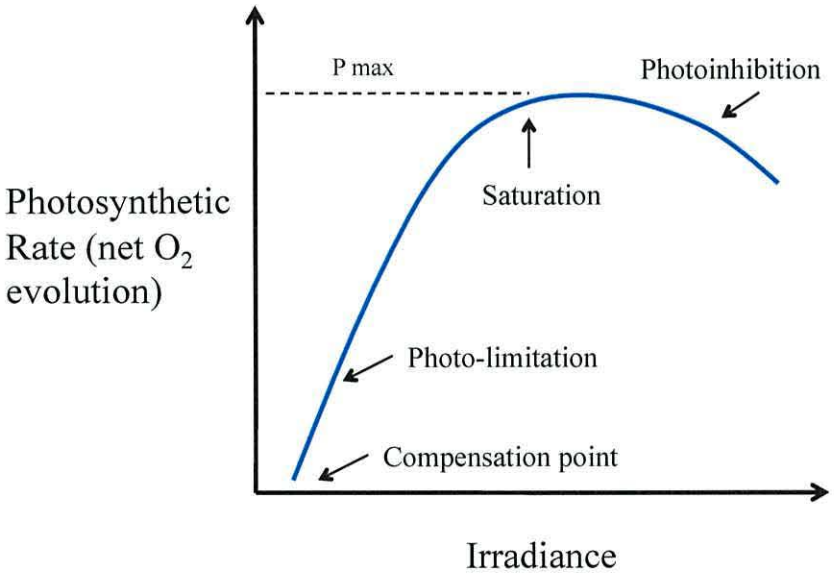


Figure 1.2. Typical relationship between photosynthesis and irradiance for microalgae. P max corresponds to the maximum rate of O_2 evolution.

However, one loss-factor which has not been discussed in any great detail in the context of mass algal cultures, to date, is the release of dissolved substances from algal cells into the surrounding water. In the natural environment, microalgae 'exude' a significant fraction of their photo-assimilated carbon into the water, typically in the

order of 5 to 30% (Carlson, 2002; Malinsky-Rushansky & Legrand, 1996; Biersmith & Benner, 1998). For a 'carbon-capture' application using intensive cultures, this release of organic matter could be a significant loss of carbon/ biomass/ energy from the process, resulting in a corresponding loss of efficiency. Furthermore, if CO_2 removal from the gas phase is used to characterize CO_2 emissions savings, un-utilised carbon could lead to erroneously high measurements of CO_2 uptake. Dissolved substances are also a substrate for bacteria, and the presence of significant concentrations of dissolved organic matter in process water could present problems for recycling or disposing of the fluid. The potential loss of dissolved substances is tested in this work under framework of the following hypothesis: *Exudation of dissolved organic matter in photobioreactor cultivations of microalgae is a significant loss in solar energy conversion.*

Despite the loss processes outlined above, microalgae are usually more productive than conventional terrestrial crops (Dismukes et al., 2008). From a first-principles perspective this seems unfounded, as they both rely upon similar photosynthetic mechanisms (Walker, 2009). However, algae are very productive because algal production systems can be engineered to make maximum use of sunlight (Molina Grima et al., 2001; Molina Grima et al., 1999) and can be supplied concentrated CO_2 (10% CO_2 in air or more) to increase performance further (Chiu et al., 2008; Douskova et al., 2009; Vunjak-Novakovic et al., 2005). Despite various theoretic calculations, data for the actual yields of microalgal cultivations per unit area of land are difficult to identify, and most research has been conducted in relatively mild climates and over short periods of time. There is a significant deficit of data concerning the areal productivity of microalgae in temperate regions in relation to ambient weather conditions and over sustained periods of time. The issue is

addressed in this work by the hypothesis that: *The cultivation of microalgae using photobioreactors is a potentially viable method of biomass production in cool-temperate regions.*

1.6. Cultivating microalgae

With a limited number of exceptions, microalgae are cultivated using technologies grouped into one of three categories: Raceways (Campbell et al., 2011; Chisti, 2007; Hase et al., 2000; Moheimani & Borowitzka, 2006; Park et al., 2011), gas-sparged photobioreactors (Chiu et al., 2008; Sierra et al., 2008; Vunjak-Novakovic et al., 2005; Zhang et al., 2001) and tubular photobioreactor systems (Travieso et al., 2001). Of the photobioreactor designs, gas-sparged systems seem to give the best energy return when analysed on a life-cycle basis (Jorquera et al, 2010). They are scalable and have been successfully used outdoors (Zhang et al., 2001; Slegers et al., 2011).

A simple summary of the basic inputs into gas-sparged photobioreactors is shown in Figure 1.3. The supply of gas provides CO_2, removes O_2 and mixes the culture. For microalgal CO_2 mitigation, such designs can be supplied directly with flue gas (Vunjak-Novakovic et al., 2005; Douskova et al., 2009). The advantage of this is higher concentrations of dissolved inorganic carbon together with lower O_2 concentrations. This improves the CO_2 fixation efficiency by promoting the carboxylase function of the enzyme RuBisCO (Douskova et al., 2009). Many studies have reported the advantages of high CO_2 concentrations for improving microalgal growth performance (Chiu et al., 2008; Fulke et al., 2010; Westerhoff et al., 2009), though several have noted that very high CO_2 concentrations reduce growth (Chiu et al., 2008; Fulke et al., 2010). Thus, power plant may offer significant advantages by

improving the growth performance of microalgae. Adding concentrated CO_2 to water increases the equilibrium concentration of carbonic acid, thus decreasing the pH (Falkowski & Raven, 2001). Saline systems and associated microalgal species may therefore be advantageous due to their high buffering capacity, because they limit the changes in pH which occur in response bubbling CO_2–rich flue gases through cultures.

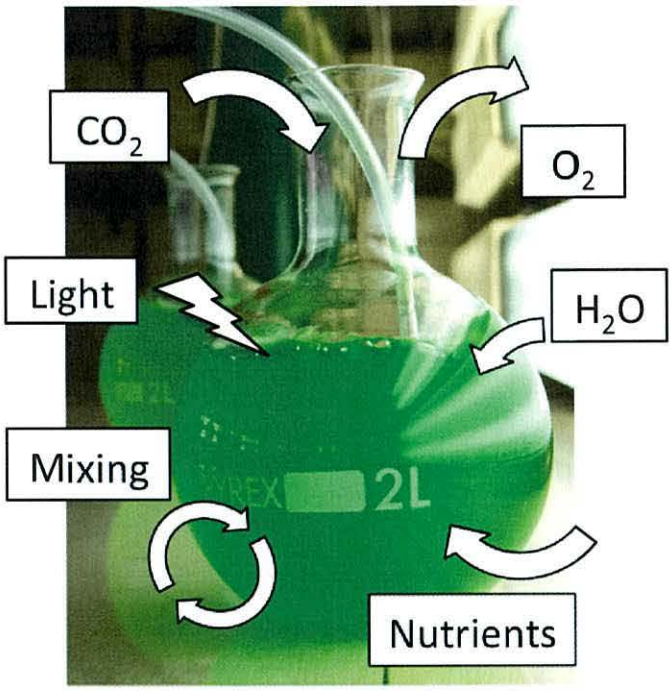


Figure 1.3. Resource and energy inputs into microalgal cultures.

In addition to the CO_2 concentration, the rate at which gas is sparged into photobioreactors impacts the mass transfer of gases and the rate of mixing (light and nutrients). Higher sparging rates result in higher growth rates and photosynthetic efficiencies (Chisti, 1989; Merchuk et al., 2000). Sparging is the dominant energy input during the cultivation of microalgae in this type of photobioreactor, consuming a significant amount of energy (Jorquera et al., 2010). Thus higher sparging rates

incur a proportionally greater energy penalty. In the literature, there is no direct evidence of the optimum sparging rate for microalgal cultivations based on energy balance, especially using different CO_2 concentrations representative of power plant flue-gas and saline/ freshwater media. This is addressed in this work by the hypothesis that: *Cultivating microalgae in photobioreactors supplied with a high power input improves productivity, but reduces the net energy production.*

1.7. Supply of inorganic nutrients (fertilisers).

A critical aspect of microalgal cultivation is the supply of inorganic nutrients to support algal growth (Fig. 1.3). Since microalgae contain a relatively large quantity of protein (Lopez et al., 2010), nitrogen may account for up to 10% of biomass (Perez-Garcia et al., 2010). Supply of nitrogen fertilisers to microalgal cultures is usually achieved using artificial compounds (Clarens et al., 2010; Chisti, 2007), which currently supports most terrestrial agriculture (Ramirez & Worrell, 2006). However, the manufacture of fertilisers consumes a large amount of energy and produces huge quantities of greenhouse gases, so it is important to minimize consumption (Dalgaard et al., 2001). Stephenson et al., (2010) identify that supply of fertilisers accounted for 12% of total energy inputs involved with microalgal cultivation in raceways, whereas Chisti (2007) calculated a value of 45% for a similar scenario. It is not clear how important the supply of fertilisers is in terms of the overall energy burdens of microalgal cultivation in photobioreactors. In addition to the amounts of nitrogen fertilizer needed, the type of nitrogen compound used may also affect the productivity of microalgal technologies (Rodrigues et al., 2010).

Cultivation strategy plays an important part in nitrogen utilization efficiency and the biochemical composition of microalgae (Converti et al., 2009). Nitrogen

limitation can be used to increase the concentration of lipids suitable for biodiesel production (Dean et al., 2010; Hsieh et al., 2010; Mairet et al., 2011; Pruvost et al., 2011; Xin et al., 2010), which also increases the calorific value (Illman et al., 2000; Scragg et al., 2002). For example, Hsieh & Wu (2009) found that by limiting the supply of urea, the lipid content of *Chlorella* could be increased from 33 to 66% mass. However, the 'trade-off' for this increased lipid content was a reduction in productivity by approximately half. Since the type of nitrogen source and cultivation conditions impact the performance of microalgae, the following hypothesis was formulated: *Cultivation of microalgae using different nitrogen sources and at different growth stages affects productivity, biochemical composition and the energy demands associated with the supply of nutrients.*

1.8. Fuels from algae

1.8.1. Energy content

The baseline value of algal biomass is its calorific (heating) value. Combusting microalgal biomass in power plant (e.g. co-firing with coal, Kadam 2002) is one method by which energy may be utilised. The disadvantages of simply combusting algae include loss of nutrients, and the need to invest energy to dry the biomass sufficiently. It is also not clear what effect combusting microalgae may have on power plant equipment (fouling boiler tubes, corrosion etc.) Miles et al., 1996; Heinzel et al., 1998; Jenkins et al., 1998.

1.8.2. Liquid transport fuels

Biodiesel has properties similar to conventional fossil-derived diesel and can be used in normal compression-ignition engines. It can be produced from chemical

upgrading (transesterification) of the lipid fraction of the microalgal biomass (Chisti, 2007). Selection of microalgae species for high lipid content and the conditions that promote lipid production (Lardon et al., 2009) is an area of significant scientific investigation.

Ethanol is produced from fermentation of the carbohydrate and protein components of biomass (Singh & Gu, 2010) and may be particularly suited to processing residual algal biomass after lipid extraction (Rojan et al., 2011). Ethanol can be used in conventional spark-ignition engines, but has a lower energy density than petroleum fuels (Sobrino et al., 2009). Ethanol is already commercially established and production currently accounts for 3% of global transport fuels. (Demirbas 2010).

1.8.3. Methane & hydrogen production using anaerobic bioprocesses

Anaerobic bioprocesses, especially digestion for methane production, are an established technology used for converting biomass (often waste) to methane and hydrogen (Singh & Gu, 2010). They have been described as 'domesticating the microbial loop' (Sialve et al., 2009). Methane can be produced from microalgal biomass efficiently (Oswald & Golueke, 1960). Potential issues influencing productivity include nitrogen content (ammonium production), salt content and cell wall characteristics of microalgal substrates (Sialve et al., 2009).

Current research supports integrated microalgae/ digestion systems where technologies are linked together to process biomass, e.g. extraction of lipids for conversion to biodiesel before anaerobic digestion of remaining (waste) biomass (Collet et al., 2011; Ras et al., 2011; Stephenson et al., 2010). Power from anaerobic digestion can be used to support systems for cultivating algae, which allows these

burdens to be offset (Stephenson et al., 2010; Harun et al., 2011). Sialve et al., (2009) showed that anaerobic digestion of all the biomass may be an energetically more efficient method of using algae with a lipid content lower than 40%. Anaerobic digestion conserves nutrients such as nitrogen, which may be recycled/ remineralised back to the algal production process (Park et al., 2010; Sialve et al., 2009). Combining biomass types such as microalgae and cellulosic material such as waste paper is termed co-digestion (Sialve et al., 2009; Yen & Brune, 2007), and can be used to improve digester performance by manipulation of the C:N ratio. Despite the use of microalgae for methane production, there is little evidence for hydrogen production from microalgal biomass. Hydrogen production is addressed in this work using the hypothesis that: *Microalgal biomass alone or co-digested with cellulosic material yields significant quantities of H_2 and/ or CH_4 gas.*

1.8.4. Microbial fuel cells

Microbial fuel cells (MFCs) supplied with algal biomass can be used to produce bioelectricity via hydrolytic and fermentative processes using micro-organisms (Velasquez-Orta et al, 2009). Generally, the MFC anode contains an organic substrate and a microbial consortium, which decomposes the substrate to protons and electrons. Reduction of oxygen at a cathode produces power (Velasquez-Orta et al., 2009). Microbial fuel cells can be used to treat waste-water streams and produce electricity (Oh et al, 2010). A photoautotrophic microalgal culture at the cathode can be used as an electron acceptor, whilst simultaneously reducing CO_2 (Powell et al., 2009), and various configurations of photosynthetic MFCs have been explored (Rosebaum et al., 2010).

1.8.5. Thermochemical processes

Chemical upgrading of whole algal biomass, or the lipid fraction, can be achieved by use of various thermochemical processes such as pyrolysis, liquefaction and gasification (Brown et al., 2010). Pyrolysis is the thermal decomposition of biomass at high temperature and pressure. The products generated are synthetic gas, tars/ oils and biochar (Pan et al., 2010; Zou et al., 2010). Nutrients are incorporated into biochar, which can then be used as a fertiliser (Grierson et al., 2008). Furthermore, ammonia may be recovered from gasification processes to re-cycle nitrogen (Brown et al., 2010). The conditions (fast, slow, temperature, pressure) can be adjusted to modify the products (Brown et al., 2010; Grierson et al., 2008).

1.9. Competing technologies.

As discussed in section 1.3, there are a variety of technologies which may compete with microalgae for CO_2 mitigation and energy production. In section 1.5 it has been detailed that the major resource required by microalgae was sunlight, which is fixed per unit area of land. As a result, microalgal processes may receive competition from other technologies for sunlight/ land area in future applications. The following introduces some relevant alternative technologies using sunlight for energy.

1.9.1. Alternative crops

Alternative energy crops may compete for land area with microalgal technologies and conventional food crops. The need to produce large quantities of biomass to satisfy future demands and government targets for renewable energy will place considerable demand on land space, especially in areas such as the EU (Krasuska et al., 2010). Compared to conventional terrestrial crops used for bio-

energy production (Table 1.1), microalgae are extremely productive both in terms of dry mass and energy content, suggesting they may compete effectively with terrestrial crops.

Table 1.1. Comparison of areal yields (dry mass and biomass energy) for selected crops.

Species	Productivity dry T ha^{-1} y^{-1}	Energy GJ ha^{-1} y^{-1}
Corn grain	7	120
Switchgrass/ Prarie grass	3.6 - 15	61 - 255
Rapeseed	2.7	73
Tetraselmis sp. (microalga)	38 - 69	700 - 1550
Spirulina sp. (microalga)	27 - 70	550 - 1435

Table adapted from Dismukes et al (2008).

1.9.2. Photovoltaic (PV) cells

Aside from other terrestrial crops, photovoltaic systems may be another key competitor with microalgae in future scenarios. Currently, commercial photovoltaic systems can convert around to 6-8% of incident solar energy to electricity (Ibrahim et al., 2010), compared to 1-3% usually achieved by microalgae (section 1.5). Thus, in terms of energy conversion, PV technologies currently offer improved land area utilisation compared to microalgae. However, PV systems produce electricity, which has different applications/ markets to biomass products. Improvements in PV technologies include hybridization of photovoltaic and thermal systems (PV/T) (Chow, 2009).

1.9.3. Artificial photosynthesis

The development of chemical processes that mimic photosynthesis (production of organic molecules from inorganic precursors and light energy) has been researched for some time (Hammarstom et al., 1998; Hammarstrom et al., 2001). The various processes envisaged show promise, but are still at the research and development stage (Kalyanasundaram & Graetzel, 2010).

1.9.4. Hydrogen via direct photolysis

Photo-induced hydrogen production from green microalgae was first observed in 1942. Many studies have attempted to refine the process of producing hydrogen by photolysis of water, but the necessary anaerobic conditions are extremely difficult to maintain in practice using photoautotrophic organisms, making this technology difficult to implement (Degrenne et al., 2010).

1.10. Synergies between microalgal cultivation and the power industry

As discussed previously, microalgal cultivation has the potential to mitigate CO_2 emissions and produce a fuel, whilst power plant offers a supply of concentrated CO_2. In addition to the CO_2 supply, power plants also offer a considerable quantity of low-grade waste heat. This heat may be useful for maintaining temperature and optimal productivity of microalgal cultivations (Shang et al., 2010). However, one important question concerns the potential dependence of each technology on the other: It is not known whether commercially and environmentally sustainable microalgal cultivation *must* be carried out in association with power plant, or whether intensive, sustainable microalgal cultivation may also be practiced independently.

Various different power plant (e.g. coal, gas) produces flue gases with a range of qualities, particularly CO_2 concentration (Westerhoff et al., 2010). Individual plant types may also be subject to various modifications aimed at reducing the environmental impacts of power generation e.g. low NO_x burners (Kurose et al., 2004) and flue gas desulphurisation (Aust, 2007). The flue gas composition varies between applications; however, a representative flue gas composition from a coal-fired power plant located in Arizona (Westerhoff et al., 2010) is shown in Table 1.2. In addition to CO_2 uptake, microalgae may also utilise NO_x (Nagase et al., 1997), although in some circumstances NO_x and SO_2 may inhibit growth (Lee et al., 2002).

Table 1.2. Flue gas composition of a typical coal-fired power plant located in Arizona

Gas	Concentration	Units
CO_2	12 to 15	%
SO_2	300	ppm
NO_x	225	ppm
O_2	7	%
Moisture	12 to 15	%
N_2	balance	%

Adapted from Westerhoff et al., (2010).

1.11. Hypotheses tested in this work

The general hypothesis tested in this work was that *cultivating microalgae is an efficient method for mitigating CO_2 emissions from power plant and for producing bio-energy*. The information presented in Chapter 1 indicates important aspects of

microalgal cultivation that must be investigated in order to advance the field. These points formed the basis of five hypotheses, which are presented below:

1) *Exudation of dissolved organic matter in photobioreactor cultivations of microalgae is a significant loss in solar energy conversion.*
2) *Cultivating microalgae in photobioreactors supplied with a high power input improves productivity, but reduces the net energy production.*
3) *Cultivation of microalgae using different nitrogen sources and at different growth stages affects productivity, biochemical composition and the energy demands associated with the supply of nutrients.*
4) *The cultivation of microalgae using photobioreactors is a potentially viable method of biomass production in cool-temperate regions.*
5) *Microalgal biomass alone, or co-digested with cellulosic material, yields significant quantities of H_2 and/ or CH_4 gas.*

Chapter 2.

General Methods

2.1. Algal strains & cultivation media

Three species of microalgae were selected for experimentation in this work: *Chlorella vulgaris*, *Dunaliella tertiolecta* and *Scenedesmus obliquus*. Each of these species have been used for mass cultivation including potential CO_2 mitigation and bio-energy technologies. In particular, the species are each tolerant to elevated levels of CO_2 (Ho et al., 2010; Tang et al., 2010; Vunjak-Novakovic et al., 2006; Watanabe & Saiki 1997). The following strains were used in this work: *Chlorella vulgaris* (CCAP 211/11B, Culture Collection of Algae and Protozoa, Oban UK), *Dunaliella tertiolecta* (SAG 13.86, Sammlung von Algenkulturen Gottingen) and *Scenedesmus obliquus* (Local ecotype, extracted from a pool at the test site and maintained in the laboratory).

2.1.1. Photobioreactors.

2.1.1.1. Light.

Light was supplied to 20 L polythene and to bubble column photobioreactors using cool white fluorescent tubes (Osram, 36 W, 1200 mm in length) arranged in parallel rows. The spectral light output of the cool white tubes is shown in Figure 2.1. The incident irradiance is reported separately in each Chapter. For a single indoor tubular photobioreactor cultivation only (Chapter 3.1), twelve metal-halide lamps were used (400W each).

2.1.1.2. 20 L Polythene photobioreactors

Photobioreactors were formed from tubular polythene material with a diameter of 160 mm and a height of 1000 mm. Photobioreactors were sparged from the base

with air supplied by an oil-less compressor using soft silicon tubing. Specific details of polythene photobioreactors and methods used are detailed in Chapter 3, 5 and 7.

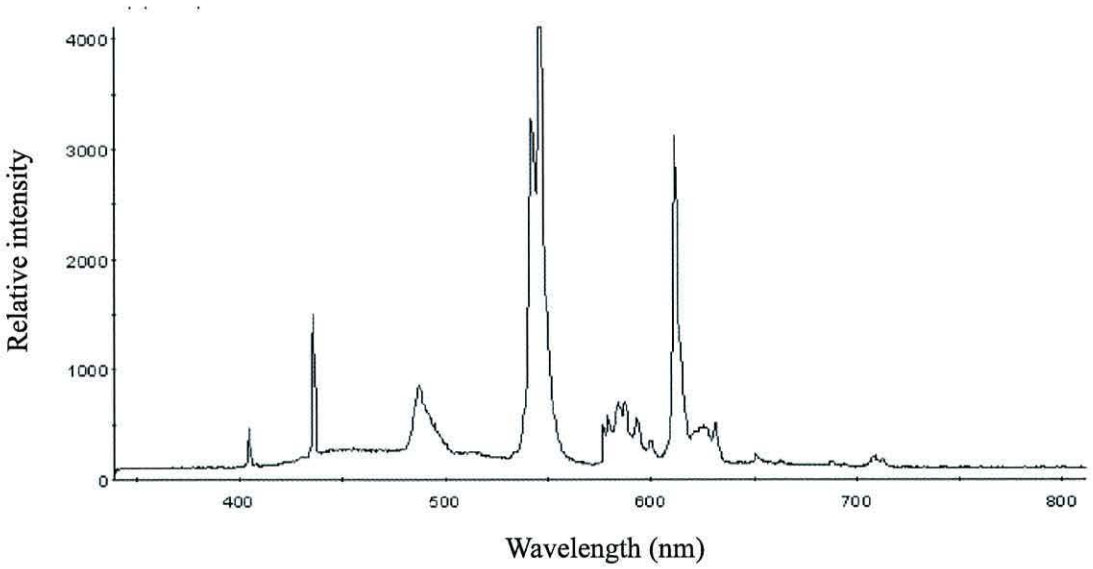


Figure 2.1. Spectral output (relative units) of cool-white fluorescent tubes used for supply of light to bubble column and 20 L polythene photobioreactors.

2.1.1.3. Bubble column photobioreactor design

Bubble column photobioreactors were designed by the author specifically to test the effect of superficial gas velocity/ power input on the growth of microalgae. Reactors comprised columns with an internal diameter of 32 mm and a height of 2000 mm. The design and use of bubble column photobioreactors is presented in Chapter 4.

2.1.1.4. Indoor tubular photobioreactor

A 1200 L tubular photobioreactor (Biofence, Varicon Aqua Ltd) was used for large scale cultivation of *Chlorella vulgaris*. The temperature of the photo-stage

culture fluid was held at 20 ±0.5°C using air conditioning. The culture was circulated through two 2.7 × 2.2 m tall photo-stages and a header tank using a centrifugal pump. Photo-stage tubes were constructed from polyethylene and measured 28 mm internal diameter. The photo-stages were illuminated by 12 high-intensity mercury lamps (400 W, 5000 K colour temperature). The mean incident irradiance (±SE) was 392 (18.34) μmol photons PAR m^{-2} s^{-1} (220 measurements). The system was operated as a pH-stat (pH 7) through the solenoid-controlled addition of CO_2. Further details are available in Chapter 3.1.

2.1.1.5. Outdoor tubular photobioreactor

A 500 L tubular photobioreactor (Biofence, Varicon Aqua Ltd) was sited outside in an un-shaded location at 53°22'58''N 4°16'01''W, using natural sunlight as the light source. The design consisted of a single photo-stage with a frontal area of 6 m^2 and a recirculation tank. A centrifugal pump circulated the culture through the photo-stage tubes at a velocity of 87 cm s^{-1}. Further details are available in Chapter 6.

2.1.2. Analysis of dry weight

The dry weight of the culture was used as the standard measure of biomass. Dry weight was analysed by vacuum filtration (-0.5 Bar) of algal culture through 47mm Whatman GF/F filters (see method development, Chapter 2.2.3 and results Chapter 3.2 for justification of selection of this filter) mounted in a Sartorius filtration assembly. The volume of cultivation filtered was adjusted to ensure a dry mass >2-3 mg retained on the filter. Depending upon the method of cultivation and growth stage, the filtered volume ranged from 50 ml (start of 20 L polythene photobioreactor cultivations) to 3 ml (end of bubble column cultivations. Sample volumes were

measured by pipette. Filters were dried for 24 hours at 80°C (see method development, Chapter 2.2.3), cooled to room temperature in a desiccator, then weighed using a 6 point balance (Mettler Toledo MX5). After filtration of *Dunaliella tertiolecta* samples, filters were washed 3 times using 5 ml aliquots of 0.5 M ammonium formate solution to remove salts retained in artificial seawater on the filter.

2.1.3. Microalgal cell counts

Microalgal cell density was measured by counting cell number with a Neaubauer Haemocytometer. Ten replicate grids were counted per sample. The cell number was adjusted by dilution to a target of between 20 and 40 cells per grid.

2.1.4. Dissolved oxygen, pH and temperature

The concentration of dissolved oxygen (% air saturation) in photobioreactors was measured using a calibrated dissolved oxygen electrode (WTW CellOx 325). The pH of the cultivation fluid was measured using a calibrated pH meter (Mettler Toledo, MP120). The temperature of indoor cultivations was measured using the pH meter thermometer.

2.1.5. Nitrate, nitrite, phosphate.

The concentration of the major dissolved inorganic nutrients, nitrate (NO_3^-), nitrite (NO_2^-), and inorganic phosphorus (PO_4^-) was determined by standard colorimetric methods (Grasshoff et al., 1983) as adapted for flow injection analysis (FIA) on a LACHAT Instruments Quick-Chem 8000 autoanalyzer (Hales et al., 2004). The accuracy of these methods was <5%, and the detection limit <0.2 μmol L^{-1}.

2.1.6. Dissolved urea analysis

The colorimetric diacetylmonoxime based method of Mulvenna & Savidge (1992) was used to measure the concentration of dissolved urea. Samples were prepared as for other dissolved constituents by filtration through Whatman GF/F filters. The method was adapted proportionally for 5 ml sample sizes. The first analytical reagent (A) was comprised by diacetylmonoxime solution (8.5g in 250ml water), to which 10ml of thiosemicarbazide solution (0.95g in 100ml water) was added. The second analytical reagent (B) was comprised by 300ml concentrated sulphuric acid, 235ml water and 0.5ml of ferric chloride solution (0.15g in 10ml water). The method was calibrated using standards of urea (Sigma-Aldrich) dissolved in ultra-pure water from 0 to 5 mg L^{-1}. Standards and samples were added in 5 ml aliquots to glass test tubes. Using a pipette, 0.357 ml of reagent (A) was added. Immediately after mixing, 1.14 ml of reagent (B) was added to each tube, mixed, and the tubes covered with foil. Tubes were immersed in a water bath at 85°C for 20 minutes. Tubes were then removed from the bath, cooled in a tray of water, before the absorption of each sample was measured at 520 nm using a spectrophotometer (Molecular Devices Spectramax Plus 384) and a 1 cm path length cell. The R^2 for the calibrations was >0.998. Samples with values outside the calibrated range (i.e. those taken at the start of the experiments) were always diluted before the analysis (before reagents were added).

2.1.7. Ammonium

Dissolved ammonium (NH^+_4) was determined using the fluorometric method of Holmes et al. (1999) using a Hitachi F2000 fluorescence spectrophotometer. 2.5 ml of sample was added to 10 ml of OPA reagent in an acid-cleaned scintillation vial and

incubated in the dark for 4 hours. The OPA reagent was made up by adding together: 80g of Sodium tetraborate in 2 L of ultra-pure water, 4g of Phthaldialdehyde in 100 ml of Ethanol and 0.08 g of Sodium sulphite in 10 ml of ultra-pure water. A 7 point calibration was made up using ammonium sulphate (0.25 to 6 μmol L^{-1}). The R^2 for the calibrations was >0.99.

2.1.8. Measurements of elemental composition (CHN)

Samples for elemental (CHN) determination were extracted by centrifugation (1000 rcf, 10 minutes) before the supernatant was removed and the sample dried at 80°C for 48 hours in an oven. A 2 to 3 mg fraction of the dried sample was weighted into a tin cap using a 6–point balance (Mettler Toledo MX5). The concentration of the elements carbon, hydrogen and nitrogen in the microalgal biomass were measured using a thermo-electron elemental analyser (Flash EA 1112). The machine was equipped with a copper/ copper oxide column, and the standards Sulphanilamide, 2,5-bis(5'-tert-butyl-2-benzoxazolyl)thiophene and L-Cystine were used for calibration. Measurements of carbon, hydrogen and nitrogen in reference material (DL-Methionine) were made regularly during sample runs (every 10-15 samples) to check the performance of the machine/ calibration. The elemental composition (by mass) of the DL-Methionine reference material was C (40.25%), H (7.43%), N (9.39%). The mean deviation of the measured values from certified values was C (±0.48), H (±0.3%), N (±0.2%).

2.1.9. Dissolved organic matter

Dissolved organic matter was characterised by the concentration of dissolved organic carbon, dissolved organic nitrogen and by the chromophoric/ coloured

component (optical density). Samples were prepared by filtration at low pressure (-0.2 bar) that was not sufficient to damage the integrity of the cells. Cell integrity after filtration was evidenced by the absence of any photosynthetic pigments in absorption spectra of the filtrate. Filters used were Whatman GF/F (0.7 μm nominal pore size) and Whatman GDX (0.45 μm nominal pore size).

2.1.9.1. Dissolved organic carbon

Dissolved organic carbon (DOC) was analysed by high temperature combustion using an MQ1001 TOC analyser (Qian & Mopper, 1996). The machine was calibrated with potassium phthalate using a 7 point calibration over the range 20-750 μmol L^{-1}. Stable reference material (Hansell Marine Laboratories, Miami) was used to check column performance and a 250 μmol L^{-1} potassium phthalate standard was incorporated within the samples to check performance. The R^2 for the calibrations was >0.99, and the mean difference of the check-standard from 250 μmol L^{-1} was 22 μmol L^{-1}.

2.1.9.2. Dissolved organic nitrogen (DON)

DON was determined by subtraction of NO_3^-, NO_2^- and NH^+_4 from the total dissolved nitrogen (TDN) analyzed using on-line peroxodisulfate oxidation coupled with ultraviolet radiation at pH 9.0 and 100°C (Kroon, 1993) with a Lachat Instruments flow-injection analyser. Due to the high levels of nitrate initially present in the culture media, DON could only be accurately determined toward the end of the growth period. Reference material (Hansell Laboratories, Miami) measured during analyses had a certified dissolved nitrogen content of 33 μmol L^{-1}. Measurements made during experiments had a mean (±SD) of 33.2 (0.76), n = 36.

2.1.9.3. Chromophoric dissolved organic matter.

Chromophoric dissolved organic matter (CDOM) was measured by optical absorption (Hulatt et al., 2009). A Shimadzu UV 1601 dual-beam spectrophotometer was used to measure the optical density of dissolved substances over the range 750 to 230 nm at 0.5 nm intervals. A reference cell containing ultra-pure water was used. Samples of cultivation fluid were filtered and transferred to a 10 cm quartz glass cell. The absorption spectrum baseline was set to zero at 750 nm.

2.1.10. Bacteria.

Bacterial cell density in the cultivation water was measured by epifluorescence microscopy using 4',6-diamidino-2-phenylindole (DAPI) to identify bacterial cells (Servais et al., 1999). Samples fixed by formaldehyde (25%) were prepared by exposing samples to DAPI at a final concentration of 5 μmol L^{-1} for 10 minutes, before filtering through 25mm Whatman® Nuclepore 0.2 μm filters. Filters were mounted on slides and examined under a UV microscope. Cells were enumerated and sized (length and width) in order to calculate the bacterial biovolume. 15 random fields of view were used to enumerate bacteria in one sample, and 30 bacteria were sized. The biovolume was used to calculate the concentration of bacterial carbon in the culture fluid using a conversion factor of 148 fg μm^{-1} (see Table 1 section 3.2). The equation presented in Chapter 3.2 was used to calculate the biovolume, and was applicable to both rod-shaped and coccoid bacterial cell forms.

2.1.11. Biochemical composition

2.1.11.1. Total lipid

Measurement of total lipids was determined gravimetrically using the Bligh & Dyer (1959) solvent extraction method. Samples for lipid analysis were extracted from cultivation fluid by centrifugation (1000 rcf, 10 minutes). Extraction of lipids was carried out using 50 ml glass centrifuge tubes, which were pre-weighed on a 4 point balance. Samples were added to tubes and re-suspended by addition of 5 ml of lysis buffer (0.5% sodium dodecyl sulphate, 5% glycerol). The cells were disrupted by sonication using a Branson 450 Digital Sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA). The sonifier was fitted with a ½" disruptor horn used in conjunction with a ⅛" tapered microtip. The frequency was 20 kHz at an amplitude of 30% (13.5 W per 10 ml sample). *Chlorella vulgaris* samples required 30 minutes of sonication, whilst *Dunaliella tertiolecta* required 1 minute. Samples were then dried (80°C, 72 hours), cooled in a desiccator, then re-weighed to obtain the dry mass of sample. Chloroform, methanol and water were then added to the tube in the proportions 1:2:0.8. Lipids were allowed to extract for 48 hours, during which time a vortex was used to repeatedly homogenise the sample. The solvent proportions were then adjusted by addition of chloroform and water to the final ratio of 2:2:1.8 chloroform: methanol: water. Subsequent phase separation was accelerated by low-speed centrifugation (350 rcf, 3 minutes). The lower chloroform/ lipid phase was removed by pipette and the chloroform partially evaporated using a heating block (50°C, 6 hours). The remaining lipid/ chloroform solution was then transferred to a microcentrifuge tube, and centrifuged (1000 rcf, 10 minutes) to remove cell debris from the liquid. The chloroform/ lipid phase was then transferred into a pre-weighed (6 point balance, Mettler Toledo MX5) glass vial and the remaining chloroform

evaporated until constant mass using a heating block (60°C). The samples were stored in a desiccator and weighed at room temperature. The percentage mass of the lipid fraction could then be obtained by; $(T_mM-T_m)/ (V_mL-V_m) \times 100$. Where T_mM is the mass of centrifuge tube and dry biomass, T_m is the mass of the empty tube, V_mL is the mass of the vial and lipid, V_m is the mass of the empty vial. Data presented in Chapter 6.2 showed that the standard deviation of replicate total lipid samples (n = 5) was 3.2 and 5.6%.

2.1.11.2. Protein

Total protein was measured using elemental nitrogen content (Section 2.1.8.). The percentage nitrogen contribution to the dry biomass was multiplied by 4.44, giving the percentage protein on a dry mass basis. Although a conversion factor of 6.25 may be assumed, the presence of non-protein cellular nitrogen means that a value of 4.44 is the best approximation, verified by practical experimentation using several methods (Lopez et al, 2010).

2.1.11.3. Heating value of biomass

The higher heating value of the microalgal biomass was measured using an oxygen bomb calorimeter (Parr 1341). Biomass was extracted by centrifugation (1000 rcf, 10 minutes). Samples were dried in an oven (80°C, 72 hrs) before they were ground to a powder using a pestle and mortar. The calorimeter was calibrated using triplicate benzoic acid standards. Replicate measurements of algal biomass presented in Chapter 6 had standard deviations of 1.0 and 2.4% of the mean values (n= 3).

2.1.11.4. Chlorophyll-*a*

The concentration of Chlorophyll-*a* was determined fluorometrically following solvent extraction. A 1 ml sample of culture was filtered through a Whatman GF/F filter and stored frozen at -80°C. Samples were analysed following extraction in 90% acetone for 20 hours using a Turner Designs 10AU fluorometer. The instrument calibration was checked with stable standards.

2.1.12. Statistical analysis

Statistical analysis was carried out using Microsoft Excel, SPSS v.16 and Minitab 14. For formal statistical tests, the relevant assumptions were verified. With the exception of a single preliminary tubular photobioreactor experiment (Chapter 3.1), algal cultivation treatments were always carried out in triplicate.

2.2. Method development.

During the research it was necessary to conduct additional experiments to validate the use of equipment. These experiments themselves did not provide conclusions pertinent to the hypotheses presented in Chapter 1, but are included below to support the data collected during this work.

2.2.1. Photosystem II performance

The performance of photosystem II was examined in this work using a Walz Water PAM-FT fluorometer, originally designed as a flow-through system. The device was configured for large culture volumes containing dilute cell suspensions, and thus was not suited to measuring samples from algal cultivations. The instrument was modified by the author by removing the flow-through assembly and fitted with a

bespoke cuvette system. The new design ensured a cuvette containing a sample cell suspension could be accurately placed in the optimal position in front of the emitter/ detector windows with an optimised light path. The design ensured that that the sample could be dark-adapted. In order to improve the light regime, the sample was diluted using filtrate from the same sample, obtained using a Whatman GDX syringe filter (0.45 μm nominal pore size). The minimal fluorescence under measuring light was maintained in the range 100 to 600 photomultiplier fluorescence units at a gain setting by appropriate dilution. In this range the cell density had negligible effect on measurements of parameters (section 2.2.2.).

2.2.2. PAM calibration.

In order to establish an appropriate working range for the instrument without significant cell density effects, rapid light curve analysis (Ralph & Gademann, 2005) was performed on samples of *Chlorella vulgaris* of a range of concentrations at three photomultiplier gain settings. The rapid light curve parameters Fv/Fm, α, Ek and ETRmax were measured (see Ralph & Gademann, 2005 for parameters & equations). The results are presented in Figure 2.2.1 and Table 2.2.1, and a photomultiplier gain setting of 5 was selected for use in experiments.

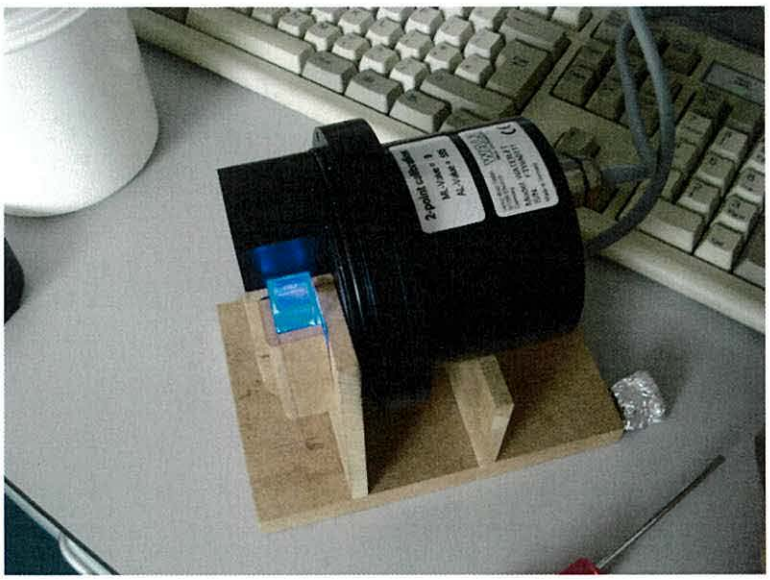


Figure 2.2. Image of adapted PAM fluorometer and cuvette holder assembly. The image was taken during a saturation pulse with the foil cap (used for dark-adaptation) removed for illustrative purposes. Fluorometer used blue LEDs for actinic light and saturation pulses.

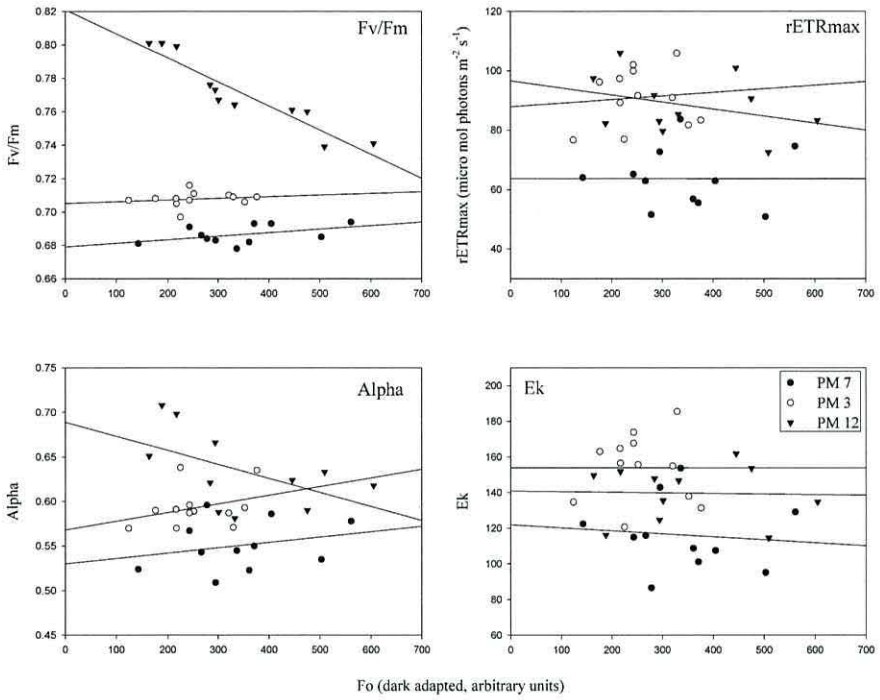


Figure 2.2.1. Graphical analysis of investigation of cell density and photomultiplier (PM) gain setting on rapid light curve parameters.

Table 2.2.1. Statistical analysis of effect of cell density and photomultiplier (PM) gain setting on rapid light curve parameters. AD= Anderson-Darling normality test statistic.

Parameter	PM Gain	Slope	F, p and *df* for slope	R^2	Effect Size	normally distributed
rETRmax	PM - 3	0.01205	F = 0.09, p = 0.775, *df* = 11	0.0086	<0.01	AD = 0.336, p = 0.444
	PM - 7	-0.00017	F = 0.00, p = 0.995, *df* = 10	<0.0001	<0.01	AD = 0.280, p = 0.571
	PM - 11	-0.02368	F = 1.16, p = 0.309, *df* = 10	0.1145	0.13	AD = 0.303, p = 0.515
alpha	PM - 3	0.0001	F = 1.19, p = 0.300, *df* = 11	0.1066	0.12	AD = 0.584, p = 0.101
	PM - 7	0.00006	F = 0.62, p = 0.450, *df* = 10	0.0649	0.07	AD = 0.189, p = 0.871
	PM - 11	-0.00016	F = 3.44, p = 0.097, *df* = 10	0.2345	0.31	AD = 0.331, p = 0.450
Ek	PM - 3	-0.00025	F = 0.00, p = 0.998, *df* = 11	<0.0001	<0.01	AD = 0.269, p = 0.613
	PM - 7	-0.01683	F = 0.09, p = 0.771, *df* = 10	0.0009	<0.01	AD = 0.268, p = 0.609
	PM - 11	-0.0034	F = 0.01, p = 0.929, *df* = 10	0.0099	0.01	AD = 0.398, p = 0.304
Fv/Fm	PM - 3	0.00001	F = 0.30, p = 0.597, *df* = 11	0.0289	0.03	AD = 0.737, p = 0.039
	PM - 7	0.00002	F = 2.34, p = 0.160, *df* = 10	0.2063	0.26	AD = 0.227, p = 0.755
	PM - 11	-0.0034	F = 64.14, p = <0.001, *df* = 10	0.8754	0.7	AD = 0.653, p = 0.064

2.2.3. Microalgal dry weight

The effect of filter type/ pore size and drying temperature on the microalgal dry weight measurement was investigated. Dry weight measurements were conducted using 5ml of *Chlorella vulgaris* cell suspension measured by calibrated pipette according to the filtration methods outlined in section 2.1.2. Whatman GF/C filters have a nominal pore size of 1.2 μm, whilst Whatman GF/F filters have a nominal pore size of 0.7 μm. Analysis was carried out using 5 replicates per treatment. The facilities available allowed three possible drying methods: Drying in an oven at 60°C, drying in an oven at 80°C and using a microwave (750W, 5 minutes). The microwave method was included for potential use at a power station site with limited drying facilities. Results are shown in table 2.2.3.

Table 2.2.2. Effect of filter type and drying temperature on the dry weight (g L^{-1}) of 5 ml *Chlorella vulgaris* cultivation retained on the filter. The drying temperature is measured in °C. Data is derived from 5 replicate samples.

Filter	Drying Temperature	Dry Weight Mean (±SD)
GF/C	60	1.201 (0.019)
GF/C	80	1.171 (0.016)
GF/C	Microwave	1.164 (0.008)
GF/F	60	1.278 (0.027)
GF/F	80	1.235 (0.012)
GF/F	Microwave	1.237 (0.011)

A two-factor analysis of variances found that there was no significant interaction between factors ($F = 0.42$, $p = 0.662$). The test assumptions of normality (Anderson Darling test = 0.328, $p = 0.505$) and approximately equal variances (Bartlett's test = 7.1, $p = 0.214$) were held. There was a significant effect of filter type ($F = 135.55$, $p<0.001$), with GF/F filters retaining a mean of 6.01% more dry matter than GF/C filters. The drying method also had a significant effect on the dry weight ($F = 17.03$, $p<0.001$). Tukey's pairwise comparisons showed that drying using a microwave did not produce a significantly different mean dry weight to the oven at 80°C, but that the oven at 60°C produced a mean dry weight measurement significantly higher than each of the other treatments, showing that some cellular moisture was retained at the lower temperature. Measurements of microalgal dry weight made during this work were made using GF/F filters dried at 80°C due to efficient biomass extraction and higher moisture removal.

Chapter 3.

Dissolved Organic Matter in Photobioreactors.

3. Overview

The following chapter is presented in two parts. Section 3.1 comprises preliminary work, whilst section 3.2 consists of a published article from a separate set of experiments. The conclusions from section 3.1 were used to inform the second set of experiments, leading to the publication. The hypothesis tested in each section of Chapter 3 was that *exudation of dissolved organic matter in photobioreactor cultivations of microalgae is a significant loss in solar energy conversion.*

3.1. Dissolved organic matter in tubular and polythene photobioreactors.

3.1.1. Introduction.

In Chapter 1 the maximum efficiency of microalgal photosynthesis and losses in the solar-to-biomass energy conversion process were discussed and it was noted that the productivity of such models is never reached in practice. One of the reasons for this may have been overlooked in the literature: In the natural environment, microalgae are widely known to release dissolved substances into the surrounding water (Carlson, 2002). Release of dissolved substances is unavoidable and occurs from every algal cell. In the context of total primary production release of dissolved substances may range from <1 up to 80% of total production (Malinsky-Rushansky & Legrand, 1996), but is commonly in the range 5-30% (Biersmith & Benner, 1998). Thus, release of dissolved substances in intensive photobioreactor cultivations might have a significant impact on biomass production efficiency. Further, release of dissolved substances by microalgae grown at high cell densities implies a potentially high organic loading to the remaining process water. This indicates that recycling or disposing of waste water from algal systems may present a problem for large-scale industrial production. In particular, the supply of water of sufficient quality may be a

significant life-cycle problem in the production of microalgae, especially using freshwater species (Yang et al., 2011). Algal exudates tend to be biologically labile, supporting bacterial communities (Lønborg & Søndergaard, 2009; Obernosterer & Herndl, 1995). However, although the presence of bacteria has frequently been acknowledged, there is relatively little evidence for the amount of bacterial biomass present in intensive microalgal cultivations.

The aim of this work was to establish whether the release of dissolved organic matter (DOM) by microalgae could be an important factor in the production of algal biomass, and to investigate whether bacteria could constitute a significant fraction of the total biomass present in large scale photobioreactor cultures.

3.1.2. Methods

3.1.2.1. Definition of dissolved & particulate matter

Conventional vacuum filtration was used to partition the dissolved and particulate phases using Whatman GF/F filters (0.7 μm nominal pore size). Biomass retained on GF/F filters was the harvestable biomass, whilst material passing through filters comprised the dissolved organic matter. This principle ensured that all the organic matter in the photobioreactor was quantified. Supporting samples of dissolved substances were obtained by using more conventional 0.45 μm filters (Whatman GDX), and were used to confirm that samples from GF/F filters were reliable (see Chapter 3.2).

3.1.2.2. 20L Polythene photobioreactors

Chlorella vulgaris (CCAP 211/11B) was grown in 20 L polythene photobioreactors in front of a light bank containing 8 cool white 36 W fluorescent

tubes (Fig. 1). Light output was measured using a Li-190 SA 2π PAR sensor (Li-Cor, USA). Mean (±SD) irradiance was 233 (±3.96) μmol photons m^{-2} s^{-1} (150 spatial measurements). The mean temperature inside each photobioreactor was 24°C, varied by no more than ±1.5°C, and was consistent to within 0.5°C between all bags at any one time. Photobiorector material was pre-washed with Milli-Q water to remove any contaminants. Air filtered to 0.3 μm (Whatman Hepa-Vent) was bubbled (0.65 vvm) through the solution using an oil-less compressor. Of six bags, three experimental bags were inoculated and three were controls (not inoculated). The inoculum was cultivated in identical media in 2 L glass flasks, and the starting cell density in the bags was adjusted to 1600 cells μL^{-1}.

3.1.2.3. 1200 L Tubular photobioreactor

Cultures were scaled up to 1200 L in a tubular photobioreactor (Biofence, Varicon Aqua Ltd) shown in Figure 1. The temperature of the culture fluid was held at 20 ±0.5°C using air conditioning. The mean incident irradiance was 392 (sd ±32) μmol photons PAR m^{-2} s^{-1} (220 measurements). The system was operated as a pH stat (pH 7) through the solenoid-controlled addition of CO_2. The culture innoculum was prepared in three 20 L polythene photobioreactors using the same media.

3.1.2.4. Nutrient media

Standard algal growth media can contain large quantities of the organic chelating agent ethylenediaminetetraacetic acid (EDTA). To avoid confounding DOC measurements with this component an inorganic nutrient medium was used for 20 L photobioreactor cultures. The inorganic nutrient media gave final concentrations of: KNO_3 (7.5 mM), $MgSO_47H_2O$ (7.5 mM), KH_2PO_4 (6 mM), $FeSO_47H_2O$ (10 μM),

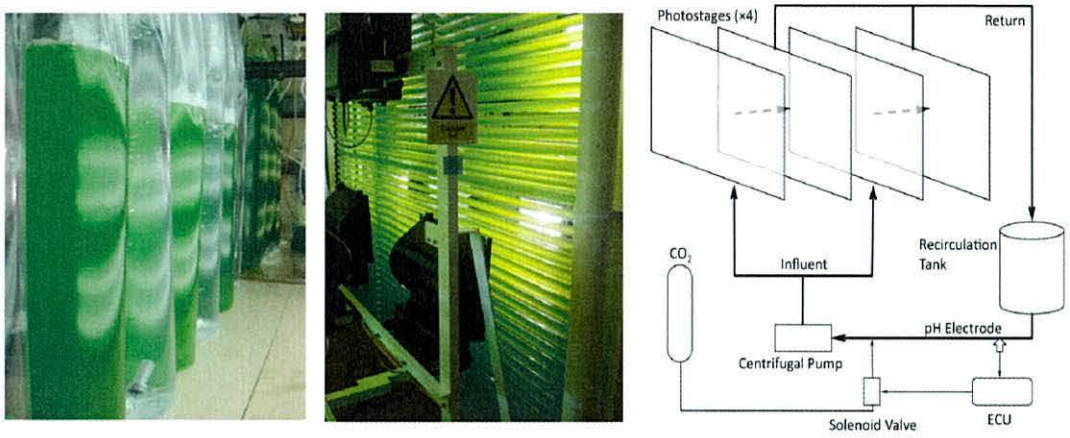


Figure 1. Apparatus used for culture of *Chlorella vulgaris*: (left) 20 L photobioreactors using inorganic media– control/ experimental systems placed alternately; (centre) A single tubular photobioreactor photostage containing growing culture; (right) A process diagram of the PBR equipment and control system.

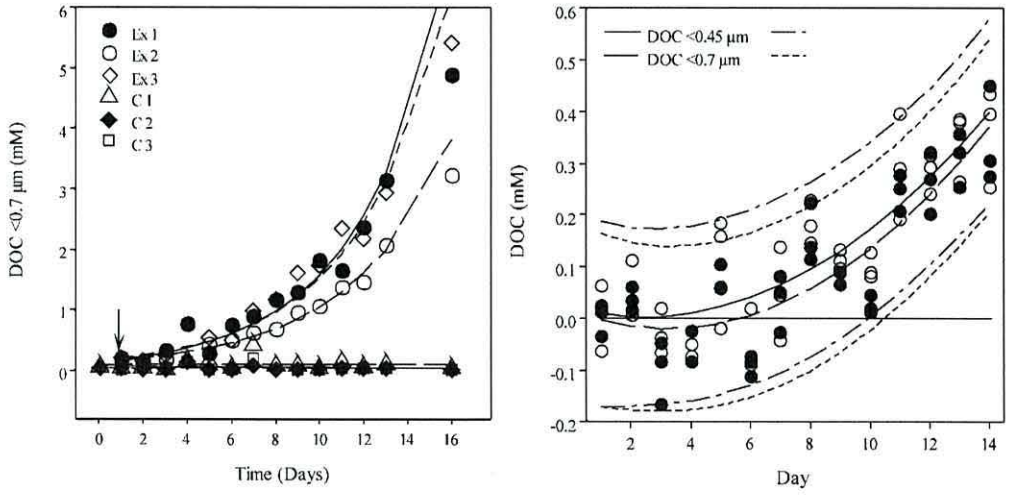


Figure 2. (a. left) DOC concentrations (<0.7 µm, mmol L^{-1}) measured in experimental (Ex1-3) and control (C1-3) 20 L cultures over 16 days described by linear (controls) and log-linear (experimental) models. Arrow shows point of inoculation. (b. right) DOC (<0.7 & <0.45 µm) concentrations in the tubular photobioreactor over 14 days, modeled by quadratic regression (±95% CI).

$ZnSO_47H_2O$ (0.1 μM), $MnCl_24H_2O$ (0.1 μM), H_3BO_3 (0.05 μM), $CuSO_45H_20$ (0.001 μM). A vitamin stock solution comprising B_{12}, B_1 and Biotin was also added to the cultures, though altogether they contributed less than 5 μmol L^{-1} organic carbon to the final culture medium.

For the tubular photobioreactor standard Jaworski media was used (Culture Collection of Algae and Protozoa, CCAP, Oban, UK) with tap-water. The system was sterilised with sodium hypochlorite prior to inoculation.

3.1.2.5. Dry weight

Dry weight measurements (n= 1 per bag per day, n= 3 per day for photobioreactor) were made by filtering 20 ml of culture through pre-weighed Whatman Ø47 mm GF/F filters as detailed in general methods.

3.1.2.6. Dissolved organic carbon measurements

Dissolved organic carbon was analysed on a MQ-1001 high temperature combustion total organic carbon analyser as detailed in general methods.

3.1.2.7. Particulate organic carbon measurements

Particulate organic carbon was measured by elemental analysis, as detailed in general methods. The particulate organic carbon (POC) concentration (mmol L^{-1}) was calculated from the carbon content (mmol g^{-1}) of the dried pellet, multiplied by its corresponding dry weight measurement (g L^{-1}). Calculations for derivation of total organic carbon (1), percentage carbon dissolved in <0.7 μm fraction (2), and percentage carbon dissolved in <0.45 μm fraction (3) are shown below:

$$t = d_{0.7} + p \quad (3.1.1)$$

$$pd_{0.7} = [d_{0.7} / t] \times 100 \quad (3.1.2)$$

$$pd_{0.45} = [d_{0.45} / t] \times 100 \quad (3.1.3)$$

Where t is the total organic carbon (mmol L^{-1}); $d_{0.7}$ is the dissolved organic carbon (<0.7μm, mmol L^{-1}); p is the particulate organic carbon concentration (>0.7μm, mmol L^{-1}); $pd_{0.7}$ is the percentage of organic carbon present as DOC (<0.7 μm); $pd_{0.45}$ is the percentage DOC present in the <0.45 μm fraction; $d_{0.45}$ is the concentration of organic carbon in the <0.45 μm fraction (mmol L^{-1}).

3.1.2.8. Bacteria

Bacterial number and biomass were determined according to the methods outlined in the general methods and Chapter 3.2 using epifluorescence microscopy.

3.1.2.9. Statistical analysis

Cultivations in 20 L photobioreactors were performed in triplicate (+triplicate controls). Each sample was derived from a replicate 20 L photobioreactor. Samples from the tubular photobioreactor were taken from a single run, with 3 samples taken on each occasion.

3.1.3. Results

3.1.3.2. DOC and POC in bag cultures

Figure 2(a) shows the change in DOC concentrations (mmol L^{-1}) in experimental and control 20 L polythene photobioreactor cultures of *Chlorella vulgaris* over 16 days. The DOC concentrations in the control bags did not change

significantly over time: Data from each replicate photobioreactor was fitted with a linear regression model to derive the slope coefficient. The mean slope (±95% CI) for the three control bags was -0.0005 (0.0045) mmol L^{-1} d^{-1} and was not significantly different to zero (1 sample t-test, t= -0.47, p = 0.685).

The concentration of DOC in experimental 20 L photobioreactors accumulated at an increasing rate, reaching a mean (±SE) of 0.822 (0.104) mmol L^{-1} by day 7. The DOC increase with time was best described by an exponential relationship (Fig. 2). Figure 2b shows the change in DOC concentration (<0.45 and <0.7μm) in the tubular photobioreactor, after subtraction of the pre-innoculation DOC concentration. A quadratic model was fitted to describe the response. Although there was a strong relationship (F= 55.7, p <0.001) significant DOC accumulation could only be detected from day 10, toward the stationary phase. In the mid–exponential phase (day 6), the predicted values for both <0.7 and <0.45 μm samples were both positive, though the 95% confidence intervals encompassed zero. However, by day 12 there was sufficient evidence to demonstrate net DOC accumulation in the tubular photobioreactor at a predicted concentration of 0.239 mmol L^{-1}.

The relationship between particulate organic carbon (>0.7 μm) and dissolved organic carbon (<0.7 μm) for 20 L photobioreactor cultures is shown in Figure 3. During the first few days of growth there was evidence of a curved trend, though for most of the growth period the relationship between DOC to POC was approximately linear. During the last 3 days of the 20 L photobioreactor cultivations there was relatively little change in POC but DOC increased.

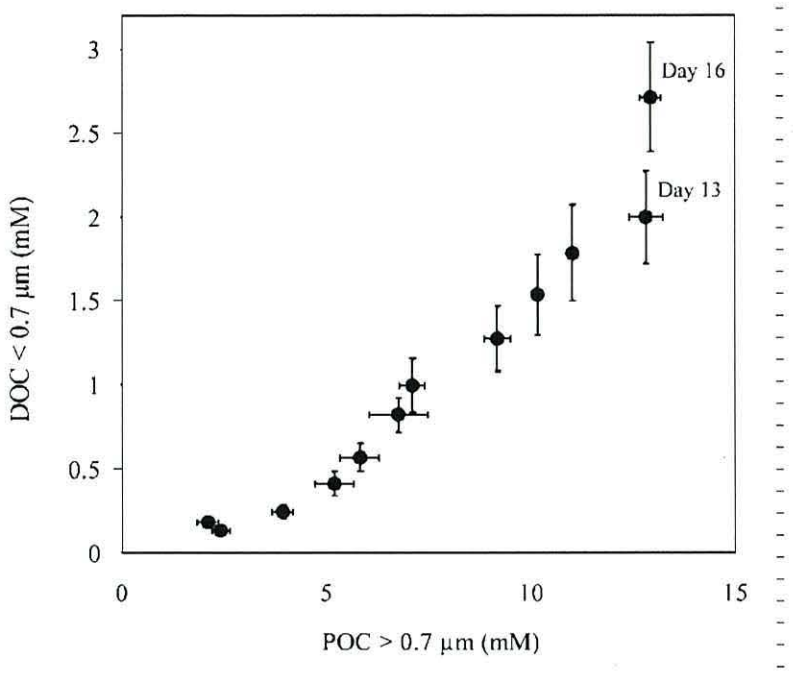


Figure 3. Particulate organic carbon (POC, mmol L^{-1}) vs. dissolved organic carbon (DOC, mmol L^{-1}) separated by a GF/F filter (0.7 µm) during batch growth in bag cultures. Data for days 13 and 16 indicated, and error bars represent standard errors, n= 3.

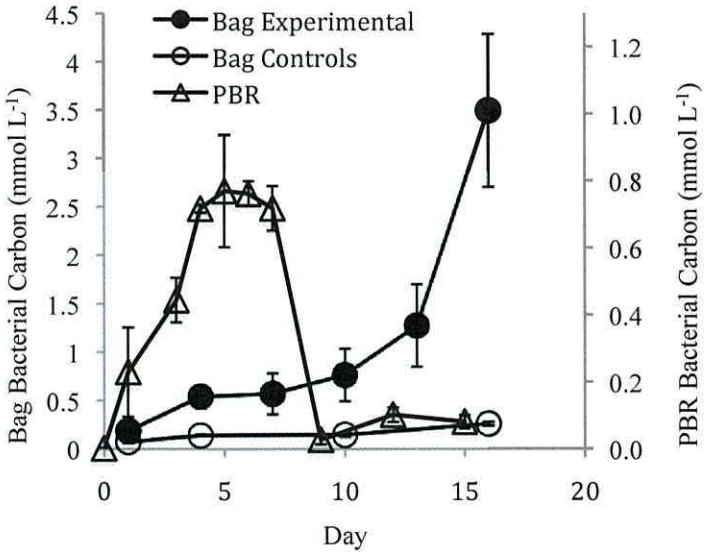


Figure 4. Bacterial carbon dynamics (mmol L^{-1}) over time in 20 L cultivations (control and experimental) and in the tubular photobioreactor (secondary axis), mean values with standard errors shown (n= 3).

3.1.3.3. Bacteria

During cultivation in 20 L photobioreactors, bacterial cell number increased (Fig. 4). The greatest increase in cell number was observed between days 13 and 16, when the cultures entered the stationary phase. Measurements showed that >99% of the cells in the cultures were at least 0.48 μm across their smallest dimension, so that most of the observed cells comprised particulate organic matter, included in the algal biomass measurement.

In the tubular photobioreactor the bacterial community behaved somewhat differently to 20 L photobioreactors. A bacterial bloom which peaked at days 5-6 was followed by an equally dramatic population crash, which then began to recover from day 12 onward. The measurements of bacterial carbon in the stationary phase corresponded to a mean of 22.0 and 32.0 fg per cell for tubular and 20 L polythene photobioreactors respectively. Most bacteria observed were free–living cells, though there were some bacterial cell aggregations around algal cells in both systems.

Up to 16 % of the total carbon fixed by the algae in the 20 L polythene and tubular photobioreactor cultures was comprised by bacteria, showing that heterotrophs can make a significant contribution to the total carbon and biomass present in microalgal cultures. Bacteria in bag cultures behaved relatively predictably, with an increase in bacterial numbers as the algal biomass increased. In contrast, bacteria in the tubular photobioreactor rapidly reached their highest biomass at days 5-6, before the population crashed. The cause of this apparent instability is not clear, since although there was organic matter present from the start, the bacterial degradability of EDTA is reported to be relatively low (Fabregas et al., 1993).

The absence of DOC during the mid exponential phase of the tubular photobioreactor culture suggests that although the algae might have produced some

DOC during this period, it was probably consumed by the bacterial population, which reached its greatest biomass at that time. The bacterial bloom may also explain why the maximum DOC concentration reached in the stationary phase (4 %) was five-fold lower than that reached in the 20 L polythene cultures (21 %).

3.1.3.4. Comparison of growth phase and system type

The concentration of carbon in the particulate, dissolved and bacterial phases is summarised in Table 1 for the mid exponential and stationary phases of the 20 L polythene and tubular photobioreactor cultures. In 20 L photobioreactors, DOC (<0.7 μm) reached 21.2 % of TOC in the stationary phase, but was lower at 10.8 % during the main growth period. The concentration of bacterial carbon present in the 20 L photobioreactors was similar to the concentration of DOC in both growth phases. Since most bacterial cells were free–living, it was concluded that algal-derived dissolved organic matter was the main substrate for growth.

3.1.4. Discussion

3.1.4.1. Dissolved organic carbon

The results show that *Chlorella* exuded measurable quantities of organic carbon into the surrounding medium during growth. The data from 20 L polythene photobioreactors compares favorably with other estimates of DOC release, which are typically in the order of 13 % (Malinsky-Rushansky & Legrand, 1996; Carlson, 2002). However, it is apparent that the relative importance of the DOC component is dependent upon the growth phase of the culture, since both absolute and relative concentrations of dissolved organic carbon were highest in the stationary phases of bag and tubular photobioreactor cultures.

Table 1. Summarised data for total, particulate, dissolved and bacterial organic carbon in 20 L and tubular photobioreactor cultures (mmol L^{-1} & % total organic carbon).

Fraction	Measurement	Bag Cultures		Photobioreactor	
		Mid-Exponential (day 7)	Stationary (day 16)	Mid-Exponential (day 6)	Stationary (day 12)
DOC <0.7	mmol L^{-1}	0.82 (0.18)	4.50 (0.66)	0.005 (0.159)[b]	0.239 (0.159)[b]
	% TOC	10.84 (1.09)	21.16 (3.02)	0.11 (3.37)[d]	3.75 (2.49)[d]
DOC <0.45	mmol L^{-1}	0.822 (0.042)	3.40 (0.57)	0.039 (0.170)[b]	0.273 (0.170)[b]
	% TOC	11.02 (1.10)	17.86 (2.36)	0.83 (3.61)[d]	4.29 (2.69)[d]
TOC	mmol L^{-1}	7.58 (1.39)	21.26 (0.12)	4.72[c]	6.37[c]
POC >0.7	mmol L^{-1}	6.75 (0.71)	16.75 (0.56)	4.71 (0.05)	6.13 (0.09)
Bacteria	mmol L^{-1}	0.56 (0.21)	3.45 (0.78)	0.76 (0.04)	0.10 (0.02)
	% TOC	8.19 (4.00)	16.28 (3.73)	16.10 (0.85)[e]	1.57 (0.31)[e]

[a] Bacterial carbon content calculated from biovolume and expressed as percent total organic carbon.
[b] Data predicted by quadratic regression (±95% CIs).
[c] TOC estimated using mean DOC + mean POC.
[d] Percentage DOC derived from DOC (±95 % CIs) divided by single TOC measurement ×100.
[e] Percentage bacterial carbon derived from mean (±SE) divided by single TOC measurement ×100.

*For 20 L photobioreactor cultivations, means and standard errors are from replicate photobioreactors (n = 3). For tubular photobioreactor cultivation, the standard error is presented only for POC and bacterial measurements. Dissolved organic carbon in the tubular photobioreactor is predicted by regression, shown with the 95% confidence limits.

In stationary phase bag cultures, the presence of one-fifth of the net total photosynthetically fixed carbon as DOC constituted a potentially important loss of biomass, reduced carbon and energy. Even in the mid-exponential phase, 11 % of the organic carbon was lost as DOC. The much higher fraction of DOC in the stationary phase of both bag cultures can be explained by limitation of one or more nutrients; evidence from work on natural phytoplankton has shown that microalgal DOM exudation is enhanced under sub–optimal conditions, particularly when a nutrient such as phosphate becomes limiting (Ji & Sherrel, 2008; Carlson, 2002).

3.1.5. Conclusions from preliminary work.

Significant amounts of dissolved organic matter accumulated in the cultures during growth. Assuming that materials exuded by *Chlorella vulgaris* had the same energy and carbon content as the particulate biomass, the data here suggests that for every MJ of particulate biomass energy recovered, 0.12 MJ could remain dissolved in the culture fluid (based on mid–exponential cell growth from 20 L photobioreactor measurements where DOC was 10.8 % of TOC). This could be a potentially important loss of efficiency for an applied microalgal process and worthy of further investigation.

Bacteria are a potentially important component in microalgal cultivations. The bacterial dynamics in the tubular photobioreactor cultivation were not as expected, and a particularly low algal cell density was achieved. For these reasons and for the purposes of adequate replication, further work should use 20 L cultivation systems for reliability.

Only *Chlorella vulgaris* was tested in this preliminary study although other species lacking a cell wall, such as *Dunaliella tertiolecta*, may have higher DOC

production rates. Thus, further research requires examination of at least another species. In addition to DOC, losses of dissolved organic nitrogen (DON) may also be important.

Bacterial measurements were derived from the whole cultivation medium, including dissolved and particulate phases. It is not clear from this research whether large concentrations of bacterial carbon were present in the dissolved phase. This should be addressed in subsequent work.

3.1.6. References

Biersmith, A., Benner, R. 1998. Carbohydrates in phytoplankton and freshly produced organic matter. Marine Chemistry. 63, 131–144.

Carlson, C. 2002. Production and Removal Processes. In Carlson, C & Hansell, D. (eds). In Biogeochemistry of Marine Dissolved Organic Matter. Academic Press London, pp 91-139.

Fabregas, J., Vazquez, V., Cabezas, B., Otero, A. 1993. Tris not only controls the pH in microalgal cultures, but also feeds bacteria. Journal of Applied Phycology. 5, 543-545.

Ji, Y., Sherrel, R. 2008. Differential effects of phosphorus limitation on cellular metals in *Chlorella* and *Microcystis*. Limnology and Oceanography. 53, 1790-1804.

Lønborg, C., Søndergaard, M., 2009. Microbial availability and degradation of dissolved organic carbon and nitrogen in two coastal areas. Estuarine Coastal and Shelf Science. 81, 513-520.

Malinsky-Rushansky, N., Legrand, C. 1996. Excretion of dissolved organic carbon by phytoplankton of different sizes and subsequent bacterial uptake. Marine Ecology Progress Series. 132, 249-255.

Obernosterer, I., Herndl, G. 1995. Phytoplankton extracellular release and bacterial growth: dependence on the inorganic N:P ratio. Marine Ecology Progress Series. 116, 247–257.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y. 2011. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresource Technology. 102, 159-165.

Bioresource Technology 101 (2010) 8690–8697

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Dissolved organic matter (DOM) in microalgal photobioreactors: A potential loss in solar energy conversion?

Chris J. Hulatt *, David N. Thomas

School of Ocean Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK

ARTICLE INFO

Article history:
Received 20 April 2010
Received in revised form 9 June 2010
Accepted 19 June 2010
Available online 14 July 2010

Keywords:
Microalgae
Dissolved organic matter
Biofuel
Bio-energy
Photobioreactor

ABSTRACT

Microalgae are considered to be a potential alternative to terrestrial crops for bio-energy production due to their relatively high productivity per unit area of land. In this work we examined the amount of dissolved organic matter exuded by algal cells cultured in photobioreactors, to examine whether a significant fraction of the photoassimilated biomass could potentially be lost from the harvestable biomass. We found that the mean maximum amount of dissolved organic carbon (DOC) released measured 6.4% and 17.3% of the total organic carbon in cultures of *Chlorella vulgaris* and *Dunaliella tertiolecta*, respectively. This DOM in turn supported a significant growth of bacterial biomass, representing a further loss of the algal assimilated carbon. The release of these levels of DOC indicates that a significant fraction of the photosynthetically fixed organic matter could be lost into the surrounding water, suggesting that the actual biomass yield per hectare for industrial purposes could be somewhat less than expected. A simple and inexpensive optical technique, based on chromophoric dissolved organic matter (CDOM) measurements, to monitor such losses in commercial PBRs is discussed.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Demand for sustainable energy technologies has refreshed scientific and commercial interest in the mass culture of microalgae. Microalgal technologies appear promising because, compared to terrestrial crops, they tend to be efficient at using solar energy to produce biomass (Mata et al., 2010; Dismukes et al., 2008). Microalgae can be incorporated into a wide variety of design-optimised structures (Ugwu et al., 2008), and the careful control of culture systems can lead to promising, potentially commercially viable, growth rates (Keffer and Kleinheinz, 2002). In particular, the use of microalgae to remove CO_2 from power plant flue gas and re-cycle the biomass as a fuel is posited as a potentially feasible technology (Jacob-Lopez et al., 2010; Zeiler et al., 1995), and the principle has been demonstrated at the pilot scale (Vunjak-Novakovic et al., 2005). Like any crop or bio-energy technology utilizing sunlight, maximizing the efficiency of the sunlight-to-biomass process is critical to ensure optimal use of the land area available, and it is vital to understand any parameters that significantly impact the process.

The mass culture of microalgae does suffer from additional process costs that are not experienced by terrestrial based biomass production systems: in particular, the supply of energy for circulating cell suspensions (Jorquera et al., 2010) and for biomass extraction or dewatering can be costly (Oliveira et al., 2009; Horiuchi et al., 2003). As a result, estimation of the feasibility of microalgal mass culture is extremely sensitive to factors such as growth rate and the biomass concentration attained at the point of harvest, so that even small changes in productivity can significantly impact the energetics and economics of the process (Chisti, 2009).

One characteristic unique to aquatic algae is the release of a fraction of photosynthetically fixed organic matter into the surrounding water during normal, healthy growth (Puddu et al., 2003). The exudation of dissolved organic matter (DOM) from algal cells has been recognised and studied in natural systems for several decades Carlson (2002), though its potential significance for microalgal bio-energy technologies does not seem to have received much consideration. Since microalgal biomass is recovered by separating the cells from the liquid phase (Molina Grima et al., 2003), a significant fraction of the photo-assimilated carbon is likely to be present as dissolved substances in the water. Any non-recovered carbon would thus constitute a loss factor in solar energy conversion or, possibly, a previously un-explored resource. Figures for algal DOM release in natural systems are widely reported, and dissolved organic carbon (DOC) values may range from zero to 80% of that fixed by photosynthesis. However, most report typical values in the range 5–30% (Malinsky-Rushansky and Legrand, 1996; Carlson, 2002; Biersmith and Benner, 1998), suggesting that a modest but nonetheless significant fraction of microalgal biomass may ultimately present in the dissolved phase.

Algal exudates are comprised of a wide range of compounds, but are generally dominated by carbohydrates. These vary from

* Corresponding author. Tel.: +44 (0)1248 382 852.
E-mail address: osp418@bangor.ac.uk (C.J. Hulatt).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.06.086

simple monosaccharides to larger heteropolysaccharides, and the chemical composition may vary considerably (Puddu et al., 2003). Nitrogenous compounds also contribute to the DOM pool (Aluwihare and Repta, 1999; Carlson, 2002), and there is considerable interest in identifying useful extracellular products from microalgae (Mishra and Jha, 2009). Exudates can also provide an excellent substrate for heterotrophic microbes, so that microalgal DOM supports heterotrophic organisms and in particular bacterial communities (Lønborg and Søndergaard, 2009; Obernosterer and Herndl, 1995): Although the presence of bacteria in mass algae cultures has been acknowledged (Scragg et al., 2002), there is still relatively little evidence for their potential significance in terms of carbon capture efficiency of photobioreactors, especially for large-scale industrial applications.

The aim of this work was to investigate whether dissolved organic substances could constitute a significant fraction of the photoassimilated carbon in microalgal cultures in order to determine whether DOM exudation could have economic implications for the efficiency of microalgal bio-energy applications. Furthermore, a rapid method for monitoring the DOM concentrations in algal cultures that could be adopted in industrial-scale operations is discussed.

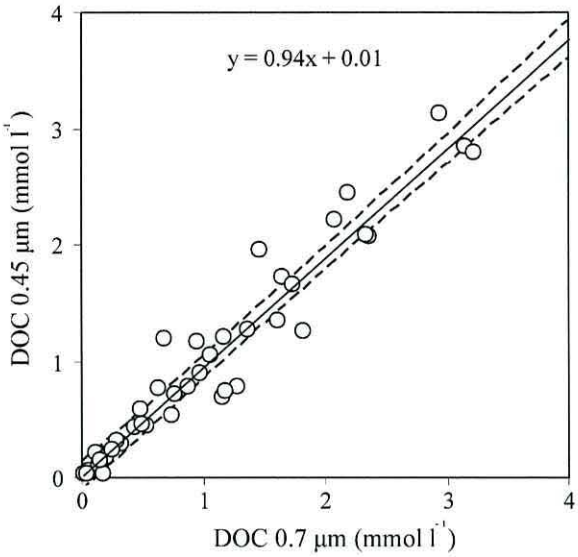


Fig. 1. Comparison of dissolved organic carbon measurements using samples filtered with 0.45 and 0.7 μm filters (nominal pore size). Samples were collected from three replicate cultures of *C. vulgaris* in a preliminary experiment.

2. Methods

2.1. Microalgal cultivations

Chlorella vulgaris (Culture Collection of Algae and Protozoa, CCAP, UK strain 211/11B) and *Dunaliella tertiolecta* (Sammlung von Algenkulturen Gottingen, SAG, strain 13.86) were cultured in 20 l cylindrical polythene photobioreactors (PBRs) of 0.16 m diameter, sparged with 0.5 vvm air supplied by an oil-less compressor. Air supplied all the inorganic carbon to the cultures as CO_2, and was filtered to 0.3 μm (Whatman® Hepa-Vent).

Dunaliella tertiolecta was cultured in artificial seawater (2ASW) using the composition of CCAP, whilst *Chlorella vulgaris* was cultured using M-8 media composition (Mandalam and Palsson, 1998). Both media were selected for their negligible initial organic loading. Media used were filtered to 0.2 μm (Milli-Q®) and analytical grade reagents used throughout. Light was provided continuously by cool white fluorescent tubes (Osram, 36 W) at an incident photon flux density of 225 μmol photons $m^2\ s^{-1}$ (measured with a LiCor 190-SA sensor). The temperature was 24 ± 1 °C. For each species, three replicate PBRs were used, plus a further three controls (no algae in the bags). Microalgae were cultivated until cessation of growth in biomass.

2.2. Analytical procedures

Microalgal dry mass was measured by vacuum filtration through, pre-weighed, 0.7 μm nominal pore size glass fibre filters (Whatman® GF/F) and oven-dried at 80 °C for 24 h. Samples of *Dunaliella* were also washed three times with a 0.5 M ammonium formate solution to remove salt.

The particulate organic carbon (POC) and Particulate Organic Nitrogen (PON) concentration in the microalgal biomass was measured using a weighed pellet of dried algae (80 °C, 72 h) from which most of the water had been previously removed by centrifugation (14,000 rpm, 10,000g). Samples were analysed using an elemental analyzer (Thermo-Electron Flash EA 1112) equipped with a copper/copper oxide column and a thermal conductivity detector. The machine was calibrated using BBOT, sulphanilamide and L-cystine standards, and DL-methionine was used routinely as a further check standard.

The definition of dissolved substances varies depending upon the filter pore size used, typically over the range 0.2–0.7 μm (Carlson, 2002). In order to examine the effect of different filters on the dissolved organic carbon and nitrogen (DOC and DON), we measured the same samples using Whatman® GFF (0.7 μm nominal pore size) and Whatman® GDX (0.45 μm pore size) filters in a preliminary experiment using *Chlorella vulgaris* (Fig. 1). The correlation between the two measurements ($r = 0.983$, $p = <0.001$) and a slope estimate of 0.95 showed that the use of GF/F filters for dissolved measurements was robust (c.f. Nayar and Chou, 2003).

Therefore the filtrate from the dry weight measurement was collected and stored frozen (−20 °C). Dissolved organic carbon (DOC) was analysed by high temperature combustion on an MQ1000 TOC analyzer according to Qian and Mopper (1996). Analyses for the major dissolved inorganic nutrients, nitrate (NO_3^-), nitrite (NO_2^-) and dissolved inorganic phosphorus (DIP), were done using standard colorimetric methodology (Hanson and Koroleff, 1983) using flow injection analysis on a Lachat Instruments Quick-Chem autoanalyzer. Dissolved ammonium (NH_4^+) was determined using the fluorimetric method of Holmes et al. (1999) using a Hitachi F2000 fluorescence spectrophotometer. The sum of the inorganic nitrogen measurements ($NO_3^- + NO_2^- + NH_4^+$) comprised the total dissolved inorganic nitrogen (DIN). Dissolved organic nitrogen (DON) was determined by subtraction of NO_3^-, and NH_4^+ from the total dissolved nitrogen (TDN) analyzed using on-line peroxodisulfate oxidation coupled with ultraviolet radiation at pH 9.0 and 100 °C (Kroon, 1993). Due to the high levels of nitrate initially present in the culture media, DON could only be accurately determined toward the end of the growth period.

In addition to the DOC and DON measurements, the optical density of the filtrate was determined using a dual beam UV–Visible spectrophotometer (Shimadzu UV 1601) using 10 cm quartz glass cuvettes. These measurements are henceforth referred to as chromophoric dissolved organic matter (CDOM), and were taken to test whether a convenient, rapid and low-cost optical technique could be used as a proxy for the more time consuming and expensive analytical determinations of DOC and DON.

Bacteria in the culture water were enumerated and sized by epifluorescence microscopy using 4′,6-diamidino-2-phenylindole (DAPI) to identify bacterial cells (Servais et al., 1999). Samples fixed by formaldehyde were prepared by exposing samples to DAPI at a final concentration of 5 μmol l^{-1} for 10 min, before filtering through 25 mm Whatman® Nuclepore 0.2 μm filters. Cells were

Table 1
Collated data from the relevant literature detailing the amount of organic carbon (fg) present in aquatic planktonic bacterial biomass per unit cell or biovolume. Values in parentheses are ranges.

Reference	Value
Fukuda et al. (1998)	21.3 (12.4–30.2) fg cell^{-1}
Smith et al. (1995)	20 fg cell^{-1}
Sturluson et al. (2008)	20 fg cell^{-1}
Nagata (1986)	106 fg μm^{-3}
Vrede et al. (2002)	146 (51–241) fg μm^{-3}
Riemann et al. (1982)	121 fg μm^{-3}
Bratbak and Dundas (1984)	220 (160–290) fg μm^{-3}

enumerated and sized (length and width) and in order to calculate bacterial carbon content the biovolume was calculated, then converted to a carbon measurement using values from the relevant literature (Table 1).

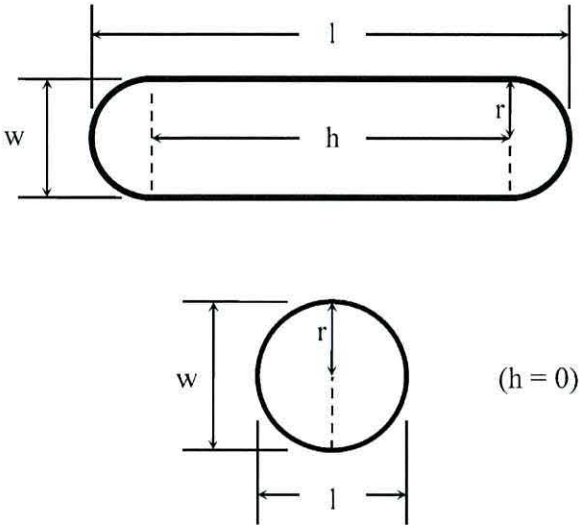


Fig. 2. Diagram showing measurements of bacterial cells used to derive carbon content using Eq. (1).

A mean value of 148 fg carbon μm^{-3} was used, and biovolume calculations (Eq. (1)) accounted for the rod-shaped and coccoid forms identified:

$$V = 2(2/3\pi r^3) + \pi r^2 h \quad (1)$$

where V is the biovolume of the cell (μm^3) and parameters are shown in Fig. 2. In addition to total bacterial measurements, selected samples were also filtered through GFF filters to examine the proportion of the bacteria which were included with the DOC measurement (i.e. in the filtrate).

3. Results and discussion

3.1. Dissolved and particulate organic carbon

The growth in POC and exudation of DOC in cultures of both microalgal species is shown in Fig. 3A and B. Cultures of *Dunaliella tertiolecta* reached a mean dry mass of 0.5 (± sd, 0.03) g l^{-1} with a corresponding POC concentration of 18.6 (± sd, 1.0) mmol l^{-1}, whilst *Chlorella vulgaris* attained a mean maximum dry mass of 2.0 (± sd, 0.3) g l^{-1}. These figures were typical values for the equipment and methods used, and comparable to typical densities reported in the literature (Chiu et al., 2008). As expected, the concentration of DOC in cultures of both species increased over time, reaching a maximum of 5.6 (± sd, 0.2) mmol l^{-1} and 3.9 (± sd, 1.5) mmol l^{-1} in *C. vulgaris* and *D. tertiolecta* cultures, respectively. There was no algal growth in the control bags and DOC concentrations in the control PBRs remained low at <0.15 mmol l^{-1} throughout the experiment, and there was no detectable change between start and end points; paired *t*-tests for *C. vulgaris* and *D. tertiolecta* treatments resulted in $t = 2.04$, $p = 0.178$, and $t = 2.66$, $p = 0.117$, respectively.

The fraction of the total organic carbon (derived from the sum of the DOC and POC) comprised by DOC is shown in Fig. 3C. There was an increase in the percentage DOC during growth of both species, although *Dunaliella tertiolecta* consistently excreted a greater fraction of its photosynthetically fixed carbon than *Chlorella vulgaris*. After 3 days the mean DOC accounted for just 2.0 (± sd, 0.3)% of TOC in cultures of *C. vulgaris*, but this was higher in *D. tertiolecta* cultures, at 4.8 (± sd, 0.7)% at day 2. By the end of each culture cycle the fraction comprised by DOC rose to 6.4 (± sd, 0.7)% in cultures of

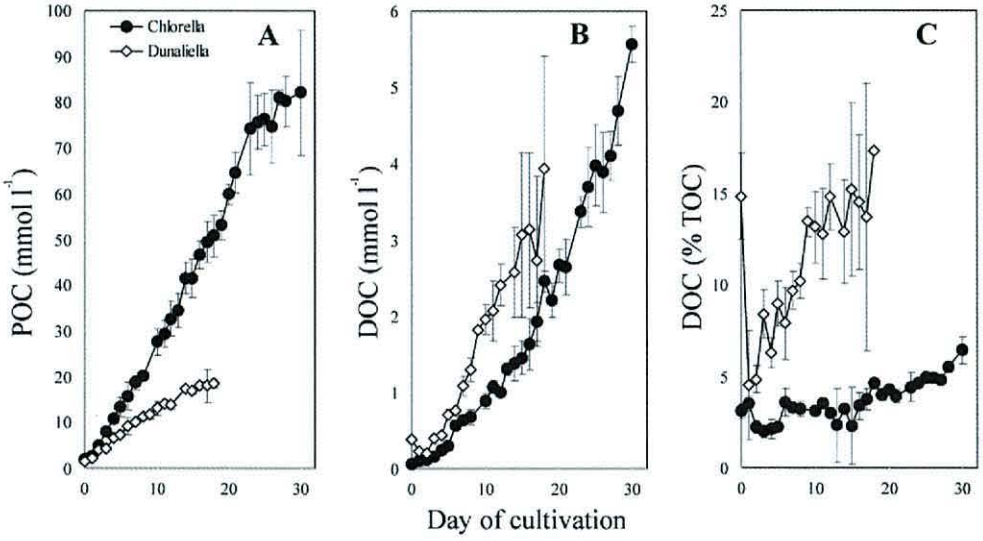


Fig. 3. (A) The concentration of particulate organic carbon (POC, mmol l^{-1}) for each species (*Chlorella vulgaris and Dunaliella tertiolecta*) over the cultivation period; (B) the concentration of dissolved organic carbon (DOC, mmol l^{-1}); (C) the proportion of total organic carbon comprised by DOC (%). Data expressed as mean of three replicate cultures and error bars represent standard deviations.

C. vulgaris, and 17.3 (± sd, 5.8)% in cultures of *D. tertiolecta*. The magnitude of DOC release measured here is within the ranges from ecological measurements (Malinsky-Rushansky and Legrand, 1996; Biersmith and Benner, 1998), illustrating that extracellular release is an unavoidable component of microalgal mass culture, even in the relatively stable environment provided by PBRs. The absolute fraction of the carbon and biomass released is clearly related to the species cultured and the stage of the growth cycle. Widespread evidence suggests that DOM exudation can be enhanced by physiological stresses, particularly the supply of nutrients (Carlson, 2002; Obernosterer and Herndl, 1995). Here, the percentage of carbon lost as DOC increased over time, suggesting that gradual depletion of inorganic nutrients may have driven some of this exudation.

3.2. Inorganic nutrients

The depletion of DIN at the end of both *Dunaliella tertiolecta* and *Chlorella vulgaris* cultures (Fig. 4) may have had a role in enhancing DOM production during the latter stages of cultivation. *D. tertiolecta* cultures exhausted DIN during the last 4 days of cultivation, though this only occurred at day 30 in *C. vulgaris* treatments. However, DOC in *D. teritolecta* cultivations still measured over 15% of TOC at day 12, indicating that nitrogen depletion was not the primary cause of exudation. *C. vulgaris* utilized only half of the available DIP over the batch cycle, so this was clearly not a limiting nutrient. In *D. tertiolecta* cultures, DIP concentrations decreased rapidly in the first 3 days, but thereafter depleted slowly until the end of the cultivations. Dissolved inorganic phosphorus was possibly concentration-limited in the last 2–4 days of cultivation, suggesting that it may have played some part in enhancing exudation toward the end of the experiment. Cultivations were carried out using standard media formulations until cessation of growth in biomass, so the DOM losses presented here are relevant to batch-operated mass algal cultivations. However, it should be noted that manipulation of nutrient supply, including possible concentration effects and other nutrients not measured, could impact DOM release rates, and is an area for further investigation. Production of DOM could be minimised through manipulation of the culture conditions by ensuring a constant excess of nutrients, achieved practically by using continuous cultivation systems. However, continuous cultivation may be counterproductive because of the commercial need to fully utilize the nutrient resource, especially as nutrient supply has been estimated to account for around 45% of the operational energy cost of microalgal mass culture (Chisti, 2009). Further, nutrient limitation is a method by which the cellular lipid content can be enhanced (Xin et al., 2010), so there could also be a conflict between minimizing DOM production whilst maximizing lipid content.

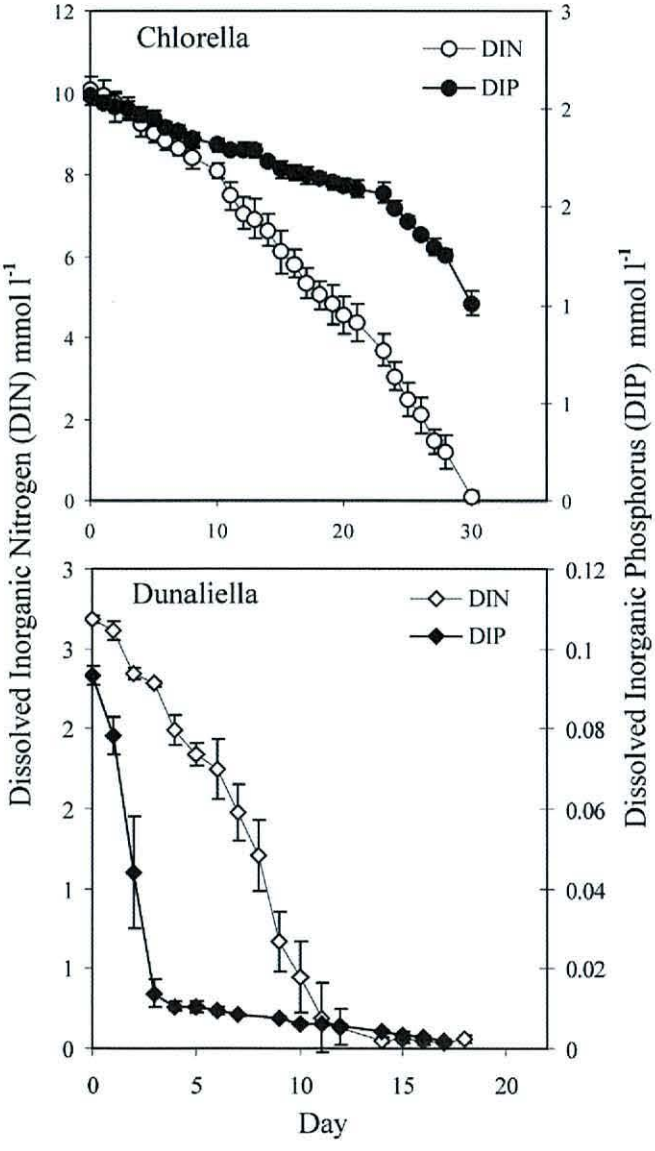


Fig. 4. The concentration of dissolved inorganic nitrogen (DIN, mmol l^{-1}) and dissolved inorganic phosphorus (DIP, mmol l^{-1}) during the batch cultivation of *Chlorella vulgaris* and *Dunaliella tertiolecta*. DIN was derived from the sum of $NO_3^- + NO_2^- + NH_4^+$.

In this work the pH and DIC concentrations were not controlled: for *Dunaliella tertiolecta*, the pH increased from 8.27 (± sd, 0.05) to 9.29 (± sd, 0.03), whilst in *Chlorella vulgaris* PBRs, the pH increased from 6.38 (± sd, 0.06) to 9.09 (± sd, 0.16) over the batch cycle. The dissolved inorganic carbon (DIC) available was thus comprised primarily by HCO_3^-, with limited dissolved CO_2 present during the first 2–3 days in the *C. vulgaris* treatments. Since both the concentration and type of inorganic carbon can impact the metabolism of microalgae (Falkowski and Raven, 2007) and references therein), it is possible it may also affect the release of DOM. For example, the use of power plant flue gas as a concentrated carbon source may not only impact growth rates, but also the rate at which DOM is released, and may be an area for further investigation. The cultivations in this work were conducted in very stable indoor conditions, and it is conceivable that growth in outdoor raceways and photobioreactors, where variable stress factors such as sunlight (including UV light stress) and temperature persist, that exudation could be somewhat higher.

3.3. Dissolved organic nitrogen

At the end of the experiments, DON peaked at 0.3 (± sd, 0.03) and 0.4 (± sd, 0.1) mmol l^{-1} in cultures of *Chlorella vulgaris* and *Dunaliella tertiolecta*, respectively. In percentage terms, this DON accounted for 15.7% (± sd, 5.1) of *D. tertiolecta* and 2.6% (± sd, 0.5) of *C. vulgaris* TON. DOM produced by both species had a higher C:N ratio than the corresponding particulate organic matter (POM) sample, concordant with the published literature (Meon and Kirchman, 2001). *C. vulgaris* POM had a C:N ratio of 6.8 (± sd, 0.2) at day 26, compared to 18.2 (± sd, 3.4) for its DOM. The discrepancy was lower for *Dunaliella* treatments as the POM C:N ratio measured 8.3 (± sd, 4.2), whilst the DOM measured 11.9 (± sd, 0.92). *D. tertiolecta* DOM was thus relatively richer in nitrogenous material than *C. vulgaris* by a factor of around 1.5, and this trend was true for all samples analysed. If it is considered that all of the nitrogen in the system was derived from that supplied as nitrate, then for

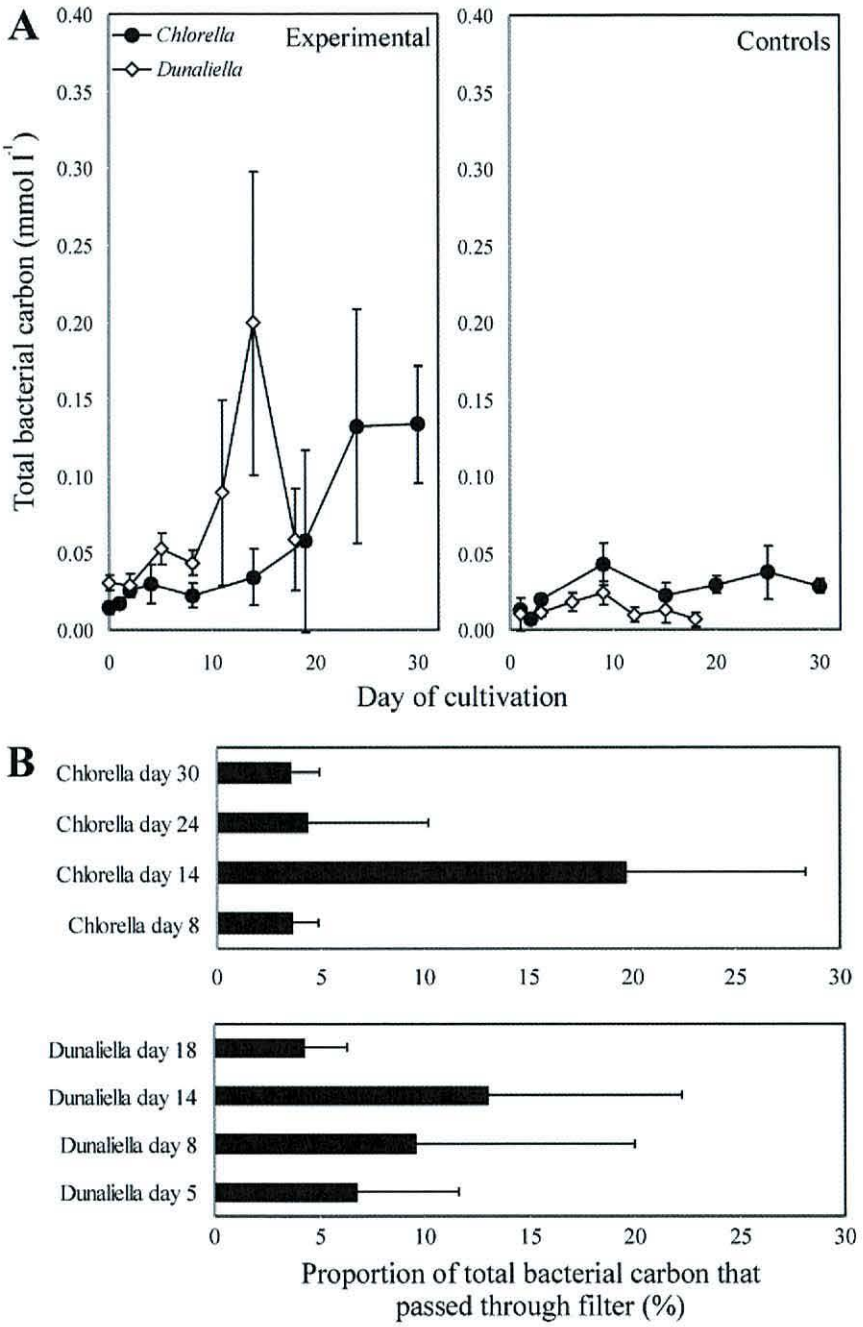


Fig. 5. (A) The total concentration of bacterial carbon (mmol l^{-1}) present in experimental and control photobioreactor cultures of *Chlorella vulgaris and Dunaliella tertiolecta* through the cultivation period; (B) the proportion (%) of the total bacterial biomass which passed through the filters into the dissolved phase through the cultivations. Values are means of three replicate photobioreactor cultures and error bars represent standard deviation.

D. tertiolecta it seems that 13.3 (± sd, 3.2)% of the nitrogen supplied as inorganic nutrients presented in the dissolved organic matter, but was much lower at 3.1 (± sd, 0.3)% for *C. vulgaris*. In common with carbon, the release of nitrogen could thus also constitute a significant loss of supplied nutrients.

3.4. Bacterial carbon

The change in total bacterial carbon observed during the growth of both microalgae, and in the controls is shown in Fig. 5A. In the controls there was evidence of growth in bacterial biomass during the trials, although this was minimal compared to the observed changes in the PBRs containing microalgae: In the *Chlorella* controls, there was a significant increase ($t = 6.83$, $p = 0.021$) of 0.016 mmol l^{-1} bacterial carbon over 30 days, whilst *Dunaliella tertiolecta* controls exhibited a slight decrease of 0.006 mmol l^{-1}, which was not significant ($t = 0.97$, $p = 0.435$). In PBRs containing *Chlorella vulgaris*, bacterial carbon increased over time, most notably between days 14 and 24, before plateauing between days 25 and 30. The maximum bacterial carbon concentration was reached at day 30, and measured 0.134 (± sd, 0.038) mmol l^{-1}. In comparison, *D. tertiolecta* bacterial carbon peaked at 0.200 (± sd, 0.098) mmol l^{-1} at day 14. Thereafter there was a decline in bacterial carbon toward the end of the experiment. Bacteria are important consumers of newly produced algal DOM (Puddu et al., 2003), and in natural systems may utilize DOC with an efficiency anywhere in

the range <0.1 to >0.6 (Kroer, 1993). The standing-stock of bacterial carbon could thus indicate a DOC consumption equivalent to <1.5 to 10+ times the amount that their biomass would suggest. Bacterial remineralisation of DOC is a loss process that is not apparent from the measurements made in the study. For example, taking a representative bacterial growth efficiency of 0.25, the amount of DOC consumed by bacteria at the end of the experiments would be in the order of 8% of that released by *C. vulgaris* and 10% of that released by *D. tertiolecta*. Adding these values to the direct measurements of dissolved losses raises the total fraction of photosynthetically assimilated carbon lost up to 7% and 19% at the end of the *C. vulgaris* and *D. tertiolecta* PBRs, respectively. Thus, a fraction of the released DOC is lost permanently from the system via bacterial activity. The proportion of the total bacterial carbon that passed through the filter as part of the DOC measurement is presented in Fig. 5B. Most of the bacteria are retained on the filter and therefore constitute a part of the POM values, with only a maximum of approximately 20% of the bacteria passing through a GFF filter during the mid part of the algal growth period (c.f. Nayar and Chou, 2003). Toward the beginning and end of the experiments values were much lower in the order of 5%. These discrepancies may be due to a combination of cell size, form, or surface adhesion. It can be concluded that the majority of the carbon (>99%) in the dissolved fraction is comprised by actual exudates, rather than by bacteria that were able to pass through the filtration process.

3.5. Summary of organic carbon pools

The relative proportions of the various carbon pools were investigated at selected parts of the growth phase, and the results are shown in Table 2. The total bacterial carbon in the cultures, including that in the dissolved and particulate phases, formed a relatively insignificant fraction of TOC, as all values were below 1%. When expressed as a percentage of their growth substrate though, bacterial carbon accounted for between 1% and 7.5% of the DOC. Dissolved bacterial carbon, that which could pass through a GFF filter, was less than 1% of DOC in all cases, showing that the dissolved fraction was comprised almost entirely by organic exudates rather than bacterial biomass which had passed through the filters. The total non-algal carbon (DOC + particulate bacterial carbon) present reached a maximum of 6.5 (± sd, 0.8)% in cultures of *Chlorella vulgaris*, and peaked at 17.6% (± sd, 5.9) in cultures of *Dunaliella tertiolecta*.

3.6. Optical measurements of DOM

Chromophoric dissolved organic matter is typically characterized by an approximately exponential increase in absorption with decreasing wavelength (Stedmon et al., 2000) and Fig. 6A shows typical absorption spectra for CDOM measured in the PBR cultures. For ease of application, it was necessary to locate a single wavelength at which the concentration of DOC was highly correlated with CDOM. This was investigated by correlating the DOC values for each PBR culture with their respective CDOM measurements over the growth cycle, using a number of different wavelengths through the UV and visible light spectrum. The results are resented in Fig. 6A as the mean r correlation coefficient of DOC versus CDOM of three replicate PBR cultivations. The correlation between CDOM and DOC was relatively consistent for *Dunaliella tertiolecta* cultures across all wavelengths tested, with r values at or above 0.9. In contrast CDOM in *Chlorella vulgaris* cultures exhibited greater wavelength dependency, but still peaked with a mean r value of 0.95, comparable to that of *D. tertiolecta*. The most suitable wavelength for comparability purposes was selected at 340 nm. The relationship between optical density (OD_{340}) and DOC was somewhat different between species (Fig. 6C), as material exuded by *C. vulgaris* (slope coefficient = 0.55) showed stronger absorption per unit carbon than *D. tertiolecta* (slope = 0.36). Multiple linear regression on square-root transformed data confirmed a highly significant difference between the two slopes ($F = 85.7$, $p = <0.001$, test assumptions held). The excellent correlation between DOC and CDOM therefore suggests that optical measurements could potentially be used provide a rapid estimate of the DOC present in mass algal cultures.

3.7. Implications for bio-energy technologies

The driving force behind the development of microalgal technologies is their higher potential productivity per unit area of land than terrestrial crops. Recent work by Zhu et al. (2008) and Williams and Laurens (2010) demonstrates the higher theoretic maximum potential photosynthetic energy efficiency of microalgae (~10%), compared to C_3 (4.6%) and C_4 plants (6%). Likewise, Weyer et al. (2010) calculated the maximum theoretic oil yield based on a similar first principles approach. These estimates, though fundamentally correct, do not account for any losses in biomass through DOM production. Taking the estimated theoretic maximum 330 t ha^{-1} yr^{-1} for mid latitudes (Williams and Laurens, 2010) as an example and assuming that exuded material has roughly similar properties to cellular biomass, the work here suggests that around 20–60 t ha^{-1} yr^{-1} could potentially be un-harvestable. Of course, this does not mean that algae are not competitive. Experimental measurements of algal biomass production consistently show higher yields than conventional crops (Dismukes et al., 2008). However, the release of dissolved matter is a significant loss that should be recognized and understood, especially when accounting for the differences between theoretic and achieved productivity.

It is worth noting that the production of waste-water rich in labile organic compounds with a potentially high chemical oxygen demand could present issues for treatment and recycling of the process water for any large scale microalgal production. Even at the moderate cell densities achieved here, DOC levels were in the same order of magnitude as domestic waste-water (Ditzig et al.,

Table 2
The relative contributions to total organic carbon by algal cells (*Chlorella vulgaris and Dunaliella tertiolecta*), bacteria and dissolved organic matter at selected points during growth (%). Values represent mean (±standard deviation) for three replicate photobioreactor cultures for each species.

Treatment	Day	POC (%TOC)	DOC (%TOC)	All bacteria (%TOC)	All bacteria (%DOC)	Dissolved bacteria (%DOC)	Total non-algal carbon (%TOC)
Chlorella	8	97.10 (0.39)	2.90 (0.36)	0.11 (0.04)	3.46 (1.57)	0.11 (0.02)	3.00 (0.39)
	14	97.13 (0.29)	2.87 (0.26)	0.08 (0.05)	2.68 (1.88)	0.60 (0.63)	2.94 (0.26)
	24	95.75 (0.27)	4.25 (0.25)	0.17 (0.10)	3.63 (2.34)	0.10 (0.08)	4.39 (0.26)
	30	93.60 (0.72)	6.40 (0.72)	0.16 (0.06)	2.42 (0.76)	0.08 (0.01)	6.54 (0.78)
Dunaliella	5	91.03 (1.25)	8.97 (1.25)	0.68 (0.15)	7.49 (0.82)	0.52 (0.39)	9.60 (1.40)
	8	89.80 (0.96)	10.20 (0.96)	0.35 (0.07)	3.43 (0.70)	0.29 (0.27)	10.52 (0.95)
	14	87.10 (2.85)	12.90 (2.85)	0.14 (0.13)	1.09 (0.96)	0.08 (0.04)	13.02 (2.84)
	18	82.70 (5.84)	17.30 (5.84)	0.31 (0.03)	1.92 (0.68)	0.08 (0.04)	17.60 (5.86)

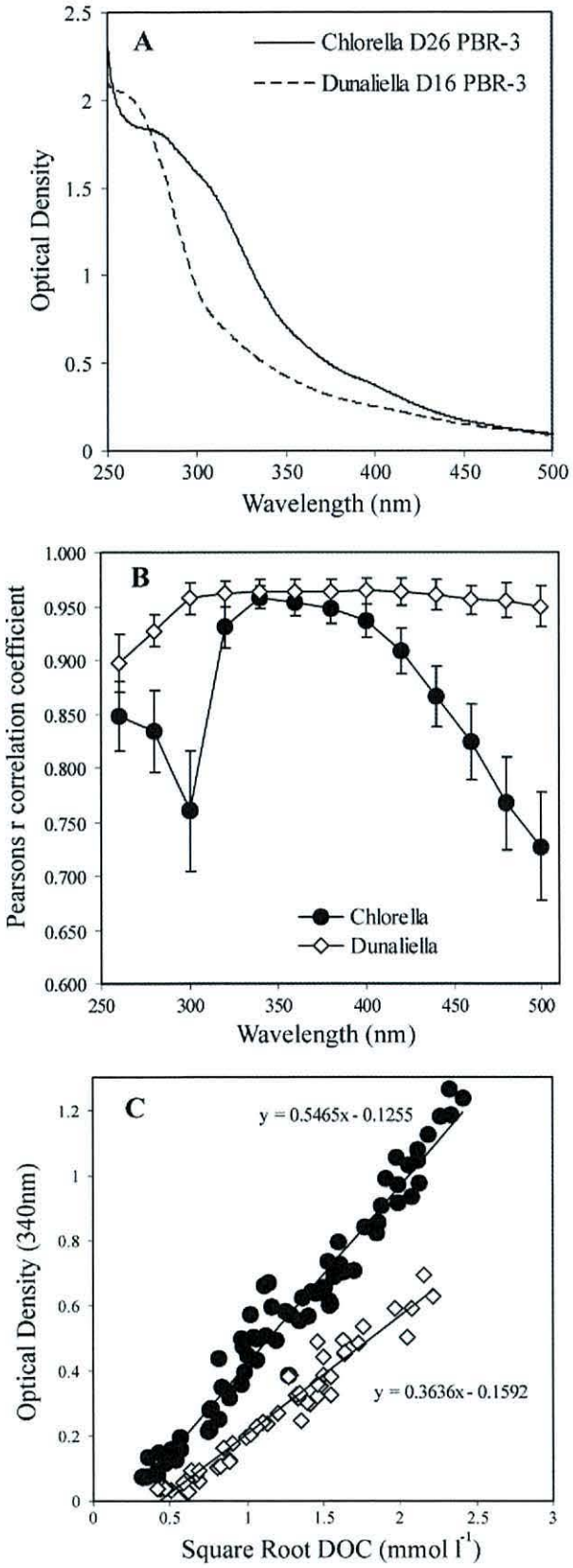


Fig. 6. (A) Representative absorption spectra (optical density) of algal culture filtrate from each species (*Chlorella vulgaris and Dunaliella tertiolecta*) measured over the range 250–500 nm; (B) Pearsons r correlation coefficient (mean ± standard deviation) for correlation between optical density and DOC (mmol l^{-1}) over the range 250–500 nm; (C) linear regression of optical density at 340 nm versus DOC concentration. DOC data was square-root transformed to improve the fit, and the model assumptions were upheld.

2007). Technologies such as microbial fuel cells, which are still in the development stages, may perhaps provide a solution to harnessing some of this energy (Pant et al., 2010). However to our knowledge there does not seem to be any readily available process that could be applied, suggesting that a significant fraction of the photosynthetically fixed biomass could be lost.

4. Conclusions

As in the natural environment, microalgae exude a significant proportion of their photosynthetic products into the water. This process has the potential to significantly reduce the theoretic production of biomass, reduced carbon and energy by microalgae. The supply of nutrients and inorganic carbon may impact the release of DOM, and requires further investigation. Bacterial biomass dynamics are a relatively un-explored component of mass microalgal cultures and could potentially be important consumers of photosynthetically fixed organic matter, even in the controlled environment of a PBR. It may be possible to use a simple, rapid and inexpensive optical technique to monitor dissolved organic matter in mass algal cultures however, in common with optical measurements of biomass, it is important to first verify the relationship in each application.

Acknowledgements

This work was supported by an Engineering and Physical Sciences Research Council (EPSRC) studentship, in collaboration with RWE power UK and Varicon Aqua Solutions Ltd., UK. We thank Martin Pilbin, Roger Bewley, Richard Hotchkiss and Joe McDonald for encouraging discussions in earlier stages of this work, and Louiza Norman and Naomi Thomas for their analytical support.

References

Aluwihare, L., Repta, D., 1999. A comparison of the chemical characteristics of oceanic DOM and extracellular DOM produced by marine algae. Mar. Ecol. Prog. Ser. 186, 105–117.

Biersmith, A., Benner, R., 1998. Carbohydrates in phytoplankton and freshly produced organic matter. Mar. Chem. 63, 131–144.

Bratbak, G., Dundas, I., 1984. Bacterial dry matter content and biomass estimations. Appl. Environ. Microbiol. 48, 755–757.

Carlson, C., 2002. Production and removal processes. In: Carlson, C., Hansell, D. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, London, pp. 91–139.

Chisti, J., 2009. Response to Reijnders: do biofuels from microalgae beat biofuels from terrestrial crops? Trends Biotechnol. 26, 351–352.

Chiu, S., Kao, C., Chen, C., Kuan, T., Ong, S., Lin, C., 2008. Reduction of CO_2 by a high-density culture of *Chlorella* sp. in a semicontinuous photobioreactor. Bioresour. Technol. 99, 3389–3396.

Dismukes, G., Carrieri, D., Bennette, N., Ananyev, G., Posewitz, M., 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr. Opin. Biotechnol. 19, 235–240.

Ditzig, J., Liu, H., Logan, B., 2007. Production of hydrogen from domestic wastewater using a bioelectrochemically assisted microbial reactor (BEAMR). Int. J. Hydrogen Energy 32, 2296–2304.

Falkowski, P., Raven, J., 2007. Aquatic Photosynthesis. Princeton University Press, New Jersey.

Fukuda, R., Ogawa, H., Nagata, T., Koike, I., 1998. Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. Appl. Environ. Microbiol. 64, 3352.

Hanson, H., Koroleff, F., 1983. Determination of nutrients. In: Grasshoff, K., Ehrhardt, M., Kremling, K. (Eds.), Methods of Seawater Analysis. Weinheim Verlag-Chemie, pp. 159–228.

Holmes, R., Aminot, R., Kerouel, B., Hocker, A., Peterson, B., 1999. A simple and precise method for measuring ammonium in marine and fresh water. Can. J. Fish Aquat. Sci. 56, 1801–1808.

Horiuchi, J., Ohba, I., Tada, K., Kobayashi, M., Kanno, T., Kishimoto, M., 2003. Effective cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control. J. Biosci. Bioeng. 95, 412–415.

Jacob-Lopez, E., Scoparo, C., Queiroz, F., 2010. Biotransformations of carbon dioxide in photobioreactors. Energy Convers. Manage. 51, 894–900.

Jorquera, O., Kiperstok, A., Sales, E. Embirucu, Ghirardi, M., 2010. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresour. Technol. 101, 1406–1413.

Keffer, J., Kleinheinz, G., 2002. Use of *Chlorella vulgaris* for CO_2 mitigation in a photobioreactor. J. Ind. Microbiol. Biotechnol. 29, 275–280.
Kroer, N., 1993. Bacterial growth efficiency on natural dissolved organic matter. Limnol. Oceanogr. 38, 1282–1290.
Kroon, H., 1993. Determination of nitrogen in water: comparison of continuous flow method with on-line UV digestion with the original Kjedahl method. Anal. Chim. Acta 276, 287–293.
Lønborg, C., Søndergaard, M., 2009. Microbial availability and degradation of dissolved organic carbon and nitrogen in two coastal areas. Estuar. Coast Shelf Sci. 81, 513–520.
Malinsky-Rushansky, N., Legrand, C., 1996. Excretion of dissolved organic carbon by phytoplankton of different sizes and subsequent bacterial uptake. Mar. Ecol. Prog. Ser. 132, 249–255.
Mandalam, R., Palsson, B., 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. Biotechnol. Bioeng. 59, 605–611.
Mata, T., Martins, A., Caetano, N., 2010. Microalgae for biodiesel production and other applications: a review. Renew. Sustain. Energy Rev. 14, 217–232.
Meon, B., Kirchman, D., 2001. Dynamics and molecular composition of dissolved organic material during experimental phytoplankton blooms. Mar. Chem. 75, 185–199.
Mishra, A., Jha, B., 2009. Isolation and characterization of extracellular polymeric substances from micro-algae *Dunaliella salina* under salt stress. Bioresour. Technol. 100, 3382–3386.
Molina Grima, E., Belarbi, E., Fernandez, F., Medina, A., Chisti, Y., 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol. Adv. 20, 491–515.
Nagata, T., 1986. Carbon and nitrogen content of natural planktonic bacteria. Appl. Environ. Microb. 52, 28–32.
Nayar, S., Chou, L.M., 2003. Relative efficiencies of different filters in retaining phytoplankton for pigment and productivity studies. Estuar. Coast Shelf Sci. 58, 241–248.
Obernosterer, I., Herndl, G., 1995. Phytoplankton extracellular release and bacterial growth: dependence on the inorganic N:P ratio. Mar. Ecol. Prog. Ser. 116, 247–257.
Oliveira, E., Rosa, G., Moraes, M., Pinto, L., 2009. Characterisation of thin layer drying of *Spirulina platensis* utilizing perpendicular air-flow. Bioresour. Technol. 100, 1297–1303.
Pant, D., Bogaert, G., Diels, L., Vanbroekhoven, K., 2010. A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. Bioresour. Technol. 101, 1533–1543.
Puddu, A., Zoppini, A., Fazi, S., Rosati, M., Amalfitano, S., Magaletti, E., 2003. Bacterial uptake of DOM released from P-limited phytoplankton. FEMS Microbiol. Ecol. 46, 257–268.
Qian, J., Mopper, K., 1996. An automated, high performance, high temperature combustion dissolved organic carbon analyzer. Anal. Chem. 68, 3090–3097.
Riemann, B., Fuhrman, J., Azam, F., 1982. Bacterial secondary production in freshwater measured by ^{3}H-thymidine incorporation method. Microb. Ecol. 8, 101–114.
Scragg, A., Illman, A., Carden, A., Shales, S., 2002. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass Bioenergy 23, 67–73.
Servais, P., Garnier, J., Demarteau, N., Brion, N., Billen, G., 1999. Supply of organic matter and bacteria to aquatic ecosystems through waste water effluents. Water Res. 33, 3521–3531.
Smith, D., Steward, G., Long, R., Azam, F., 1995. Bacterial mediation of carbon fluxes during a diatom bloom in a mesocosm. Deep-Sea Res. Part II Top. Stud. Oceanogr. 42, 75–97.
Stedmon, C.A., Markager, S., Kaas, H., 2000. Optical properties and signatures of chromophoric dissolved organic matter (CDOM) in Danish coastal waters. Estuar. Coast Shelf Sci. 51, 267–278.
Sturluson, M., Nielsen, T., Wasmann, P., 2008. Bacterial abundance, biomass and production during spring blooms in the northern Barents Sea. Deep-Sea Res. Part II Top. Stud. Oceanogr. 55, 2186–2198.
Ugwu, C., Aoyagi, H., Uchiyama, H., 2008. Photobioreactors for mass cultivation of algae. Bioresour. Technol. 99, 4021–4028.
Vrede, K., Heldal, M., Norland, S., Bratbak, G., 2002. Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton. Appl. Environ. Microbiol. 68, 2965–2971.
Vunjak-Novakovic, G., Kim, Y., Wu, X., Berzin, I., Merchuk, J., 2005. Air-lift bioreactors for algal growth on flue gas: mathematical modeling and pilot-plant studies. Ind. Eng. Chem. Res. 44, 6154–6163.
Weyer, K., Bush, D., Darzins, A., Willson, B., 2010. Theoretical maximum algal oil production. Bio. Energy Res. doi:10.1007/s12155-009-9046-x.
Williams, P., Laurens, L., 2010. Microalgae as biodiesel and biomass feedstocks: review and analysis of the biochemistry, energetics and economics. Energy Environ. Sci. doi:10.1039/b924978h.
Xin, L., Hong-Ying, H., Ke, G., Ying-Xue, S., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresour. Technol. 101, 5494–5500.
Zeiler, K., Heacox, D., Toon, S., Kadam, K., Brown, L., 1995. The use of microalgae for assimilation and utilization of carbon dioxide from fossil fuel-fired power plant flue gas. Energy Convers. Manage. 36, 707–712.
Zhu, X., Long, S., Ort, D., 2008. What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? Curr. Opin. Biotechnol. 19, 153–159.

Chapter 4.

Productivity, Carbon Dioxide Uptake and Net Energy Balance of Bubble Column Photobioreactors.

4. Overview

The photobioreactors presented in this chapter were specifically designed by the author to test the effect of power input on productivity and net energy balance. The hypothesis tested was that *high power inputs increase productivity, but to the detriment of the net energy return (energy invested for sparging vs. biomass energy output).* Chapter 4.1 introduces the photobioreactor design and equipment used, whilst Chapter 4.2 comprises a published article. Supplementary information including mass transfer data, calculations, nutrient and DOC measurements for the published article are included thereafter.

4.1. Bubble column photobioreactor design

Bubble column photobioreactors were designed specifically to operate using a range of appropriate superficial gas velocities/ power inputs. The superficial gas velocity is the gas flow rate ($m^3\ s^{-1}$) normalised to the cross sectional area of the column (m^2), and has units of ms^{-1} (see methods, Chapter 4.2). As a result, the photobioreactor was designed with a small diameter in order that sufficient gas could be supplied from a cylinder. However a significant volume of culture was also required in order to obtain sufficient sample. As a result, bubble columns were of a relatively tall design. The optimal configuration was derived as 32 mm internal diameter, with a height of 2000 mm. Reactors were constructed from 3 mm wall thickness acrylic. The bases were machined from nylon and formed a conical shape in order that any cell suspension settling on the base would be re-directed to the sparging jet, retaining the cells in suspension. This feature was particularly important for the lowest power inputs (10 Wm^{-3}).

The supply of gas was controlled by rotameter (Fig. 4.1 A), and was provided by calibration-standard, pre-mixed from a cylinder via a dual-stage pressure regulator (Fig 4.1 B). Photobioreactors filled with cultivation medium before inoculation are shown in Fig. 4.1 C, and after 2 days growth (Fig. 4.1 D). The exhaust gas was vented outdoors using nylon tubing. Fig 4.1 F shows the cell density after 2 days of cultivation (*Chlorella vulgaris* and *Dunaliella tertiolecta* alternate, left to right) using simulated coal-fired power plant flue gas (12% CO_2). The experiment comprised a full factorial design using three replicate cultivations per treatment. Treatment replicates were rotated between photobioreactors.

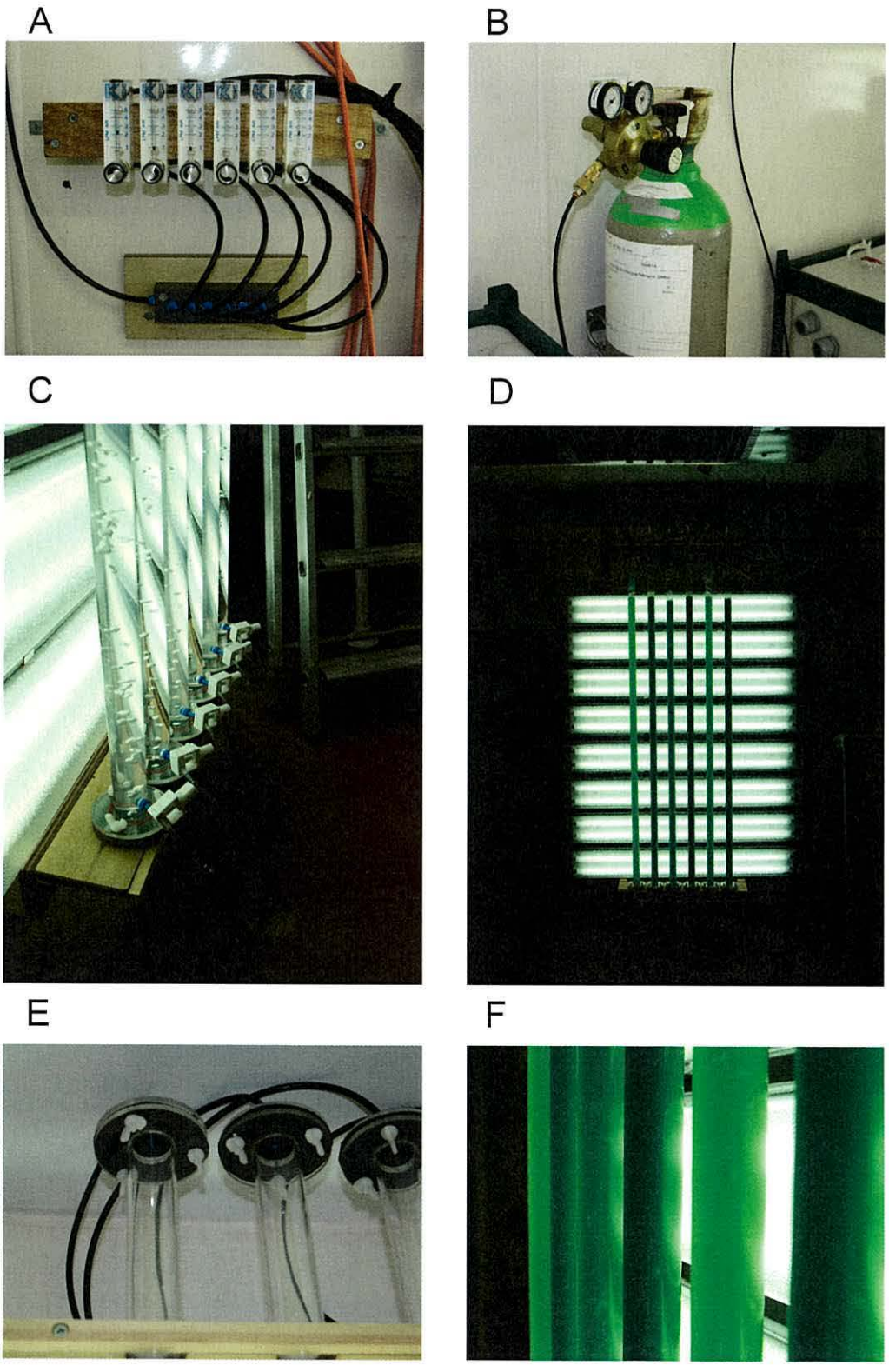


Figure 4.1. Images of bubble column photobioreactors used to test the effect of power input on net energy output. Each image is described in the text.

Author's personal copy

Bioresource Technology 102 (2011) 5775–5787

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Productivity, carbon dioxide uptake and net energy return of microalgal bubble column photobioreactors

Chris J. Hulatt [a,*], David N. Thomas [a,b]

[a] School of Ocean Sciences, College of Natural Sciences, Bangor University, Askew Street, Menai Bridge, Isle of Anglesey LL59 5AB, UK
[b] Finnish Environment Institute (SYKE), Marine Centre, P.O. Box 140, FI-00251 Helsinki, Finland

ARTICLE INFO

Article history:
Received 3 December 2010
Received in revised form 1 February 2011
Accepted 4 February 2011
Available online 4 March 2011

Keywords:
Microalgae
Photobioreactor
Energy balance
Life cycle
Carbon dioxide

ABSTRACT

This work examined the energy return of *Chlorella vulgaris* and *Dunaliella tertiolecta* cultivated in a gas-sparged photobioreactor design where the power input for sparging was manipulated (10, 20, and 50 W m^{-3}). Dry weight, organic carbon and heating values of the biomass were measured, plus a suite of variables including Fv/Fm and dissolved oxygen. A model for predicting the higher heating value of microalgal biomass was developed and used to measure the energetic performance of batch cultivations. High power inputs enhanced maximum biomass yields, but did not improve the energy return. Cultivation in 10 W m^{-3} showed up to a 39% higher cumulative net energy return than 50 W m^{-3}, and increased the cumulative net energy ratio up to fourfold. The highest net energy ratio for power input was 19.3 (*D. tertiolecta*, 12% CO_2, 10 W m^{-3}). These systems may be a sustainable method of biomass production, but their effectiveness is sensitive to operational parameters.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Microalgae have considerable potential as a biomass feedstock for renewable energy technologies because they offer, in general, high yields per unit area of land (Dismukes et al., 2008). Many species produce high cellular lipid concentrations suitable for conversion to biodiesel (Chisti, 2007), and their ability to utilise concentrated CO_2 from industrial waste gases makes them attractive for mitigation purposes (Jacob-Lopes et al., 2010). Part of the appeal is that microalgal biomass can be produced in engineered structures such as raceways and photobioreactors, which can be optimised for maximum productivity (Ugwu et al., 2008). However, unlike terrestrial crops, it is these same systems that require an extra energy investment which, when analysed over the life cycle of the product, can have a significant impact on the process efficiency (Stephenson et al., 2010; Jorquera et al., 2010).

The efficiency of microalgal technologies has been determined using a number of parameters including; biomass yield, CO_2 uptake (Douskova et al., 2009; Jacob-Lopes et al., 2010) and lipid production (Chiu et al., 2008). Recent assessments have focused on the demands or burdens of achieving these yields including; energy input, greenhouse gas emission, land use and water consumption (Brune et al., 2009; Clarens et al., 2010; Jorquera et al., 2010; Lardon et al., 2009; Stephenson et al., 2010). It is widely known that producing commercial biofuels consumes significant amounts of energy and that this must be accounted for to assess their overall performance, including how well they compete with alternatives (Chisti, 2008). Several authors have examined the performance of different microalgal production systems using life-cycle assessment (LCA): Jorquera et al. (2010) identified that the tubular PBR systems in their study offered a biomass net energy ratio of 0.2, a flat-plate design offered an energy return of 4.51 and a raceway model produced the best energy return overall at 8.34. A positive energy balance (>1) for the entire process is required in order for the implementation of microalgal technologies to be justified (Lardon et al., 2009), and there is a need to develop photobioreactors which both efficiently use light and minimise energy inputs (Reyna-Velarde et al., 2010). Although life-cycle assessments indicate current photobioreactor technologies have lower net energy ratios than open ponds, well-designed photobioreactors clearly have potential to generate positive energy returns (Jorquera et al., 2010; Lehr and Posten, 2009), have a number of process control advantages (Ugwu et al., 2008) and have potential to be further improved (Kumar et al., 2010).

Though comparing algal cultivation technologies is useful, the wide variety of photobioreactors, scales and operational parameters tested in the literature (Ugwu et al., 2008), indicates that energy balances vary considerably between applications. Furthermore, there has been relatively little optimisation of the most promising PBR designs (Kumar et al., 2010), suggesting their potential has not been reached. As a result most LCAs conclude that the assumptions of process efficiencies and technology alternatives

* Corresponding author. Tel.: +44 01248 38 2852.
E-mail address: osp418@bangor.ac.uk (C.J. Hulatt).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.02.025

Author's personal copy

in their models can make analyses somewhat sensitive (Stephenson et al., 2010; Lardon et al., 2009). It is therefore important to design experiments aimed at improving the performance of closed photobioreactors, so that fair comparisons can be made, for example, between photobioreactors and raceways. It is necessary to optimise the relationship between energy inputs and outputs.

One of the most significant issues associated with producing algal biomass using photobioreactors is supplying the power required to run algal production systems, in order to maintain culture conditions within certain parameters, e.g., mixing cells, removing oxygen and supplying CO_2 (Jorquera et al., 2010; Reyna-Velarde et al., 2010; Stephenson et al., 2010). Life-cycle assessments have identified the process of algal cultivation as the most important energy-consuming step, and that the power requirement is often the dominant factor during cultivation (Jorquera et al., 2010; Stephenson et al., 2010).

This work investigated the effect of power input on the biomass produced by two species of microalgae, *Chlorella vulgaris* and *Dunaliella tertiolecta* (both commonly used for large scale algal production) cultivated using air and flue gas concentrations of CO_2 in a bubble column photobioreactor design. This design was selected as an experimental system because gas-sparged designs have the most promising energetic performance of closed photobioreactors, based on recent life-cycle assessment (Jorquera et al., 2010). A method for predicting the higher heating value of microalgal biomass was also developed, and was then used to examine the energy balance of biomass production in this type of cultivation system.

2. Methods

2.1. Photobioreactor design and operation

The two microalgal species used were the freshwater *C. vulgaris* (Culture Collection of Algae and Protozoa 211/11B) and marine *D. tertiolecta* (Sammlung von Algenkulturen Gottingen, 13.86). For comparability, the nutrient concentrations were standardised between the two species, with media adjusted proportionally to 10 mmol L^{-1} NO_3^-. This as far as possible avoided any differences in biomass carrying capacity between the two species treatments. The nutrient media used for *D. tertiolecta* was according to Carballo-Cardenas et al. (2003), but with the bicarbonate removed to prevent a potential confound in inorganic carbon availability (mg L^{-1}): KNO_3 (1010), $NaH_2PO_4{\cdot}H_2O$ (138), $Na_2EDTA{\cdot}2H_2O$ (14.4), $FeCl_3{\cdot}6H_2O$ (3.95), $CuSO_4{\cdot}7H_2O$ (0.04), $ZnSO_4{\cdot}7H_2O$ (0.05), $CoCl_2{\cdot}6H_2O$ (0.01), $MnCl_2{\cdot}4H_2O$ (0.24), Na_2MoO_4 (0.01). Media for *D. tertiolecta* was adjusted to a salinity of 30 using artificial seawater formulation (Culture Collection of Algae and Protozoa, Oban, UK). *C. vulgaris* was cultured using M8 formulation (Mandalam and Palsson, 1998): KNO_3 (1010), KH_2PO_4 (249), $Na_2HPO_4{\cdot}2H_2O$ (88), $CaCl_2{\cdot}2H_2O$ (4.4), Fe EDTA (3.4), $FeSO_4{\cdot}7H_2O$ (44), $MgSO_4{\cdot}7H_2O$ (135), $Al_2(SO4)_3{\cdot}18H_2O$ (1.2), $MnCl_2{\cdot}4H_2O$ (4.4), $CuSO_4{\cdot}5H_2O$ (0.6), $ZnSO_4{\cdot}7H_2O$ (1.1).

Photobioreactors consisted of bubble columns 2000 mm high and Ø32 mm (Fig. 1). The incident irradiance was 350 ± 10 μmol photons PAR m^2 s^{-1}, supplied with continuous light from cool white tubes placed to one side of the reactors. The temperature of the bioreactors was controlled (26 ± 0.5 °C) by air conditioning. The bioreactors contained 1.4 L fluid and gas was supplied pre-mixed (CO_2 + 20% oxygen + nitrogen balance, BOC special products, UK). The CO_2 concentrations used during the study were chosen to replicate air (0.04%) and two types of simulated flue gas (SFG); combined cycle gas turbine plant flue gas (4%) and coal fired plant flue gas (12%). Gas flow rates were controlled equivalent to superficial gas velocities (U_{sg}) of 0.001, 0.002 and 0.005 ms^{-1}, and each treatment was carried out in triplicate. The term U_{sg} is used rather than the actual flow rate because it is (i) scale-independent and (ii) required for calculation of power input.

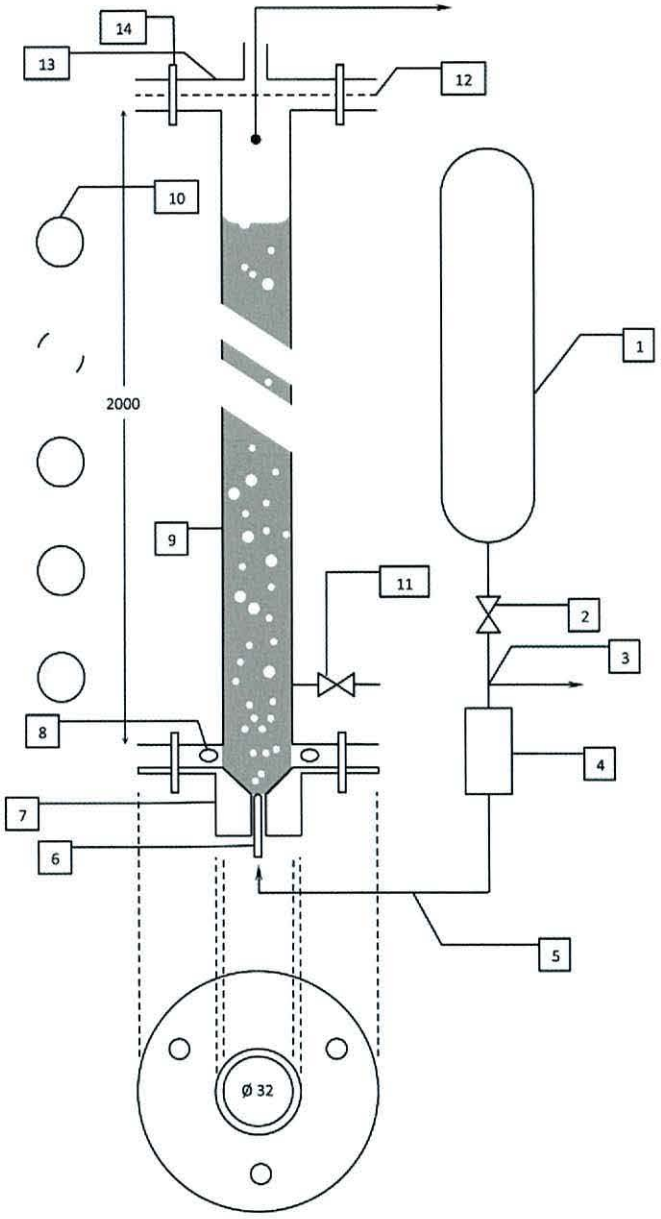


Fig. 1. Diagram showing reactor assembly and components used during cultivation. The components were: (1) gas cylinder, (2) regulator assembly, (3) manifold to six replicate reactors, (4) rotameter, (5) airline, (6) gas jet, (7) base body, (8) silicon gasket, (9) reactor body (3 mm acrylic tube, 32 mm ID), (10) cool white fluorescent lamps (×16, 36 W, Osram), (11) sampling port, (12) gasket, (13) lid assembly, and (14) nylon bolt.

2.2. Analysis of biomass properties

Biomass dry weight (g L^{-1}) was measured daily by vacuum filtration of cultivation samples through Whatman GF/F filters (0.7 μm nominal pore size) before drying in an oven at 80 °C for 24 h. Samples for measurement of particulate organic carbon, hydrogen and nitrogen in the biomass were collected every 48 h by centrifugation. Biomass was centrifuged at 1000 rcf for 10 min before the supernatant was removed and the sample dried at 80 °C for 48 h. Elemental analysis was conducted using a Thermo-Electron elemental analyser configured for CHNS

Author's personal copy

determination using a copper/copper oxide column. The standards sulphanilamide, 2,5-bis(5′-tert-butyl-2-benzoxazolyl)thiophene and L-cystine were used for calibration. Measurements of carbon, hydrogen and nitrogen in the reference material (DL-methionine) varied on average ±0.48%, 0.30% and 0.20% from the certified values. The concentration of particulate organic carbon (POC, mmol L^{-1}) was calculated by multiplying the concentration (mmol g^{-1}) by the dry mass (g L^{-1}).

Following 10 days of cultivation, the biomass contained in the photobioreactors was collected by centrifugation (1000 rcf, 10 min), before drying the pellet at 80 °C for 72 h. The higher heating value (HHV) was measured using a Parr 1341 oxygen bomb calorimeter calibrated with benzoic acid (accuracy ±0.5%). Due to the mass (approximately 1 g) of sample required, only endpoint samples could be measured. A regression model was used to derive HHV values for other data points using the elemental composition (see Section 3.5).

2.3. Calculation of growth rates and efficiency

Maximum productivity was calculated by using the equation $y = (b_2 - b_1)/t$. Where y is the dry weight productivity (g L^{-1} d^{-1}), b_1 and b_2 are the biomass dry weight concentrations at time points 1 and 2 (g L^{-1}), and t is the time (days) between b_1 and b_2. For SFG treatments, y was calculated between days 2–4 for *D. tertiolecta*, and between days 4–6 for *C. vulgaris*. For 0.04% treatments y was calculated over a longer period to improve accuracy, from days 2 to 6.

The maximum biomass concentration was determined either at peak density or where any subsequent value was <5% higher. The averaged growth rate describes the mean (g L^{-1} d^{-1}) achieved over a complete batch cycle, to maximum density. The maximum CO_2 uptake rate was calculated as for the maximum productivity using the carbon concentration (g L^{-1}) in the culture. The efficiency of CO_2 uptake was calculated as $E = (f/m) \times 100$. Where E is the CO_2 removal efficiency of the whole device (%), f is the maximum fixation rate (gCO_2 d^{-1} for the whole device) and m is mass of CO_2 flowed through the device per day (gCO_2 d^{-1}), calculated from the CO_2 concentration in the aeration gas and flow rate.

The photosynthetic efficiency was calculated as: $(E_{in}/E_{out}) \times 100$. Where E_{in} is the amount of PAR light energy received by 1 m^2 of photobioreactor frontal area per day (kJ m^{-2} d^{-1}). The PAR light energy was calculated by dividing the photon irradiance measurements (Section 2.1) by a value of 4.6 (Watanabe and Hall, 1996) to obtain units of W m^{-2}, then expressing this in kJ m^{-2} d^{-1}. The parameter E_{out} (kJ m^{-2} d^{-1}) was calculated by dividing the growth in bio-energy (kJ L^{-1} d^{-1}) during the maximum growth period (calculation detailed in Section 3.6) by the frontal area of 1 L of reactor volume (frontal area was 0.04 m^2 L^{-1}).

2.4. Accessory measurements

The maximum quantum yield of photosystem II (Fv/Fm) was measured every 48 h using the saturation pulse method with a pulse amplitude modulation fluorometer (Walz Water–PAM, Walz GmBH). Dissolved oxygen was measured every 48 h using a portable oxygen electrode (WTW CellOx 325), as was the pH (Mettler Toledo MP120). The following dissolved nutrients were analysed according to Grasshoff et al. (1983): NO_3^-, NO_2^-, NH_4^+ (collectively dissolved inorganic nitrogen, DIN) and dissolved inorganic phosphorus (DIP). Dissolved organic carbon (DOC) in the filtrate (<0.7 μm nominal pore size) was analysed by high temperature combustion using a MQ1001 TOC analyser. The machine was calibrated using potassium phthalate using a seven point calibration over the range 20–750 μmol L^{-1}. Stable reference material (Hansell Laboratories, Miami) was used to check column performance and a 250 μmol L^{-1} potassium phthalate standard was incorporated within the samples to check performance (the average deviation was 22 μmol L^{-1}). The total organic carbon (TOC) in each reactor could thus be defined by TOC = POC (mmol L^{-1}) + DOC (mmol L^{-1}). Chlorophyll a was measured every 48 h using 20 h extraction in 90% acetone and a Turner Designs 10AU fluorometer.

2.5. Energy calculations

The calculation of power input was derived according to Sierra et al. (2008). In the calculations, a freshwater density of 1000 kg m^{-3} and a seawater density of 1020 kg m^{-3} was used. The power requirement was calculated according to Eq. (1).

$$P_G/V_L = \rho_L g U_{sg} \quad (1)$$

where P_G/V_L is the power supplied per unit volume (W m^{-3}), ρ_L is the liquid density (kg m^{-3}), g is the rate of gravitational acceleration (9.8 ms^{-2}) and U_{sg} is the superficial gas velocity (ms^{-1}), Eq. (2).

$$U_{sg} = V_G/A \quad (2)$$

where V_G is the gas flow rate (m^3 s^{-1}) and A is the cross sectional area of the column (m^2).

2.6. Calculation of mass transfer coefficient

The gas–liquid mass transfer coefficient for oxygen $k_L a_L(O_2)$ (s^{-1}) was measured using the dynamic gassing-in (oxygen absorption) method (Chisti, 1989; Contreras et al., 1998). A dissolved oxygen electrode (WTW CellOx 325) was fitted at 90° into the side of the reactor half way up the column. The electrode tip protruded 10 mm horizontally into the liquid. The liquid was purged of oxygen using pure nitrogen, then sparged with air at the same superficial gas velocities as the experimental treatments. The rise in O_2 concentration over time was measured until the dissolved oxygen concentrations were almost at equilibrium with the gas phase. After prolonged sparging with air, the stable equilibrium value was recorded. The coefficient $k_L a_L(O_2)$ was calculated as the slope of linear regression of Eq. (3).

$$\mathrm{Ln}((C^* - C_0)/(C^* - C)) = k_L a_L (t - t_0) \quad (3)$$

where C^* is the dissolved oxygen concentration at equilibrium with air, C_0 is the dissolved oxygen concentration at the onset of air-sparging (time = 0) and C is the dissolved oxygen concentration at a sample time point ($t - t_0$) during sparging (Reyna-Velarde et al., 2010). Measurements were made in triplicate for each U_{sg}, for both fresh and saline nutrient media used in experiments. As a final check the data was compared with established relationships (see Supplementary information and references therein). Measured values agreed very well with calculated values (Fig. S1), and are presented in Table 1. The $k_L a_L(CO_2)$ was estimated by taking the square root of the diffusivity ratio of both gases, multiplying $k_L a_L(O_2)$ by 0.91 (Contreras et al., 1998).

2.7. Statistical analysis

Experiments comprised a complete factorial design with each treatment in triplicate. For statistical analysis of the data SPSS v.16 was used. Use of three-factor analysis of variances was verified by checking data for approximate homogeneity of variances and normally-distributed residuals. The multiple linear regression model was examined for residual normality, the distribution of fitted values and tests for collinear effects.

Table 1
Summary statistics for growth of *Dunaliella tertiolecta* and *Chlorella vulgaris* in bubble column photobioreactors. The biomass higher heating values (HHV) for the end of the experimental runs are given. Measured data is presented as mean (±standard deviation).

Treatments						Biomass yield				Carbon uptake		Bio-energy
Species	CO_2 (%)	U_{sg} (ms^{-1})	Power ($W m^{-3}$)	Energy demand ($kJ L^{-1} d^{-1}$)	Mass transfer coefficient $k_La_L(O_2)/k_La_L(CO_2)$ (s^{-1})	Maximum growth rate ($g L^{-1} d^{-1}$)	Maximum biomass ($g L^{-1}$)	Day of maximum	Averaged growth rate ($g CO_2 L^{-1} d^{-1}$)	Maximum net CO_2 uptake rate ($g CO_2 d^{-1}$)	Device CO_2 efficiency at maximum rated (%)	HHV (endpoint) ($kJ g^{-1}$)
Dunaliella	12	0.001	10.0	0.9	0.0012 (0.0003)/0.0011 (0.0003)	0.71 (0.26)	2.85 (0.32)	7	0.41 (0.05)	1.29 (0.41)	8.8 (2.8)	20.5 (1.2)
Dunaliella	12	0.002	20.0	1.7	0.0031 (0.0002)/0.0028 (0.0002)	0.79 (0.12)	3.17 (0.33)	7	0.45 (0.05)	1.40 (0.19)	4.7 (0.6)	19.8 (0.6)
Dunaliella	12	0.005	50.0	4.3	0.0061 (0.0013)/0.0055 (0.0012)	0.83 (0.13)	3.19 (0.39)	7	0.46 (0.06)	1.51 (0.19)	2.1 (0.3)	20.0 (0.2)
Dunaliella	4	0.001	10.0	0.9	0.0012 (0.0003)/0.0011 (0.0003)	0.51 (0.05)	3.03 (0.56)	7	0.43 (0.08)	0.90 (0.14)	18.4 (2.9)	19.5 (0.8)
Dunaliella	4	0.002	20.0	1.7	0.0031 (0.0002)/0.0028 (0.0002)	0.66 (0.16)	3.33 (0.76)	7	0.48 (0.11)	1.18 (0.30)	12.0 (3.1)	19.7 (0.2)
Dunaliella	4	0.005	50.0	4.3	0.0061 (0.0013)/0.0055 (0.0012)	0.73 (0.16)	3.60 (0.74)	7	0.51 (0.11)	1.31 (0.30)	5.3 (1.2)	18.8 (2.0)
Dunaliella	0.04	0.001	10.0	0.9	0.0012 (0.0003)/0.0011 (0.0003)	0.02 (0.03)	0.16 (0.26)	6	0.02 (0.03)	0.02 (0.05)	49.3 (111.7)	na
Dunaliella	0.04	0.002	20.0	1.7	0.0031 (0.0002)/0.0028 (0.0002)	0.03 (0.02)	0.33 (0.21)	6	0.03 (0.02)	0.05 (0.04)	49.8 (38.4)	na
Dunaliella	0.04	0.005	50.0	4.3	0.0061 (0.0013)/0.0055 (0.0012)	0.07 (0.02)	0.72 (0.21)	6	0.07 (0.02)	0.12 (0.04)	47.9 (16.5)	18.4 (0.7)
Chlorella	12	0.001	9.8	0.8	0.0018 (0.0004)/0.0016 (0.0004)	0.49 (0.23)	2.78 (1.08)	9	0.31 (0.12)	0.86 (0.37)	5.8 (2.5)	23.7 (1.1)
Chlorella	12	0.002	19.6	1.7	0.0037 (0.0003)/0.0034 (0.0003)	0.53 (0.16)	3.04 (0.74)	9	0.34 (0.08)	0.95 (0.26)	3.2 (0.9)	23.4 (0.5)
Chlorella	12	0.005	49.0	4.2	0.0077 (0.0010)/0.0070 (0.0009)	0.60 (0.11)	3.79 (0.50)	10	0.38 (0.05)	1.12 (0.15)	1.5 (0.2)	23.4 (1.1)
Chlorella	4	0.001	9.8	0.8	0.0018 (0.0004)/0.0016 (0.0004)	0.41 (0.09)	2.70 (0.19)	9	0.30 (0.02)	0.72 (0.08)	14.6 (1.7)	22.3 (1.0)
Chlorella	4	0.002	19.6	1.7	0.0037 (0.0003)/0.0034 (0.0003)	0.42 (0.12)	3.18 (0.38)	9	0.35 (0.04)	0.83 (0.21)	8.5 (2.1)	23.5 (0.7)
Chlorella	4	0.005	49.0	4.2	0.0077 (0.0010)/0.0070 (0.0009)	0.47 (0.06)	3.62 (0.25)	9	0.40 (0.03)	0.93 (0.11)	3.8 (0.5)	24.3 (1.2)
Chlorella	0.04	0.001	9.8	0.8	0.0018 (0.0004)/0.0016 (0.0004)	0.03 (0.01)	0.19 (0.03)	8	0.02 (0.01)	0.03 (0.01)	60.4 (17.9)	na
Chlorella	0.04	0.002	19.6	1.7	0.0037 (0.0003)/0.0034 (0.0003)	0.05 (0.01)	0.24 (0.05)	10	0.02 (0.01)	0.03 (0.01)	35.2 (6.2)	na
Chlorella	0.04	0.005	49.0	4.2	0.0077 (0.0010)/0.0070 (0.0009)	0.10 (0.04)	0.58 (0.22)	10	0.06 (0.02)	0.09 (0.04)	35.5 (15.2)	19.1 (0.4)

Author's personal copy

Author's personal copy

3. Results and discussion

3.1. Biomass yield and CO_2 uptake

Fig. 2a illustrates the growth in dry mass over the batch cycle. The maximum dry weights obtained by SFG treatments ranged from 2.7 to 3.6 g L^{-1}, each obtained using *C. vulgaris* cultivated using 4% CO_2 (Table 1). These values are comparable to the published literature (Jacob-Lopes et al., 2010), and are in the range expected considering the initial nutrient concentrations. Differences in the maximum productivity and maximum dry weight between SFG treatments were investigated using a three-factor analysis of variances summarised in Table 2. There were no significant interaction effects in each test ($p \geqslant 0.466$), so main effects were examined independently. Both species and CO_2 concentrations significantly influenced the maximum productivity ($p \leqslant 0.02$). Mean growth rates were increased at higher U_{sg}, but the effect was not significant ($p = 0.118$). The 12% CO_2 treatment produced significantly higher maximum productivity than 4% CO_2, with a mean difference of 0.12 g L^{-1} d^{-1} (±95% confidence limit 0.10). *D. tertiolecta* treatments had significantly higher maximum growth rates than *C. vulgaris* (mean difference 0.22 g L^{-1} d^{-1}).

The maximum dry weight was significantly greater at higher U_{sg} ($p = 0.022$). Tukeys' pairwise comparisons amongst U_{sg} treatments showed that the 0.005 ms^{-1} treatment produced significantly higher maximum dry weight than the 0.001 ms^{-1} treatment (mean difference 0.71 g L^{-1} ± 95% confidence limit 0.59). However, there was no significant improvement in maximum dry weight when operated using 0.005 ms^{-1} over 0.002 ms^{-1}. The effect of U_{sg} on microalgal growth has been well-documented, and the results can be attributed to the improved mixing (both for light regime and nutrients), plus improved mass transfer of gases at increased U_{sg} (Merchuk et al., 2000).

Compared to the SFG treatments, 0.04% CO_2 resulted in low productivity. The 0.005 ms^{-1} treatments grew acceptably, and productivity was comparable with other studies using air concentrations of CO_2 (Chiu et al., 2008). However, the 0.001 and 0.002 ms^{-1} treatments for *D. tertiolecta* and *C. vulgaris* were severely carbon-limited with maximum productivity <0.05 g L^{-1} d^{-1} (Table 1).

In the 4% and 12% treatments for both *C. vulgaris* and *D. tertiolecta*, the amount of CO_2 (g L^{-1} d^{-1}) that was incorporated into the cellular material varied similarly to the dry weight measurements (Table 1). The maximum rate at which carbon was incorporated into biomass was achieved by *D. tertiolcta* cultivated in 12% CO_2 at 0.005 ms^{-1} CO_2, measuring 1.51 (SD ± 0.19) g CO_2 L^{-1} d^{-1}. The corresponding CO_2 removal efficiency (the proportion of the CO_2 gas flowing through the photobioreactor which was incorporated into microalgal biomass) for the whole photobioreactor device was 2.1 (SD ± 0.3)%. For *C. vulgaris*, maximum CO_2 uptake also occurred in 12% CO_2 at 0.005 ms^{-1} and measured 1.12 (SD ± 0.15) g CO_2 L^{-1} d^{-1}. Douskova et al. (2009) cultivated *C. vulgaris* in a similar photobioreactor design to that used here and obtained a maximum CO_2 uptake rate in their control treatments of 3.0 g CO_2 L^{-1} d^{-1}. The higher CO_2 fixation rate in their systems can be explained by the 3.5-fold higher incident irradiance used in their experiments. Douskova et al. (2009) also identified that the CO_2:O_2 ratio in the sparging gas significantly impacted growth, and this explains why growth rates in 12% CO_2 were significantly higher than those in 4% CO_2 (Tables 1 and 2). The CO_2 removal efficiencies in SFG treatments were improved by utilising low U_{sg} but produced lower growth rates (Table 1), concordant with Merchuk et al. (2000). Calculation shows that the CO_2 removal efficiency in similar photobioreactor design using real flue gas was 5.8% (Douskova et al., 2009), so the removal efficiencies obtained in this work are comparable. As expected, the highest removal efficiencies were observed in carbon-limited air treatments, consistently 50% in the case of *D. tertiolecta*, but never exceeded 60% for *C. vulgaris* (Table 1). The highest removal efficiency for the SFG treatments (18.4 ± SD 2.9%) was obtained by *D. tertiolecta* grown in 4% CO_2 at 0.001 ms^{-1}, due to a comparatively low CO_2 mass flow. In the 0.001 ms^{-1} treatments, some settlement of the cell suspension at the base of the reactor was observed for both species, but was less significant in *D. tertiolecta* treatments. This did not cause any appreciable issues with the health of the cultures, but suggests that 0.001 ms^{-1} is around the lower limit that was feasible for this reactor design.

3.2. Dissolved oxygen and pH

The concentration of dissolved oxygen was related to the balance between the rate of photosynthetic O_2 production and removal by sparging. As expected, maximum dissolved oxygen concentrations were greater in low U_{sg} treatments, reaching a mean maximum of approximately 210% air saturation in the *D. tertiolecta* 0.001 ms^{-1} SFG treatments (Fig. 2b). This effect is explained by the greater mass transfer capability of the photobioreactors operated at higher U_{sg}. Mass transfer measurements in Table 1 show that mean $k_La_L(O_2)$ was 4.3 and 5.2-fold higher at 0.005 ms^{-1} compared to 0.001 ms^{-1} for *C. vulgaris* and *D. tertiolecta* cultivation media, respectively. Mean maximum dissolved oxygen values for *C. vulgaris* SFG treatments were lower than the corresponding *D. tertiolecta* treatments (Fig. 2b), and this discrepancy can be related to the higher maximum productivity of *D. tertiolecta*.

The pH dynamics are shown in Fig. 3a. The initial (t_0) pH values within species treatments are a result of gas–liquid equilibrium conditions for the different CO_2 concentrations tested (photobioreactors were sparged for several hours before inoculation). At equilibrium, high gas CO_2 concentrations increase the partial pressure of dissolved CO_2 and the concentration of carbonic acid, reducing pH (see Falkowski and Raven (2007) for carbonate dynamics). The SFG treatments exhibited an initial rise in pH from t_0 to days 4 and 6 for *D. tertiolecta* and *C. vulgaris* treatments, respectively. Stabilisation of the pH corresponded exactly to the point of nitrate/DIN exhaustion for each SFG treatment (Supplementary information Fig. S2). Subsequently the pH of these treatments remained at around pH 7, even during low productivity (and thus low inorganic carbon uptake) at the end of the cultivations. This indicates that the partial pressure of CO_2 in the liquid was close to equilibrium with the gas phase during this period; i.e., $k_La_L(CO_2)$ values (Table 1) were sufficient to avoid significant depletion of CO_2 in the medium. Uptake of nitrate increases alkalinity and pH via OH^- production (Goldman and Brewer, 1980). Thus the initial pH rise in SFG treatments can predominantly be explained by uptake of nitrate, followed by relatively stable gas–liquid CO_2 dynamics thereafter. However, cultivation of *D. tertiolecta* using 4% CO_2 at low U_{sg} (0.001 and 0.002 ms^{-1}, Fig. 3a) resulted in a peak in pH values during maximum productivity (days 2–4), which was not consistent with the 0.005 ms^{-1} or 12% CO_2 treatments. The effect was attributed to depletion of dissolved inorganic carbon significantly below equilibrium levels, caused by rapid growth, lower gas (4%) CO_2 concentrations and lower $k_La_L(CO_2)$ values, and may have contributed to lower biomass production observed in these treatments (Table 1).

The pH of *D. tertiolecta* 0.04% CO_2 cultivations remained stable during cultivation due to the high buffering capacity of the artificial seawater media. In contrast, *C. vulgaris* 0.04% CO_2 treatments experienced significant and variable increases in pH (Fig. 3a). The highest pH values were associated with higher growth and biomass concentrations, which can be attributed to depletion of dissolved inorganic carbon and nitrate assimilation in freshwater media with a low buffering capacity.

Author's personal copy

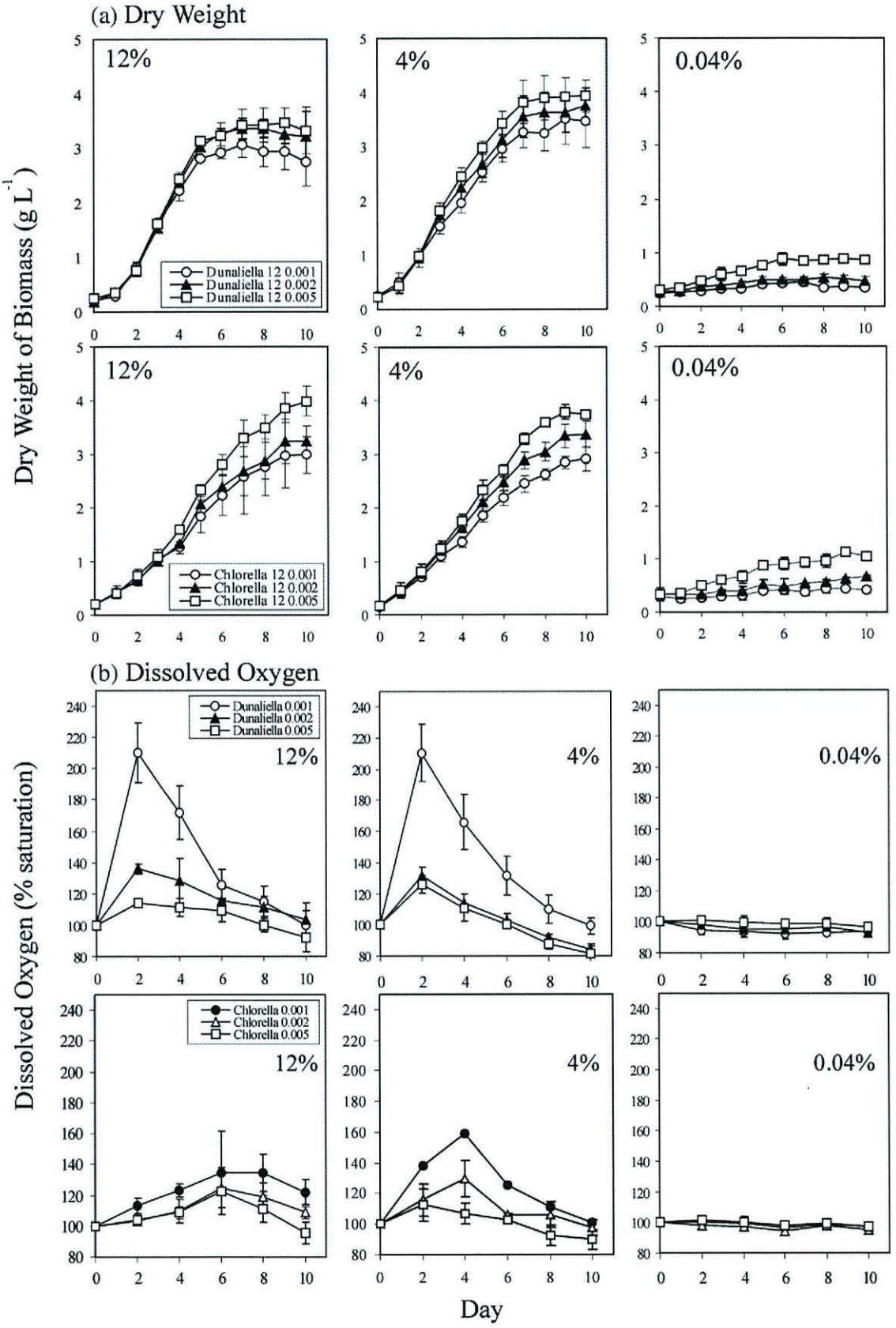


Fig. 2. (a) Growth in dry weight (g L^{-1}) of *Dunaliella tertiolecta* and *Chlorella vulgaris* in batch cultivations at different U_{sg} and CO_2 concentrations; (b) concentration of dissolved oxygen (% air saturation) during cultivation. Error bars are standard deviation.

Author's personal copy

Table 2
Results of 2 three-factor analysis of variances for the effect of algal species, CO_2 concentration and superficial gas velocity on the biomass maximum growth rate and maximum cell density. In each case the assumptions of approximate normality and approximately equal variances were upheld.

	F	p	Significant
Maximum dry weight growth rate			
Species	19.2	<0.001	*
CO_2 concentration	6.3	0.020	*
U_{sg}	2.3	0.118	
Species × CO_2 concentration	0.1	0.733	
Species × U_{sg}	0.4	0.686	
CO_2 concentration × U_{sg}	0.0	0.977	
Species × CO_2 concentration × U_{sg}	0.2	0.827	
Maximum dry weight			
Species	0.0	0.952	
CO_2 concentration	0.3	0.579	
U_{sg}	4.5	0.022	*
Species × CO_2 concentration	0.6	0.466	
Species × U_{sg}	0.7	0.507	
CO_2 concentration × U_{sg}	0.0	0.976	
Species × CO_2 concentration × U_{sg}	0.2	0.833	

* denotes a significant effect at the 5% level.

3.3. Photosynthetic characteristics, inorganic nutrients and chlorophyll

Changes in Fv/Fm during cultivation are shown in Fig. 3b. Fv/Fm measures the maximum quantum yield of charge separation in photosystem II and, although it is not directly coupled to carbon fixation and growth, may be used as an indicator of physiological stress (Obata et al., 2009; Parkhill et al., 2001). Values obtained from SFG treatments during maximum productivity (0.55–0.7) were in the range expected for healthy cells (Parkhill et al., 2001), but there was a gradual decline in Fv/Fm during batch cultivation, including the period of maximum productivity. Values toward the end of the stationary phase reduced to 0.3–0.4 for SFG treatments and differences between treatments were minimal (Fig. 3b). Since incident light and temperature were stable, the decline in Fv/Fm may be largely attributed to rapid growth and nutrient consumption in batch cultivations: microalgae must assimilate a wide range of macro and micro-nutrients at sufficient rate to achieve balanced growth and batch production does not ensure steady-state conditions, leading to reduction in Fv/Fm values (Parkhill et al., 2001).

In the SFG treatments, DIN was consumed by day 6 (Fig. S2). *D. tertiolecta* SFG treatments also exhausted all of the DIP by day 4, but *C. vulgaris* remained DIP-replete throughout cultivation (Fig. S3). The difference in DIP available is due to the different composition of the nutrient media used: the molar N:P ratio in the media used for *D. tertiolecta* was 6.9-fold greater. Treatments using 0.04% CO_2 had sufficient inorganic nitrogen and phosphorus throughout the cultivation period as the biomass concentrations reached were not sufficient to exhaust the DIN and DIP.

The chlorophyll-*a*: carbon ratio (g g^{-1}, Chl*a*:C) was also calculated for *D. tertiolecta* (see Supplementary information). During the maximum productivity period of *D. tertiolecta* cultivated in SFG, Chl*a*:C ranged from mean values of 0.03–0.10. However, the Chl*a*:C ratio of *D. tetrtiolecta* decreased throughout cultivation in 0.04% CO_2 treatment (Fig. 4a), reaching 0.005 (±SD 0.002) at 0.005 ms^{-1}. This photoacclimation response can be attributed to excess light in relation to the availability of CO_2, and relates to the sub-optimal biomass yields obtained in these treatments. Treatments receiving 0.04% CO_2 also showed a decline in Fv/Fm over the cultivation period and combined with their depleted chlorophyll-*a* concentrations, shows that availability CO_2 could not sustain efficient photosynthetic performance in these treatments.

3.4. Dissolved organic carbon

Not all the CO_2 assimilated by microalgae ultimately presents as cellular biomass in microalgal cultivations. Some photosynthetically fixed carbon is lost from algal cells to the surrounding medium by passive diffusion during normal growth, but higher rates of exudation may be observed when algal cells are subjected to stress factors e.g., following nutrient depletion. The concentration of DOC is potentially important because it is an excellent substrate for bacteria and may be an important loss factor in carbon uptake (Hulatt and Thomas, 2010). Data for both particulate and dissolved organic carbon (POC and DOC) is available in Fig. 4b, and as Supplementary information (Fig. S4). During the period of maximum productivity, DOC comprised 3–5% of total organic carbon (TOC) in both *C. vulgaris* and *D. tertiolecta* SFG treatments. Toward the end of the growth period, *C. vulgaris* showed little increase in the percentage of the carbon released as DOC, however *D. tertiolecta* showed increased DOC losses up to 19% (12% CO_2, 0.001 ms^{-1}). The latter DOC concentration measured 20 (±SD 5.8) mmol L^{-1}, which is approximately the same quantity of carbon found in the cells of an algal suspension of 0.5 g L^{-1}. Production of DOC by *C. vulgaris* cultivated in 0.04% CO_2 was enhanced over SFG treatments, with DOC reaching up to 16.6 (±SD 2.6)% of TOC by the end of cultivation. Since the biomass concentrations in these treatments were constrained by the supply of carbon, it is postulated that these values may be due to glycolic acid production. Exudation of this compound is associated with photorespiration, and high excretion rates may be observed in low CO_2 and/or high O_2 conditions (Beardall et al., 2003). Dissolved organic carbon thus varied from <5% to 20% of the total organic carbon fixed in the PBR device, and was related to the species and growth stage of the algae.

In the literature there are a variety of different methods by which CO_2 uptake has been measured, e.g., ΔpCO_2 concentration in/out of a photobioreactor (Sydney et al., 2010), measure directly the amount of carbon fixed as biomass (Jacob-Lopes et al., 2008), or a combination (Jacob-Lopes et al., 2010). Sydney et al. (2010) presented data implying 12–30% of the carbon retained in the system was not incorporated into cellular biomass. Further, Chiu et al. (2008) used a similar carbon balance method and reported CO_2 reduction several times higher than the biomass measurements would suggest. Since there are a variety of potential fates for CO_2 introduced to a PBR (Jacob-Lopes et al., 2010), the data available here indicates that direct measurements of harvested material could be more reliable for estimating the CO_2 fixed, especially where it is important to assess the environmental impacts of photobioreactors and possible fate of waste waters produced from the systems. In particular, if microalgae are to be used for CO_2 mitigation and the biomass is re-cycled as a fuel, it is the net energy gain which is important and not the CO_2 fixation rate itself. This is because the environmental benefits are derived from using the biomass to displace fossil fuels (Campbell et al., 2010).

3.5. Proximate analysis of biomass higher heating value (HHV)

After 10 days of growth, treatments resulted in a range of biomass energy values comparable to the literature for nutrient replete and starved biomass (Scragg et al., 2002). The lowest HHVs were observed in endpoint samples of the air treatments (19.1 ± SD 0.4 and 18.4 ± SD 0.7 kJ g^{-1}) for *C. vulgaris* and *D. tertiolecta*, respectively) shown in Table 1. These were carbon-limited systems with excess nutrients. Treatments using SFG exhausted nutrients and this stimulated a range of HHVs. The greatest values were observed in *C. vulgaris*, 4% CO_2, U_{sg} 0.005, and measured 24.3 (±SD 1.2) kJ g^{-1}. A regression model for predicting the HHV of a range of non-algal biomass types using the CHN composition was

Author's personal copy

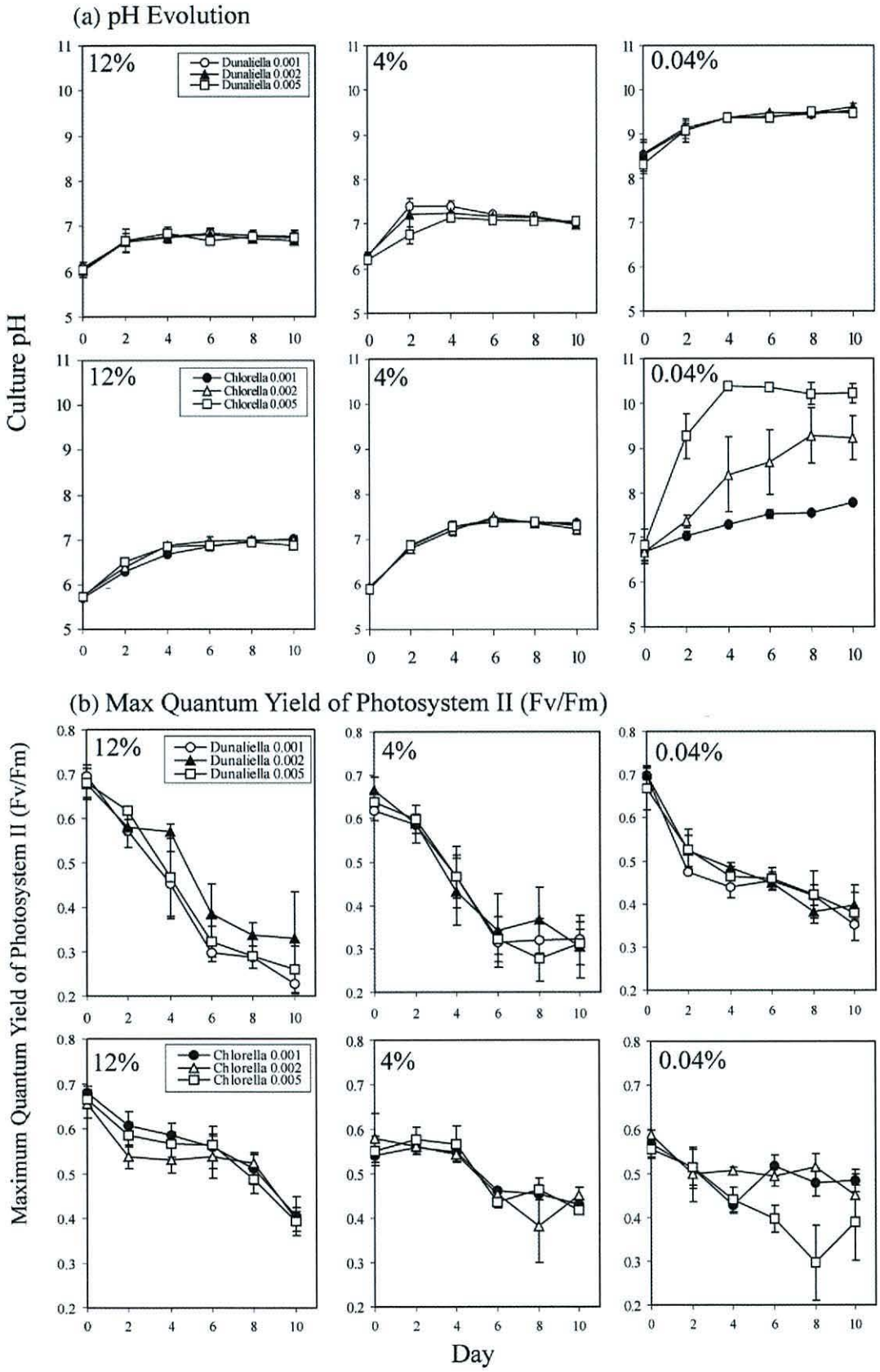


Fig. 3. (a) Culture fluid pH evolution over time; (b) changes in maximum quantum yield of photosystem II (Fv/Fm) during batch cultivation. Error bars are standard deviation.

presented by Friedl et al. (2005). When applied to the microalgae samples here there was strong correlation, but the model under-predicted HHV by ~10%. Thus, a new analysis was conducted here using multiple linear regression: Fig. 5 shows the correlation between the measured values and those predicted by the derived regression Eq. (4) below.

Author's personal copy

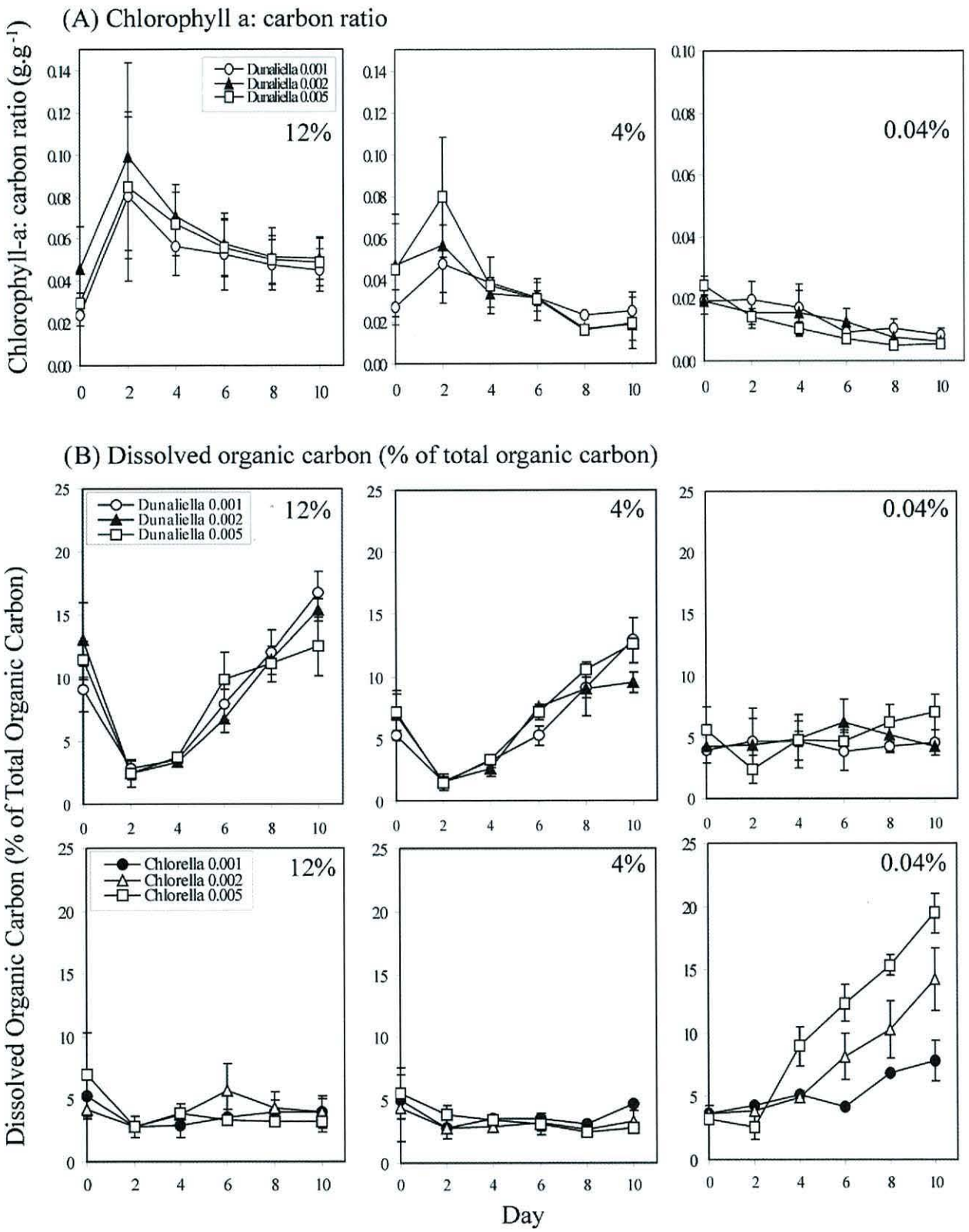


Fig. 4. (a) Ratio of chlorophyll a: carbon during cultivation of *Dunaliella tertiolecta*; (b) changes in the proportion of dissolved organic carbon in the reactor as a percentage of total organic carbon during cultivations. Error bars are standard deviation.

$$\text{Predicted HHV} = -4.90 + 0.069\ \text{N} + 0.533\ \text{C} + 0.226\ \text{H} \quad (4)$$

where the elements carbon, hydrogen and nitrogen (CHN) are measured as % mass, and HHV is kJ g^{-1}. The single equation resulted from pooling all samples together. The analysis was also investigated by treating *D. tertiolecta* and *C. vulgaris* samples separately (inset), but confidence limits indicated there was no appreciable difference. The analysis of variances for the relationship found $F = 87.1$, $p < 0.001$, df 37 and the test assumptions were held. Multi-collinearity did not cause problems with the analysis (variance

Author's personal copy

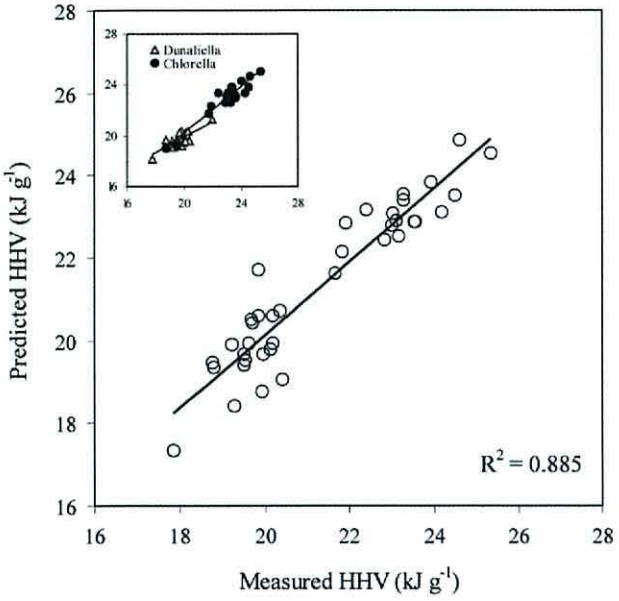


Fig. 5. Correlation between measured higher heating values for microalgal biomass (kJ g^{-1}) and the higher heating value (kJ g^{-1}) predicted by the elemental composition. Inset shows models where species are treated separately.

inflation factor <2.38). Considering the moderate range of HHV values, the strong correlation ($R^2 = 0.885$) indicated the method was suitable for subsequent proximate analysis. Proximate analysis describes a relationship but does not explain the complex biochemistry of microalgal cells, so the model was also tested on *Scenedesmus* samples ($n = 6$). It was found that on average the model predicted the energy content ±0.51% of calorimeter values, showing that the model may be applied to other work.

3.6. Energy returns over the batch cycle

The amount of biomass energy (kJ L^{-1}) at each sample point in the growth cycle was calculated as the product of the biomass dry weight (g L^{-1}) multiplied by the corresponding predicted HHV (kJ g^{-1}), derived using Eq. (4). The cumulative amount of energy consumed at any given point (kJ L^{-1}) was calculated from the power consumption (Eq. (1) and expressed in kJ L^{-1} d^{-1} in Table 1), multiplied by the total number of days run. The cumulative net bio-energy return was then obtained by subtracting the energy input from the bio-energy output. Fig. 6 shows the resultant net energy returned from the various treatments over batch cultivations, and Table 3 shows equivalent summary statistics for power output and net energy ratio.

Table 3 shows that where maximum net power outputs (W m^{-3}) were similar, the low power input treatments showed superior net energy ratios (NERs). For example, the *D. tertiolecta* treatments using 12% CO_2 had similar max net power outputs of 183 (±58) and 175 (±27) W m^{-3} respectively. However, differences become apparent when expressed as a net energy ratio: utilisation of 0.001 ms^{-1}/10 W m^{-3} over 0.005 ms^{-1}/50 W m^{-3} produced a fourfold higher NER. When data was averaged over the batch cycle to day 8, similar patterns in power output and NER were observed, but values were somewhat lower due to inclusion of sub-optimal parts of the growth cycle.

In a study using a flat plate gas-sparged PBR, Reyna-Velarde et al. (2010) estimated a power output (bio-energy production) of 35.8 W m^{-3}, countered by a power input of roughly 1/3 this value (NER = 2.9). In this work, the NER based on cumulative data to day 8 peaked at 8.8 (±SD 1.1) for *D. tertiolecta*, 0.001 ms^{-1}, 12% CO_2. The corresponding NER during maximum productivity was 19.3 (±SD 5.8). These values are considerably higher than reported in the published literature for closed photobioreactors, and it is important to re-iterate that these are values for power input only.

Fig. 5 and Table 3 show that treatments using 0.04% (air) concentrations of CO_2 were all energy-negative, and utilised around 50% of the CO_2 available (*D. tertiolecta*). Even if algae could utilise close to 100% of the CO_2 in the gas flowing through the system (e.g. micro-diffuser to increase interfacial area), mass balance shows that they could only roughly double their growth rate, which would barely be sufficient to overcome the energy demand for power input alone in this type of photobioreactor. Moreover cells experienced physiological stress in this treatment, detected by PAM measurements and Chla:C ratios. Photobioreactors sparged with air-concentrations of CO_2 in this manner thus seem incapable of providing a significant positive energy return and as such do not seem feasible as a bio-energy technology: the supply of concentrated CO_2 to these photobioreactors is not only important for improving yield, but is the only way of generating a significant positive energy balance.

The photosynthetic efficiency of these cultivations during the period of maximum growth varied in SFG treatments from 3.5% to 7.4% for *C. vulgaris* (0.001 ms^{-1}, 4% CO_2) and *D. tertiolecta* (0.005 ms^{-1}, 12% CO_2) respectively (Table 3). These values are based on PAR energy only, and comparison with the literature indicates efficient light energy conversion in these cultivations (Watanabe and Hall, 1996). Naturally, the light energy conversion efficiency recorded in 0.04% CO_2 treatments was much lower, between 0.3% and 1.8%. An additional experiment was performed using both species and smaller bubbles produced by a diffuser (12% CO_2, 0.002 ms^{-1}) to investigate whether there was any improvement in yield. There was no significant improvement in either maximum biomass attained or growth rate ($t \leqslant 0.38$, $p \geqslant 0.73$). It was concluded that the SFG results obtained in this study were not sensitive to this feature of the reactor design.

To conclude, low power inputs using SFG could result in a high energy return, high NERs and make efficient use of CO_2, at the cost of slightly lower absolute biomass yields and photosynthetic efficiencies. Literature values for power input in gas-sparged photobioreactors (including life-cycle assessments) are commonly in the order of 50–70 W m^{-3} (Lehr & Posten, 2009; Jorquera et al., 2009). The data presented here shows that, in this design, excellent growth and energy return could be achieved at power inputs 2.5 to 5-fold lower than commonly cited. This suggests that the power input could potentially be minimised to lower levels than commonly cited and consequently improving the energy return.

3.7. Additional energy impacts associated with power input

In this work the theoretical power input was used for scale-up purposes. However there are other efficiency-reducing steps involved with supply of power to a photobioreactor. The efficiency losses of pump/blower systems used to supply gas and the supply of energy to those pumps has additional, potentially large impacts. For example a worst case scenario may be a fossil fuel power plant used to provide electricity (~40% efficient) to drive a pump system (efficiency un-defined and scale-dependent) which would result in an energy burden >2.5 times higher than that at the photobioreactor. However, the systems proposed for growing algae on a large scale have not yet been refined, and some authors have reported that energy could be recovered from waste algal biomass (Stephenson et al., 2010), which could offset much of these additional energetic costs.

Author's personal copy

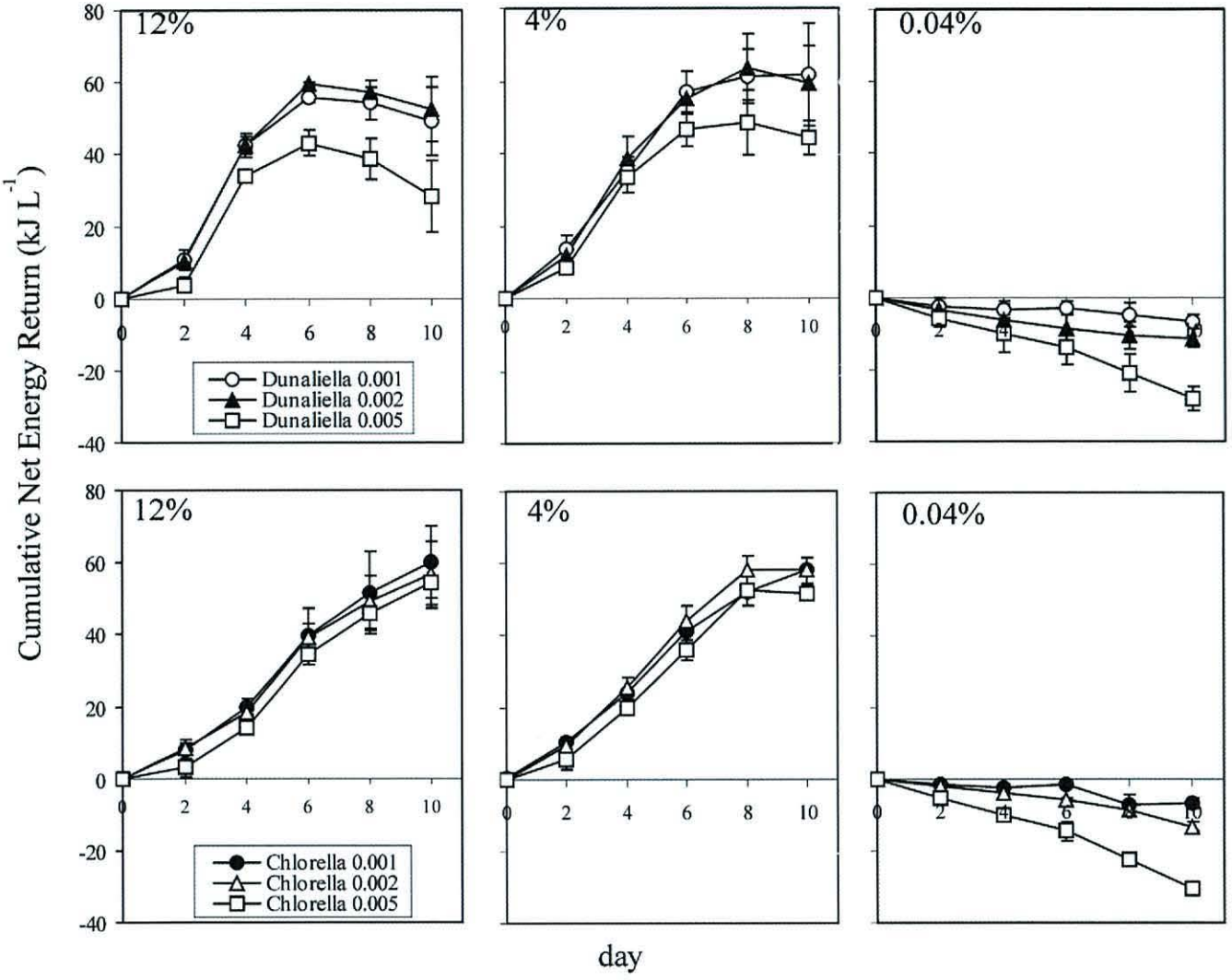


Fig. 6. The cumulative net bio-energy concentration (kJ L^{-1}) in the photobioreactors after subtraction of the energy consumed by the supply of gas. Error bars are standard deviation.

Table 3
Summary statistics for energetic performance for *Dunaliella tertiolecta* and *Chlorella vulgaris* in photobioreactors based on bio-energy output and power input only. Parameters at maximum growth correspond to measurements made over the period (days) indicated. Measurements to day 8 compare the average performance over the batch cycle to day 8. The net power output (W m^{-3}) is the balance between sparging power input and the rate of biomass energy production (Reyna-Velarde et al., 2010). The net energy ratio (NER) values for power input vs. bio-energy output were calculated according to Jorquera et al. (2010). The photosynthetic efficiency (PE, %, based on PAR energy only) is calculated for the period of maximum growth. Values in parentheses are standard deviations.

Treatments			At maximum growth				Averaged to day 8	
Species	Power input (W m^{-3})	% CO_2	Period (days)	NER	Net power (W m^{-3})	PE (PAR only)	NER	Average net power (W m^{-3})
D. tertiolecta	10	12	2–4	19.3 (5.8)	183 (58)	6.33 (1.92)	8.8 (1.1)	78 (11)
D. tertiolecta	20	12	2–4	10.4 (1.4)	188 (28)	6.83 (0.92)	5.2 (0.4)	83 (7)
D. tertiolecta	50	12	2–4	4.5 (0.5)	175 (27)	7.40 (0.88)	2.1 (0.3)	56 (14)
D. tertiolecta	10	4	2–4	13.5 (2.0)	125 (20)	4.42 (0.65)	9.9 (1.9)	87 (19)
D. tertiolecta	20	4	2–4	8.8 (2.1)	157 (42)	5.81 (1.39)	5.6 (1.1)	92 (23)
D. tertiolecta	50	4	2–4	3.9 (0.8)	146 (42)	6.42 (1.36)	2.4 (0.4)	70 (22)
D. tertiolecta	10	0.04	2–6	0.8 (0.5)	−10 (12)	0.54 (0.34)	0.3 (0.8)	−19 (23)
D. tertiolecta	20	0.04	2–6	0.2 (0.4)	−19 (23)	0.30 (0.46)	0.2 (0.5)	−15 (9)
D. tertiolecta	50	0.04	2–6	0.5 (0.3)	−29 (26)	1.77 (0.90)	0.4 (0.3)	−30 (13)
C. vulgaris	9.8	12	4–6	12.3 (5.3)	116 (52)	4.13 (1.72)	8.6 (2.9)	74 (28)
C. vulgaris	19.6	12	4–6	7.1 (1.8)	120 (36)	4.59 (1.18)	4.6 (0.9)	71 (18)
C. vulgaris	49.0	12	4–6	3.3 (0.4)	117 (19)	5.44 (0.61)	2.4 (0.2)	66 (12)
C. vulgaris	9.8	4	4–6	10.9 (0.8)	97 (8)	3.50 (0.26)	8.7 (0.4)	76 (4)
C. vulgaris	19.6	4	4–6	6.4 (1.5)	107(29)	4.15 (0.95)	5.3 (0.5)	84 (10)
C. vulgaris	49.0	4	4–6	2.9 (0.4)	92 (20)	4.63 (0.65)	2.5 (0.2)	76 (10)
C. vulgaris	9.8	0.04	2–6	1.0 (0.7)	0 (7)	0.67 (0.45)	0.3 (0.3)	−7 (3)
C. vulgaris	19.6	0.04	2–6	0.4 (0.1)	−11 (1)	0.55 (0.05)	0.4 (0.2)	−12 (3)
C. vulgaris	49.0	0.04	2–6	0.5 (0.2)	−27 (11)	1.47 (0.72)	0.3 (0.1)	−32 (5)

Author's personal copy

3.8. Scale-up and integration

One of the critical issues involving photobioreactor design is the ability to scale up to industrial production. In this work, it was necessary to use small scale systems in a controlled environment in order to measure effects. The bubble column design used here can be related to larger scale gas-sparged column arrays and to flat plate reactors, which are another geometric form of the same design principle. These designs have significant potential for scale-up (Slegers et al., 2011).

This work demonstrates a systems-oriented method by which microalgal biomass production was optimised to improve the economic and environmental performance of such technologies. As such the experimental energy balance approach can be applied to other microalgal cultivation technologies, e.g., to optimise cultivation conditions and harvest points, thereby minimising the negative impacts of microalgal technologies. The actual net energy yield will be important for bio-energy production per unit area of land or volume of culture, whereas the net energy ratio will be important where the energy for power input is recovered from the process, for example where anaerobic digestion of residual biomass is used to recycle energy back into the process (Stephenson et al., 2010); if the ratio is not high enough, there will be insufficient energy generated to offset the demands of production.

At industrial scales, continuous production systems may be used in place of batch systems. However, batch cultivation may be required in order to increase lipid production via nutrient limitation. The maximum rates of bio-energy production recorded in this work are representative of the values achievable by continuous production in the same conditions, whereas the averaged values are representative of energy return over a batch production cycle.

Here we only examined the effect of power input, although there are other burdens which should significantly impact the energetic efficiency of producing algal biomass. These include supply of CO_2, nutrients and reactor materials. Light energy is not considered a burden as such and was not used in the energy balance in this work. This is because sustainable systems will need to utilise natural solar energy. The next development steps should investigate the relationship between energy inputs and bio-energy outputs in outdoor cultures with variable light and temperature regime. For example, it may be necessary to develop a flexible control system capable of adjusting the sparging rate to compensate for large fluctuations in light and temperature in outdoor systems, to optimise mixing and mass transfer, whilst minimising energy inputs.

4. Conclusions

The results of this study show that there may be little practical use in pursuing the highest absolute biomass production using high power input photobioreactors. The optimal treatment is that which provides the highest net return; both net energy and net energy ratios may be important. This might be considered intuitive, though to date few studies have focused on this issue, especially for a single photobioreactor design. The evidence from these laboratory measurements indicates this type of closed photobioreactor design has potential as an environmentally sustainable method of producing microalgal biomass. However, the range of results obtainable from a single photobioreactor design shows that it may be unreliable to compare alternative technologies without significant optimisation.

Acknowledgements

Work supported by an Engineering and Physical Sciences Research Council (EPSRC) PhD studentship in collaboration with RWE npower, UK and Varicon Aqua Solutions Ltd., UK. We thank Richard Hotchkiss, Martin Pilbin, Roger Bewley, Joe Mcdonald and David Bowers for valuable discussions, Louiza Norman and Naomi Thomas for analytical support, and independent reviewers for their constructive comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.02.025.

References

Beardall, J., Quigg, A., Raven, J., 2003. Oxygen consumption: photorespiration and chlororespiration. In: Larkum, A., Douglas, S., Raven, J. (Eds.), Photosynthesis in Algae. Kluwer Academic, The Netherlands, pp. 157–181.

Brune, D., Lundquist, T., Benemann, J., 2009. Microalgal biomass for greenhouse gas reductions: potential for replacement of fossil fuels and animal feeds. J. Environ. Eng. – ASCE 135, 1136–1144.

Campbell, P., Beer, T., Batten, D., 2010. Life cycle assessment of biodiesel production from microalgae in ponds. Bioresour. Technol. 102, 50–56.

Carballo-Cardenas, E., Tuan, P., Janssen, M., Wijffels, R., 2003. Vitamin E (alpha-tocopherol) production by the marine microalga *Dunaliella tertiolecta* and *Tetraselmis suecica* in batch cultivation. Biomol. Eng. 20, 139–147.

Chisti, Y., 1989. Airlift Bioreactors. Elsevier, New York.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Chisti, Y., 2008. Biodiesel from microalgae beats bioethanol. Trends Biotechnol. 26, 126–131.

Contreras, A., Garcia, F., Molina, E., Merchuk, J., 1998. Interaction between CO_2 mass transfer, light availability, and hydrodynamic stress in the growth of *Phaeodactylum tricornutum* in a concentric tube airlift photobioreactor. Biotechnol. Bioeng. 60, 317–325.

Chiu, S., Kao, C., Chen, C., Kuan, T., Ong, S., Lin, C., 2008. Reduction of CO_2 by a high-density culture of *Chlorella* sp. in a semicontinuous photobioreactor. Bioresour. Technol. 99, 3389–3396.

Clarens, A., Resurrecion, E., White, M., Colosi, M., 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ. Sci. Technol. 44, 1813–1819.

Dismukes, G., Carrieri, D., Bennette, N., Ananyev, G., Posewitz, M., 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr. Opin. Biotechnol. 19, 235–240.

Douskova, I., Doucha, J., Livansky, K., Machat, J., Novak, P., Umysova, D., Zachleder, V., Vitova, M., 2009. Simultaneous flue gas bioremediation and reduction of microalgal biomas production costs. Appl. Microbiol. Biotechnol. 82, 179–185.

Falkowski, P., Raven, J., 2007. Aquatic Photosynthesis. Princeton University Press, New Jersey.

Friedl, A., Padouvas, E., Rotter, H., Varmuza, K., 2005. Prediction of heating values of biomass fuel from elemental composition. Anal. Chim. Acta 544, 191–198.

Goldman, J., Brewer, P., 1980. Effect of nitrogen source and growth rate on phytoplankton-mediated changes in alkalinity. Limnol. Oceanogr. 25, 352–357.

Grasshoff, K., Ehrhardt, M., Kremling, K., 1983. Methods of Seawater Analysis. Verlag-Chemie, Weinheim.

Hulatt, C., Thomas, D., 2010. Dissolved organic matter in microalgal photobioreactors: a potential loss in solar energy conversion? Bioresour. Technol. 101, 8690–8697.

Jacob-Lopes, E., Lacerda, L., Franco, T., 2008. Biomass production and carbon dioxide fixation by *Aphanothece microscopica* Nageli in a bubble column photobioreactor. Biochem. Eng. J. 40, 27–34.

Jacob-Lopes, E., Scoparo, C., Queiroz, M., Franco, T., 2010. Biotransformations of carbon dioxide in photobioreactors. Energ. Convers. Manage. 51, 894–900.

Jorquera, O., Kiperstock, A., Sales, E., Embirucu, M., Ghirardi, M., 2010. Comparative energy life-cycle analysis of microalgal biomass production in open ponds and photobioreactors. Bioresour. Technol. 101, 1406–1413.

Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., Malcata, F., Langenhove, H., 2010. Enhanced CO_2 fixation and biofuel production via microalgae: recent developments and future directions. Trends Biotechnol. 28, 371–380.

Lardon, L., Helias, A., Sialve, B., Steyer, J., Bernard, O., 2009. Life-cycle assessment of biodiesel production from microalgae. Environ. Sci. Technol. 43, 6475–6481.

Lehr, F., Posten, C., 2009. Closed photo-bioreactors as tool for biofuel production. Curr. Opin. Biotechnol. 20, 280–285.

Mandalam, R., Palsson, B., 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. Biotechnol. Bioeng. 59, 605–611.

Merchuk, J., Gluz, M., Mukmenev, I., 2000. Comparison of photobioreactors for cultivation of the red microalga *Prophyridium* sp. J. Chem. Technol. Biotechnol. 75, 1119–1126.

Obata, M., Toda, T., Taguchi, S., 2009. Using chlorophyll fluorescence to monitor yields of microalgal production. J. Appl. Phycol. 21, 315–319.

Parkhill, J., Maillet, G., Cullen, L., 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37, 517–529.

Reyna-Velarde, R., Cristiani-Urbina, E., Hernandez-Melchor, D., Talasso, F., Canizares-Villanueva, R.O., 2010. Hydrodynamic and mass transfer characterization of a flat-panel airlift photobioreactor with high light path. Chem. Eng. Process. 49, 97–103.

Author's personal copy

Scragg, A., Illman, A., Carden, A., Shales, S., 2002. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass Bioenerg. 23, 67–73.

Sierra, E., Acien, F., Fernandez, J., Garcia, J., Gonzalez, C., Molina, E., 2008. Characterization of a flat plate photobioreactor for the production of microalgae. Chem. Eng. J. 138, 136–147.

Slegers, P., Wijffels, R., Straten, G., Boxtel, A., 2011. Design scenarios for flat panel photobioreactors. Appl. Energy. doi:10.1016/j.apenergy.2010.12.037.

Stephenson, A., Kazamia, E., Dennis, J., Howes, C., Scott, S., Smith, A., 2010. Life-cycle assessment of potential algal biodiesel production in the United Kingdom: a comparison of raceways and air-lift tubular bioreactors. Energ. Fuel 24, 4062–4077.

Sydney, E., Sturm, W., Carvalho, J., Thomaz-Soccol, V., Larroche, C., Pandey, A., Soccol, C., 2010. Potential carbon dioxide fixation by industrially important microalgae. Bioresour. Technol. 101, 5892–5896.

Ugwu, C., Aoyagi, H., Uchiyama, H., 2008. Photobioreactors for mass cultivation of algae. Bioresour. Technol. 99, 4021–4028.

Watanabe, Y., Hall, D., 1996. Photosynthetic production of the filamentous cyanobacterium *Spirulina platensis* in a cone-shaped helical tubular photobioreactor. Appl. Microbiol. Biotechnol. 44, 693–698.

5. Supplementary Information.

5.1. Mass Transfer.

Mass transfer measurements were conducted as described in manuscript methods. The oxygen absorption technique assumes a well-mixed liquid phase and stable gas composition (Contreras et al, 1998). Slight deviations of measured and predicted values may be expected considering differences in reactor geometry and fluid properties (Chisti & Jauregui-Haza, 2002). Higher k_La_L values were obtained in freshwater media, consistent with Ruen-ngam et al (2008).

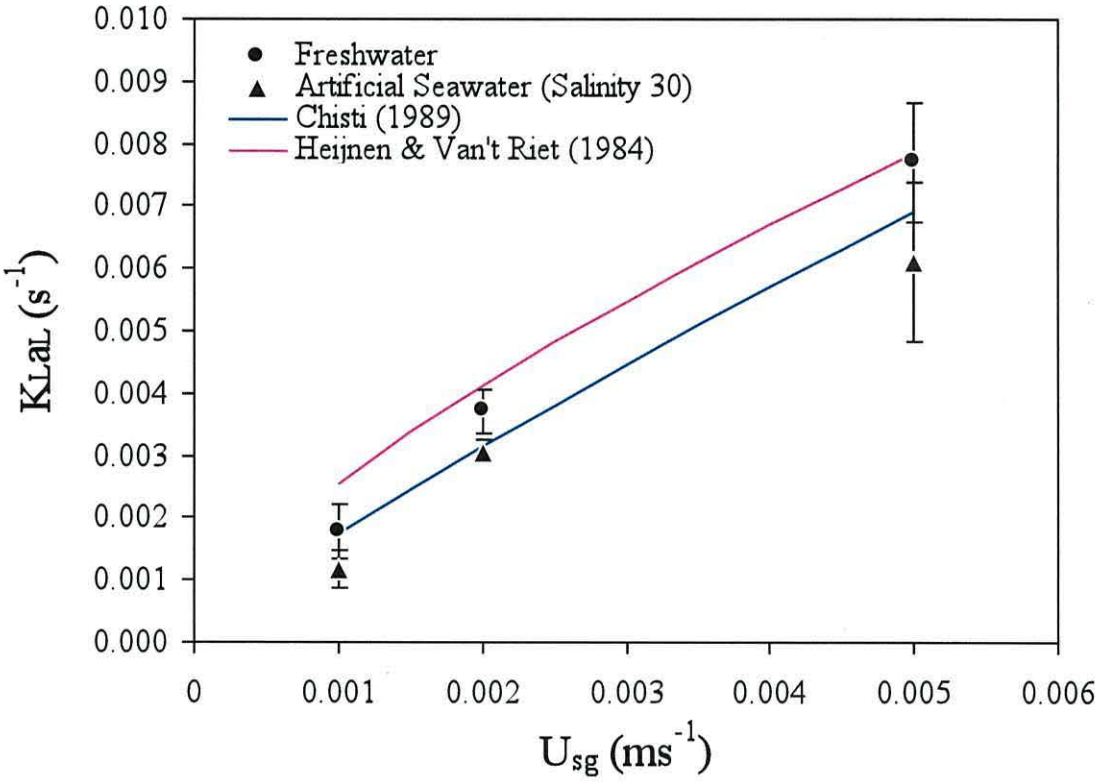


Figure S1. Measured and predicted values of the mass transfer coefficient $k_La_L(O_2)$ (s^{-1}) in bubble column photobioreactors operated at superficial gas velocities (U_{sg}) used in experimental work. Data collected for the artificial seawater and freshwater media used in experiments. Solid lines are values predicted using the empirical relationships from Chisti (1989) and Heijnen & Van't Riet (1984). Experimental data is mean of 3 replicates ±standard deviation.

Equations used in figure S1:

$k_L a_L = 0.32\ U_{sg}^{0.7}$ (Heijnen & Van't Riet, 1984)

$k_L a_L = 2.39\times10^{-4}\ (P_G/V_L)^{0.86}$ (Chisti, 1989)

References for supplementary information only.

Chisti, Y., Jauregui-Haza, U., 2002. Oxygen transfer and mixing in mechanically agitated bioreactors. Biochem. Eng. J. 10, 143-153.

Heijnen & Van't Riet (1984), cited in: Sanchez-Miron, A., Camacho, F., Gomez, A., Grima, E., Chisti, Y., 2000. Bubble-column and airlift photobioreactors for algal culture. J. AIChE. 46, 1872-1887.

Ruen-ngam, D., Wongsuchoto, P., Limpanuphap, A., Charinpanitkul, T., Pavasant, P. 2008. Influence of salinity on bubble size distribution and gas-liquid mass transfer in airlift contactors. Chem. Eng. J. 141, 222-232.

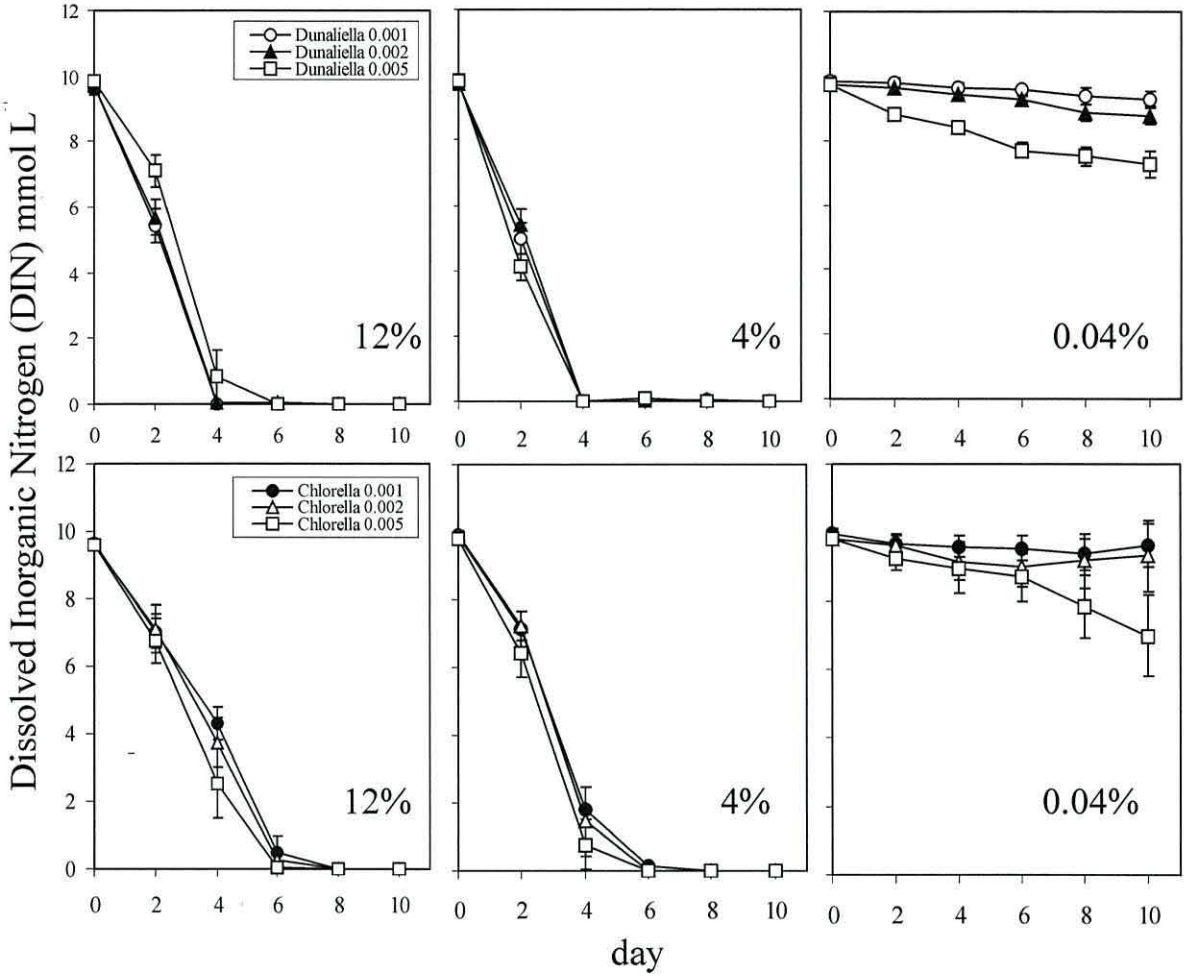


Figure S2. Dissolved inorganic nitrogen (mmol L^{-1}) dynamics in batch cultivations. DIN was derived from the sum of NO_3^-, NO_2^- and NH_4^+.

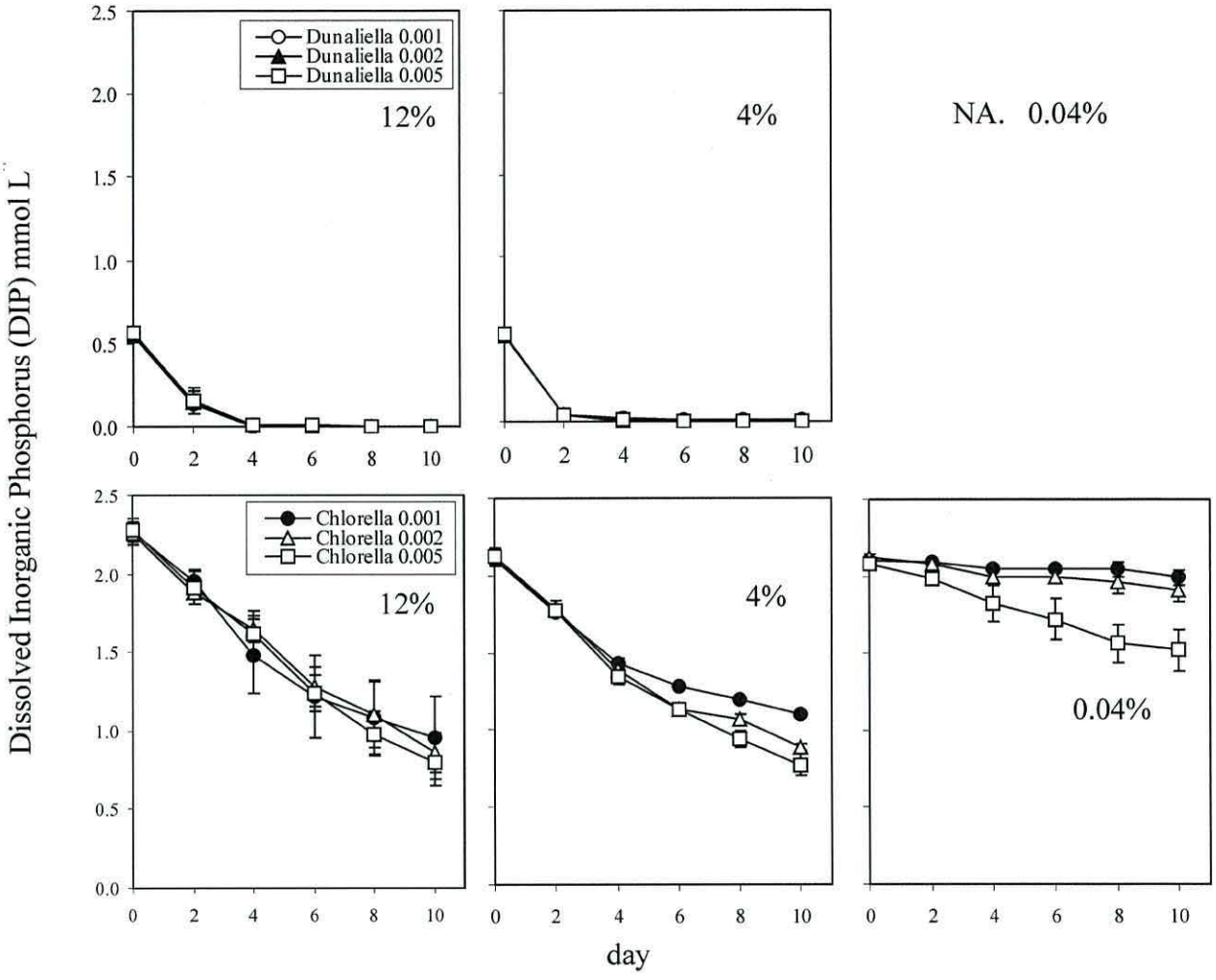


Figure S3. Dissolved inorganic phosphorus (mmol L^{-1}) dynamics in batch cultivations.

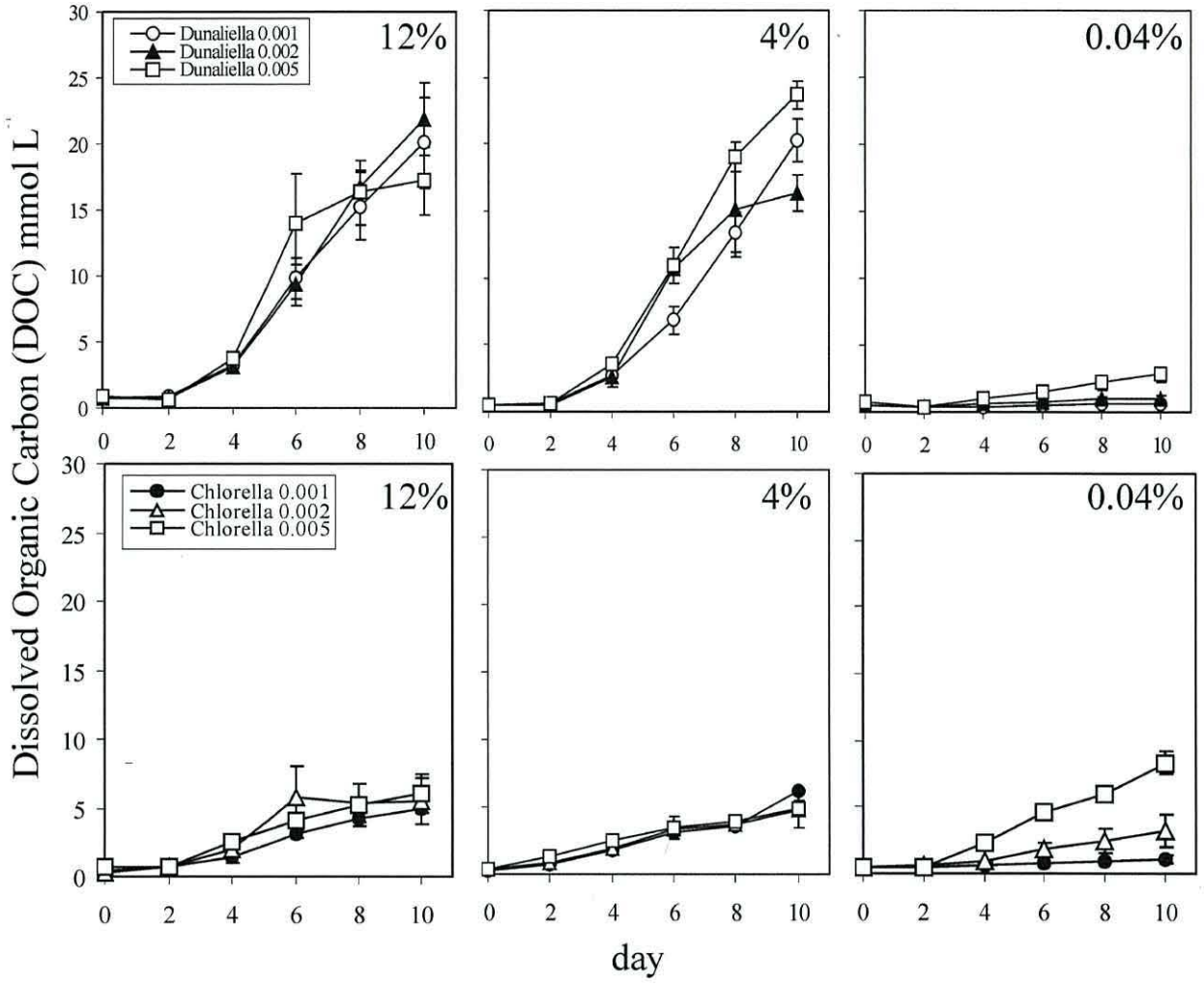


Figure S4. Changes in the concentration of dissolved organic carbon present in each treatment over the batch cycle (mmol L^{-1}).

Chapter 5.

Energy Demands of Nitrogen Fertiliser Supply in Mass Microalgal Cultivation.

5.1. Overview

The objective of the work presented in the following chapter was to test the hypothesis that *cultivation of microalgae using different nitrogen sources and at different growth stages affects productivity, biochemical composition and the energy demands associated with the supply of nutrients,* as discussed in Chapter 1.

Energy demands of nitrogen fertiliser supply in mass cultivation of two commercially important microalgal species, *Chlorella vulgaris* and *Dunaliella tertiolecta.*

Chris J. Hulatt[a*], Aino-Maija Lakaniemi[b], Jaakko A. Puhakka[b], David N. Thomas[a,c]

[*]Corresponding Author; tel (+44) 01248 38 2852; osp418@bangor.ac.uk.

[a]School of Ocean Sciences, College of Natural Sciences, Bangor University, Askew Street, Menai Bridge, Anglesey, U.K. LL59 5AB.

[b]Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland.

[c]Finnish Environment Institute (SYKE), Marine Centre, PO Box 140, FI-00251 Helsinki, Finland.

1. Introduction

In order to reduce the environmental impacts of fossil fuel usage (Fargione et al, 2008), to provide energy security and to meet interntional and national-set government targets (Bauen, 2006), there is an urgent need to develop sustainable energy technologies. Biomass energy derived from crops has the potential to fulfil at least some of this requirement, and already has commercial applications in the transport fuel and power industry (Hughes et al., 2000; Sanchez & Cardona, 2008).

One potential method of producing biomass for energy and greenhouse gas mitigation that is not currently in viable large-scale commercial production (Lardon et al., 2010), is to cultivate microalgae in place of conventional terrestrial crops (Chisti, 2007; Dismukes et al., 2008). Microalgae have several advantages over their terrestrial counterparts including; high areal productivities, high cellular concentrations of lipids that can be converted to bio-diesel, and the ability to cultivate microalgae on land not suitable for conventional agriculture (Dismukes et al., 2008; Griffiths & Harrison, 2009). In fact, it has been calculated that the requirement for large areas of land for growing terrestrial energy crops could mean that microalgae are the only feasible solution for meeting biomass production quotas (Chisti, 2007).

The potential utilisation of microalgal technologies is often justified by the environmental benefits, particularly reduction in greenhouse gases released to the atmosphere. However, all commercial bio-fuels currently face serious challenges in terms of their life cycle 'net benefits', when the energetic and environmental costs incurred during production are accounted for (Hill et al., 2006; Jorquera et al., 2010; Lardon et al., 2009; Stephenson et al., 2010). As a result, the most significant improvements in algal technologies are likely to come from managing and improving the energetic and environmental burdens of their production.

A fundamental issue that has been largely overlooked in the most recent drive towards commercial microalgal biomass production is the need to supply large amounts of essential nutrients to grow microalgae in any cost effective, commercially viable way (Chisti, 2007; Stephenson et al., 2010): If microalgae are to be used for large-scale transport fuel production and/ or power plant flue-gas CO_2 mitigation, it is vital to consider the demands on fertiliser supply, especially the nitrogen and phosphorus sources. In modelling studies, it is logically assumed that nutrients will be supplied from manufactured sources (Clarens et al., 2010; Chisti, 2007), because this is the mechanism that currently supports the majority of global agriculture (Ramirez & Worrell, 2006). It has been estimated that the fertiliser energy input for microalgal biomass production may account for 12 to 45% of all fossil energy inputs (Stepehenson et al., 2010, Chisti, 2008).

Supply of nitrogen to microalgal cultivations is very significant because i) the manufacture of nitrogen fertiliser via ammonia (Haber-Bosch process) is much more energy intensive than, for example, production of phosphates (Dalgaard et al., 2001) and ii) Microalgal biomass typically has a high protein content (up to 60%) with an optimal molar carbon: nitrogen ratio of approximately 6.6:1 (Geider & Roche 2002; Lopez et al., 2010; Redfield, 1958). This compares to terrestrial crops which have lower nitrogen demands, their C:N ratios typically ranging between 20:1 and 120:1 (Friedl et al., 2001). Thus the fertiliser demands for algae are potentially significantly higher than for other crops. Production of phosphorus fertilisers requires around 5 times less energy per unit mass than nitrogen fertilisers (Dalgaard et al., 2001), and microalgae require phosphorus in quantities around seven times lower than nitrogen (Geider & Roche, 2002; Redfield, 1958). Thus the energy consumption of nitrogen supply may be estimated to be roughly 30 to 40 times higher than that of phosphorus.

In a similar context, supply of other trace nutrients has been regarded as negligible, and many of these are already present in natural water supplies (Lardon et al., 2009). Life-cycle assessment of microalgal technologies invariably places importance on minimising the consumption of nutrients. For example, Clarens et al., (2010) identified the need to supply waste streams, Lardon et al., (2010) identified the requirement to starve the biomass of nitrogen, whilst Stephenson et al., (2010) found that recycling the nutrients via anaerobic digestion greatly improved process efficiency.

Though some cyanobacteria are capable of fixing their own nitrogen from N_2, most commercially valuable microalgae must be provided with external sources of nitrogen compounds. Typically, nitrogen is supplied to autotrophic algal cultures in the form of nitrate (NO_3^-) and/or ammonium (NH_4^+) (Chiu et al., 2008; Soletto et al., 2005; Tam & Wong, 1996). However, some species are capable of utilising urea (Hsieh & Wu, 2009), which may be useful for commercial applications due to its lower cost than nitrate compounds (Soletto et al., 2005). Assimilation of amino-acids from the culture broth is possible (Perez-Garcia et al., 2010), but heterotrophic cultivation strategies rely upon another source of organic matter to support them, and are not considered in this work. The transport of nitrogen compounds into algal cells, reduction and subsequent building of amino-acids is integrated with photosynthesis and carbon metabolism (Falkowski & Raven, 2007). Nitrogen availability strongly affects biochemical composition, including the concentration of cellular lipids, and nitrogen-limitation is a strategy for producing lipid-rich biomass (Xin et al., 2010).

The fate of nitrogen compounds in microalgal mass cultures has received relatively little consideration. The release of dissolved substances, including nitrogenous compounds has been widely recognised (Carlson, 2002). However, the

role of bacteria in utilising this source of organic matter is still poorly understood, especially in mass algal cultivations.

The purpose of this research was to i) measure the productivity of *Chlorella vulgaris* and *Dunaliella tertiolecta* cultivated using different nitrogen sources (nitrate, ammonium and urea; ii) To measure the nitrogen dynamics and growth of microalgae in intensive bubble column photobioreactors using flue-gas concentrations of CO_2 and; iii) To relate the growth performance of microalgae to the commercial demands and energy implications of fertiliser usage. The effects on growth, calorific value and biochemical composition (lipid, protein) content are also presented and discussed.

2. Methods

2.1 Cultivation conditions

Two experimental photobioreactor systems were used in this study: Large 20 L polythene bioreactors, and smaller scale 1.4 L bubble column reactors. It is important to note that in the descriptions that follow the nutrient, light and growth conditions were quite different between the two systems.

2.1.1. Polythene photobioreactors (20 litres)

Cultivation was carried out using polythene photobioreactors, diameter 160 mm, sparged with air (0.038% CO_2 approx) at 10 L min^{-1} (Hulatt & Thomas 2010). Light was provided at an incident irradiance of 225 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (Li-Cor 190SA sensor) using a bank of 8 cool white fluorescent tubes (Osram, 36W ea.). *Chlorella vulgaris* and *Dunaliella tertiolecta* were each cultured in batch conditions for 14 days using nitrate, urea and ammonium as the nitrogen sources. There were thus 6 treatments in a complete

factorial design, each carried out in triplicate. The inoculum consisted of exponentially growing cells supplied with nitrate; typically no adaptation is needed for the nitrogen species tested (Fabregas et al., 1989). The temperature was 24 ±2°C during cultivation. Nutrient media for *Chlorella vulgaris* and *Dunaliella tertiolecta* were according to 8M composition (Mandalam & Palsson, 1998) and 2ASW (Culture Collection of Algae and Protozoa, Oban, UK) respectively, and both were adjusted in proportion to a common 1.33 mmol L^{-1} nitrogen. Molar N:P ratios for *Dunaliella tertiolecta* and *Chlorella vulgaris* media were 24.7 and 4.3 respectively. Media was prepared using 0.2 μm filtered water (Millipore). Medium for *Dunaliella tertiolecta* was adjusted to a salinity of 30 using artificial seawater (Ultramarine Synthetica, according to the Culture Collection of Algae and Protozoa, Oban, UK).

2.1.2. Intensive bubble columns (1.4 litres).

Cultivation was carried out using purpose-designed bubble column photobioreactors, detailed in Hulatt & Thomas (2011). Briefly, reactors had an internal diameter of 32 mm, and received light from cool white fluorescent tubes placed on one side only, at an incident irradiance of 350 μmol photons m^{-2} s^{-1} photosynthetically active radiation (lamps as above). Four treatments are presented in this work: *Chlorella vulgaris* and *Dunaliella tertiolecta* each cultivated using air (0.04% CO_2) and simulated coal-fired plant flue gas (12% CO_2), each in triplicate. Gas was sparged at a rate of 0.24 L min^{-1}, supplied pre-mixed from a cylinder (BOC special products, UK).

Nutrient medium for *Chlorella vulgaris* was according to Mandalam & Palsson (1998), whilst the composition for *Dunaliella tertiolecta* was according to Carballo-Cardenas et al (2004). For comparability, the nutrient media were each standardised

proportionally to a common 10 mmol L^{-1} concentration of inorganic nitrogen (Hulatt & Thomas, 2011). For *Dunaliella tertiolecta* cultivations, media was adjusted to a salinity of 30 using artificial seawater as above. Temperature was controlled at 26 ±0.5°C using air conditioning in the laboratory.

2.2. Analytical methods

2.2.1. Biomass Properties

The microalgal dry weight was analysed by vacuum filtration using Whatman GF/F filters, before drying at 80°C for 24 hours. The initial and final filter weights were measured using a 6 point balance, and dry mass calculated as $d= (w_i-w_f)/ v$, where d is the dry weight (g L^{-1}), w_i and w_f are the dry filter weights before and after filtration respectively and v is the culture volume filtered (L). Cell counts were conducted using a haemocytometer.

The elemental composition (carbon, hydrogen, nitrogen) of the biomass was measured using a Thermo-Electron Elemental Analyser (EA1112) configured for CHNS determination using a thermal conductivity detector. Samples were extracted by centrifugation of culture (1000 rcf, 10 mins) and dried at the same temperature as the dry weight measurements (80°C) for 48 hours. Samples comprised 2-3 mg of dried material measured using a 6 point balance. Measurements were calibrated using Sulphanilamide, BBOT and L-Cystine standards. DL-Methionine was used a regular check standard between every 10-15 samples. The mean difference to the certified values of the check standard was 0.48, 0.30 and 0.20 % for the elements C (40.25%), H (7.43%) and N (9.39%) respectively. The protein composition of the algal biomass was calculated by multiplying the total elemental nitrogen content by 4.44: A value of 6.25 is commonly used as a protein approximation, but the presence of intracellular

nitrogen in non-protein forms means 4.44 is a more accurate approximation (Lopez et al., 2010). The elemental composition (carbon, hydrogen, nitrogen) was also used to calculate the higher heating value of the biomass by proximate analysis using a multiple regression model Hulatt & Thomas (2011). The equation used was; HHV (kJ g^{-1}) = -4.9 + 0.069(N) + 0.533(C) + 0.226(H).

The total lipid content was measured using the Bligh & Dyer (1959) solvent method. A biomass pellet for a single lipid sample was collected by centrifugation of 250 ml of culture (1000 rcf, 10 min), which was stored without drying at -80°C. For measurement, samples were re-suspended to a volume of 5 ml; deionised water was sufficient for *Dunaliella tertiolecta*, but a lysis buffer (0.5% sodium dodecyl sulphate, 5% glycerol) was required for *Chlorella vulgaris*. Cells were disrupted in pre-weighed glass centrifuge tubes using sonication (Branson Digital Sonifier) at a power input of 13.5W per 10 ml of sample. *Dunaliella tertiolecta* required only brief (1 min) treatment to ensure complete disruption (checked using microscope), but *Chlorella vulgaris* required 30 minutes and cooling in an ice bath to ensure an equivalent result. Biomass was then dried (80°C, 72 h), weighed at room temperature and the lipid extracted using chloroform: methanol: water in the proportion 1:2:0.8. Adjustment of the ratio to 2:2:1.8 followed by low speed centrifugation (500 rcf, 3 min) ensured phase separation. The chloroform/ lipid phase was transferred to a micro-centrifuge tube and centrifuged at 11,000 rcf for 5 minutes, to remove any remaining cell debris. The sample was then placed in a pre-weighed vial and the chloroform evaporated using a heating block (60°C). The vial plus lipid mass was weighed at room temperature. Lipid weight was expressed as a percentage of the dry weight.

2.2.2. Nitrogen and phosphorus measurements

The term 'fertiliser nitrogen' is used in this work to describe nutrients supplied to the algae (nitrate, ammonium and urea). For cultures using 20 L photobioreactors with alternative nitrogen sources, the concentration of dissolved nitrate, ammonium, urea and nitrite were measured in each treatment every 48 hours. The sum of these measurements comprised the total dissolved nitrogen available to the growing algae.

For cultures using intensive bubble columns, nitrate was the only supplied nitrogen source. However, ammonium and nitrite were also measured in these cultures every 48 hours. In addition, dissolved organic nitrogen (DON, substances exuded by the algae) was measured at days 8 and 10 in flue gas treatments only. Analysis at other times was not possible due to the presence of high concentrations of nitrate that reduced analytical accuracy.

Dissolved nutrients in filtrates were measured after filtration through Whatman GF/F filters. Analysis of NO_3^-, NO_2^- and PO_4 was conducted using standard colorimetric methods (Grasshof et al, 1983) using a Lachat Instruments Quick-Chem flow-injection autoanalyser. Dissolved ammonium was analysed using the method of Holmes et al (1999) using a Hitachi F2000 fluorescence spectrophotometer. Dissolved urea was measured using the diacetylmonoxime method of Mulvenna & Savidge (1992), adapted for 5 ml sample sizes.

2.2.3. Dissolved organic matter

Dissolved organic matter was measured using samples filtered through Whatman GF/F filters (0.7 μm nominal pore size). In 20 L photobioreactors, dissolved organic carbon (DOC) was measured using using TOC-5000 Analyzer (Shimadzu) according to the Finnish standard SFS-ISO 8245. DOC samples from

bubble column photobioreactors was measured using a MQ1001 TOC analyzer according to Qian & Mopper (1996). Dissolved organic nitrogen (DON) was measured at days 8 & 10 of the bubble column experiments by subtraction of dissolved inorganic nitrogen species from the total dissolved nitrogen, the latter measured by peroxodisulphate oxidation and UV radiation at pH 9 and 100°C (Kroon 1993).

2.2.4. Photosystem II Fv/Fm

The performance of photosystem II was measured by the maximum quantum yield (Fv/Fm) using a pulse amplitude modulation (PAM) fluorometer (Water PAM, Walz, Germany) according to the saturation pulse method.

2.2.4. Temperature, dissolved oxygen & pH

Temperature, dissolved oxygen and pH were each measured using HACH HQ40d portable multi meter; temperature and pH with a HACH PHC101-03 IntelliCAL^{TM} probe and DO with a HACH LDO101-03 IntelliCAL^{TM} probe

2.2.5. Bacterial measurements.

Bacterial abundance was determined by staining with DAPI and examination using epifluorescence microscopy. The method is described in Hulatt & Thomas (2010).

2.2.6. Statistical Analysis.

The dry weight productivity was calculated using the equation $y = (d_i - d_f)/t$, where y is the growth rate (g L^{-1} d^{-1}), d_i and d_f are the concentrations (g L^{-1}) at two

time points, and t is the time between both measurements (days). Nitrogen uptake rates were calculated from the dissolved measurements in the same manner, using the total dissolved nitrogen concentration (μmol L^{-1}). Treatments compared where appropriate with two-sample and paired t-tests using Minitab v.14. An effect was considered significant at the 5% level.

3. Results and discussion

3.1. Nitrogen sources.

3.1.1.Growth and nitrogen uptake of *Dunaliella tertiolecta.*

Dunaliella tertiolecta cultures using ammonium, nitrate and urea reached 0.48 to 0.54 g L^{-1} dry weight at day 14 (Table 1). These values are comparable to the published literature considering the light path length, air concentration of CO_2 (0.04%) and irradiance (Chen et al., 2011). The productivity and cell density were comparable to similarly operated photobioreactors (Chiu et al., 2008) and to high-rate algal ponds (Lardon et al., 2009). Data presented in Fig. 1a indicates that dry weight at day 14 was lowest in the ammonium treatment. A similar pattern was observed by Xin et al., (2010) in cell counts of *Scenedesmus* cultivated using nitrate *vs.* ammonium. Uptake of nitrogen from the medium ranged from 181 (sd±10) to 274 (sd±14) μmol N L^{-1} d^{-1} for *Dunaliella tertiolecta*, shown in Table 1. Uptake of nitrogen in the urea treatment was significantly greater than the ammonium treatment ($t= 9.36$, $p= 0.003$), although there was no significant difference in nitrogen uptake between ammonium and nitrate ($t= 2.29$, $p= 0.15$), or between nitrate and urea ($t= 3.13$, $p= 0.052$).

Table 1. Effect of treatment (species and nitrogen source) on maximum dry weight growth rate (obtained between days 2 and 6), maximum dry weight density (at day 14) and maximum nitrogen uptake rate (during maximum growth). Data is mean (± standard deviation).

Species	N Source	Dry Weight Growth Rate days (2-6) $g\ L^{-1}\ d^{-1}$	End $g\ L^{-1}$	N uptake Rate days 2-6 $\mu mol\ d^{-1}$
Dunaliella tertiolecta	Nitrate	0.033 (0.010)	0.52 (0.07)	219 (27)
Dunaliella tertiolecta	Urea	0.038 (0.007)	0.52 (0.06)	274 (14)
Dunaliella tertiolecta	Ammonium	0.038 (0.004)	0.48 (0.02)	181 (10)
Chlorella vulgaris	Nitrate	0.046 (0.003)	0.53 (0.03)	229 (13)
Chlorella vulgaris	Urea	0.052 (0.012)	0.54 (0.06)	247 (32)
Chlorella vulgaris	Ammonium	/	/	/

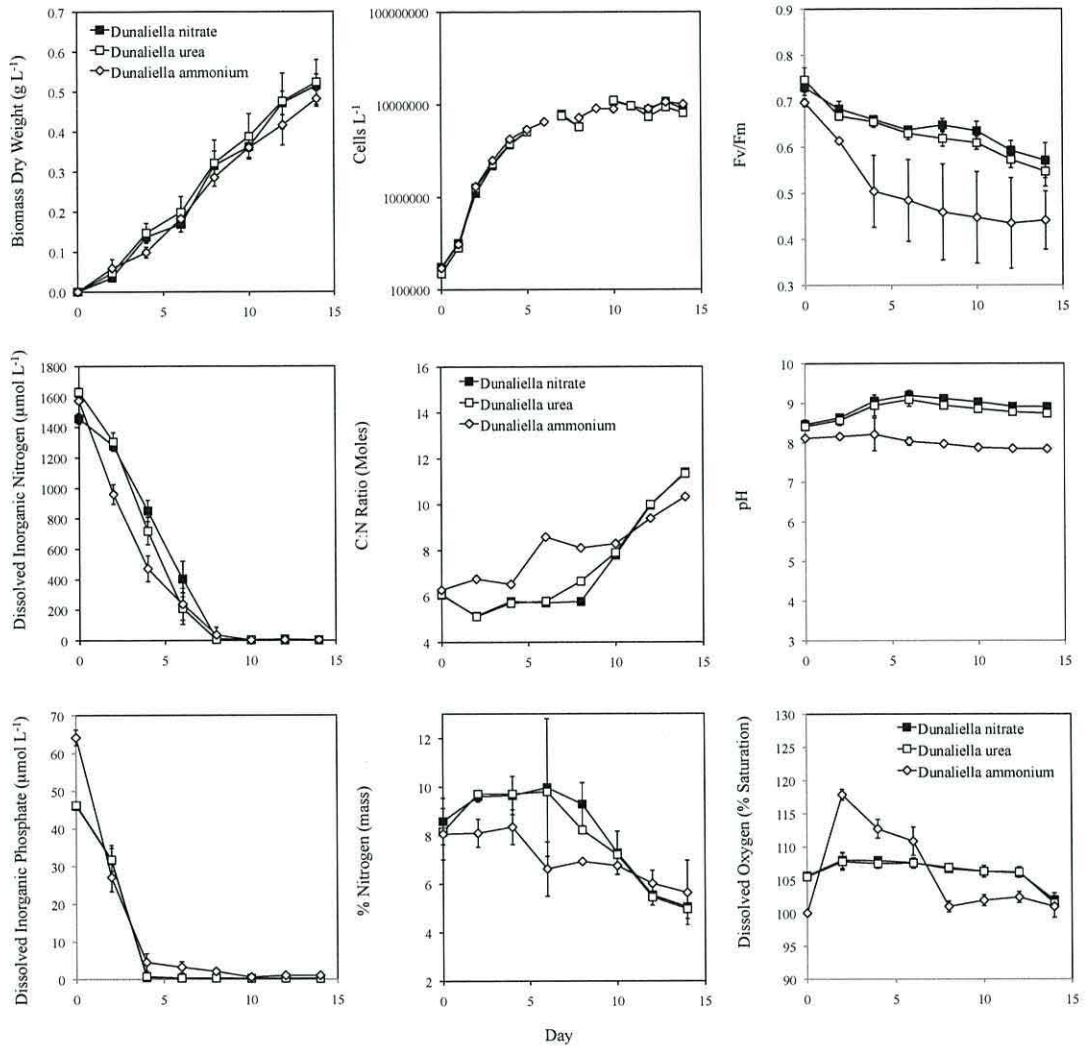


Figure 1a. Parameters measured during cultivation of *Dunaliella tertiolecta* in 20 L photobioreactors using either nitrate, urea or ammonium as the nitrogen source. For each point, n = 3, and error bars are standard deviations.

3.1.2. Growth and nitrogen uptake of *Chlorella vulgaris.*

Growth of *Chlorella vulgaris* supplied with ammonium was healthy for the first two days, but invariably collapsed between 3 and 4 days from the start of cultivation despite three repeated attempts (n= 9 photobioreactors). Loss of the cultures was associated with acidification, caused by Nitrification and/ or uptake of NH_4^+ (see section 3.1.7). The maximum dry weight attained by *Chlorella vulgaris* treatments was approximately the same in nitrate and urea treatments (Table 1, Fig 1b). The mean maximum growth rate (days 2-6) using nitrate was 12% lower than with urea, although there was no significant difference between treatments (t= 0.84, p= 0.489). Table 1 shows that the maximum uptake rate of dissolved nitrogen (days 2-6) in nitrate and urea treatments was comparable, there was no significant difference between treatments (t= 0.9, p= 0.462). When all treatments of both species were combined, there was positive correlation between maximum growth rates and maximum uptake of fertiliser nitrogen (R^2= 0.322).

Despite the similar growth in dry weight, cell densities were consistently higher in *Chlorella vulgaris* supplied with urea compared to *Chlorella vulgaris* supplied with nitrate. Further investigation found that *Chlorella vulgaris* supplied with urea produced smaller cells (mean 4.05 ±sd 0.26 ng $cell^{-1}$, day 6) than cultivation on nitrate (mean 6.35 ±sd 0.96 ng $cell^{-1}$, day 6). However, the mean difference was not significant (t = 3.99, p = 0.057).

To summarise, there was very little difference in growth performance between nitrogen source treatments. Cultivation of either species using urea offered a small but non-significant advantage over nitrate in terms of maximum growth rate and/ or maximum biomass attained. In the context of industrial production, there thus seems little to choose between use of urea, nitrate and (in the case of *Dunaliella tertiolecta*)

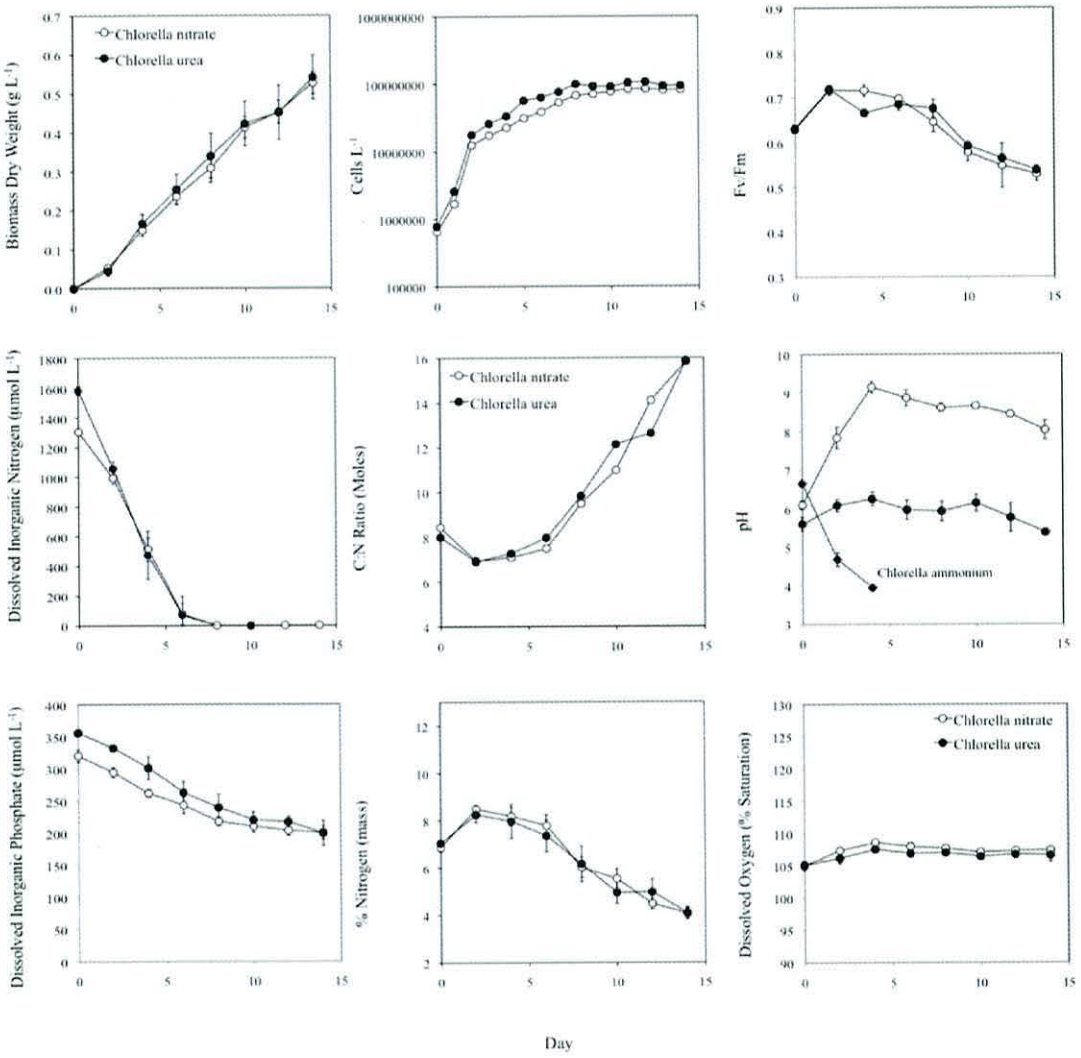


Figure 1b. Parameters measured during cultivation of *Chlorella vulgaris* in 20 L photobioreactors using either nitrate or urea as the nitrogen source. For each point, n = 3, and error bars are standard deviations.

ammonium. Ammonium is unlikely to be an appropriate nitrogen source for *Chlorella vulgaris* (and likely many other freshwater species) where high cell densities and un-buffered solutions are used. Selection of nitrogen fertiliser source could be solely in terms of environmental impacts and economics.

3.1.3. *Dunaliella tertiolecta* biomass composition.

All treatments for both species remained nitrogen-replete between days 2 to 6, and this is reflected in the nitrogen content and C:N ratios presented in Fig. 1a. The nitrogen content of *Dunaliella tertiolecta* during nutrient-replete growth (day 4) ranged from 8.35 (sd ±0.72) to 9.77 (sd ±2.13) %. Sources of nitrogen in the media were removed (<5 μmol L^{-1}) between days 6-8 in all treatments. By the end of the cultivation period, the nitrogen content of *Dunaliella tertiolecta* biomass was depleted to approximately half of the optimal values (Fig. 1a).

Corresponding biochemical properties for specific parts of the cultivations are shown in Table 2. Day 4 corresponds to nutrient-replete conditions, day 8 to the point of nitrogen exhaustion in the media and day 14 to the nitrogen-deprived stage at the end of the experiment. The protein content decreased from 37.1 to 42.5% at day 4 to 22.1 to 25% by day 14. The calorific values showed a very minimal increase over the same period, and there was little evidence for any lipid accumulation toward the end of the experiment (Table 1). Thus, by deduction, the primary response to nitrogen limitation by *Dunaliella tertiolecta* was to assimilate carbohydrates rather than lipids. Chen et al (2011) identified values of 15 to 18 % lipid per unit dry mass for nutrient replete and deprived *Dunaliella tertiolecta* biomass respectively. Values from this work were very comparable, in the range 14.6 to 18.6 %.

3.1.4. *Chlorella vulgaris* biomass composition.

The mean nitrogen content of *Chlorella vulgaris* at day 4 was 8.18 (0.40) and 7.97 (0.71) % for nitrate and urea treatments respectively (Fig 1b). Protein concentrations (Table 2) followed a similar dynamic to the *Dunaliella tertiolecta* cultures. However, both the calorific value and lipid content increased in response to nitrogen limitation in the *Chlorella vulgaris* treatments (Table 2). Paired t-tests between days 4 and 14 identified a significant increase in lipid content ($t \geq 2.8$, $p \leq 0.047$) and heating value ($t \geq 3.7$, $p \leq 0.004$). Considering samples for both species contained below 40% lipid, evidence from Sialve et al., (2008) suggests that it would be energetically favourable to subject the whole biomass to anaerobic digestion, rather than to first extract the lipid for biodiesel production.

Both species cultivated in nutrient-replete conditions thus had very similar crude biochemical properties, but responded differently to nitrogen limitation. Whilst *Dunaliella tertiolecta* predominantly produced carbohydrates and had a relatively constant higher heating value, *Chlorella vulgaris* assimilated lipids in response to nitrogen limitation and the consequently had enhanced heating values, making this species more favourable for commercial lipid production in this respect.

Apart from species-specific differences in response to nitrogen limitation, the different responses of the two species might be attributable to the nutrient media compositions used. For example, *Dunaliella tertiolecta* utilised all of the available inorganic phosphorus within 4 days of cultivation, but *Chlorella vulgaris* remained phosphorus-replete throughout (Fig. 1a & 1b). Part of this discrepancy in phosphate utilisation may be the accumulation of polyphosphates by *Dunaliella tertiolecta*, meaning that phosphate depletion in the medium might have a negligible effect on growth (Pick & Weiss, 1991; Chen et al., 2011). Differences in nutrient balance may

Table 2. Biochemical properties (lipid, protein and heating value) at days 4, 8 and 14 of the cultivation period. Day 4 corresponds to nutrient-replete growth, day 8 the approximate point of nitrogen exhaustion and day 14 the experiment end point. Data is mean (± standard deviation).

Species	N Source	Day 4			Day 8			Day 14		
		protein %	lipid %	HHV KJ g^{-1}	protein %	lipid %	HHV KJ g^{-1}	protein %	lipid %	HHV KJ g^{-1}
Dunaliella tertiolecta	Nitrate	42.5 (3.8)	16.0 (8.7)	22.5 (0.4)	41.2 (4.7)	14.9 (1.8)	21.5 (0.8)	22.4 (2.1)	14.6 (1.2)	23.2 (0.3)
Dunaliella tertiolecta	Urea	43.1 (1.3)	18.2 (0.8)	22.0 (0.1)	36.5 (6.5)	18.2 (0.8)	21.1 (0.6)	22.1 (2.3)	17.9 (3.4)	22.1 (0.4)
Dunaliella tertiolecta	Ammonium	37.1 (3.2)	18.6 (8.8)	22.7 (0.9)	30.7 (0.4)	18.6 (8.8)	22.8 (0.3)	25.0 (5.9)	14.7 (0.7)	22.9 (0.5)
Chlorella vulgaris	Nitrate	36.3 (1.8)	21.0 (1.6)	23.8 (1.2)	26.7 (1.9)	18.4 (1.6)	23.1 (1.3)	18.1 (1.0)	27.0 (1.5)	26.6 (0.5)
Chlorella vulgaris	Urea	35.4 (3.2)	20.9 (2.3)	23.6 (0.3)	27.3 (3.3)	22.3 (5.7)	24.5 (1.2)	18.1 (1.1)	27.9 (3.6)	26.8 (0.9)
Chlorella vulgaris	Ammonium	/	/	/	/	/	/	/	/	/

cause significant physiological effects on cell biochemistry, including lipid, protein and carbohydrate content, plus the classes of those compounds (Geider & Roche 2002). The media used in this work remained standard, but there is the potential to adjust the dominant limiting nutrients in future work.

Growth in dry weight of both species continued at a similar rate toward the end of the experiments (Fig. 1a & 1b), despite depletion of nutrients and subsequent impacts on biochemical composition. It has been assumed that either microalgae can be grown in nitrogen sufficient conditions at high growth rates, or in nitrogen depleted conditions at low growth rates (Lardon et al., 2009). Extreme nitrogen (and other nutrients e.g. phosphorus) limitation will undoubtedly impact productivity, however the data here for batch cultivations shows that the nitrogen content of algal biomass could be depleted to around half its optimal value without sacrificing dry mass production. This implies that the optimal harvest point for microalgal cultivation may be somewhere in the early nitrogen-limited phase.

3.1.5. Dissolved organic carbon and bacterial cell number

The concentration of DOC in 20 L photobioreactor cultivations using alternative nitrogen sources is shown in Figure 2. These figures are absolute values, but note that the urea treatments contained 0.665 mmol L^{-1} of added organic carbon at the start. Taking this into account, the difference in DOC concentrations between treatments was minimal, although production of DOC in the ammonium treatment between days 2 to 6 was higher than in the corresponding nitrate and urea treatments. The highest DOC concentration in *Dunaliella tertiolecta* treatments was attained in the ammonium treatments with mean 1.69 (SD±0.47). The mean DOC value measured 9% of the total organic carbon in the system. For *Chlorella vulgaris*, the

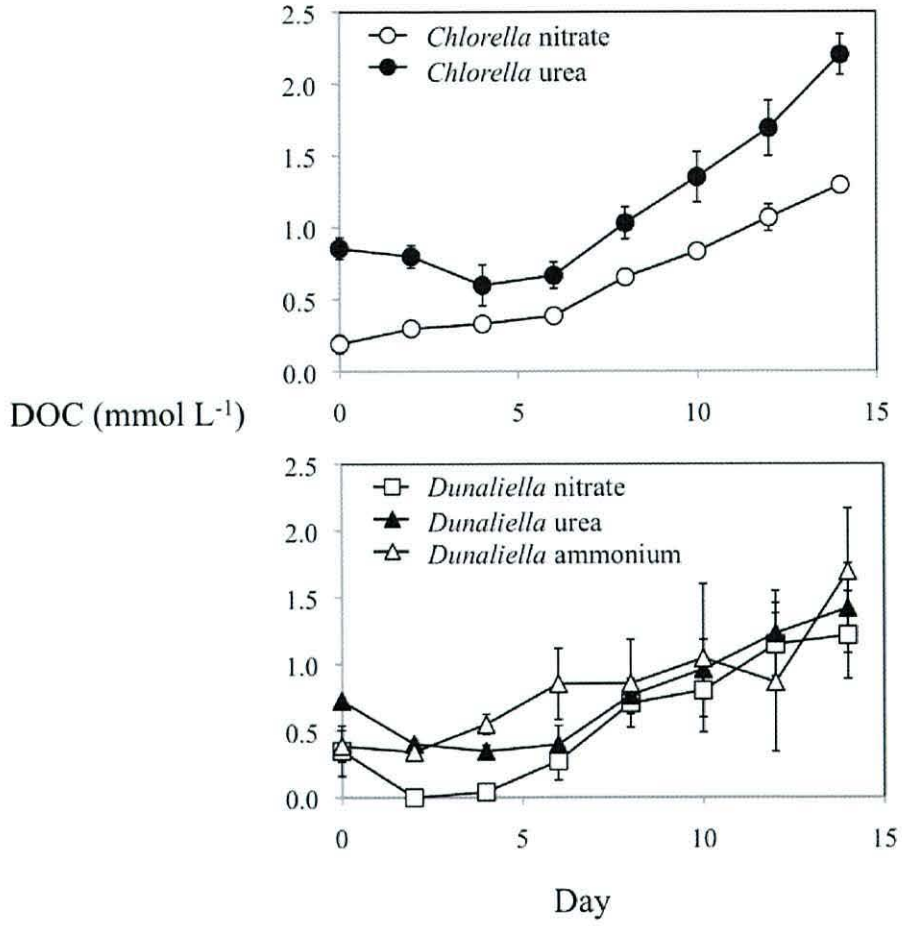


Figure 2. The concentration of DOC (mmol L^{-1}) in 20 L photobioreactor cultivations of *Chlorella vulgaris* (Nitrate, Urea) and *Dunaliella tertiolecta* (Nitrate, Urea, Ammonium). Data points are mean of 3 replicates, error bars are standard deviations.

highest DOC concentrations were observed at the end of cultivation using urea (Fig. 2). In this case, the mean DOC accounted for 7.4% of the organic carbon in the photobioreactor.

Bacterial cell numbers in cultures of each species are shown in Fig. 3. Comparable bacterial dynamics were observed in nitrate and urea treatments for each species, with bacterial cell number increasing over the duration of the experiment. In contrast, bacterial cell numbers in the *Dunalliella tertiolecta* ammonium treatment were much higher than the corresponding nitrate and urea treatments, which may have been stimulated by the higher DOC concentrations observed previously. By the end of the experiments, bacterial cells numbered 1.1×10^{11} (sd $\pm3.2\times10^{10}$) cells L^{-1} in the ammonium treatment, compared to just 2.4×10^{10} (sd $\pm4.0\times10^{9}$) and 2.2×10^{10} (sd $\pm7.6\times10^{9}$) cells L^{-1} in the urea and nitrate treatments. Bacteria were mostly observed as free-living single cells, although some bacterial cell aggregations were observed. Bacteria may only be a small fraction of the total biomass present in healthy algal cultures, but will be important for recycling organic nitrogen compounds to biologically utilisable forms.

3.1.6. *Dunaliella tertiolecta* accessory measurements.

Changes in the maximum quantum yield of photosystem II (Fv/Fm) during cultivation of *Dunaliella tertiolecta* are shown in Fig. 1a. Initial values ranged from 0.70 (sd ±0.01) to 0.75 (sd ±0.03) indicating healthy, efficient photosynthetic performance (Parkhill et al., 2001). Fv/Fm values declined with time, reaching 0.44 (0.06) to 0.57 (0.04) by the end of the experiment. There was little evidence for any difference in Fv/Fm dynamics between nitrogen treatments. In order to test whether Fv/Fm could be used as real-time measurement of nitrogen stress, Fv/Fm values were

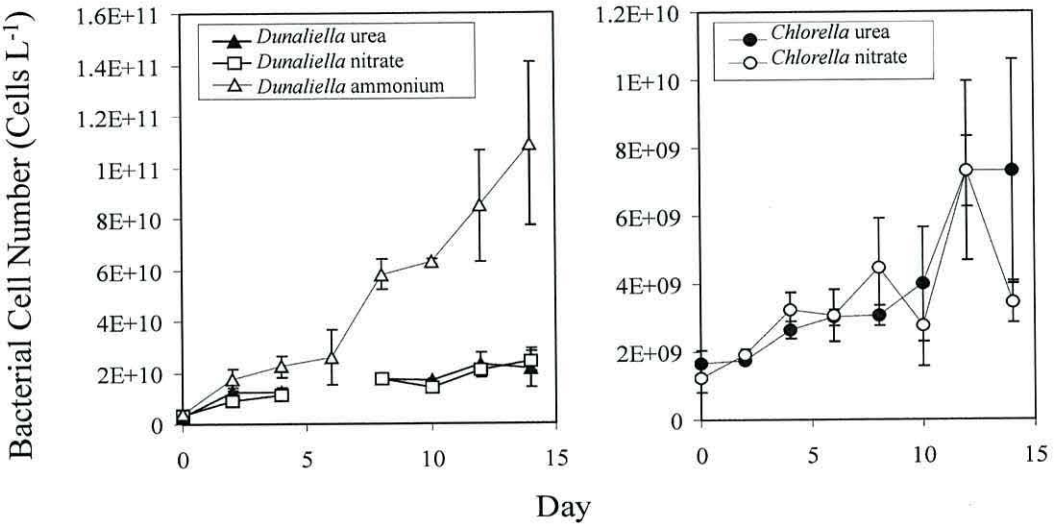


Figure 3. Bacterial cell number (cells L^{-1}) in cultures of *Chlorella vulgaris* and *Dunaliella tertiolecta* supplied with alternative nitrogen sources. Data points are mean of 3 replicates, error bars are standard deviations.

correlated with the biomass nitrogen content by combining all *Dunaliella tertiolecta* treatments together. There was positive correlation, but the relationship was not especially strong (R^2=0.287), suggesting that application of this method would be limited, even in such controlled conditions. Fv/Fm values for ammonium source cultivations were lower than the nitrate and urea treatments (Fig 1a).

Changes in the pH of *Dunaliella tertiolecta* treatments were minimal due to the relatively high buffering capacity of the saline media. Cultivation using ammonium lead to a mild acidification (mean ΔpH -0.27), whilst cultivation using urea and nitrate lead to an increase in mean pH of 0.68 and 0.73 respectively (Fig.1a).

Dissolved oxygen concentrations in 20 L photobioreactors containing either species remained <20% above air saturation throughout cultivation (Fig. 1a), indicating that dissolved oxygen had little impact on cultivation performance. These values are a result of efficient removal due to the high sparging rate employed.

3.1.7. *Chlorella vulgaris* accessory measurements.

The Fv/Fm dynamics in cultivations of *Chlorella vulgaris* followed a similar trend to those of *Dunaliella tertiolecta* (Fig. 1b). There was little difference between nitrate and urea treatments. Changes in the culture pH were as shown in Fig.1b, including the *Chlorella vulgaris*/ ammonium treatment that failed to grow beyond day 4. Cultivation of *Chlorella vulgaris* using urea caused an increase in mean pH of 0.65 units, whilst cultivation in nitrate led to an increase of 3.05. The pH of microalgal cultivations is modified both by the uptake of inorganic carbon from the medium (increases pH), nitrification of ammonium treatments, and also by microalgal uptake of nitrogen compounds. Uptake of nitrogen compounds follows charge balance: Ammonium uptake leads to H^+ production (decreases pH), whilst uptake of NO_3^-

produces OH^- (increases pH) (Goldman & Brewer, 1980). Since urea uptake should not affect the pH significantly (Goldman & Brewer, 1980), the increase in pH in urea treatments of both species can be attributed to the depletion of dissolved inorganic carbon. The pH changes in nitrate treatments of both species were higher than the equivalent urea treatments and can be attributed to the additive effect of nitrate uptake plus inorganic carbon depletion.

The rapid acidification (pH 3.95 ±0.01 after 4 days) observed in the *Chlorella vulgaris*/ ammonium treatment was attributed to nitrification by bacteria and/ or NH_4^+ uptake coupled with the low buffering capacity of the freshwater medium. Nitrification may be an important process when using ammonium as a nitrogen source. Evidence from Fig. 2 showed strong bacterial growth in the *Dunaliella tertiolecta* ammonium treatment, suggesting that nitrification of ammonium to nitrite (equation 1.) and nitrite to nitrate (equation 2.) may have contributed to a decrease in alkalinity and pH.

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+ \quad (1)$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (2)$$

Buffering solutions, or acid/ base addition, may be used to stabilise the pH of microalgal cultures (Tam & Wong, 1996). However, this is unlikely to be practical for large-scale algal production involving considerable volumes of liquid medium, together with additional cost, and was not considered an appropriate avenue for further investigation. Cultivating freshwater species to high cell density using ammonium as the sole nitrogen source thus seems impractical for commercial production.

3.2. Bubble Columns

3.2.1 Growth and nitrogen uptake in bubble columns

Growth expressed in dry weight of *Chlorella vulgaris* and *Dunaliella tertiolecta* cultivated intensively in bubble columns using nitrate as the nitrogen source are shown in Fig. 4. Treatments using simulated flue gas (12% CO_2) grew rapidly, at maximum rates of 0.60 (sd ±0.11) and 0.83 (sd ±0.13) g L^{-1} d^{-1} for *Chlorella vulgaris* (days 4 to 6) and *Dunaliella tertiolecta* (days 2 to 4) respectively. Treatments cultivated in 0.04% CO_2 were carbon–limited, and never reached the carrying capacity of the available nitrogen and phosphorus (Fig. 4). Growth rates in air were 0.10 (sd ±0.04) and 0.07 (sd ±0.02) g L^{-1} d^{-1} for *Chlorella vulgaris* and *Dunaliella tertiolecta,* respectively.

Dunaliella tertiolecta and *Chlorella vulgaris* cultivated in 12% CO_2 had mean nitrogen uptake rates of 3.14 (sd ±0.79) and 1.26 (sd ±0.88) mmol N L^{-1} d^{-1} during the period of maximum growth, so that >99.9% of the available nitrogen was removed between days 4 to 6 (Fig. 4). Cultures of *Dunaliella tertiolecta* and *Chlorella vulgaris* using 0.04% CO_2 assimilated inorganic nitrogen from the medium at a rate of 0.21 (sd±0.17) and 0.14 (sd±0.16) mmol N L^{-1} d^{-1}. The higher heating value of *Chlorella vulgaris* treatments increased in response to nitrogen limitation, but as in the 20 L photobioreactor experiments the effect on *Dunaliella tertiolecta* was negligible (Fig. 3).

3.2.2. DOC & DON

The concentrations of DOC and DON during batch cultivations using 12% CO_2 are shown in Fig 5. The concentration of DOC increased over the batch cycle, reaching 17.19 (4.37) and 6.03 (2.49) mmol L^{-1} at the end of *Dunaliella tertiolecta*

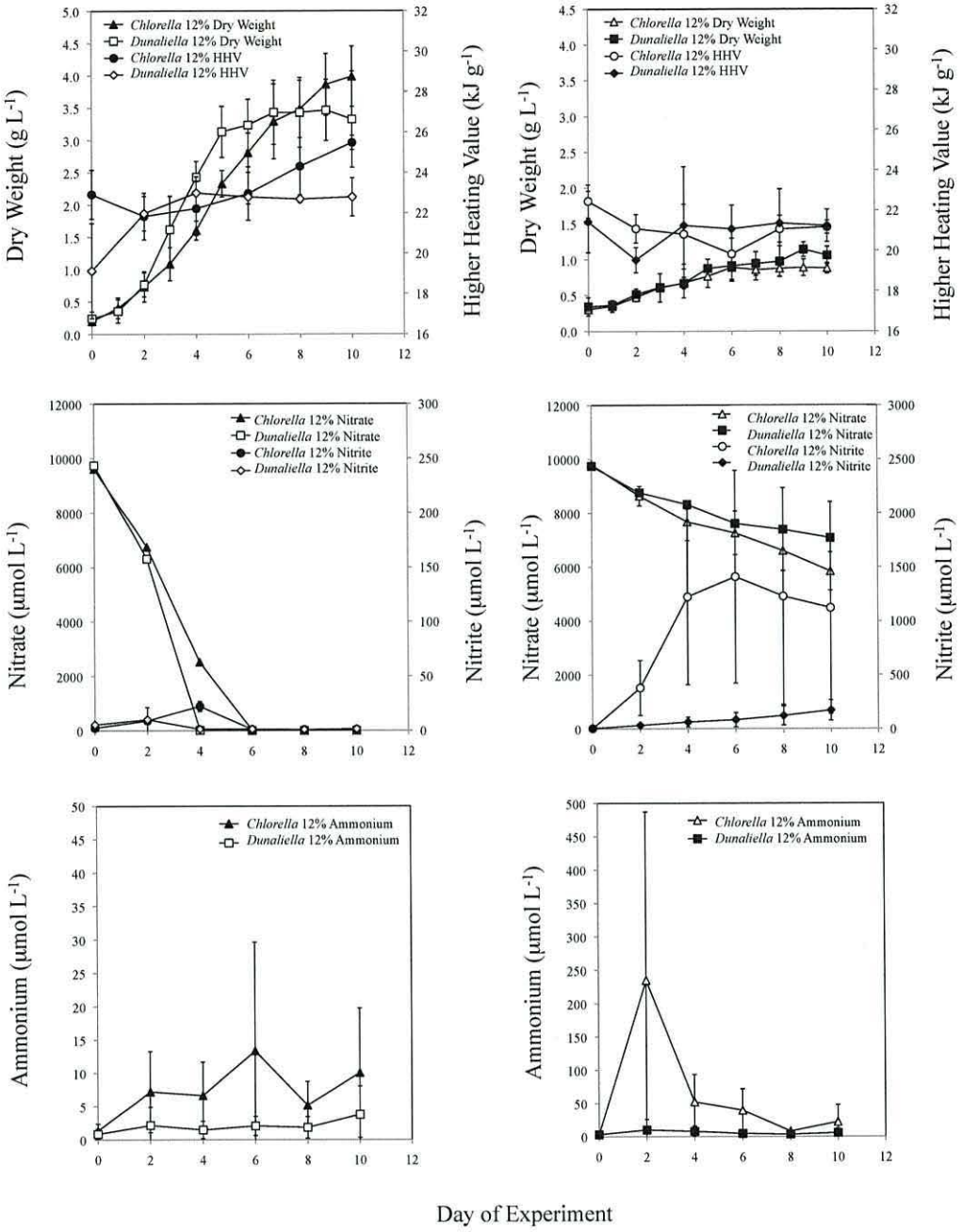


Figure 4. Growth performance of *Chlorella vulgaris* and *Dunaliella tertiolecta* cultures in bubble column experiments using simulated flue gas (12%) and air CO_2 concentrations (0.04%). Data points are mean (n= 3), error bars are standard deviations.

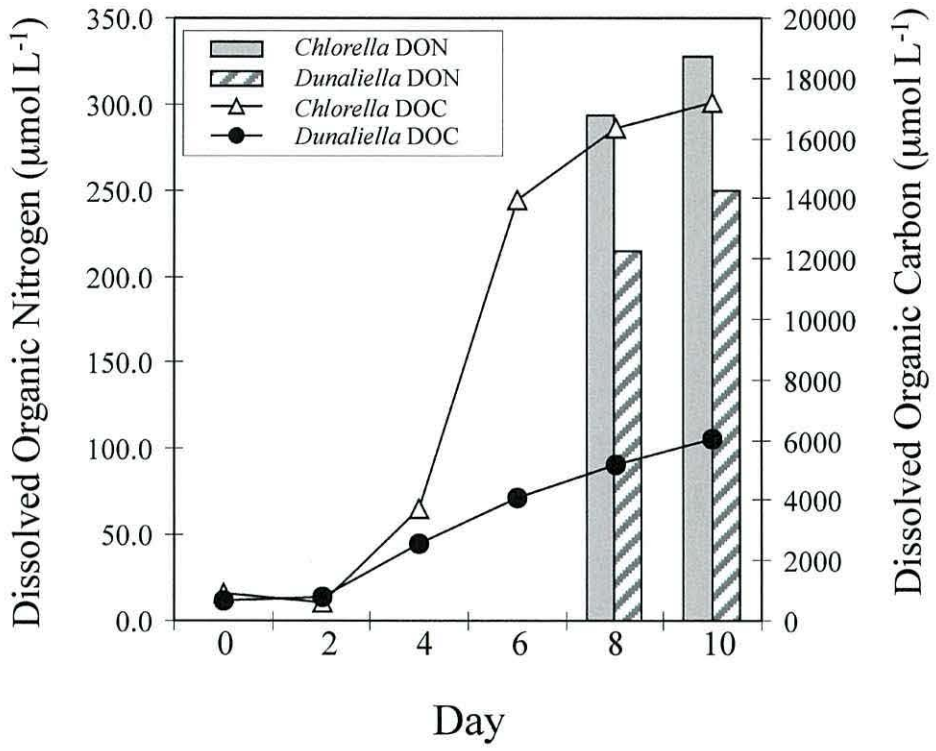


Figure 5. Dissolved organic carbon and dissolved organic nitrogen in bubble column cultures of *Chlorella vulgaris* and *Dunaliella tertiolecta* supplied with simulated flue gas (12% CO_2). Data is mean of 3 replicates.

Table 3. Fossil energy required to produce 1 kg of fertiliser elemental nitrogen (MJ kg^{-1}) as reported for various fertiliser types in the literature.

MJ/kg Nitrogen	End Product	Reference
35	Ammonium nitrate [a]	Ahlgren et al, 2010
2.3-3.7	Ammonium nitrate [b]	Ahlgren et al, 2010
approx 40-50	Ammonia, urea, ammonium nitrate, calcium ammonium nitrate [c]	Ramirez & Worrell, 2006
43-78 (norm 50)	*Nitrogen [d]	Dalgaard et al 2001 (references therein)
50	*Nitrogen [e]	Kongshaug 1998 (cited in Lewandowski & Schmidt, 2006)
41	Ammonium nitrate [f]	Williams et al 2010
49	Urea [f]	Williams et al 2010
43	Ammonium sulphate [f]	Williams et al 2010

[a] Natural gas plant, modelled for fossil energy
[b] Biogas plant, modelled for fossil energy
[c] Based on most recent data (2000-on)
[d] Not including diesel contribution to application, all references pre-1991
[e] Cumulative primary energy consumption
[f] Includes burdens of producing packing and delivering

Table 4. Energetic performance of bubble column photobioreactors over the batch production cycle (kJ L^{-1}).

Species	Day	Gross	minus Nitrogen Burden	minus Nitrogen and Power Burden ($50Wm^{-3}$)	minus Nitrogen and Low-Power Burden ($15Wm^{-3}$, Theoretic)
Chlorella vulgaris	0	0	0	0	0
	2	11.6	9.3	-0.2	6.7
	4	31.1	25.9	6.7	20.8
	6	59.7	52.7	24.3	45.1
	8	79.7	72.7	35.9	62.6
	10	96.9	89.9	44.5	77.2
Dunaliella tertiolecta	0	0	0	0	0
	2	12.4	10.3	1.7	7.8
	4	51.3	44.9	27.6	39.7
	6	69.1	62.1	36.2	54.4
	8	73.2	66.2	31.7	55.8
	10	71.4	64.4	21.3	51.5

*Gross energy is the biomass energy contained in the photobioreactor. Subsequent columns indicate the effect of subtracting the nitrogen energy demand, the nitrogen and power input demand. The right-hand column indicates the combined effect of nitrogen fertiliser and power input, if the sparging power input could be reduced from 50 to 15 Wm^{-3} whilst maintaining the same bio-energy productivity.

and *Chlorella vulgaris* culture experiments, respectively. At day 10 the concentration of DON reached 0.25 (0.04) and 0.33 (0.08) mmol L^{-1} for the same species, corresponding to mean C:N ratios of 69.0:1 and 18.4:1. The C:N ratio of dissolved organic matter in 12% CO_2 cultures of *Chlorella vulgaris* at day 10 (18.4:1) was approximately the same as the corresponding cellular C:N ratio at the same point (18.5:1). In contrast, the dissolved substances exuded by *Dunaliella tertiolecta* at the same point had a C:N ratio of 69.0:1, almost 5 fold higher than the cellular C:N ratio (14.1:1).

An important difference between species is that *Chlorella vulgaris* possesses a rigid cell wall, whilst *Dunaliella tertiolecta* does not. This physical difference may be the cause of higher concentrations of DOC released by *Dunaiella tetiolecta*, and may also explain the differences in C:N ratio of dissolved substances exuded by either species. The difference in C:N ratio may also be due to differences in the relative utilisation of DOC and DON by heterotrophic bacteria (section 3.1.5).

3.2.3. Nitrite & ammonium dynamics

The presence of bacteria (section 3.1.5) and release of DON from cells suggests that a fraction of the nitrogen initially supplied as a fertiliser is cycled within the cultivation system. Both nitrite and ammonium are products of bacterial metabolism of organic nitrogenous substrates, and Fig. 4 shows the concentration of each of these compounds during cultivation in bubble columns. In 12% CO_2 treatments, where nitrogen was utilised rapidly, there was limited accumulation of ammonium and nitrite ($\leq$22 μmol L^{-1}).

Bacteria are an important component of microalgal cultures, where they may compete for fertiliser nitrogen, re-cycle algal-derived organic nitrogen to forms that

can be easily utilised by phototrophs (Patil et al., 2010) and assimilate a fraction. They also have other roles in supplying essential vitamins to algae (Croft et al., 2005). However, the implications of bacterial activity for applied microalgal cultivation has received little research to date, and clearly is an area for further experimentation.

3.3. Industrial scale nitrogen consumption and energy demands.

3.3.1. Nitrogen mass requirements; areal and industrial perspectives.

The nitrogen content of nutrient-replete microalgae is relatively constant, and has received widespread recognition in the field of phytoplankton ecology. The Redfield ratio (C:N:P, molar) of 106:16:1 (6.63:1 for C:N) is the universal approximation for nutrient-replete phytoplankton elemental composition (Geider & Roche 2002; Klausmeier et al., 2008; Redfield, 1958). The ratio is an average, so nitrogen-replete C:N ratios for individual species lie close to, but slightly above and below the Redfield approximation (see section 3.1.3 for *Dunaliella tertiolecta* and *Chlorella vulgaris*). Since microalgae comprise a relatively constant 50% (±5%) carbon by mass, the maximum predicted nitrogen and phosphorus requirement equates to approximately 0.088 kg elemental nitrogen (and 0.012 kg elemental phosphorus) per kg of dry microalgal biomass. Assuming that the nitrogen in algal biomass could be depleted to around 50% of its optimal value without significantly impacting productivity (evidence from 20 L photobioreactors), then this would double the nitrogen utilisation efficiency.

3.3.2. How important is the nitrogen energy burden?

One of the main questions arising from life cycle assessments concerns identifying the dominant energy burdens involved with microalgal cultivation, and thereby identifying which components should be targeted for improvement. The

relationship between different aspects of microalgal cultivation is complex and case-specific. (see Lardon et al., 2009; Jorquera et al., 2009, Clarens et al., 2010, Stephenson et al., 2010). Supply of fertiliser nitrogen has been suggested as the major variable determining the sustainability of microalgal technologies (Lardon et al., 2009; Clarens et al., 2010). However others have suggested it is the supply of power for cultivation mixing that is the dominant burden (Stephenson et al., 2010; Jorquera et al., 2009). The following examines the relative importance of power input and fertiliser requirement in the intensive bubble column reactors.

The relative impacts of fertiliser nitrogen and power input on energy production by *Chlorella vulgaris* and *Dunaliella tertiolecta* in bubble column photobioreactors (section 3.2) using 12% CO_2 was calculated. The energy consumption for gas-sparging was calculated according to Sierra et al., (2008) at 50 Wm^{-3} (4.3 kJ L^{-1} d^{-1}). This is in the usual range for gas-sparged reactors (Reyna-Velarde et al., 2010). Table 4 shows the gross energy (kJ L^{-1}) production (dry weight multiplied by the higher heating value) during batch cultivation of *Chlorella vulgaris* and *Dunaliella tertiolecta*, plus the cumulative effect of subtracting the nitrogen fertiliser energy demand (uptake from the medium multiplied by the energy demand for nitrogen fertiliser production (50 kJg^{-1}) Table 3) and the sum of the nitrogen demand and power consumption combined. At day 10, the supply of nitrogen to *Chlorella vulgaris* and *Dunaliella tertiolecta* reduced the gross energy production by 7.2 and 9.8%. However in this reactor configuration, the supply of power far outweighed the impact of nutrient energy consumption. At day 4 of *Dunaliella tertiolecta* cultivation, consumption of fertiliser nitrogen accounted for a peak of 27.1% of both energy burdens combined. After days 4-6, when nitrogen was exhausted from the media, power became even more dominant and accounted for 86% of the energy consumed

in production in both species cultivations at day 10. It is possible to operate gas-sparged reactors using power inputs between 2.5 and 5 fold lower than commonly used (Hulatt and Thomas, 2011; Zhang et al., 2001). Furthermore, by reducing the power input to 15 Wm^{-3} (as opposed to the 50 Wm^{-3}) the fertiliser energy demand would equal the power demand during nutrient-replete growth (days 2-6), but drop to 50 % of the power demand by day 10 (Table 4). This calculation assumes the same gross energy production is possible in low power input, although in practice it may be a little lower (Hulatt & Thomas, 2011).

3.3.3. Optimal harvest point

Several approaches to nitrogen utilisation have been proposed, although the research was targeted at lipid production rather than nitrogen consumption. Lardon et al., (2009) modelled two scenarios where microalgae were grown in either nitrogen replete, or nitrogen–deprived medium, finding that the latter case was energetically favourable for producing lipids. The optimal harvest point needs to be established, in order to minimise the requirement for fertiliser inputs and energy into microalgal production systems. Clearly the nitrogen utilisation efficiency is important, but so too is its impact on growth rate, photosynthetic efficiency, lipid content and calorific value. Also, the energy efficiency of microalgal cultivation is sensitive to the energy input for mixing/ circulating the culture (Hulatt & Thomas, 2011), so that this factor will also interact with each of the others. The relative impacts of each of the parameters are presented in Table 5. The impacts of nitrogen limitation on photosynthetic efficiency are not well described within the context of microalgal biomass production. This is important because microalgal technologies are usually

Table 5. Relative advantages (+) and disadvantages (-) of batch and continuous production processes on performance of microalgal cultivations.

	Continuous production	Batch Production
Nitrogen Utilisation Efficiency	-	+
Heating Value	-	+
Lipid Content	-	+
Productivity	+	-
Photosynthetic Efficiency	+	-
Power Input per Unit Biomass	+	-

justified on this point, and any negative impacts effectively reduce competitiveness with other crops.

One advantage microalgae have over conventional crops is that fertilisers supplied should not be lost via run-off processes. Losses of ammonia from microalgal cultivations to the atmosphere via volatilisation are possible, although Vonshak & Richmond (1988) showed that this was insignificant. Denitrification of nitrate may occur (Vonshak & Richmond, 1988), but this is unlikely to be significant in well-oxygenated microalgal cultivations.

3.3.5. Alternative nitrogen fertiliser supply

In this work we considered that supply of fertilisers would be by artificial fossil-energy sources. However, there are a number of technologies available which may improve process efficiency. Nutrients may be re-cycled by anaerobic bioprocesses used to produce energy carriers (CH_4 & H_4) (Lakaniemi et al., (in submission); Sialve et al., 2009) e.g. in integrated systems (Oswald & Golueke, 1960; Del Schamphelaire & Verstraete 2009). For example, ammonium concentrations at the end of anaerobic processes may reach 20-30 mmol L^{-1} (Carver et al., 2010). Nutrients may also be recycled via residues from thermochemical conversion processes, particularly pyrolysis and gasification (Grierson et al., 2008; Brown et al., 2010). Nutrients may be supplied via waste water streams (Clarens et al., 2010). However, this may present some problems due to security of supply (quantity, nutrient loading) and availability (transporting large volumes of water with very dilute nutrients is inefficient).

5. References.

Bauen, A. 2006. Future energy sources and systems- Acting on climate change and energy security. Journal of Power Sources. 2, 893-901.

Bligh, E., Dyer, W. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Physiology and Pharmacology. 37, 911-917.

Brown, T., Duan, P., Savage, P. 2010. Hydrothermal liquefaction and gasification of *Nannochloropsis* sp. Energy and Fuels. 24, 3639-3646.

Carballo-Cardenas, E., Tuan, P., Janssen, M., Wijffels, R., 2003. Vitamin E (alpha-tocopherol) production by the marine microalga *Dunaliella tertiolecta* and *Tetraselmis suecica* in batch cultivation. Biomolecular Engineering. 20, 139–147.

Carlson, C., 2002. Production and removal processes. In: Carlson, C., Hansell, D. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, London, pp. 91–139.

Carver, S., Hulatt, C., Thomas, D., Tuovinen, O. 2010. Thermophilic, anaerobic co-digestion of microalgal biomass and cellulose for H_2 production.

Chen M., Tang., H, Ma., H, Holland., T, Ng., K, Salley, S. 2011. Effects of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology. 102, 1649-1655.

Chisti, Y. 2007. Biodiesel from microalgae. Biotechnology Advances. 25, 294-306.

Chiu, S., Kao, C., Chen, C., Kuan, T., Ong, S., Lin, C., 2008. Reduction of CO_2 by a high-density culture of *Chlorella sp.* in a semicontinuous photobioreactor. Bioresource Technology. 99, 3389–3396.

Clarens, A., Resurrecion, E., White, M., Colosi, M. 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environmental Science and Technology. 44, 1813-1819.

Croft, M., Lawrence, A., Raux-Deery, E., Warren, M., Smith, A. 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature. 438, 90-93.

Dalgaard, T., Halberg, N., Porter, J. 2001. A model for fossil energy use in Danish agriculture used to compare organic and conventional farming. Agriculture, Ecosystems and Environment. 87, 51-65.

De Schamphelaire., Verstraete, W. 2009. Revival of the Biological Sunlight-to-Biogas Energy Conversion System. Biotechnology and Bioengineering. 103, 296-304.

Dismukes, G., Carrieri, D., Bennette, N., Ananyev, G., Posewitz, M., 2008. Aquatic phototrophs: Efficient alternatives to land-based crops for biofuels. Current Opinion in Biotechnology. 19, 235–240.

Fabregas, J., Abalde, J. Herrerco, C. 1989. Biochemical composition and growth of the marine microalga *Dunaliella tertiolecta* (Butcher) with different ammonium nitrogen concentrations as chloride, sulphate, nitrate and carbonate. Aquaculture, 83, 289-304.

Falkowski, P., Raven, J., 2007. Aquatic Photosynthesis. Princeton University Press, New Jersey.

Fargione, J., Hill, J., Tilman, D., Polasky, S., Hawthorne, P. 2008. Land Clearing and the Biofuel Carbon Debt. Science. 319, 1235-1238.

Friedl, A., Padouvas, E., Rotter, H., Varmuza, K., 2005. Prediction of heating values of biomass fuel from elemental composition. Analytica Chimica Acta. 544, 191–198.

Geider, R., Roche, J. 2002. Redfield revisited: Variability of C:N:P in marine microalgae and its biochemical basis. European Journal of Phycology. 37, 1-17.

Goldman, J., Brewer, P. 1980. Effect of nitrogen source and growth rate on phytoplankton-mediated changes in alkalinity. Limnology and Oceanography. 25, 352–357.

Grasshoff, K., Ehrhardt, M., Kremling, K. 1983. Methods of seawater analysis, 2nd Edition. Verlag Chemie, Wienheim, Germany, pp. 419.

Grierson, S., Strezov, V., Ellem, G., Mcgregor, R., Herbertson, J. 2009. Thermal characterization of microalgae under slow pyrolysis conditions. Journal of Analytical and Applied Pyrolysis. 85, 118-123.

Griffiths, M., Harrison, S. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. 21, 493-507.

Hsieh, C., Wu, W. 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresource Technology. 100, 3921-3926.

Hill, J., Nelson, E., Tilman, D., Polasky, S., Tiffany, D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol fuels. Proceedings of the National Academy of Sciences of the United States of America. 25, 11206-11210.

Holmes, R., Aminot, R., Kerouel, B., Hocker, A., Peterson, B. 1999. A simple and precise method for measuring ammonium in marine and fresh water. Canadian Journal of Fish and Aquatic Science. 56, 1801–1808.

Hughes, E. 2000. Biomass cofiring: economics, policy and opportunities. Biomass & Bioenergy. 19, 457-465.

Hulatt, C., Thomas, D. 2010. Dissolved organic matter (DOM) in microalgal photobioreactors: A potential loss in solar energy conversion? Bioresource Technology. 101, 8690-8697.

Hulatt, C., Thomas, D. 2011. Productivity, carbon dioxide uptake and net energy return of microalgal bubble column photobioreactors. Bioresource Technology. Doi: 10.1016/j.biortech.2011.02.025.

Jorquera, O., Kiperstock, A., Sales, E., Embirucu, M., Ghirardi, M. 2010. Comparative energy life-cycle analysis of microalgal biomass production in open ponds and photobioreactors. Bioresource Technology. 101, 1406-1413.

Klausmeier, C., Litchman, E., Daufresne, T. Levin, S. 2008. Phytoplankton Stoichiometry. Ecological Research. 23, 479-485.

Kroon, H. 1993. Determination of nitrogen in water: comparison of continuous flow method with on-line UV digestion with the original Kjedahl method. Analytica Chimica Acta. 276, 287–293.

Lopez, C., Garcia, M., Fernandez, F., Bustos, C., Chisti, Y., Sevilla, J. 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresource Technology. 101, 7587-7591.

Lakaniemi, A-M., Hulatt, C., Thomas, D., Tuovinen, O., Puhakka, J. (In Submission). Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. Biotechnology for Biofuels.

Lardon, L., Helias, A., Sialve, B., Steyer, J., Bernard, O. 2009. Life-Cycle Assessment of Biodiesel Production from Microalgae. Environmental Science and Technology. 43, 6475–6481.

Mandalam, R., Palsson, B., 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. Biotechnology and Bioengineering. 59, 605–611.

Mulvenna, P., Savidge, G. 1992. A modified manual method for the determination of urea in seawater using diacetylmonoxime reagent. Estuarine, Coastal and Shelf Science. 34, 429-438.

Oswald, W., Golueke, C. 1960. Biological transformations of solar energy. Advances in Applied Microbiology. 2, 223-262.

Perez-Garcia, O., Escalante, F., de-Bashan, L., Bashan, Y. 2010. Heterotrophic cultures of microalgae: Metabolism and potential products. Water Research. Doi: 10.1016/j.watres.2010.08.037.

Pick U, Weiss M. 1991. Polyphosphate hydrolysis within acidic vacuoles in response to amine-induced alkaline stress in the halotolerant alga *Dunaliella salina*. Plant Physiology. 97, 1234-1240.

Qian, J., Mopper, K. 1996. An automated, high performance, high temperature combustion dissolved organic carbon analyzer. Analytical Chemistry. 68, 3090–3097.

Ramirez, C., Worrell, E. 2006. Feeding fossil fuels to the soil: An analysis of energy embedded in technological learning in the fertilizer industry. Resources, Conservation and Recycling. 46, 75-93.

Redfield, A. 1958. The biological control of chemical factors in the environment. American Scientist. 206, 205-221.

Sanchez, O., Cardona, C. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technology. 99, 5270-5295.

Sialve, B., Bernet, N., Bernard, O. 2009. Anaerobic digestion as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances. 27, 409-416.

Sierra, E., Acien, F., Fernandez, J., Garcia, J., Gonzalez, C., Molina, E. 2008. Characterization of a flat plate photobioreactor for the production of microalgae. Chemical Engineering Journal. 138, 136-147.

Soletto, D., Binaghi, L., Lodi, A., Carvalho, J., Converti, A. 2005. Batch and fed-batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. Aquaculture. 243, 217-224.

Stephenson, A., Kazamia, E., Dennis, J., Howes, C., Scott, S., Smith, A. 2010. Life-cycle assessment of potential algal biodiesel production in the United Kingdom: a comparison of raceways and air-lift tubular bioreactors. Energy Fuels. 24, 4062–4077.

Tam, N., Wong, Y. 1996. Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. Bioresource Technology. 57, 45-50.

Vonshak, A., Richmond, A. 1988. Mass production of the blue-green alga *Spirulina*: An overview. Biomass. 15, 233-247.

Xin, L., Hong-ying, H., Ying-xue, S. 2010. Effects of different nitrogen and phosphorus concentrations on the nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp.

Zhang, K., Miyachi, S., Kurano, N. 2001. Photosynthetic performance of a cyanobacterium in a vertical flat-plate photobioreactor for outdoor microalgal production and fixation of CO_2. Biotechnology Letters. 23, 21-26.

Chapter 6.

Energy Efficiency of an Outdoor Tubular Photobioreactor Sited at Mid-Temperate Latitude.

6. Overview

The objective of the work presented in the following chapter was to measure the productivity of a microalgal photobioreactor in outdoor conditions using natural sunlight over spring and summer. The hypothesis tested was that *the cultivation of microalgae using photobioreactors is a potentially viable method of biomass production in cool-temperate regions.* The test location was novel due to its latitude (53°N). Establishing the long-term productivity of a photobioreactor at the test location would inform the design of future technologies used to cultivate algae.

6.1.1. Outdoor cultivation

The photobioreactor, a Biofence (Varicon Aqua, UK) was used to test productivity. The photobiorector was constructed outside in August 2009 (Figure 6.1 A). Initial attempts to cultivate *Chlorella vulgaris* proved unsatisfactory. However, the system was colonized by a local strain of *Scenedesmus obliquus* that, despite freezing temperatures (Fig. 6.1 B) grew steadily to a cell density of approximately 0.25 g L^{-1} during the winter. The species survived fluid temperatures to just above freezing, at ambient air temperatures of -5°C. In these conditions Fv/Fm measured 0.2-0.3. Subsequent freezing temperatures caused failure of the manifold seals and the culture was lost. However, a sample was removed and maintained in the laboratory. In February 2010, the culture was scaled-up to three 20 L polythene photobioreactor cultivations and the photobioreactor was re-inoculated on 11.03.10.

After a lag-phase of approximately 1 month, the culture grew strongly (Chapter 6.2), and maintained healthy continuous growth throughout spring and summer, until the experiment was stopped on 05.09.10. Some problems with bio-fouling were observed (Fig. 6.1 C), which was rectified by turning the tubes. Cell density was

maintained sufficiently high to ensure no light passed through the tubes (Fig. 6.1 D). The photobioreactor was un-shaded (Fig. 6.1 E & F).

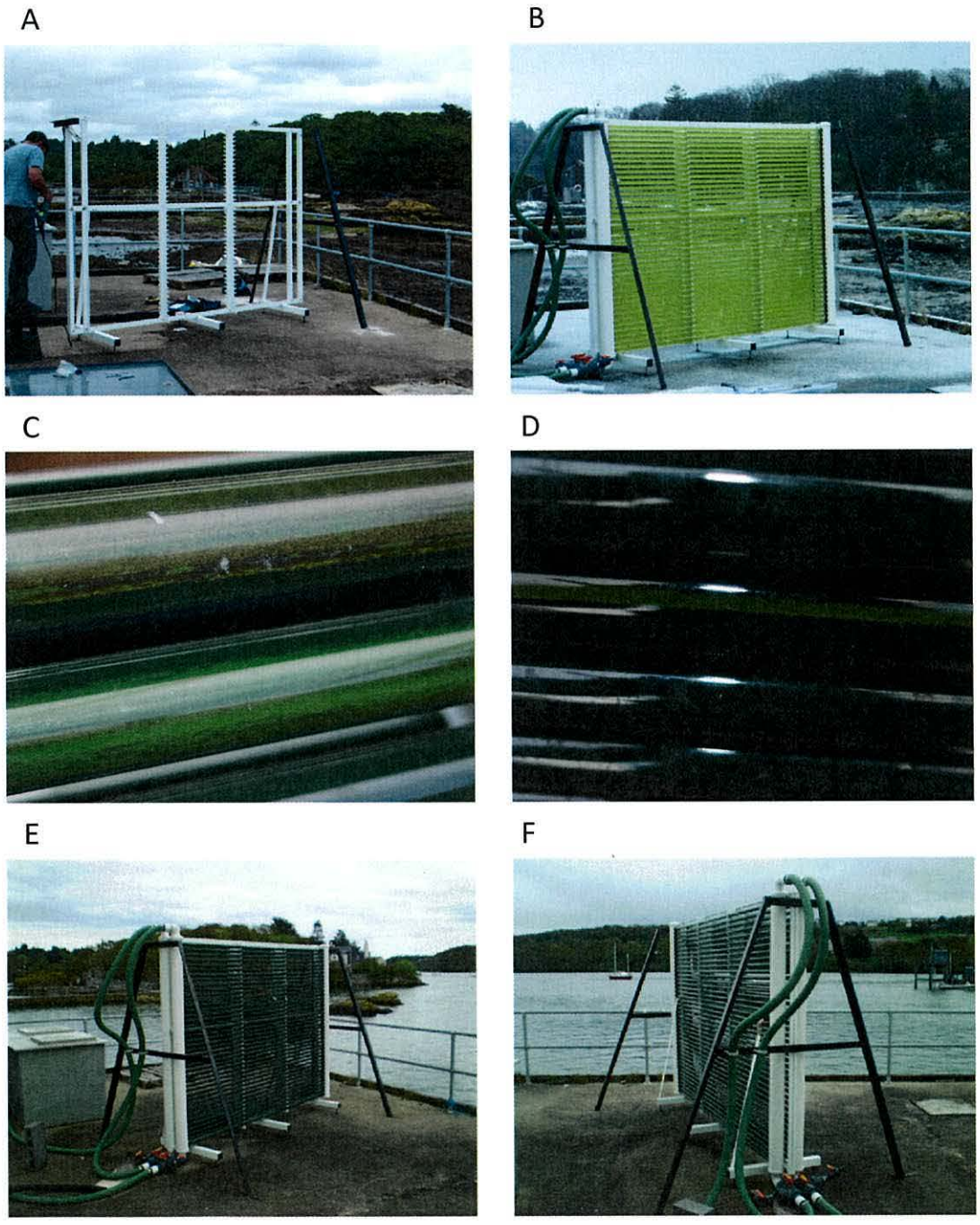


Figure 6.1. Images of the outdoor Biofence photobioreactor, described in the text. Images A & C to F are by the author, Image B courtesy of D.N. Thomas.

6.1.2. Physical data

Weatherproof data-loggers (Onset HOBO UA-002-64) were used to record light and temperature measurements during the experiments every 5 minutes. Photodiodes of the light sensors measured a broad spectrum of light (200-1200 nm) in Lux. Measurements were converted to μmol photons PAR m^{-2} s^{-1} by calibration against a Li-Cor Li-190SA sensor. Data was collected across different weather patterns (rain to direct sunlight), time of day and season. The calibration between data-loggers and Li-Cor sensor was linear (Fig. 6.1.2.), equation 6.1.1.

$$PAR = (0.0139 \times Lux) + 127.05 \qquad (6.1.1.)$$

Where PAR is the photosynthetically active radiation (μmol photons PAR $m^{-2}s^{-1}$) and Lux is the datalogger irradiance (Lux). The R^2 for the calibration was >0.999. The PAR energy (MJ m^{-2}) was calculated by dividing the photon irradiance (μmol photons PAR m^{-2} s^{-1}) by the conversion factor 4.57 (Zhang et al., 2001, cited in section 6.2.).

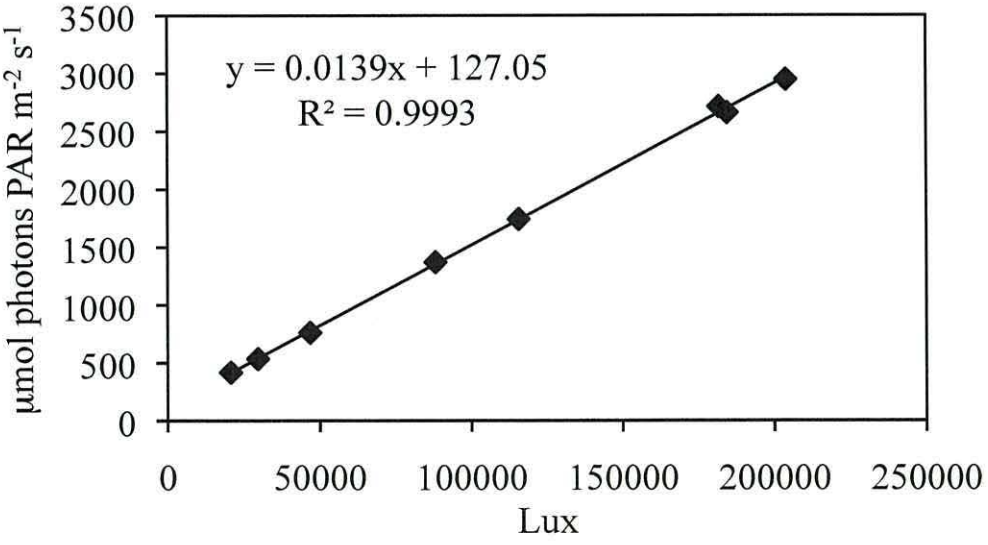


Figure 6.1.2. Calibration between data-logger measurements of irradiance (Lux) and measurements of photosynthetically active radiation (μmol photons PAR m^{-2} s^{-1}).

Author's personal copy

Bioresource Technology 102 (2011) 6687–6695

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Energy efficiency of an outdoor microalgal photobioreactor sited at mid-temperate latitude

Chris J. Hulatt [a,*], David N. Thomas [a,b]

[a] School of Ocean Sciences, College of Natural Sciences, Bangor University, Askew Street, Menai Bridge, Anglesey LL59 5AB, UK
[b] Finnish Environment Institute (SYKE), Marine Centre, P.O. Box 140, FI-00251 Helsinki, Finland

ARTICLE INFO

Article history:
Received 17 January 2011
Received in revised form 23 March 2011
Accepted 29 March 2011
Available online 2 April 2011

Keywords:
Microalgae
Photobioreactor
Energy efficiency
Biofuel
Scenedesmus

ABSTRACT

This work examined the energetic performance of a 6-month semi-continuous cultivation of *Scenedesmus obliquus* in an outdoor photobioreactor at mid-temperate latitude, without temperature control. By measuring the seasonal biomass production (mean 11.31, range 1.39–23.67 g m^{-2} d^{-1}), higher heating value (22.94 kJ g^{-1}) and solar irradiance, the mean seasonally-averaged photosynthetic efficiency (2.18%) and gross energy productivity (0.27 MJ m^{-2} d^{-1}) was calculated. When comparing the solar energy conversion efficiency to the energy investment for culture circulation, significant improvements in reactor energy input must be made to make the system viable. Using the data collected to model the energetic performance of a substitute photobioreactor design, we conclude that sustainable photobioreactor cultivation of microalgae in similar temperate climates requires a short light path and low power input, only reasonably obtained by flat-panel systems. However, temperature control was not necessary for effective long-term cultivation.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Microalgae are a promising source of renewable bio-energy: They typically have high areal productivities (Chisti, 2007), can utilise waste water and can be cultivated on land not suitable for conventional agriculture (Clarens et al., 2010). A potential use for microalgal technologies is for the recycling of CO_2 in flue gas produced by the electricity generation industry (Vunjak-Novakovic et al., 2005). In this role, algal growth can be accelerated by the availability of concentrated CO_2, whilst emissions of CO_2 to the atmosphere are offset by producing a renewable biomass fuel.

A key aspect of microalgal physiology, and the main reason they may be favoured over terrestrial crops, is the relatively high efficiency with which they convert solar energy to the chemical energy of biomass (Williams and Laurens, 2010). Calculation of the theoretic maximum efficiency of solar energy conversion (total solar energy to primary photosynthetic products) for microalgae identifies a maximum value of around 10%. However, metabolic activities such as lipid and protein production alone can easily halve this, and measured values are typically 1–3% (Williams and Laurens, 2010).

It is difficult to simulate outdoor conditions in a laboratory, so it is therefore important to gain practical evidence for microalgal productivity in the field, especially in different geographic regions and over sustained periods of time (Scott et al., 2010; Silva et al., 2009). Microalgal productivity varies with geographic location and prevailing weather conditions (Ugwu et al., 2008), and most research on outdoor production has been conducted in regions with a relatively mild climate such as Spain (Camacho et al., 1999), Australia (Moheimani and Borowitzka, 2006), and Israel (Richmond and Wu, 2001). These locations give optimal light and temperature environments leading to high growth rates. However there is also a need to investigate microalgal performance in other locations to assess how suitable such technologies may be. In particular, data for outdoor microalgae production in mid-temperate latitudes is very scarce.

Low-grade waste heat from industrial plant (Shang et al., 2010) may be available at low environmental and economic cost in many industrial scenarios where growing microalgal biomass is being considered. However, a major aspect of photobioreator design which has received little attention is whether or not it is beneficial to control the temperature of microalgal cultivations (i.e. extra biomass energy out *vs.* extra energy in). It is simply not clear from published reports whether supply of heat is essential for cultivation of microalgae and/or provides net benefits, especially in cooler regions of the world (Carlozzi and Sacchi, 2001; Moheimani and Borowitzka, 2006; Shang et al., 2010).

Microalgae have been cultivated in a wide range of engineered systems (raceways, closed photobioreactors), and there is much debate concerning how different technologies compare (Jorquera et al., 2010; Stephenson et al., 2010). Raceway production of algal

* Corresponding author. Tel.: +44 1248 38 2852.
E-mail address: osp418@bangor.ac.uk (C.J. Hulatt).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.03.098

Author's personal copy

biomass has relatively low energetic and monetary costs, but in turn has relatively low volumetric productivities. Closed photobioreactors, such as tubular systems, may have volumetric productivities around 30 times higher than raceways (Chisti, 2007). However, for bio-energy crops the most relevant comparison is per unit area, and in this case the performance of photobioreactors and raceways may be more comparable (Mata et al., 2010).

From an experimental perspective, the advantage of closed photobioreactors is that they offer an excellent platform for measuring microalgal production in a controlled environment (Ugwu et al., 2008). This avoids problems with contamination (Mata et al., 2010) and allows the maintenance of a stable physico-chemical environment (e.g. pH, nutrients, and low evaporation), which in turn allows accurate and reliable long-term assessments of performance to be made (Kunjapur and Eldridge, 2010; Morweiser et al., 2010). These same advantages may also favour closed photobioreactors for commercial systems.

It is essential that microalgal bio-energy technologies produce more energy than they consume (Das and Obbard, 2011; Jorquera et al., 2010). In particular, recent theoretical work has shown that the apparent productivity advantages of closed photobioreactors may be nullified by the much higher energy requirements of these systems for mixing and circulating cultivations (Stephenson et al., 2010). In fact, closed photobioreactors, in some circumstances, may be net energy consumers rather than producers (Das and Obbard, 2011; Jorquera et al., 2010). However, there is little evidence for the balance between energy production and supply within the same experimental system, and there is an urgent need to ascertain the limits of microalgal productivity, so that cultivation systems can be designed accordingly.

This study examines the energetic performance of a tubular photobioreactor using natural sunlight and no temperature control. It identifies the energy conversion efficiency of a productive, long-term seasonally averaged microalgal cultivation and compares it with the energy invested in circulating and mixing the culture. This allowed the calculation of performance range (energy input) in which cultivation systems must operate in this type of environment. Subsequently, this data was used to define a system which, in terms of power input, is capable of achieving a positive energy return.

2. Methods

2.1. Cultivation

Cultivation was carried out at a south-facing un-shaded plot on at a site located at 53°22′58″N 4°16′01″W (sea level), over the spring and summer of 2010. The freshwater microalga, *Scenedesmus obliquus*, was isolated from a pool at the same location and maintained in the laboratory. The ecotype was selected as it was already adapted to the local conditions, because the species is robust, grows rapidly and has been commonly used for research into mass algal cultures and renewable energy technologies (Mandal and Mallick, 2009; Mata et al., 2010; Silva et al., 2009). The nutrients were supplied (mg L^{-1}) according to Jaworski formulation (Culture Collection of Algae and Protozoa, Oban, UK): $Ca(NO_3)_2{\cdot}4H_2O$ (20), KH_2PO_4 (12.4), $MgSO_4{\cdot}7H_2O$ (50), $NaHCO_3$ (15.9), $EDTAFeNa_2$ (2.25), $EDTANa_2$ (2.25), H_3BO_3 (2.48), $MnCl_2{\cdot}4H_2O$ (1.39), $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$ (1), cyanocobalamin (0.04), thiamine HCl (0.04), Biotin (0.04), $NaNO_3$ (80), $Na_2HPO_4{\cdot}12H_2O$ (36). Nutrients were supplied to the photobioreactor every few days to maintain nitrate > 2 mmol L^{-1}. Dissolved inorganic nitrogen ($NO_3^- + NO_2^- + NH_4^+$) and phosphorus were measured regularly as a check using standard colorimetric methodology. There was no nitrogen or phosphorus limitation throughout the cultivation. The inoculum for the reactor was prepared using three 20 L polythene bag photobioreactors grown and maintained in the laboratory (18 °C, 250 μmol photons photosynthetically active radiation (PAR) m^{-2} s^{-1}).

2.2. Photobioreactor

The experimental photobioreactor (Varicon Aqua Ltd., UK) used to measure productivity consisted of a horizontal tubular system with a 6 m^2 (frontal area) photo-stage placed vertically. The culture was circulated between the photo-stage and tank using a centrifugal pump (Fig. 1). The total system volume was 500 L, the internal diameter of the transparent tubes was 28 mm and water was recycled at a rate of 220 L min^{-1}. The flow pattern was such that the average velocity of the fluid flowing through the transparent tubes was 87 cm s^{-1}. Plastic beads were circulated in the fluid to prevent bio-film build-up on the internal surfaces. The system was operated as a pH-stat at pH 7.0 (±0.2) by addition of pure CO_2. This is the optimum pH for cultivation of many *Scenedesmus* strains (Nalewajko et al., 1997). The bioreactor was operated semi-continuously and nutrients were added to maintain nutrient-replete conditions throughout cultivation. The water used for cultivation was untreated tap water.

2.3. Biomass and biochemical composition

Samples for biomass properties were taken every 1–4 days at 16:00 h, depending upon growth rate. Biomass dry weight and elemental composition (carbon and nitrogen) was analysed as previously described (Hulatt and Thomas, 2010). Biomass higher heating values (HHV) and lipid content were measured using samples taken on days 128 and 169. The biomass higher heating value (HHV) was measured by combusting approximately 1 g (dry weight) samples in a Parr 1341 oxygen bomb calorimeter, which was calibrated using benzoic acid ($n = 3$), accuracy ± 0.5%. Total lipids were analysed using gravimetric analysis/solvent extraction method: Biomass was prepared by sonication to >99% cell rupture: a Branson 450 Digital Sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA) was used, fitted with a ½ disruptor horn used in conjunction with a 1/8 tapered microtip. The frequency was factory set to 20 kHz. A 30% amplitude (corresponding to 13.5 W per 10 ml sample) was the optimum power to disrupt the cells. After extraction, phase separation and centrifugation, the lipid/chloroform phase was vacuum filtered through Whatman® GF/F filters (0.7 μm nominal pore size) to remove remaining particulates. The protein content was measured using the total elemental nitrogen content, by multiplying the concentration of nitrogen (% mass) by the conversion factor 4.44 (Lopez et al., 2010).

2.4. Temperature and light measurements

Data-loggers (Onset HOBO UA-002-64) were used to record the irradiance incident on the face of the photobioreactor, the scattered and reflected light received by the reverse face, the ambient air temperature in the shade and the temperature of the culture fluid. The scattered light received by the rear face of the photobioreactor accounted for 20.2% of the total irradiance, averaged over the cultivation period. Data-loggers recorded every 5 min throughout the cultivation period and were calibrated *in situ* against a Li-Cor 190SA sensor to convert the light reading to μmol PAR m^{-2} s^{-1}. This was done by measuring values from both instruments positioned side-by-side during various weather conditions and time events (rain to direct sunlight, morning to evening) from March to September (the R^2 value for the light calibration was >0.999). The manufacturer reports temperature accuracy of ±0.54 °C. Where PAR energy was required, measurements were obtained by divid-

Author's personal copy

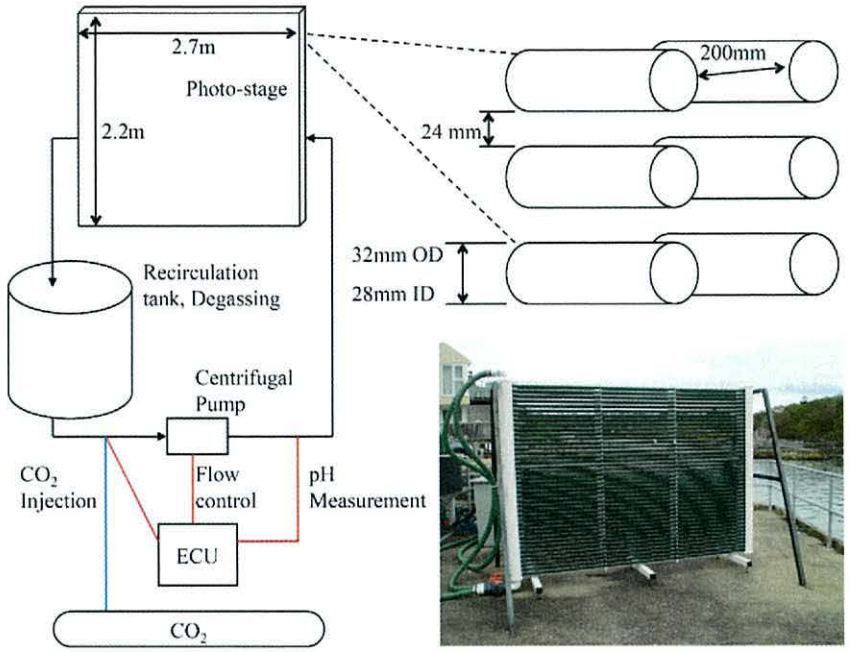


Fig. 1. Diagram of experimental apparatus, photo-stage construction and image of cultivation obtained during May 2010. Carbon dioxide was supplied from cylinders using a pH stat system moderated by an electronic control unit (ECU). Photo-stage tubes were constructed of clear polythene, 2 mm wall thickness, and were arranged in two rows to intercept the maximum amount of incident light.

ing PAR by the conversion factor 4.57 (Zhang et al., 2001), and expressed as the equivalent daily energy input MJ m^{-2} d^{-1}.

2.5. Standardisation of parameters

Productivity measurements and light energy inputs were normalised to 1 m^2 of reactor frontal area. This therefore permitted calculation of photosynthetic efficiency using the solar energy input. Productivity expressed in this manner is a scalable and interchangeable measurement. In calculations of photosynthetic efficiency, the direct irradiance on the front of the photobioreactor (MJ m^{-2} d^{-1}) was used.

2.6. Quantum yield and dissolved oxygen measurements

On selected days during cultivation, repeated measurements were made in order to identify any diurnal stresses. In each case, five sets of measurements were made at 3.5 h intervals between 07:00 h and 21:00 h. The photosynthetic performance (maximum quantum yield, Fv/Fm) was measured using a pulse amplitude modulation (PAM) fluorometer (Water-PAM, Walz, GmbH). Dissolved oxygen was measured at the outlet of the photo-stage using an oxygen meter (WTW CellOx 325).

3. Results and discussion

3.1. Biomass density, biochemical properties and temperature dynamics

The photobioreactor was inoculated on 11.03.10, but there was little growth in biomass during the first 25–30 days, until mid-April (Fig. 2). This growth, which is indicated (a) in Fig. 2, coincided with an increase in mean daily irradiance and temperature. The biomass (g L^{-1}) doubling time was around 20 days during the low-growth period. However, after point (a) indicated in Fig. 2a, the biomass doubling time rose to 2–3 days. This indicates a shift in physiology in response to light and temperature rather than a slow-start due to low inoculation biomass concentrations. During subsequent semi-continuous cultivation, the dry weight of biomass was maintained in a target range between 1.5 and 3.5 g L^{-1} (mean dry weight over whole growth period was 2.45 g L^{-1}). Maintenance of biomass density in this range ensured a light-limited system (lower limit), but also minimised the impact of respiration (upper limit), see Section 3.4 for verification. The same culture remained healthy throughout cultivation, however, there were several events where the CO_2 supply was interrupted and these outages are shown shaded in the dry weight density measurements in Fig. 2.

The molar carbon:nitrogen (C:N) ratio, plus the percentage carbon and nitrogen composition of the dry biomass, was stable throughout cultivation (Fig. 2). This was a direct result of the nutrient-replete conditions employed, and shows there was no evidence for nutrient-induced stress. The mean nitrogen content of the biomass was 7.94% (±sd 0.87), resulting in a corresponding mean C:N ratio (moles) of 7.35 (±sd 0.77). The nitrogen content of algal biomass is a potentially important parameter for the sustainability of microalgal systems, because it determines both the demand for nitrogen supply and the biodegradability of the biomass for possible anaerobic digestion; a high nitrogen content may have a negative impact on digestion efficiency through ammonium production (Sialve et al., 2009). The mean protein content during cultivation was 35.25 (±sd 3.86)% mass.

The higher heating value (HHV) and total lipid content of the biomass were comparable on both sampling occasions (11.05.10 and 21.06.10, Fig. 2), and two-sample *t*-tests confirmed that there was no significant difference in lipid content or HHV ($t = 1.34$, $p = 0.311$; $t = 0.88$, $p = 0.415$ respectively). Lipid concentrations of 13.25% (±sd 0.42) and 13.59% (±sd 0.76) by weight were extremely close (±~1%) to those reported in the published literature for *Scenedesmus* sp. maintained in nutrient-replete conditions (Mandal and Mallick, 2009; Mata et al., 2010; Silva et al., 2009). Maintenance of nutrient-replete conditions was necessary in this work to measure maximum production, though it should be noted that

Author's personal copy

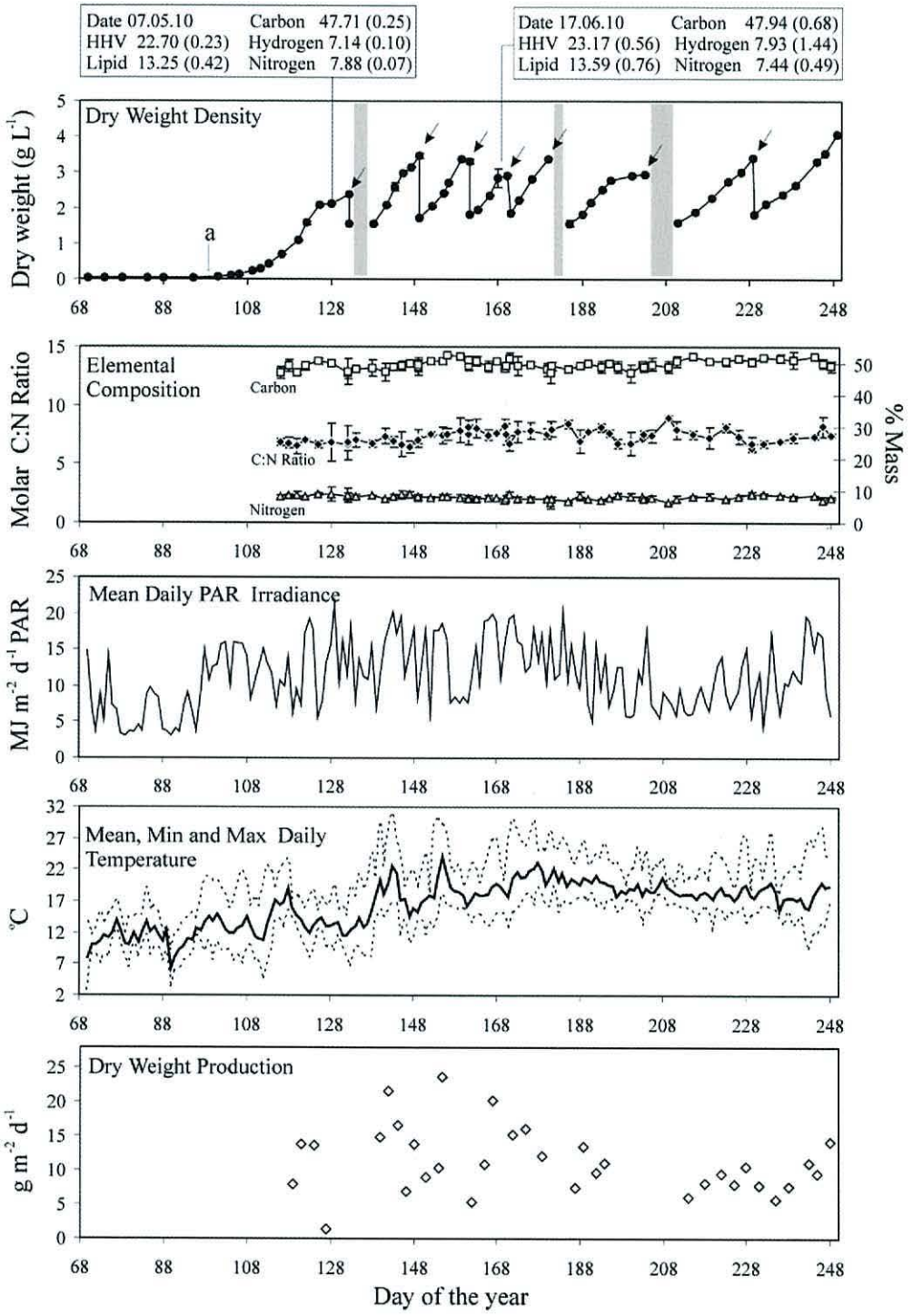


Fig. 2. Productivity data for *Scenedesmus obliquus* cultivated in an outdoor tubular photobioreactor from March to September 2010. Dry weight density is measured by g L^{-1}, $n = 3$, error bars are standard deviation. Mean parameters (±standard deviation) indicated in boxes on dry weight density measurements are: HHV, kJ g^{-1} ($n = 3$); Lipid, % dry weight ($n = 5$); carbon, hydrogen & nitrogen, % dry weight ($n = 3$). The C:N ratio for the elemental composition is expressed on a molar basis, the absolute concentrations are expressed a % dry mass ($n = 3$, error bars are standard deviations). The daily PAR irradiance is the amount of photosynthetically active radiation energy received by 1 m^2 of the front of the photobioreactor per day (PAR = approx. 45.7% of energy in full solar spectrum), expressed as MJ m^{-2} d^{-1}. The mean, minimum and maximum daily temperatures are shown (°C), as is the areal dry weight productivity, expressed per 1 m^2 of reactor frontal area (g m^{-2} d^{-1}).

nitrogen limitation strategies may prove beneficial for commercial applications (Lardon et al. 2009). Considering the lipid content of the strain used and research by (Sialve et al., 2009), it may be energetically more efficient to subject the whole biomass to anaerobic digestion, rather than to extract the lipid for biodiesel production.

Measurements of the higher heating values were mean 22.70 ± sd 0.23 and mean 23.17 ± sd 0.56.

The mean daily temperature ranged from 10.8 to 24.1 °C and the period July to September was characterised by relatively high, stable average temperatures. The peak water temperature was

Author's personal copy

30.36 °C on day 171, and the highest diurnal ΔT was 16.75 °C recorded on day 138 (Fig. 2).

3.2. Daily responses to light and temperature

In order to examine the potential impact of short-term light and temperature stresses on productivity, measurements of photosystem II performance (Fv/Fm) and dissolved oxygen (% saturation) were made every 3.5 h on selected days. Two examples of these daily investigations, one taken on a cloudy and the other on a clear day, are shown with corresponding irradiance, culture temperature and air temperature measurements in Fig. 3. On the representative cloudy day, Fv/Fm decreased minimally from a morning (07:00 h) value of 0.738 (±sd 0.003) to 0.726 (±sd 0.006) at 14:00 h, and values were all within the range expected of healthy, photosynthetically efficient cells (Parkhill et al., 2001). Dissolved oxygen reached 143% of air saturation, and the maximum diurnal ΔT of the culture fluid was 7.4 °C.

In contrast, the example clear day showed higher maximum dissolved oxygen concentrations of 170%. There was also a greater ΔT of 13.7 °C, and the irradiance incident on the front of the reactor measured over 2000 μmol photons PAR m^{-2} s^{-1} for 7 h. As a result, there was a greater drop in mean Fv/Fm from 0.708 to 0.669, approximately fourfold higher than on the cloudy day. However, although the observed drop in quantum yield indicates a degree of physiological stress, this was only a 5.5% decrease in maximum photosystem II efficiency, and thus represents minimal stress to the photosynthetic apparatus. In fact, such small changes have been observed in stable outdoor cultures at around the optimal cell density (Qiang et al., 1996).

Photoinhibition and temperature stresses commonly have significant impacts on the photosynthetic efficiency of outdoor mass algal cultivation (Fernandez et al., 2003). The apparent stability observed in this study may be largely due to the light: dark ratio (35% of the system volume was in the photo-stage structure at any one time), biomass concentration and the bulk volume of culture (minimises light over-exposure and temperature fluctuations via heating capacity of water).

3.3. Areal productivity

Daily productivity was measured after day 115, when the biomass concentration reached the light-limited stage. The mean productivity expressed per m^2 of reactor frontal area was 11.31 g (dry weight) m^{-2} d^{-1} for the whole semi-continuous cultivation period (April–September), but ranged from 1.39 to 23.67 g (dry weight) m^{-2} d^{-1} (Fig. 2). Despite the comparatively lower light levels and temperatures in this study, productivity values were typical figures

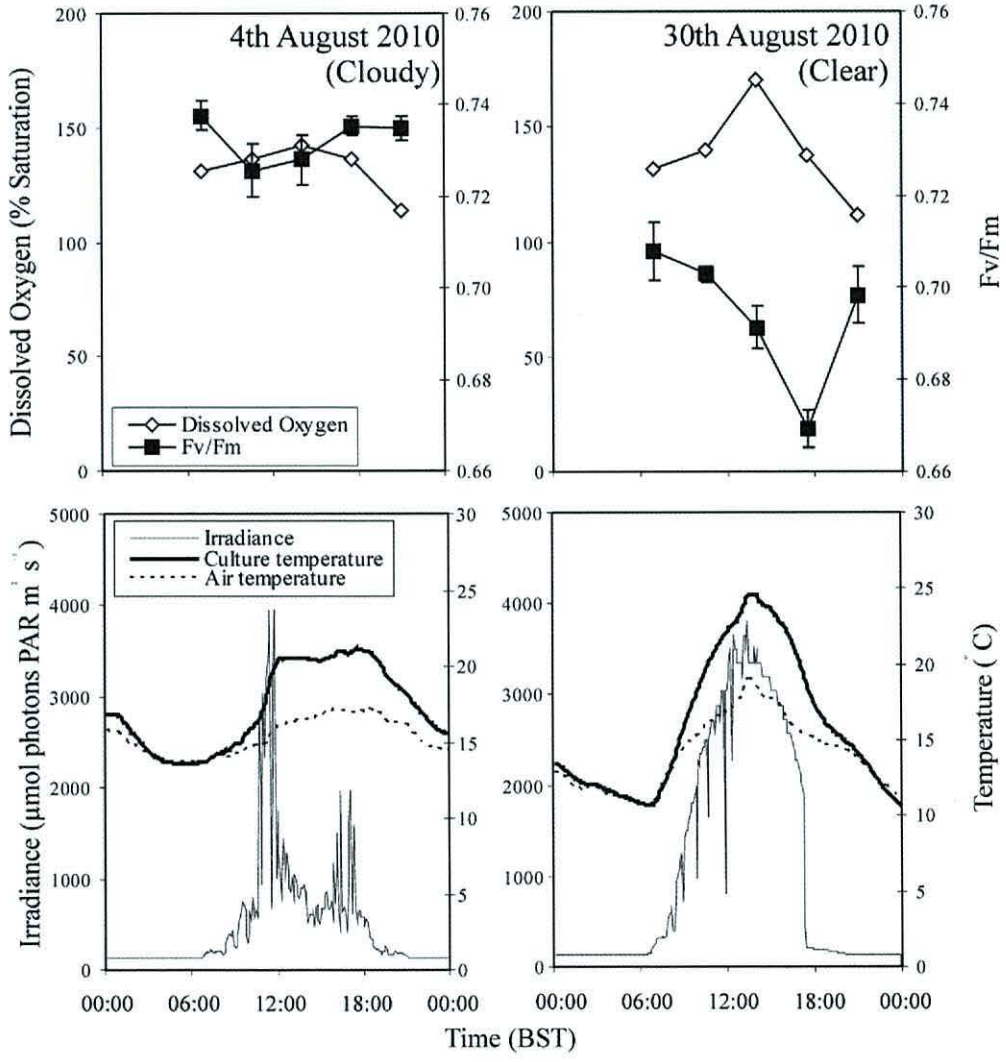


Fig. 3. Changes in Fv/Fm and dissolved oxygen (% air saturation) obtained over a typical cloudy and clear day during the cultivation. The corresponding fluid temperature, air temperature and irradiance (μmol photons PAR m^{-2} s^{-1}) are shown in panels below.

Author's personal copy

expected of long-term (>3 month) outdoor cultivations and are comparable to measurements from Spain, Italy, Mexico and Japan (see Moheimani and Borowitzka, 2006 and references therein). Areal production measurements also fall into the range of values reported by Mata et al. (2010), showing that data is representative of outdoor cultivation systems. The summer-time productivities described by Moheimani and Borowitzka (2006) for raceways in Australia were approximately twice as high as those obtained here, although the total solar irradiance received was also approximately twice as great in their study.

Most productivity models for microalgal growth use values in the order of 15–30 g (dry weight) $m^{-2}\,d^{-1}$ (Campbell et al., 2011). In this work, the maximum productivity of 23.67 g $m^{-2}\,d^{-1}$ was achieved during periods of clear skies and long days (Fig. 2), but was not sustained throughout the growth period. This implies that models for cultivating algae in geographic locations such as tested here should be revised accordingly. When examined on a monthly basis (Table 1) the highest mean productivities were observed during June, where a mean monthly average of 14.26 (5.82) g $m^{-2}\,d^{-1}$ dry weight production was recorded.

Using the mean higher heating value (22.94 kJ g^{-1}), lipid concentration (13.42%), carbon content (49.52%) and nitrogen content (7.94%), the respective production of these components was calculated for the whole (April–September) cultivation period: The mean areal bio-energy production was 0.27 (range 0.05–0.73) MJ $m^{-2}\,d^{-1}$, the mean lipid productivity was 1.6 (range 0.3–4.4) g $m^{-2}\,d^{-1}$ and the mean CO_2 fixation rate was 21.4 (range 3.6–58.2) g CO_2 $m^{-2}\,d^{-1}$. The mean daily nitrogen requirement, determined by biomass nitrogen, was 0.9 (range 0.2–2.4) g $m^{-2}\,d^{-1}$. The mean monthly biomass, energy and lipid production is shown in Table 1.

3.4. Irradiance and photosynthetic efficiency

During cultivation the mean daily PAR energy input was 12.18 MJ $m^{-2}\,d^{-1}$ and ranged from 4.23 to 21.43 (mean 55.81, range 19.44–97.72 mol photons PAR $m^{-2}\,d^{-1}$), shown in Fig. 2. The highest energy input generally occurred between days 105 and 195 (mid-April to mid-July). There was very strong and significant correlation between PAR irradiance and bio-energy production ($r = 0.724$, $p < 0.001$), and the relationship is shown in Fig. 4. The photosynthetic efficiency (PE) for each sample point was calculated by $PE = 100(E_{out}/E_{in})$, where E_{out} is the biomass output energy (MJ $m^{-2}\,d^{-1}$) and E_{in} is the PAR irradiance input energy (MJ $m^{-2}\,d^{-1}$). The average PE was 2.18% (±sd 0.68). There was no significant effect of mean daily irradiance on PE ($r = 0.046$, $p = 0.8$) over the range 7.0–19.6 MJ $m^{-2}\,d^{-1}$ (Fig. 5a), and monthly-averaged figures were stable (Table 1). Thus high mean energy inputs did not significantly reduce the efficiency of the device. This agrees with the Fv/Fm measurements (Fig. 3, Section 3.2), which also demonstrated that even on the brightest days there was little reduction in photosynthetic efficiency due to photo-inhibition and changes in temperature.

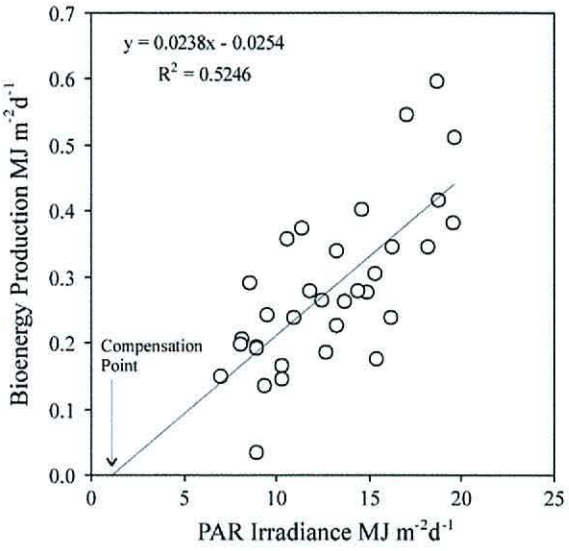


Fig. 4. The relationship between PAR energy input (MJ $m^{-2}\,d^{-1}$) and bio-energy output (MJ $m^{-2}\,d^{-1}$) during the cultivation. The compensation point, the light energy input where net production is zero, is indicated.

Backward extrapolation of the regression in Fig. 4 produced an intercept of −0.025 MJ $m^{-2}\,d^{-1}$, indicating a mean compensation light energy input of 1.2 MJ $m^{-2}\,d^{-1}$. The compensation level is that required to sustain net-zero growth, and is attributable to the 'maintenance requirement' or 'dark-losses' due to cellular metabolism. On average, the estimated loss of energy through maintenance measured 9.0% of net production and 8.3% of gross production. Chisti (2007) identified that the maintenance requirement may account for up to 25% of gross production in mass algal cultures. The lower values obtained in this study may reflect a different temperature regime (Gons and Mur, 1980), and/or because the *Scenedesmus obliquus* strain used was well-adapted to the environmental conditions.

The energetic relationship between PAR and total solar radiation is relatively stable; on average PAR accounts for approximately 45.7% of total irradiance (Nagaraja, 1984). Thus, the energy conversion efficiency of the device was 1.00% of the full solar spectrum, which is approximately 10% of the absolute theoretic maximum for microalgal photosynthesis and compares well with practical estimates (Williams and Laurens, 2010). It is useful to benchmark this value against efficiencies of 6–8% obtainable using commercial photovoltaic systems (Ibrahim et al., 2011), which may ultimately compete for land area in future applications. However the output of algal technologies is biomass/fuels, whilst photovoltaic technologies produce electricity. Thus selection is also dependent upon the product, not simply the energy conversion efficiency.

The biomass density is an important parameter in the cultivation of microalgae. Optically dilute cultivations waste solar energy

Table 1
Mean productivity and energetic parameters by month for the photobioreactor. The photosynthetic efficiency (PE) is the % of the PAR irradiance energy received which is converted into harvestable biomass energy, both related to a 1 m^2 frontal area of the photobioreactor. The number of samples used to compute parameters for each month are indicated (n), and values in parentheses are standard deviations.

	Biomass (g $m^{-2}\,d^{-1}$)	Lipid (g $m^{-2}\,d^{-1}$)	Bio-energy (MJ $m^{-2}\,d^{-1}$)	PAR irradiance (MJ $m^{-2}\,d^{-1}$)	PE (%)	n
April	10.89 (4.24)	1.46 (0.57)	0.25 (0.10)	9.42 (1.22)	2.51 (1.27)	2
May	12.03 (6.26)	1.61 (0.84)	0.28 (0.14)	14.74 (3.45)	2.00 (0.97)	8
June	14.26 (5.82)	1.91 (0.78)	0.33 (0.13)	15.69 (3.50)	2.22 (0.57)	8
July	10.39 (2.57)	1.39 (0.34)	0.24 (0.06)	11.77 (1.63)	2.24 (0.52)	4
August	8.90 (1.83)	1.19 (0.25)	0.20 (0.04)	10.51 (2.36)	2.19 (0.32)	11
September	11.85 (3.39)	1.59 (0.45)	0.27 (0.08)	13.35 (3.97)	2.44 (1.37)	2

Author's personal copy

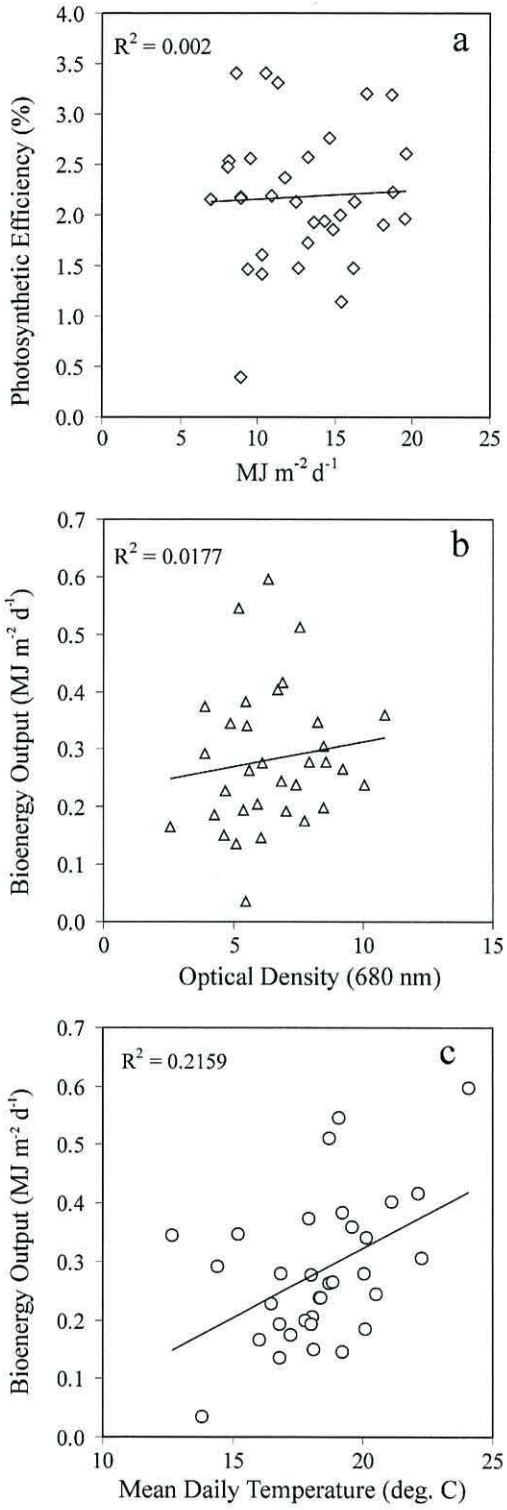


Fig. 5. (a) Relationship between PAR energy input (MJ m^{-2} d^{-1}) and PAR photosynthetic efficiency (%) during the cultivation period; (b) relationship between the optical density (680 nm) and the bio-energy output of the system (MJ m^{-2} d^{-1}); and (c) relationship between temperature (°C) and the bio-energy output of the system (MJ m^{-2} d^{-1}).

by allowing some light to pass through the medium without absorption by photosynthetic pigments, whilst very dense cultivations reduce productivity by causing excessive respiration losses. This subject may require further investigation in relation to the variations in solar irradiance experienced by outdoor cultivations (Pruvost et al., 2010). In the operational range used in this work, there was no relationship between the optical density (680 nm) and the areal dry weight productivity (MJ m^{-2} d^{-1}) (Fig. 5ab), and subsequent correlation analysis found $r = 0.136$, $p = 0.450$. This shows that the cell density range used did not appreciably affect the performance of the system, and so the reactor was operated within a suitable cell density range to make best use of the energy available.

There was positive correlation between mean temperature (°C) and bio-energy production (MJ m^{-2} d^{-1}). Though the relationship was weaker than that for irradiance (Fig. 5ac), it was still highly significant ($r = 0.465$, $p = 0.006$). This relationship would be expected for an organism within its normal tolerance range since photosynthesis, especially carbon assimilation, is enzymatically mediated. However in an outdoor system such as this, light and temperature are related to each other because light, to an extent, drives temperature. Mean temperature and mean light values on the days sampled were weakly collinear ($r = 0.278$, $p = 0.117$). Thus it seems that temperature had an effect on growth, but it is difficult to exactly determine causality because a fraction of the variability apparently explained by temperature is co-incidence. Interestingly, no temperature controls were necessary for long-term effective cultivation of *Scenedesmus obliquus* with acceptable photosynthetic efficiency. The only reliable method to study the magnitude of the temperature effect outdoors would be to run replicate photobioreactors with different protocols of temperature control side-by-side. Amongst the many options for temperature control, Moheimani and Borowitzka (2006) showed that warming of outdoor raceways in the morning improved daily production by 11–21%, while Carlozzi and Sacchi (2001) used complete temperature control (30 °C) for the outdoor cultivation of *Rhodopseudomonas* for their tubular system. However, it is not clear what the energy losses from such systems might be; the use of temperature control must be more than offset by an equivalent increase in photosynthetic efficiency/bio-energy output. This is clearly an area for future research. Passive temperature controls such as greenhouses and insulating polythene structures could be used effectively, but interfere with the energy efficiency of the system by altering light transmission characteristics.

3.5. Power consumption and energy balance

Though there are many energy demands involved in cultivating microalgae in photobioreactors, the energy supply for mixing/circulating the culture of is generally the dominant factor (Das and Obbard 2011, Jorquera et al., 2010; Stephenson et al., 2010). The pump system used to circulate the culture through the photobioreactor used in this work was not optimised for energy input, but is probably typical of many photobioreactor systems currently employed. In order to fairly determine the total power requirement, the theoretic energy consumption was calculated for a range of flow rates, to characterise the reactor performance in this design configuration. The relationship between volumetric flow rate (m^3 s^{-1}) and power consumption (Watts) was determined by measurement of the pressure drop (ΔP, N m^{-2}) through the device. The relationship between ΔP and flow rate is shown in Fig. 6, where backward extrapolation confirms a static head pressure equivalent to the reactor height. The power consumption was calculated as: Watts (N m s^{-1}) = ΔP (N m^{-2}) × Flow (m^3 s^{-1}). At the flow rate used, this system had a power input of 281 W and a volume of 500 L, thus the volumetric power input for this system was 562 W m^{-3}. Jorquera et al. (2010) report values in the order of 2000–3000 W m^{-3} for typical tubular photobioreactors and the discrepancy is mainly due to differences in bulk fluid volume.

The power input was expressed per m^2 of photo-stage for comparison with biomass production (Fig. 6). This equated to a daily

Author's personal copy

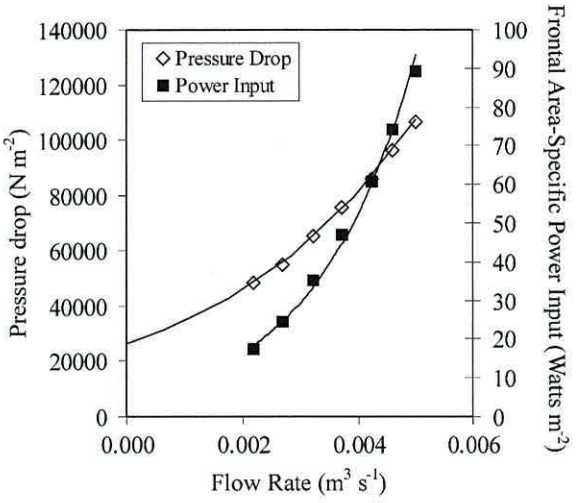


Fig. 6. Relationship between measurements of flow rate ($m^3 s^{-1}$) and pressure drop ($N m^{-2}$) for the photobioreactor used. The intercept corresponds to the static head. The relationship between flow rate ($m^3 s^{-1}$) and the calculated power input is also shown. Power input for the whole system was normalised per unit area of reactor frontal area ($W m^{-2}$).

energy input of 4.06 MJ $m^{-2} d^{-1}$, and compares poorly with the average daily bio-energy output of 0.27 MJ $m^{-2} d^{-1}$. Moreover, the areal energy input was approximately 1.5 times as great as the theoretic limits of photosynthesis for this location (assuming the mean daily light input of 26.7 MJ $m^{-2} d^{-1}$ total solar irradiance and a maximum photosynthetic efficiency of 10%). When expressed as a net energy ratio (NER) the mean value was 0.06 (±sd 0.03), so on a per-unit-area basis this reactor configuration consumed 15 times more energy in circulating the culture than it produced as biomass.

In order to reduce the energy consumption of this reactor design and achieve NER = 1, the photo-stage could be mounted horizontally (to remove the static head) and the flow rate reduced tenfold to around 25 litres per minute. It is important to note that this assumes the same productivity could be maintained. An alternative tube-cleaning method would be required. There may also be some potential to improve the flow dynamics to reduce the pressure drop, to re-arrange the tube layout and cycling the flow rate through lower velocities (e.g. at night) would improve the average daily energy consumption. Such energy-minimising techniques analogous to this have recently been proposed (Das and Obbard, 2011). An advanced system could use real-time irradiance measurements to efficiently manage flow rate for maximum net energy return. However even if NER > 1 could be achieved for this part of the process, other inefficiencies such as pump design efficiency, dewatering the biomass, reactor materials, and nutrient supply would still need to be accounted for where necessary.

Tubular photobioreactors such as the design employed here offer a very reliable method of producing contaminant-free cultures at high cell densities, are able to accurately and efficiently control the internal environment and have very low water losses through evaporation. This type of system thus seems very well-suited to producing high quality algal supplies for aquaculture, high-value pharmaceuticals/neutraceuticals and providing a quality inoculum for larger-scale bio-energy activities. They are also excellent experimental platforms for investigating microalgal performance on a large-scale. However, the necessity to move relatively large masses of liquid through narrow tubes at the velocity needed to satisfy growth and cleaning requirements inevitably incurs a high energy penalty. The evidence shows that the type of system employed here was not a sustainable method of producing *Scenedesmus* under the characteristic spring/summer conditions in temperate latitudes; an algal cultivation system with lower energy inputs would be required. Naturally, different outdoor tubular photobioreactors may have a variety of design configurations (Carlozzi and Sacchi, 2001; Fernandez et al., 2003; Tredici and Zitelli, 1998), and will be more effective in regions receiving higher mean light inputs (higher productivities to offset energy demands), or in arid regions where water evaporation is a serious problem for open systems.

3.6. Improving the energy efficiency of outdoor microalgal cultivations

Having established that the tubular system tested here is unlikely to meet bio-energy/CO_2 mitigation sustainability criteria, even with significant optimisation (Section 3.5), the obvious next step is to identify a system capable of operating within the efficiency limits imposed by microalgal photosynthesis. The tubular system used in this work could be substituted for a gas-sparged flat plate photobioreactor, which typically have lower power inputs than tubular systems (Jorquera et al., 2010). For flat plate systems, the power input ($W m^{-3}$), light path (cm) and frontal area: volume ratio are each related by geometry. This means that the energy demand for power input per unit frontal area of the reactor can be modelled easily, arriving at a design with lower energy inputs than the long-term measured photosynthetic energy conversion obtained in this work (i.e. 0.27 MJ $m^{-2} d^{-1}$). Normal power inputs for gas-sparged reactors are in the order of 50 W m^{-3} (Sierra et al., 2008), but it may be possible to use 10 to 20 Wm^{-3} (Zhang et al., 2001). Representative power inputs of 20 and 50 W m^{-3} are each modelled in Table 2. In gas-sparged photobioreactors the power input can be calculated as a product of the sparging rate and the liquid density, and is independent of the exact design used (see Sierra et al., 2008 for calculations). Further, for a given areal productivity, the volumetric productivity will vary inversely with the light path. Note that the assumption is that the same solar-to-biomass energy conversion efficiency could be achieved in the substitute photobioreactor compared to the tubular design tested here.

Table 2 shows that a flat plate photobioreactor operated at 50 W m^{-3} at the test site would need a light path of 6 cm or less in order to achieve a predicted net energy ratio > 1 for power input. Comparatively, a similar reactor operated at 20 W m^{-3} would require a light path length of less than 15 cm to achieve the same result. The conclusion is that a flat plate photobioreactor system operated at low power input with a short light path may be a fea-

Table 2
Modelled relationship between light path (cm) and the frontal-area specific power input (MJ $m^{-2} d^{-1}$) of a potential flat plate photobioreactor operated at two different sparging power inputs, 50 and 20 W m^{-3}. The predicted net energy ratio (NER) is calculated based on the long-term seasonally-averaged photosynthetic energy conversion rate obtained in this work (0.27 MJ $m^{-2} d^{-1}$).

Light path (cm)	MJ $m^{-2} d^{-1}$ at 50 W m^{-3} sparging rate	NER for 50 W m^{-3}	MJ $m^{-2} d^{-1}$ at 20 W m^{-3} sparging rate	NER for 20 W m^{-3}
2	0.09	3.13	0.03	7.81
3	0.13	2.08	0.05	5.21
4	0.17	1.56	0.07	3.91
5	0.22	1.25	0.09	3.13
6	0.26	1.04	0.10	2.60
7	0.30	0.89	0.12	2.23
8	0.35	0.78	0.14	1.95
9	0.39	0.69	0.16	1.74
10	0.43	0.63	0.17	1.56
11	0.48	0.57	0.19	1.42
12	0.52	0.52	0.21	1.30
13	0.56	0.48	0.22	1.20
14	0.60	0.45	0.24	1.12
15	0.65	0.42	0.26	1.04
16	0.69	0.39	0.28	0.98

Author's personal copy

sible method of producing a sustainable source of bio-energy at the test site and in similar temperate regions. This may conversely be regarded as an engineering method of improving volumetric productivity. Naturally, other energy burdens still need to be accounted for as outlined in Section 3.5, but energy ratios in Table 2 clearly demonstrate the potential viability of a photobioreactor system for temperate regions (without temperature control), and this may be a starting point for designing future experiments.

It is important to note that there is a complex relationship between the design of a photobioreactor and its ability to convert solar energy into microalgal biomass, evidenced by the wide range of designs and productivities in the literature (e.g. Tredici and Zitelli, 1998). The work presented here provides valuable data which can be used to inform further experimentation. However, we identify that there is a need to develop and improve the relationship between photobioreactor energy inputs and bio-energy outputs in outdoor conditions. To our knowledge very little work has addressed this issue (most research has concentrated on volumetric biomass production alone), and such information is urgently required in order to advance the field.

4. Conclusions

The mean long-term bio-energy productivity of the test photobioreactor using *Scenedesmus obliquus* at a mid-temperate location was 0.27 MJ m^{-2} d^{-1}. Artificial heating was not required for high cell density and areal productivity. The relationship between energy inputs and outputs is complex but the study highlights the need to operate reactors within the limits of microalgal photosynthesis and growth dynamics. The energy efficiency data from the study was used to recommend adaptations for gas-sparged photobioreactors increasing energy efficiency and thereby potentially achieving positive energy returns in the test environment.

Acknowledgements

This research was supported by an Engineering and Physical Sciences Research Council (EPSRC) industrial studentship in collaboration with RWE npower UK and Varicon Aqua Solutions UK. The authors thank Richard Hotchkiss, Joe McDonald, Martin Pilbin and Roger Bewley. The authors thank two anonymous reviewers for their helpful comments.

References

Camacho, F., Gomez, A., Fernandez, F., Sevilla, J., Grima, E., 1999. Use of concentric-tube airlift photobioreactors for microalgal outdoor mass cultures. Enzyme Microb. Technol. 24, 164–172.

Campbell, P., Beer, T., Batten, D., 2011. Life cycle assessment of biodiesel production from microalgae in ponds. Bioresour. Technol. 102, 50–56.

Carlozzi, P., Sacchi, A., 2001. Biomass production and studies on *Rhodopseudomonas palustris* grown in an outdoor, temperature controlled, underwater tubular photobioreactor. J. Biotechnol. 88, 239–249.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Clarens, A., Resurreccion, E., White, M., Colosi, L., 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ. Sci. Technol. 44, 1813–1819.

Das, P., Obbard, J., 2011. Incremental energy supply for microalgae culture in a photobioreactor. Bioresour. Technol. 102, 2973–2978.

Fernandez, F., Hall, D., Guerrero, E., Rao, K., Molina Grima, E., 2003. Outdoor production of *Phaeodactylum tricornutum* biomass in a helical reactor. J. Biotechnol. 103, 137–152.

Gons, H., Mur, L., 1980. Energy requirements for growth and maintenance of *Scenedesmus protuberans* Fritsch in light-limited continuous cultures. Arch. Microbiol. 125, 9–17.

Hulatt, C., Thomas, D., 2010. Dissolved organic matter in microalgal photobioreactors: a potential loss in solar energy conversion? Bioresour. Technol. 101, 8690–8697.

Ibrahim, A., Othman, M., Ruslan, M., Mat, S., Sopian, K., 2011. Recent advances in flat plate photovoltaic/thermal (PV/T) solar collectors. Renew. Sust. Energy Rev. 15, 352–365.

Jorquera, O., Kiperstock, A., Sales, E., Embirucu, M., Ghirardi, M., 2010. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresour. Technol. 101, 1406–1413.

Kunjapur, A., Eldridge, R., 2010. Photobioreactor design for commercial biofuel production from microalgae. Ind. Eng. Chem. Res. 49, 3516–3526.

Lardon, L., Helias, A., Sialve, B., Steyer, J., Bernard, O., 2009. Life-cycle assessment of biodiesel production from microalgae. Environ. Sci. Technol. 43, 6475–6481.

Lopez, C., Garcia, M., Fernandez, F., Bustos, C., Chisti, Y., Sevilla, J., 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresour. Technol., 7587–7591.

Mandal, S., Mallick, N., 2009. Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. Appl. Microbiol. Biotechnol. 84, 281–291.

Mata, T., Martins, A., Caetano, N., 2010. Microalgae for biodiesel production and other applications: a review. Renew. Sust. Energ. Rev. 14, 217–232.

Morweiser, M., Kruse, O., Hankamer, B., Posten, C., 2010. Developments and perspectives of photobioreactors for biofuel production. Appl. Microbiol. Biotechnol. 87, 1291–1301.

Moheimani, N., Borowitzka, M., 2006. The long-term culture of the coccolithophore *Pleurochrysis carterae* (Haptophyta) in outdoor raceway ponds. J. Appl. Phycol. 6, 703–712.

Nagaraja, R., 1984. Photosynthetically active components of global solar radiation: measurements and model computations. Meteorol. Atmos. Phys. 34, 354–364.

Nalewajko, C., Colman, B., Olaveson, M., 1997. Effects of pH on growth, photosynthesis, respiration, and copper tolerance of three Scenedesmus strains. Environ. Exp. Bot. 37, 153–160.

Parkhill, J., Maillet, G., Cullen, L., 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J. Phycol. 37, 517–529.

Pruvost, J., Van Vooren, G., Le Gouic, B., Couzinet-Mossion, A., Legrand, J., 2010. Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. Bioresour. Technol. 102, 150–158.

Qiang, H., Guterman, H., Richmond, A., 1996. Physiological characterisitcs of *Spirulina platensis* (cyanobacteria) cultured at ultrahigh cell densities. J. Phycol. 32, 1066–1073.

Richmond, A., Wu, Z., 2001. Optimisation of a flat plate glass reactor for mass production of *Nannochloropsis* sp. outdoors.. J. Biotechnol. 85, 259–269.

Scott, S., Davey, M., Dennis, J., Horst, I., Howe, C., Lea-Smith, D., Smith, A., 2010. Biodiesel from algae: challenges and prospects. Curr. Opin. Biotechnol. 21, 277–286.

Shang, H., Scott, J., Shepherd, S., Ross, G., 2010. A dynamic thermal model for heating microalgae incubator ponds using off-gas. Chem. Eng. Sci. 65, 4591–4597.

Sierra, E., Acien, F., Fernandez, J., Garcia, J., Gonzalez, C., Molina, E., 2008. Characterisation of a flat plate photobioreactor for the production of microalgae. Chem. Eng. J. 138, 136–147.

Silva, T., Reis, A., Medeiros, R., Oliveira, A., Gouveia, L., 2009. Oil production towards biofuel from autotrophic microalgae semicontinuous cultivations monitorized by flow cytometry. Appl. Biochem. Biotechnol. 159, 568–578.

Stephenson, A., Kazamia, E., Dennis, J., Howes, C., Scott, S., Smith, A., 2010. Life-cycle assessment of potential algal biodiesel production in the United Kingdom: A comparison of raceways and air-lift tubular bioreactors. Energ. Fuel. 24, 4062–4077.

Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion as a necessary step to make microalgal biodiesel sustainable. Biotechnol. Adv. 27, 409–416.

Tredici, M., Zitelli, G., 1998. Efficiency of sunlight utilisation: tubular vs. flat photobioreactors. Biotechnol. Bioeng. 57, 187–197.

Ugwu, C., Aoyagi, H., Uchiyama, H., 2008. Photobioreactors for mass cultivation of algae. Bioresour. Technol. 99, 4021–4028.

Vunjak-Novakovic, G., Kim, Y., Wu, X., Berzin, I., Merchuk, J., 2005. Air-lift bioreactors for algal growth on fuel gas: mathematical modeling and pilot-plant studies. Ind. Eng. Chem. Res. 44, 6154–6163.

Williams, P., Laurens, L., 2010. Microalgae as biodiesel and biomass feedstocks: review and analysis of the biochemistry, energetics and economics. Energy Environ. Sci. 3, 554–590.

Zhang, K., Miyachi, S., Kurano, N., 2001. Photosynthetic performance of a cyanobacterium in a vertical flat-plate photobioreactor for outdoor microalgal production and fixation of CO_2. Biotechnol. Lett. 23, 21–26.

Chapter 7.

Anaerobic Co-digestion of Microalgal Biomass and Cellulose for Bio-hydrogen.

7. Overview.

The hypothesis tested in this work was that *microalgal biomass digested alone or co-digested with cellulosic material yields significant quantities of H_2 and/ or CH_4 gas.* Production of methane by anaerobic digestion of microalgal biomass has been reported for some time (Oswald & Golueke, 1960). However, evidence for H_2 production is scarce. Co-digestion is a method by which biomass digestion efficiency can be improved, so research also examined the effect of combining different proportions of microalgal biomass with cellulose. Cellulosic material was used as the co-substrate as it is also of significant interest for bio-energy production (Berndes et al, 2001). Chapter 7.1 introduces the experiments, Chapter 7.2 is a published article.

7.1. Biomass Production and First Enrichment

7.1.1. Biomass Production

Chlorella vulgaris and *Dunaliella tertiolecta* biomass was produced in autotrophic conditions in 20 L polythene photobioreactors sparged with air at 0.5 vvm (Fig. 7.1). Microalgal growth medium for *Dunaliella tertiolecta* comprised Walnes medium, whilst that for *Chlorella vulgaris* was Jaworski medium (see Culture Collection of Algae and Protozoa, Oban, UK for details). Cultivation conditions were nutrient-replete. Biomass was harvested by flocculation and centrifugation (Fig 7.1). *Chlorella vulgaris* was harvested by addition of chitosan solution (8 g chitosan, 100 ml acetic acid, 1.9L water) to the cultivation, followed by correction of pH with 3M NaOH to pH 7-8. *Dunaliella tertiolecta* was harvested by pH correction alone (Horiuchi et al., 2003) by adding 3M NaOH to pH 9-10. Biomass was collected by siphon, centrifuged at 1000 rcf for 10 minutes, the supernatant removed, and the material stored frozen at -80°C.

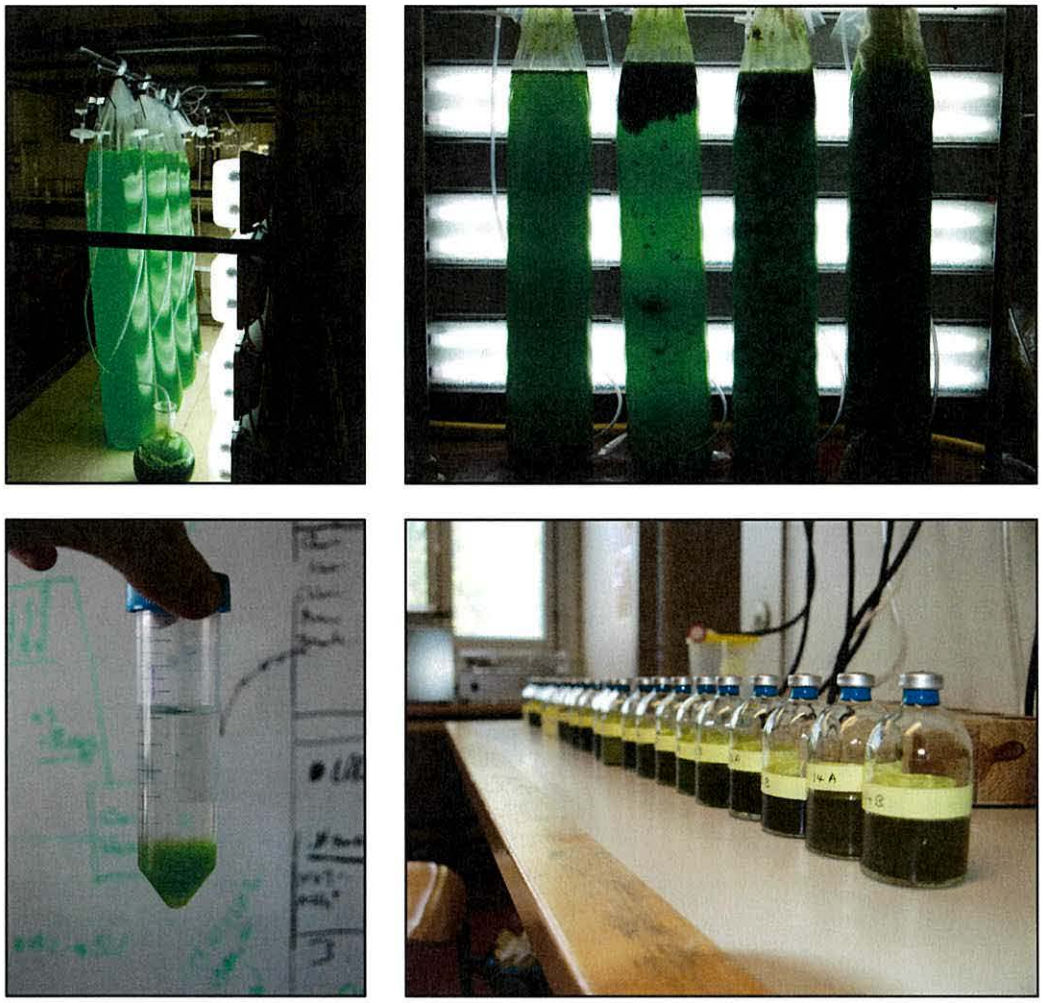


Fig. 7.1. Cultivation in 20L photobioreactors (top left); different stages in development of flocs (top right); result of flocculation in a 50 ml tube (bottom left); Experimental anaerobic serum bottles used for digestion experiments (bottom right).

7.1.2. Co-digestion

Co-digestion can be used to increase performance by modification of C:N ratios. Various substrates may be used (Sialve et al., 2009; Yen & Brune, 2007), but cellulosic material is of particular interest for bio-energy technologies (Lo et al., 2009; Zhu & Pan, 2009). The chemical BESA (bromoethanesulfonic acid) was added

to selected treatments to inhibit possible methanogens, and thermophillic conditions (60°C) were used throughout. For detailed methods see Chapter 7.2.

7.1.3. Gas results of first enrichment

Cultivations were carried out in batch anaerobic serum bottles (Fig. 7.1) according to methods detailed in Chapter 7.2. It was found that that co-digestion of *Dunaliella tertiolecta* biomass with cellulose produced high yields of H_2 (Table 7.1). Considering that the inoculum microbe consortium was enriched on cellulose rather than microalgal biomass, data indicated significant potential for H_2 production, especially from co-digested materials. No methane was produced during the enrichments.

Table 7.1. Gas yields (H_2 and CO_2, mmol g^{-1} volatile suspended solids) at the end of first experiment (10 days incubation). UNTR indicates absence of BESA.

Treatment	H_2 (mmol g^{-1})	CO_2 (mmol g^{-1})
No substrate, innoculated, UNTR	0	0
No substrate, innoculated, BESA	0	0
No innoculum, *Chlorella*	106.5	58.3
No innoculum, *Dunaliella*	0	0
100% Cellulose, UNTR	145.2 (3.7)	256.4 (17.0)
100% Cellulose, BESA	178.8 (11.2)	267.4 (4.1)
100% *Chlorella*, UNTR	82.9 (42.3)	37.0 (8.4)
100% *Chlorella*, BESA	65.4 (16.2)	81.7 (30.4)
100% *Dunaliella*, UNTR	44.9 (0.3)	20.7 (1.3)
100% *Dunaliella*, BESA	20.9 (29.6)	16.5 (8.5)
50/50 *Chlorella*/Cellulose, UNTR	67.9 (22.5)	21.7 (2.7)
50/50 *Chlorella*/Cellulose, BESA	72.3 (7.1)	50.0 (8.2)
50/50 *Dunaliella*/Cellulose, UNTR	166.0 (7.6)	128.0 (5.4)
50/50 *Dunaliella*/Cellulose, BESA	180.0 (7.2)	145.2 (3.5)

7.1.4. References

Berndes, G., Azar, C., Kaberger, T., Abrahamson, D. 2001. The feasibility of large-scale lignocellulose-based bioenergy production. Biomass & Bioenergy. 20, 371-383.

Horiuchi, J., Ohba, I., Tada, K., Kobayashi, M., Kanno, T., Kishimoto, M. 2003. Effective cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control. Journal of Bioscience and Bioengineering. 95, 412-415.

Lo, Y., Huang, C., Fu, T., Chen, C., Chang, J. 2009. Fermentative hydrogen production from hydrolysed cellulosic feedstock prepared with a thermophilic anaerobic bacterial isolate. International Journal of Hydrogen Energy. 34, 6189-6200.

Oswald, W., Golueke, C. 1960. Biological transformations of solar energy. Advances in Applied Microbiology. 2, 223-262.

Sialve, B., Bernet, N., Bernard, O. 2009. Anaerobic digestion as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances. 27, 409-416.

Yen, H., Brune, D. 2007. Anaerobic co-digestion of algal sludge and waste-paper to produce methane. Bioresource Technology. 98, 130-134.

Zhu, J., Pan, X. 2010. Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation. Bioresource Technology. 101, 4992-5002.

Biodegradation
DOI 10.1007/s10532-010-9419-z

ORIGINAL PAPER

Thermophilic, anaerobic co-digestion of microalgal biomass and cellulose for H_2 production

Sarah M. Carver · Chris J. Hulatt · David N. Thomas · Olli H. Tuovinen

Received: 19 April 2010 / Accepted: 14 September 2010
© Springer Science+Business Media B.V. 2010

Abstract Microalgal biomass has been a focus in the sustainable energy field, especially biodiesel production. The purpose of this study was to assess the feasibility of treating microalgal biomass and cellulose by anaerobic digestion for H_2 production. A microbial consortium, TC60, known to degrade cellulose and other plant polymers, was enriched on a mixture of cellulose and green microalgal biomass of *Dunaliella tertiolecta*, a marine species, or *Chlorella vulgaris*, a freshwater species. After five enrichment steps at 60°C, hydrogen yields increased at least 10% under all conditions. Anaerobic digestion of *D. tertiolecta* and cellulose by TC60 produced 7.7 mmol H_2/g volatile solids (VS) which were higher than the levels (2.9–4.2 mmol/g VS) obtained with cellulose and *C. vulgaris* biomass. Both microalgal slurries contained satellite prokaryotes. The *C. vulgaris* slurry, without TC60 inoculation, generated H_2 levels on par with that of TC60 on cellulose alone. The biomass-fed anaerobic digestion resulted in large shifts in short chain fatty acid concentrations and increased ammonium levels. Growth and H_2 production increased when TC60 was grown on a combination of *D. tertiolecta* and cellulose due to nutrients released from algal cells via lysis. The results indicated that satellite heterotrophs from *C. vulgaris* produced H_2 but the *Chlorella* biomass was not substantially degraded by TC60. To date, this is the first study to examine H_2 production by anaerobic digestion of microalgal biomass. The results indicate that H_2 production is feasible but higher yields could be achieved by optimization of the bioprocess conditions including biomass pretreatment.

Keywords *Dunaliella tertiolecta* · *Chlorella vulgaris* · Cellulose degradation · Anaerobic digestion · Hydrogen production

S. M. Carver (✉) · O. H. Tuovinen
Department of Microbiology, Ohio State University,
484 W. 12th Ave., Columbus, OH 43210, USA
e-mail: carver.84@gmail.com

S. M. Carver · O. H. Tuovinen
Department of Chemistry and Bioengineering,
Tampere University of Technology,
P.O. Box 541, 33101 Tampere, Finland

C. J. Hulatt · D. N. Thomas
School of Ocean Sciences, College of Natural Sciences,
Bangor University, Menai Bridge,
Anglesey LL59 5AB, UK

D. N. Thomas
Marine Research Centre, Finnish Environment Institute,
P.O. Box 140, 00251 Helsinki, Finland

Published online: 28 September 2010

Introduction

Microalgal biomass ties into multiple areas of bioenergy production, such as photosynthetic H_2 and anaerobic biogas production. Microalgae can produce H_2 by coupling photosynthesis with hydrogenases or

nitrogenases present in intracellular membranes (Benemann 2000; Melis and Happe 2001; Ghirardi et al. 2009). Similar biophotolysis can also occur in cyanobacteria. The efficiency of converting light energy into chemical energy in H_2 ranges between 3 and 15%, but under ambient environmental conditions conversions are less than 3% (Benemann 2000; Ghirardi et al. 2009). Direct H_2 production from microalgae as a source of sustainable energy is unlikely due to low efficiencies.

Multiple approaches have been tested for microalgae-coupled H_2 production. Some microalgae, such as the often studied green alga *Chlamydomonas reinhardtii*, can be grown under conditions known to encourage intracellular accumulation of starch (Ike et al. 1996, 1997; Kawaguchi et al. 2001). The biomass is then digested through acid or heat hydrolysis and fed to starch hydrolyzing heterotrophs, such as *Rhodobacter sphaeroides,* which can produce H_2 fermentatively (Ike et al. 1996). Kawaguchi et al. (2001) tested a three-phase approach whereby *C. reinhardtii* biomass was first grown to accumulate starch, followed by conversion of the starch to lactic acid by bacteria, and lastly, the fermentative production of H_2 from lactic acid by undefined bacteria. These approaches have produced H_2 yields too low to be feasible for industrial applications (Levin et al. 2004).

Microalgae have been used in wastewater treatment, specifically during secondary treatment processes to assimilate nutrients into biomass (Kojima and Lee 2001). Several decades ago, bulk microalgal biomass from wastewater treatment was recognized as a readily accessible feedstock for anaerobic digestion (Golueke et al. 1957; Oswald and Golueke 1960). Hernández and Córdoba (1993) demonstrated that biogas could be produced from *Chlorella vulgaris* biomass upon anaerobic digestion. Of the total biogas, 68–76% was CH_4 and total gas yields ranged between 0.40 and 0.45 l/g COD removed. Yen and Brune (2007) showed the feasibility of anaerobic co-digestion of waste paper and microalgal sludge, yielding up to 1.6 l (ca. 70 mmol) CH_4/l·d. Effluents from anaerobic digesters, especially those in the olive oil industry, have also been used as a medium for microalgal production (Hodaifa et al. 2008; Cordóba et al. 2008). Currently, the economic sustainability of biodiesel production from microalgal lipids is believed to be dependent on anaerobic digestion of residual biomass for additional biofuels (Sialve et al. 2009).

Biodiesel production from microalgae has four major phases: mass growth of microalgal biomass in photobioreactors, dewatering of biomass, lipid extraction, and processing of the lipid fraction for biodiesel production (Lardon et al. 2009; Mata et al. 2010). Residual biomass following lipid extraction has no further use for biodiesel production but could provide a feedstock for additional, sustainable energy production via anaerobic digestion processes. The ratio of carbon to nitrogen in the microalgal biomass is relatively low (<10) and could prove to be an issue for anaerobic digestion (Parkin and Owen 1986). Therefore, additional biodegradable C-rich compounds may be beneficial as a co-substrate with spent algal biomass (Hernández and Córdoba 1993; Yen and Brune 2007). Plant biomass, especially cellulose, provides an abundant feedstock that has been extensively studied and promoted in the sustainable energy field (Perlack et al. 2005; Department of Energy 2007). As cellulose feedstock is C-rich, it provides an ideal co-substrate for anaerobic digestion of microalgal biomass. Anaerobic digestion can be directed toward CH_4 or H_2 production but only H_2 provides a potential energy source that is sustainable and carbonless (Ren et al. 2009).

The purpose of this study was to examine the feasibility of H_2 production via anaerobic digestion of microalgal biomass. *Dunaliella tertiolecta* and *Chlorella vulgaris*, both green algae, were grown for mass harvest in this study. A thermophilic, cellulolytic microbial consortium was initially enriched with mixtures of the feedstocks, cellulose and microalgal biomass, before final testing for biogas and metabolite production.

Materials and methods

Cultivation and harvesting of microalgae

The freshwater microalga *Chlorella vulgaris* (strain 211/11B, Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, Oban, Argyll, UK) and marine *Dunaliella tertiolecta* (strain 13.86, Sammlung von Algenkulturen des Instituts für Pflanzenwissenschaften der Universität Göttingen, Germany) were selected for this study. Both green algae have been the subject of recent research endeavors and discussed as potential sources of biofuel (Hulatt and Thomas 2010).

C. vulgaris has a typical eukaryotic algal cell wall, whereas, *D. tertiolecta* is devoid of a rigid cell wall (Sialve et al. 2009; Ben-Amotz et al. 2010).

The algae were cultured autotrophically in 20 l photobioreactors. Photobioreactors were cylindrical (∅ 0.16 m), constructed of polyethylene, and sparged with 0.3 μm filtered air at 0.5 l/l·min (Whatman Hepa-Vent). Light was provided by cool white fluorescent tubes and the incident photosynthetic photon flux density averaged 225 μmol photons of photosynthetically active radiation m^2/s. *C. vulgaris* was grown in Jaworski's medium (http://www.ccap.ac.uk/media/documents/JM.pdf) prepared with Milli-Q water. *D. tertiolecta* was cultured in Walne's medium (http://www.ccap.ac.uk/media/documents/Walnes.pdf) made of sterilized seawater (~3.5% salinity).

Microalgal biomass was harvested via flocculation and centrifugation. For *D. tertiolecta*, the pH was adjusted with NaOH to approximately pH 9.5 for flocculation (Horiuchi et al. 2003). *C. vulgaris* was flocculated by adding 0.08 g chitosan/l and adjusting the pH to 7.0. Biomass was concentrated by centrifugation at 1,000×*g* for 10 min and removing the supernatant. The thick slurry was stored at −20°C. The pH of each slurry was adjusted to pH 7.0. Slurry volatile solid (VS) concentrations were 0.094 and 0.14 g/l for *D. tertiolecta* and *C. vulgaris*, respectively.

Microbial consortium

The microbial consortium, designated TC60, originated from the interior of a compost pile and subcultures had been maintained with cellulose. The culture can also grow on hemicellulose, pectin and starch. The identification of the dominant species based on 16S rRNA gene sequences is presently in progress. Based on qualitative PCR-DGGE analysis, the dominant species vary with substrate and other incubation conditions.

The TC60 culture was maintained anaerobically (N_2 headspace) in medium that contained (per liter): 2 g trypticase, 1 g yeast extract, 4 g Na_2CO_3, 0.23 g K_2HPO_4, 0.18 g KH_2PO_4, 0.36 g NH_4Cl, 0.04 g NaCl, 0.09 g $MgSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2 \cdot 2H_2O$, 0.25 g cysteine-HCl, 0.25 g $Na_2S \cdot 9H_2O$, 2 mg $CoCl_2 \cdot 6H_2O$, 0.16 mg Na_2SeO_4, and 0.09 mg $NiCl_2 \cdot 6H_2O$. Cellulose (Sigmacell, Type 20) was purchased from Sigma-Aldrich. Microalgal biomass and cellulose were added to a combined concentration of 4 g volatile solids (VS)/l. Cultures (50 ml) in 125 ml serum bottles were inoculated (10% v/v) and degassed prior to incubation at 60°C with 180 rev/min. Samples were withdrawn anaerobically, centrifuged at 16,000×*g* for 10 min, and the fractions were stored at −20°C until further analysis.

Four consecutive enrichment passages were completed before the fifth enrichment was analyzed in detail. The initial four enrichment stages were incubated in duplicate as follow: *D. tertiolecta* to cellulose (1:1 VS/VS), *D. tertiolecta* only, and the same two conditions with *C. vulgaris*. Appropriate controls included *D. tertiolecta* without TC60, *C. vulgaris* without TC60, TC60 with cellulose, and TC60 with medium only. The fourth enrichment of 1:1 microalgal biomass to cellulose was used to inoculate 1:2, 2:1, and 1:0 (VS/VS) ratios of substrate in the fifth enrichment. Each condition was tested in duplicate. At the end of the incubation, total and volatile solids (TS and VS, respectively) were measured according to the Standard Methods (Eaton et al. 2005). For the fifth enrichment, TS and VS were measured prior to incubation and after 10 days.

Analytical methods

Supernatants for HPLC analysis of short chain fatty acids (SCFAs) were cleaned via solid phase extraction (C18-T), diluted with Milli-Q water, and filtered through a 0.2 μm PTFE filter (Pall). A guard column, 5 cm × 4.6 mm ID (Supelguard H) and a cation-exchange column, 30 cm × 7.8 mm ID (Supelcogel C-610H) were used with an autosampler (Spectra-Physica AS 3000) and a UV detector set to 210 nm (Spectra-Physics SP100). A Beckman 114 M HPLC pump maintained a flow rate of 0.5 ml/min of the mobile phase, 0.1% *o*-phosphoric acid (Peu et al. 2004). Run times were 65 min per sample with 100 μl injections at 70 min intervals. Chromatographs were analyzed through a computer interface equipped with the Clarity Chromatography Station (DataApex).

Overpressures were measured with a sterile syringe immediately after removal from the incubator and headspace samples were manually injected into the GC. Gases were analyzed with a Shimadzu GC-2014 equipped with a thermal conductivity detector and a Porapak N (2 m length × 2 mm ID) column (Sigma-Aldrich). The carrier gas, nitrogen, was maintained at

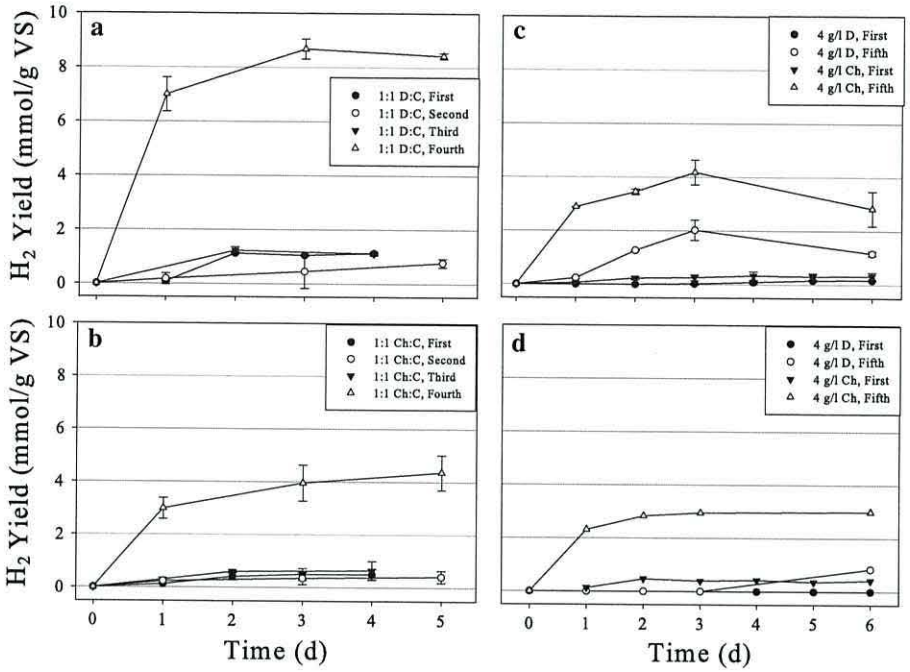


Fig. 1 Improvement of H_2 yields over sequential enrichment cultures. **a** 1:1 (VS/VS) *D. tertiolecta* to cellulose with TC60. **b** 1:1 *C. vulgaris* to cellulose with TC60; **c** First and fifth enrichments of TC60 with only microalgal biomass. **d** first and fifth enrichments of microalgal biomass without TC60. *Vertical bars* indicate the standard error. *C* cellulose, *Ch C. vulgaris*, *D D. tertiolecta*

20 ml/min. Temperatures were 110°C for the injector and detector while the column oven was kept at 80°C. The chromatographs were analyzed with GC Solution Analysis software (Shimadzu).

The ammonium concentration in supernatants was measured fluorimetrically according to Holmes et al. (1999). Samples were diluted with Milli-Q water and analysis was conducted using a Hitachi F2000 fluorometer. Excitation was measured at 360 nm and emission at 420 nm.

Solids for C and N analysis were dried at 80°C for 72 h followed by measurement with a Thermo Electron FlashEA 1112 analyzer (Thermo Scientific). The instrument was calibrated using sulfanilamide, 2,5-bis(5-*tert*-butyl-benzoxazol-2-yl)thiophene, L-cystine, and DL-methionine as standards.

Results and discussion

Enrichment improves H_2 production

By the fourth enrichment, headspace H_2 and CO_2 levels had increased (Fig. 1). CH_4 was not detected under any experimental conditions in this work. Slight changes in gas yields were seen between TC60 enrichments when grown on cellulose. The TS and VS levels were relatively constant from one enrichment to another (16.0–17.8 g TS/l and 8.2–9.3 g VS/l for *Dunaliella*; 9.2–11.2 g TS/l and 3.8–5.3 g VS/l for *Chlorella*). Following enrichment, H_2 production increased from 0.2 to 2.1 mmol H_2/g VS for *D. tertiolecta* and 0.3 to 4.2 mmol H_2/g VS for *C. vulgaris* when inoculated with TC60 (Fig. 1c). H_2 levels also increased when the microalgal biomass was not inoculated with TC60 (Fig. 1d). Autoclaved anaerobic controls showed no gas production, ruling out abiotic H_2 production.

Microscopic examination revealed a diverse microbial population associated with the microalgal biomass controls after enrichment. Both the *Dunaliella* and *Chlorella* slurries contained bacteria and protozoa, which included microorganisms capable of producing H_2 under the thermophilic conditions. Heterotrophic contamination may have been associated with the original stock cultures or introduced during the cultivation and handling of microalgal biomass.

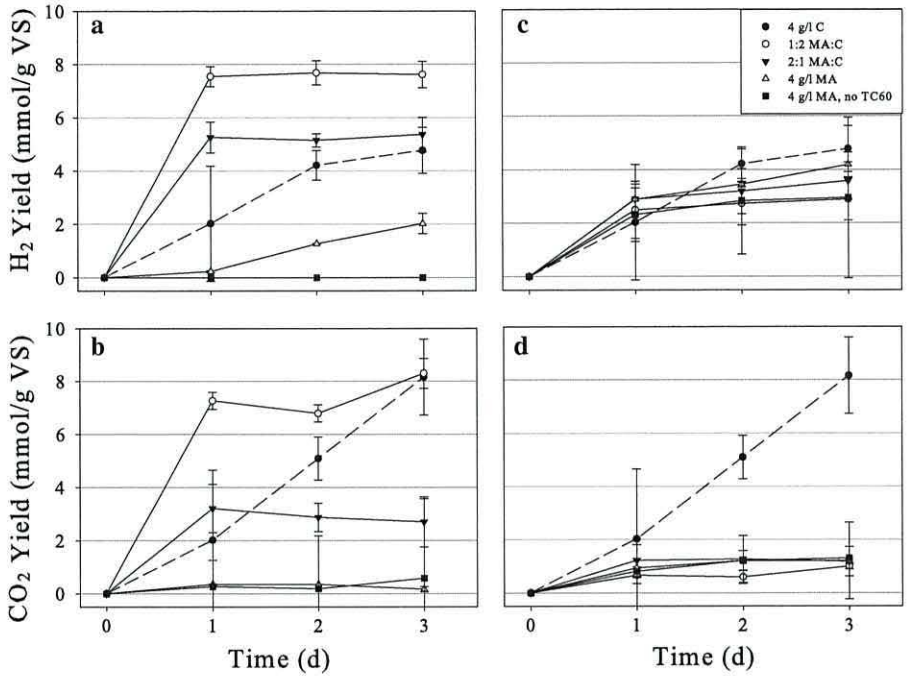


Fig. 2 Cumulative gas yields during the first three days of the fifth enrichment of TC60 with different substrate conditions. **a** H_2 and **b** CO_2 yields for samples with *D. tertiolecta;* **c** H_2 and **d** CO_2 yields for samples with *C. vulgaris.* As a reference, gas yields for cellulose-fed TC60 are included (*dashed line*). H_2 and CO_2 yields are included for uninoculated microalgal biomass. *C* cellulose; *MA* microalgal biomass

Analysis of the fifth enrichment

The fourth enrichment of the 1:1 (VS/VS) ratio of microalgal biomass to cellulose was used to inoculate the fifth enrichment, which received 1:2, 2:1, or 1:0 ratios of microalgal biomass to cellulose. Most gas production occurred within the first three days under all conditions. In addition to H_2, CO_2 was monitored in order to observe the growth of TC60. Direct measurement of microalgal or bacterial biomass was not possible due to unknown quantities of incomplete hydrolysis products and lysed cells.

As shown in Fig. 2, cellulose-fed TC60 yielded 5.2 mmol H_2 and 8.9 mmol CO_2/g VS by day 3. When grown on a ratio of 1:2 *D. tertiolecta* biomass and cellulose, the H_2 levels increased whereas the CO_2 levels were relatively constant and similar to those in cellulose-fed cultures (Fig. 2a, b). The H_2:CO_2 ratio increased from 0.8 with cellulose to 1.1 (Table 1). With a 2:1 ratio, the gas yields were comparable to those in cellulose-fed cultures (7.7 mmol H_2 and 8.6 mmol CO_2/g VS) and the H_2:CO_2 ratio increased to 1.8. TC60 fed only *D. tertiolecta* without cellulose yielded a very high H_2:CO_2 ratio but individual gas yields were relatively low (Table 1; Fig. 2). When *D. tertiolecta* biomass was not inoculated, both the H_2 and CO_2 yields remained low. These results indicated that *D. tertiolecta* biomass serves as an additional source of nutrients for TC60 and enhanced H_2

Table 1 H_2:CO_2 ratios (± standard error) for TC60 grown on various substrates

Condition	H_2:CO_2	
	Day 2	Day 6
No added substrate	1.98	1.16
4 g/l cellulose	0.83 ± 0.02	0.61 ± 0.01
1:2 *Dunaliella*:cellulose	1.13 ± 0.01	0.90 ± 0.02
2:1 *Dunaliella*:cellulose	1.81 ± 0.25	1.24 ± 0.20
4 g/l *Dunaliella*	3.69 ± 0.03	1.93 ± 0.10
4 g/l *Dunaliella* (no TC60)	0.00	0.82
1:2 *Chlorella*:cellulose	4.71 ± 0.59	1.67 ± 0.40
2:1 *Chlorella*:cellulose	3.03 ± 1.45	1.41 ± 0.07
4 g/l *Chlorella*	3.00 ± 0.84	1.60 ± 0.02
4 g/l *Chlorella* (no TC60)	2.35	2.32

production when cellulose was present. The heterotrophs associated with the *D. tertiolecta* biomass were relatively inactive under conditions in this study. Heterotrophs in the *D. tertiolecta* culture are most likely halophiles or at least highly salt-tolerant (~3.5% salinity) and their activity would be negligible in low salinity medium.

In the *C. vulgaris*-fed TC60 culture, growth remained low according to the CO_2 levels, and the H_2 levels under all conditions were within standard error measurements, averaging approximately 3.0 mmol H_2 and 2.5 mmol CO_2/g VS (Fig. 2d). These H_2 levels were comparable to those observed when TC60 was fed cellulose. Microscopic examination showed a diverse prokaryotic population in the *C. vulgaris* slurry. H_2 was produced without inoculation of TC60, indicating anaerobic activity of satellite heterotrophs associated with *C. vulgaris*. Due to the comparable H_2 yields regardless of the substrate ratio, it is concluded that these heterotrophs used organic compounds in the medium, e.g., yeast extract, tryptic case, and microalgal excreta.

The ammonium and SCFA concentrations were also monitored in the anaerobic digestion experiments. Formation of ammonium from protein degradation has been cited as a possible concern for microalgal digestion (Tam and Wong 1996; Yen and Brune 2007; Sialve et al. 2009). However, ammonium concentrations, up to 17.7 mM, did not have adverse effects on growth of TC60 and gas production (data not shown). When TC60 was fed cellulose, the concentrations of ammonium remained below 10 mM (Table 2). Without cellulose or microalgal biomass, the ammonium yields increased to 30 mM. Ammonium concentrations increased with the concentration of *D. tertiolecta* biomass, indicating enhanced ammonification of N-containing compounds in the medium. When the ratio of *D. tertiolecta* to cellulose was 1:2, the ammonium concentration was 16.3 mM and increased to 21.5 mM when TC60 was supplied with *D. tertiolecta* without cellulose. With *C. vulgaris* biomass, ammonium concentrations (24.3–27.4 mM) were relatively high under all experimental conditions. These results indicated ammonification by satellite heterotrophs regardless of TC60 inoculation.

The concentrations and trends in SCFA profiles varied with experimental conditions. When TC60 was grown only on cellulose, the dominant SCFAs were lactate (22.1 mM), butyrate (18.1 mM) and acetate (10.9 mM) (Table 2). In the presence of *D. tertiolecta* or *C. vulgaris* biomass, lactate concentration was <4 mM, whereas both acetate and butyrate levels increased (Table 2; Fig. 3). Lactic acid fermentation was suppressed by the presence of microalgal biomass, therefore allowing for increased H_2 yields. In the presence of *D. tertiolecta* and cellulose, the SCFA profiles shifted towards acetate and butyrate pathways which are coupled with hydrogenases.

Total SCFA concentrations increased with the amount of *C. vulgaris* biomass (Table 2). This association appeared to be the result of heterotrophic microorganisms present in the microalgal slurry rather than TC60. The controls without TC60 showed acid production at levels four times higher with *C. vulgaris* than with the *D. tertiolecta* control (Fig. 3). The

Table 2 Maximum lactate, acetate, butyrate, and ammonium concentrations (± standard error) during the 5th enrichment

Condition	Maximum metabolite concentration (mM)				
	Lactate	Acetate	Butyrate	Total	Ammonium
No added substrate	0.24	7.53	17.64	37.65	28.89
4 g/l cellulose	22.06 ± 2.86	10.93 ± 2.74	18.10 ± 0.69	67.33	9.84 ± 5.70
1:2 *Dunaliella*:cellulose	3.75 ± 1.55	16.34 ± 3.32	19.75 ± 1.97	66.33	16.31 ± 1.88
2:1 *Dunaliella*:cellulose	1.38 ± 0.89	15.48 ± 0.80	17.89 ± 0.74	50.71	18.28 ± 1.44
4 g/l *Dunaliella*	1.06	8.17 ± 0.84	16.37 ± 0.40	50.97	21.52 ± 0.59
4 g/l *Dunaliella* (no TC60)	1.13	5.28	3.51	23.90	14.86
1:2 *Chlorella*:cellulose	0.70	8.82 ± 1.12	22.61 ± 2.80	53.62	24.31 ± 2.82
2:1 *Chlorella*:cellulose	0.82 ± 0.02	10.43 ± 2.13	29.42 ± 2.11	63.30	26.84 ± 2.45
4 g/l *Chlorella*	1.88	11.32 ± 1.49	35.36 ± 0.85	72.80	27.45 ± 3.80
4 g/l *Chlorella* (no TC60)	0.97	12.68	34.09	80.19	25.76

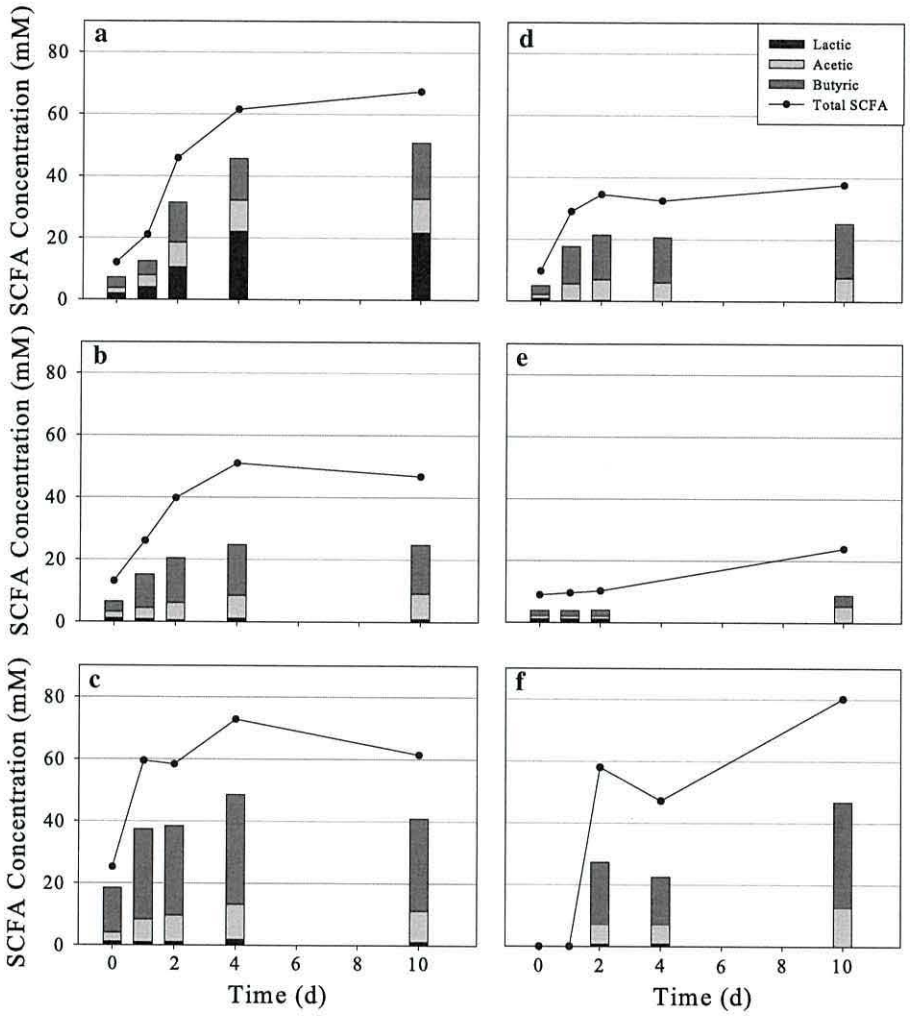


Fig. 3 Changes in lactate, acetate, butyrate and total SCFA concentrations over time for single substrate conditions. **a** 4 g/l cellulose with TC60. **b** 4 g/l *D. tertiolecta* with TC60. **c** 4 g/l *C. vulgaris* with TC60. **d** TC60 without added substrate. **e** 4 g/l *D. tertiolecta* without TC60. **f** 4 g/l *C. vulgaris* without TC60

relatively high levels of SCFAs in the presence of *C. vulgaris*, even without TC60 inoculation, suggest that the heterotrophs in the algal slurry were active in producing butyrate (50% of total SCFA). *D. tertiolecta* without TC60 had lower acid production than TC60 grown in medium only, indicating utilization of acetate and butyric acid by the satellite organisms.

Assessment of microalgal biomass digestion

To assess the biodegradability of microbial biomass, samples of solids were analyzed for C, N, TS, and VS concentrations. These results showed relatively little change in either the TS or VS over incubation. The initial C concentration in the solids was comparable across all substrate ratios in *C. vulgaris*-fed samples, suggesting that the biomass remained more or less intact under all conditions. In contrast, the initial C content of *D. tertiolecta*-fed samples varied (Fig. 4). *D. tertiolecta* cells were prone to lysis as seen microscopically, releasing soluble nutrients and lowering the C concentration in the solid fraction. Therefore, the *Dunaliella*-fed TC60 cultures contained cytosolic compounds which contributed to the increased gas production and reduced lag time. The lack of a rigid cell wall and tendency of lysis of *Dunaliella* biomass when removed from high salinity medium are useful attributes for anaerobic digestion.

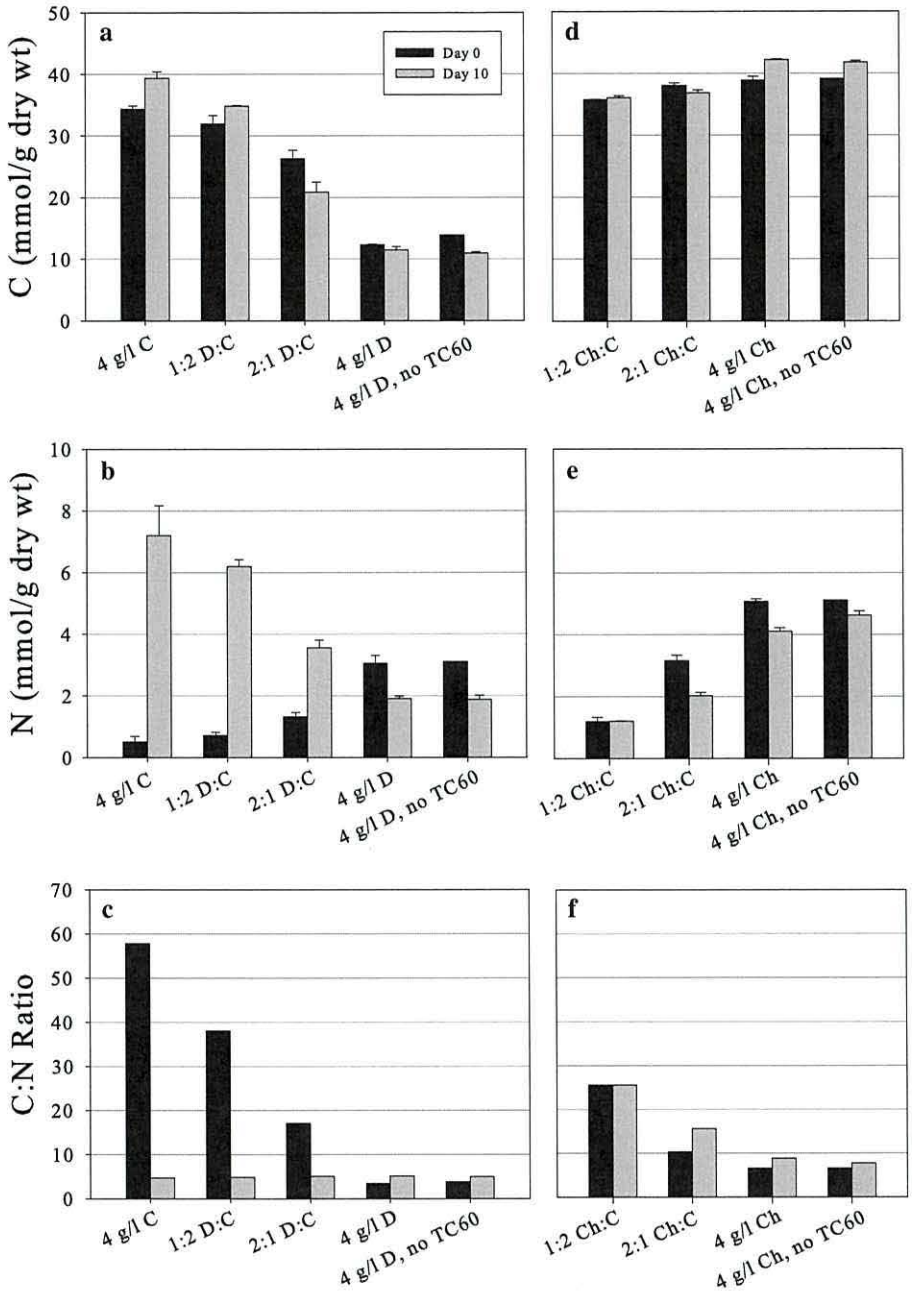


Fig. 4 C and N analysis of solid fractions and the corresponding C:N ratios for *D. tertiolecta* (**a–c**) and *C. vulgaris* (**d–f**). The cultures were inoculated with TC60 unless otherwise noted. *C* cellulose, *Ch C. vulgaris, D D. tertiolecta*

The C content in the solid fraction decreased after incubation in samples with a combination of *D. tertiolecta* and cellulose (Fig. 4). When TC60 was fed *D. tertiolecta* only, the C content did not change after 10 days of incubation, indicating negligible digestion of the insoluble biomass. These results suggest that the soluble compounds rather than the solid fraction of the *D. tertiolecta* biomass were the source for increased gas production by TC60. The level of C in solids did not change after 10 days of incubation in samples containing *C. vulgaris* (Fig. 4). *C. vulgaris* biomass appeared to suppress cellulose

utilization by TC60, consistent with the low production of gas and other indicators of poor growth.

After incubation, the N content of solids increased from 0.7 to 6.2 mmol/g dry wt and 1.3 to 3.6 mmol/g dry wt for 1:2 and 2:1 *D. tertiolecta* to cellulose, respectively (Fig. 4). This trend was consistent with the utilization of cellulose for biomass synthesis by TC60 during anaerobic digestion. When only *D. tertiolecta* was fed to TC60, the N content in the solids did not increase, again indicating that TC60 only used cellulose and soluble microalgal nutrients as substrates. When TC60 was grown in the presence of *C. vulgaris* biomass, the level of N was relatively unaffected (Fig. 4), consistent with the apparent recalcitrance of *Chlorella* biomass to anaerobic digestion.

When TC60 was grown on cellulose, decreases in the C:N ratio of solids were attributed to biomass growth. C content stayed constant whereas the N levels in solids increased by day 10. Similarly, decreases in the C:N ratios were also seen in solids from TC60 cultures fed 1:2 and 2:1 of *D. tertiolecta* to cellulose (Fig. 4). Ratios did not change in TC60 fed *D. tertiolecta* only or in uninoculated *D. tertiolecta*, agreeing with the lack of metabolic activity in these samples. In the case of *Chlorella*, the C:N ratios of solids showed relatively little change from day 0 to 10. These data suggested that growth of TC60 and satellite heterotrophs took place at the expense of soluble substrates. Metabolic data indicated that it was the satellite heterotrophs, not TC60, that were the active microorganisms in *C. vulgaris*-fed samples. Microscopic examination showed that the *C. vulgaris* biomass remained intact and accounts for a large percentage of the dry weight. Therefore, the microalgal biomass would mask the relatively minor increase of N due to satellite heterotrophic growth.

Conclusions

This study focused on H_2 generation through anaerobic degradative metabolism and dark fermentative pathways. The overall energy balance of the bioprocess was not compiled because this was an initial feasibility study with no optimization. The calorific yields calculated from the maximum H_2 yields were equal to 1.86 and 1.01 kJ/g VS for the 1:2 *D. tertiolecta* to cellulose and 4 g/l *C. vulgaris*, respectively. These yields indicate major differences in the biodegradability of the two algal biomass substrates. The marine algae, *Dunaliella tertiolecta*, lysed readily and thereby provided additional nutrients for cellulolytic activity and H_2 accumulation by TC60. Heterotrophs associated with the marine species were deemed to have a negligible effect on the digestion. In contrast, the freshwater *Chlorella vulgaris* biomass remained recalcitrant and suppressed TC60 activity. In spite of the thermophilic conditions, heterotrophs associated with *Chlorella* biomass produced H_2 yields similar to those obtained with TC60. Hydrolytic pretreatment of microalgal slurries was not tested for *Chlorella* biomass. The yields obtained in this study indicate the need for improvement of H_2 yields through biomass pretreatment and process optimization.

Acknowledgments Partial funding for this study was received from the Finland Distinguished Professorship Program (402/2006) of the Finnish Agency for Technology and Innovation (S.M. Carver and O.H. Tuovinen), USDA National Needs Graduate Fellowship Program (S.M. Carter), Academy of Finland (D.N. Thomas), and Engineering and Physical Sciences Research Council Studentship in conjunction with RWE Power (U.K.) and Varicon Aqua Ltd. (C.J. Hulatt and D.N. Thomas).

References

Ben-Amotz A, Polle JEW, Rao DVS (2010) The alga *Dunaliella*: biodiversity, physiology, genomics and biotechnology. Science, Enfield

Benemann JR (2000) Hydrogen production by microalgae. J Appl Phycol 12:291–300

Cordóba LT, Bocanegra ARD, Llorente BR, Hernández ES, Echegoyen FB, Borja R, Bejines FR, Morcillo MFC (2008) Batch culture growth of *Chlorella zofingiensis* on effluent derived from two-stage anaerobic digestion of two-phase olive mill solid waste. Electron J Biotechnol. doi:10.2225/vol11-issue2-fulltext-1 11(2):1–8

Department of Energy (2007) Roadmap for bioenergy and biobased products in the U.S. Biomass Research and Development, Technical Advisory Committee, Washington DC ADA494661

Eaton AD, Clesceri LS, Rice EW, Greenberg AE, Franson MAH (2005) Standard methods for the examination of water and wastewater. American Public Health Association, New York

Ghirardi ML, Dubini A, Yu J, Maness PC (2009) Photobiological hydrogen-producing systems. Chem Soc Rev 38:52–61

Golueke CG, Oswald WJ, Gotaas HB (1957) Anaerobic digestion of algae. Appl Microbiol 5:47–55

Hernández EPS, Córdoba LT (1993) Anaerobic digestion of *Chlorella vulgaris* for energy production. Resour Conserv Recycl 9:127–132

Hodaifa G, Martínez ME, Sánchez S (2008) Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*. Bioresour Technol 99:1111–1117

Holmes RM, Aminot A, Kérouel R, Hooker BA, Peterson BJ (1999) A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Can J Fish Aquat Sci 56:1801–1808

Horiuchi J, Ohba I, Tada K, Kobayashi M, Kanno T, Kishimoto M (2003) Effective cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control. J Biosci Bioeng 95:412–415

Hulatt CJ, Thomas DN (2010) Dissolved organic matter (DOM) in microalgal photobioreactors: a potential loss in solar energy conversion? Bioresour Technol 101:8690–8697

Ike A, Saimura C, Hirata K, Miyamoto K (1996) Environmentally friendly production of H_2 incorporating microalgal CO_2 fixation. J Mar Biotechnol 4:47–51

Ike A, Toda N, Hirata K, Miyamoto K (1997) Hydrogen photoproduction from CO_2-fixing microalgal biomass: application of lactic acid fermentation by *Lactobacillus amylovorus*. J Ferment Bioeng 84:428–433

Kawaguchi H, Hashimoto K, Hirata K, Miyamoto K (2001) H_2 production from algal biomass by a mixed culture of *Rhodobium marinum* A-501 and *Lactobacillus amylovorus*. J Biosci Bioeng 91:277–282

Kojima H, Lee YK (2001) Photosynthetic microorganisms in environmental biotechnology. Springer, Hong Kong

Lardon L, Hélias A, Sialve B, Steyer J-P, Bernard O (2009) Life-cycle assessment of biodiesel production from microalgae. Environ Sci Technol 43:6475–6481

Levin DB, Pitt L, Love M (2004) Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 29:173–185

Mata TM, Martins AA, Caetano NS (2010) Microalgae for biodiesel production and other applications: a review. Renew Sustain Energy Rev 14:217–232

Melis A, Happe T (2001) Hydrogen production. Green algae as a source of energy. Plant Physiol 127:740–748

Oswald WJ, Golueke CG (1960) Biological transformation of solar energy. Adv Appl Microbiol 2:223–262

Parkin GF, Owen WF (1986) Fundamentals of anaerobic digestion of wastewater sludge. J Environ Eng 112: 867–920

Perlack RD, Wright LL, Turhollow AF, Graham RL, Stokes BJ, Erbach DC (2005) Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. U.S. Department of Energy DOE/GO-102005-2135

Peu P, Béline F, Martinez J (2004) Volatile fatty acids analysis from pig slurry using high-performance liquid chromatography. Int J Environ Anal Chem 84:1017–1022

Ren N, Wang A, Cao G, Xu J, Gao L (2009) Bioconversion of lignocellulosic biomass to hydrogen: potential and challenges. Biotechnol Adv 27:1051–1060

Sialve B, Bernet N, Bernard O (2009) Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol Adv 27:409–416

Tam NFY, Wong YS (1996) Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media. Bioresour Technol 57:45–50

Yen HW, Brune DE (2007) Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresour Technol 98:130–134

Chapter 8.

General Discussion.

8. Introduction

The overall hypothesis tested in this work was that *microalgal cultivation is a potentially effective method for power-plant CO_2 mitigation and bio-energy production.* The research conducted and evidence presented in Chapters 3 to 7 is discussed below in context with the specific hypotheses developed in Chapter 1.

8.1. Dissolved organic matter is a loss in solar energy conversion.

Research presented in Chapter 3 supported the hypothesis that *microalgae in mass algal cultivations exude a significant fraction of their photo-assimilated carbon into the surrounding water*. Although various loss terms in microalgal productivity have been accounted for (Chapter 1), the process of exudation seems to have received no recognition to date in explaining the discrepancy between theoretic productivity and achieved biomass yields in microalgal mass cultures. In Chapter 3.2, it was shown that by the end of batch cultivations, dissolved organic carbon accounted for 6.4 and 17.3% of total organic carbon present in *Chlorella vulgaris* and *Dunaliella tertiolecta* cultivations respectively. Comparable percentages of 4.7% and 12.9% in cultures of *Chlorella vulgaris* and *Dunaliella tertiolecta* respectively were identified in intensive bubble column cultivations using simulated flue gas (4% CO_2, 0.001ms^{-1}, Chapter 5.2), so the supply of concentrated CO_2 from power plant flue gas did not seem to have a significant impact on DOC production relative to air CO_2 concentrations.

In natural systems dissolved organic matter supplies bacteria with a growth substrate (Obernosterer & Herndl, 1995), and the same effect is demonstrated in photobioreactor cultures. Due to utilization/ respiration by bacteria, actual losses from algal cells will always be higher than measurements of the DOC pool suggest. However, bacterial populations in applied algal systems have received relatively little

attention, and studies on the various interactions and roles of microalgae and bacteria are required.

One potential problem with microalgal technologies is the need for large volumes of water, meaning that recycling cultivation fluid will be important (Yang et al., 2011). Supply of freshwater in particular is a problem for almost any bio-energy crop (Hill et al., 2006), though for microalgal technologies the use of seawater (at coastal locations) and appropriate species may dramatically reduce the impacts (Tredici, 2010; Yang et al., 2011). It will be necessary to ensure that any waste water remaining from microalgal cultivation is of sufficient standard that it can be either re-used or introduced back into natural watercourses.

The fact that exuded matter is a relatively dilute solution means that it should be difficult to utilize microalgal DOM. Even at relatively high exudation rates and high cell density (*Dunaliella tertiolecta*, Chapter 3.2.), DOC measurements suggest a DOM loading of around 0.5 g L^{-1} dry weight. An energetically favourable and practical solution to utilise such dissolved materials in bulk water masses is not readily available. In Chapter 3.2 the possible use of microbial fuel cells was suggested. These may utilise waste-water streams, where they reduce the organic loading and produce electricity (Oh et al., 2010). Since most bacteria were harvested with the algal biomass (Chapter 3.2), the favourable aspect of waste water from microalgal cultivations is that dissolved substances are predominantly algal exudates rather than bacterial or recalcitrant material (Chapter 3.2), so may be well-suited to bio-processes.

Nutrient, especially nitrogen, limitation is an important strategy to improve the biochemical properties (lipid content, heating value) of microalgae (Hsieh et al., 2010; Pruvost et al., 2011; Xin et al., 2010 and Chapter 5). However, this process also

increases DOM release rates (Carlson, 2002; Pete et al., 2010). Thus, further research may be required to measure DOM production in nutrient-limited cultivations.

In terms of the sunlight-to-bio-energy conversion process, the potential energetic penalties incurred by losses of DOM may be difficult to control, aside from selection of species (e.g. *Chlorella vulgaris*) that tend to exude lower proportions of DOM. However, there are a number of aspects of photobioreactor design that have an important effect on energy efficiency, and these aspects do seem to have significant potential for improvement. One of the most important energy burdens of closed photobioreactors identified in Chapter 1 was the supply of power for mixing, circulating and exchange of gases to microalgal photobioreactors. This was used to form the hypothesis that: *Cultivating microalgae in photobioreactors supplied with a high power input improves productivity, but reduces the net energy production.*

8.2. Power input affects the energy return from photobioreactors.

Evidence from Chapter 4 supported the hypothesis that adjustment of the power input impacted the productivity and energy balance of photobioreactors. The research is significant because microalgal technologies must efficiently use the available light whilst maximizing the net energy output. However, high photosynthetic efficiency and maximum energy return do not necessarily occur in the same treatment (Table 3, Chapter 4.2). It was found that reducing the power input from 50 to 10-20 Wm^{-3} for cultivation of *Dunaliella tertiolecta* improved the net energy output (kJ L^{-1}) during the period of maximum productivity and over the batch cycle. In contrast, *Chlorella vulgaris* showed little difference in net energy output (kJ L^{-1}) between power input treatments.

A second energy-return parameter is the net energy ratio, the amount of bio-energy produced compared to the energy invested. In Chapter 4 it was shown that even where reducing the power input did not impact the net energy output (kJ L^{-1}), there was still an improvement in the net energy ratio, as would be expected. This is important because in order to improve the sustainability of microalgal technologies, it will be necessary to recover energy from waste biomass. Stephenson et al., (2010) indicate that it may be possible to recover energy by anaerobic digestion (i.e. after lipid extraction etc.) to offset the energy demands of the process itself. A high net energy ratio would thus be advantageous in this case, and may even be preferable to a high net output (kJ L^{-1}).

Work presented in Chapter 4 used cultivation of microalgae under controlled conditions (light, temperature) in order to examine the desired effects. In Chapter 6, measurements of outdoor productivity emphasized the importance of minimizing the energy inputs in order to achieve sustainable microalgal technologies. Since potential industrial applications will rely on natural light (Chapter 1), the irradiance incident on outdoor photobioreactors may peak at values several times higher than tested in the indoor experiments (Chapter 4). As a result, the optimal power input may not necessarily remain constant during the day. Further, there is little demand for gas exchange at night and perhaps only the requirement to keep most cells in suspension during darkness, so that the absolute minimum power input would suffice. Growth is also temperature dependent (Park et al., 2011), so the optimal sparging rate may also vary with the temperature of the cultivation fluid. Using this information, a basic control system may be derived (Fig. 8.1), where real time irradiance and temperature measurements are used to compute the optimum power input/ sparging rate, which is adjusted automatically.

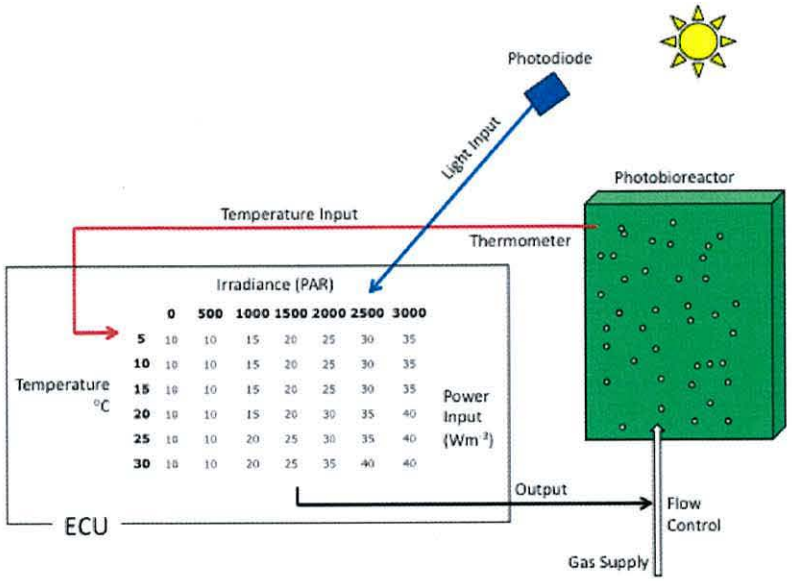


Fig. 8.1. Process diagram for a conceptual control system for optimized sparging. Inputs for light and temperature determine the sparging rate, providing optimum mass transfer and mixing based on energy return. ECU = electronic control unit.

Considering the amount of energy required to construct and particularly to run microalgal photobioreactors (Jorquera et al., 2010; Stephenson et al., 2010), it is important to establish the productivity of outdoor systems in order to design sustainable systems: The total energy investments over the life-cycle of a photobioreactor must be significantly lower than the bio-energy generated. Since microalgal productivity is sensitive to geographic location, the hypothesis that *the cultivation of microalgae using photobioreactors is a potentially viable method of biomass production in cool-temperate regions* was formulated and tested in Chapter 6.

8.3. Microalgal photobioreactors are productive using natural sunlight in a cool-temperate climate.

Data presented in Chapter 6 provided support for the hypothesis that microalgae may be grown productively in a cool temperate climate. The semi-continuous cultivation of *Scenedesmus obliquus* in an outdoor tubular photobioreactor was sustained for 6 months, with a mean productivity of 11.2 g m^{-2} d^{-1} from April to September. Considering the latitude (53°N), experimental duration and lack of temperature control, the corresponding PAR photosynthetic efficiency of 2.18% was in the expected, achievable range (Williams & Laurens, 2010; Park et al, 2011). Tredici (2010) identified that sustained (2-3 month) productivity often reaches 15 to 25 g $m^{-2}d^{-1}$, but that commercial year-round productivity of species such as *Dunaliella* typically falls below 10 g $m^{-2}d^{-1}$. Based on this evidence, the productivities obtained in Chapter 6 seem to reflect the literature evidence. Further, microalgal productivity is highly sensitive to the geographic location. Figure 8.2 indicates the yearly biomass production (T ha^{-1} y^{-1}) of microalgal production across the globe. Note that the UK and North-Western Europe are predicted to be amongst the lower biomass-producing regions, with estimated yearly productivities in the range 80 to 120 T ha^{-1} y^{-1}.

Unfortunately it was shown that the energy consumption for circulating the outdoor culture exceeded the bio-energy productivity 15 fold. Even if the energy ratio could be increased above 1 by extensive system modification, additional energy and CO_2 burdens such as reactor materials, supply of nutrients, water and CO_2 would likely render the process unsustainable. In fact, although tubular photobioreactors have been widely discussed in the literature (Ugwu et al., 2008), there does not seem to be any experimental evidence to justify the use of tubular photobioreactors for CO_2 mitigation/ bio-energy applications. Two recent studies have also shown that tubular

photobioreactors are unlikely candidates, and that the primary reason for this was culture circulation (Jorquera et al., 2010; Stephenson et al., 2010). The major point raised by the research is the need to select and design photobioreactors so that a sufficiently high energy return can be achieved.

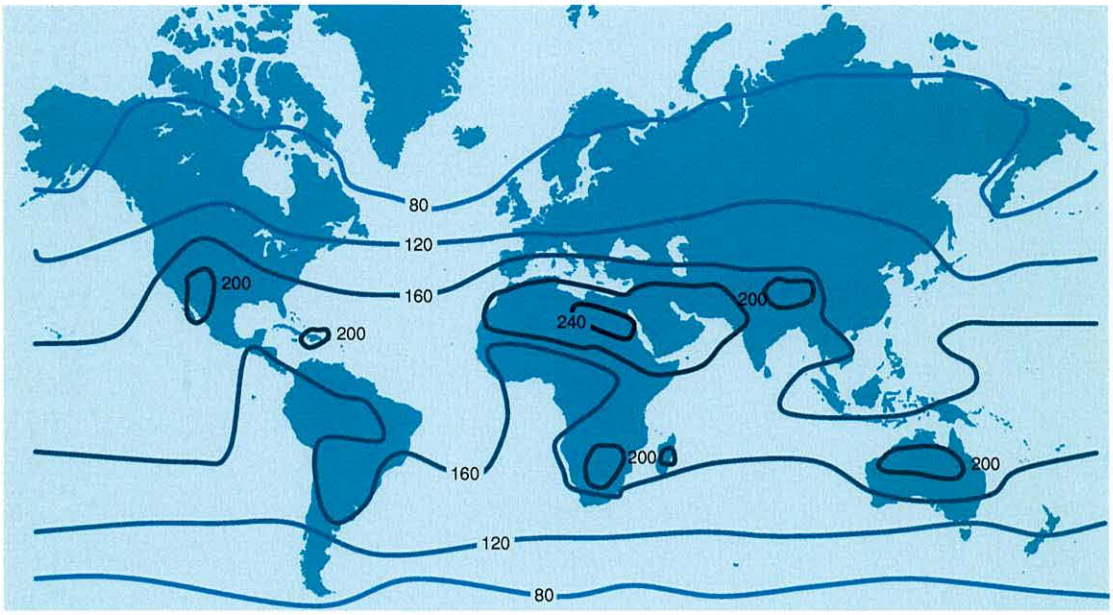


Figure 8.2. From Tredici (2010). Predicted microalgal biomass production across the globe (T ha^{-1} y^{-1}) based on a photosynthetic efficiency of 5%.

Most experimental research to date has focused on optimizing the biomass output of experimental photobioreactors. From the opposite perspective, life-cycle assessments have addressed the overall energetic and global warming contributions of different microalgal systems, in order to establish whether such technologies are environmentally sound. However, LCAs are based on rather limited design configurations from literature that has not necessarily optimized the energy return of

the reactors. For example, Jorquera et al., (2010) used a value of 53 Wm^{-3} for the energy input of their flat plate photobioreactor model. However such sparging inputs are unlikely to be necessary for much of the day (Chapter 4), and the energy input for sparging is also related to the cultivation depth/ light path (Chapter 6). Thus there is a need to accurately ascertain the design parameters which lead to maximum energy return *before* or *in conjunction with* LCA.

In addition to the supply of power to photobioreactors, the supply of fertilisers also presents a significant energy burden for every bio-energy crop, but is particularly important for microalgae due to their high protein/ nitrogen content (Chapter 1). This information was used to form the hypothesis that *cultivation of microalgae using different nitrogen sources and at different growth stages affects productivity, biochemical composition and the energy demands associated with the supply of nutrients.*

8.4. Nitrogen source does not affect growth, but cultivation dynamics affect biochemical composition and nitrogen utilisation.

Data presented in Chapter 5 showed that there was no observable support for the hypothesis that the nitrogen source affected the productivity of *Chlorella vulgaris* and *Dunaliella tertiolecta* cultivated in 20L photobioreactors. This result may be useful for industrial microalgal production, as microalgae may make use of various waste streams containing nitrogenous compounds without significant impacts on productivity (Clarens et al., 2010). The bubble column photobioreactors in Chapter 4 demonstrated that a fraction (mean 2.5 to 3.3%) of the nitrogen assimilated by the microalgae presented as dissolved substances to the surrounding medium. Further, cultivations using 0.04% CO_2 demonstrated accumulation of nitrite and ammonium in

the cultivation fluid. This indicates that exudation of DON and bacterial re-cycling of fertiliser nitrogen is a potentially important process in mass algal cultivations, and is worthy of future investigation.

Research is required to establish the best cultivation/ harvesting strategy for microalgae. This is because many factors are inter-linked, and the outcome may be a compromise between different goals. For example, two conflicting issues commonly cited in the literature are the need to maximise photosynthetic efficiency (Zijffers, 2010; Tredici, 2010), but also to maximise lipid content by limiting the availability of nutrients, thereby reducing growth (Hsieh et al., 2010). Fig. 8.3 indicates some of the compromises that must be taken into account.

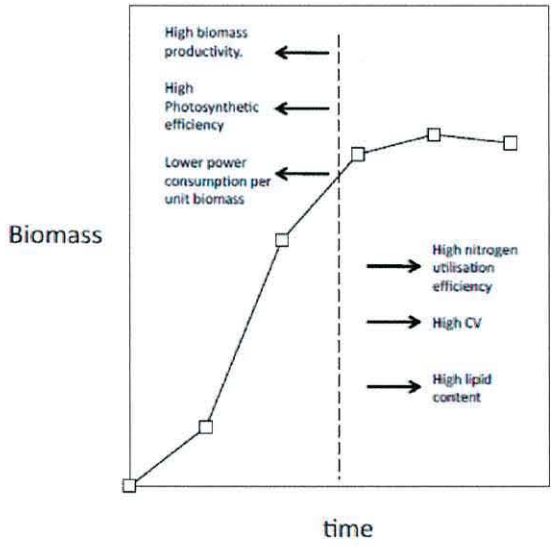


Figure 8.3. Selected compromises involved with determination of the optimal harvest point during batch cultivation of microalgae in photobioreactors.

Several approaches to nitrogen utilisation have been proposed, though in both instances the research was targeted at lipid production rather than nitrogen

consumption. Lardon et al., (2009) modelled two scenarios where microalgae were grown in either nitrogen replete, or nitrogen–deprived medium, finding that the latter case was energetically favourable for producing lipids. It has been suggested that separating the nitrogen-replete and stressed phases would be more efficient (Scott et al., 2010). However, this seems redundant because the nitrogen-stressed phase would still require sunlight, mixing etc and therefore at best be only equally as efficient as conventional batch culture. Stephenson et al., (2010^{b}) confirmed that for lipid production it was much better to use batch cultivation and allow the microalgae to be subjected to nitrogen stress naturally by gradual nutrient depletion.

Nitrogen utilisation is important, because not only does it have a significant impact on the energy burden of microalgal production, but may also impact the efficiency with which microalgal biomass is processed. In Chapter 1, various bio and thermo-chemical processes were discussed which were capable of recycling the nutrients back to the microalgal production process (Park et al., 2010; Sialve et al., 2009; Grierson et al., 2008). The efficiency of anaerobic bioprocesses may be sensitive to the high C:N ratio of microalgal biomass (Yen & Brune, 2007). This was tested in this work using the hypothesis that *microalgal biomass alone or co-digested with cellulosic material yields significant quantities of H_2 and/ or CH_4 gas.*

8.5. Anaerobic co-digestion of microalgal biomass and cellulose enhances hydrogen yields.

Research in Chapter 7 showed that production of H_2 was possible from microalgal biomass, and that relatively high production rates were observed in substrates co-digested with cellulose. The utilisation of anaerobic bioprocesses for waste-biomass nutrient and energy recovery should form an important part of

microalgal production systems (Sialve et al., 2009; Oswald & Golueke, 1960). These technologies are commercially established. However, despite the need to integrate microalgal production with anaerobic digestion, there is little literature evidence for the successful integration of both processes, especially on an industrial scale. Also, it needs to be established whether or not sufficient H_2 (or CH_4) can be generated by anaerobic bioprocesses to offset the demands of algal biomass production. Combining processes will be an important aspect of efficient, environmentally sound microalgal cultivation (Ras et al., 2011). There are many different permutations and combinations with which the cultivation of microalgae may be combined with other processes such as anaerobic digestion and fermentation. Figure 8.4 illustrates one such example (Singh & Gu, 2010).

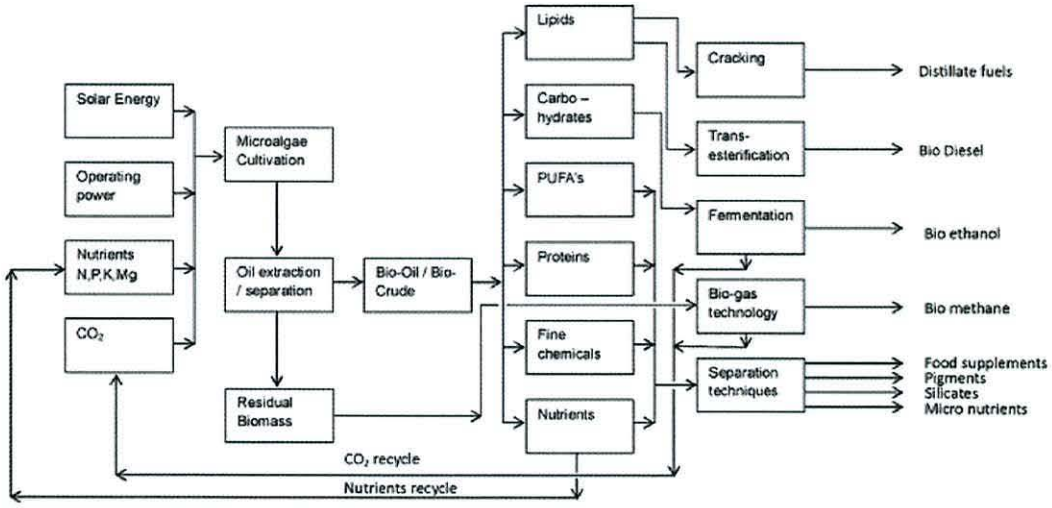


Figure 8.4. Integration of microalgal production with downstream processes plus recycling of essential components.

8.6. Microalgae, CO_2 mitigation and power plant.

One important question is whether it is necessary to have a point source of CO_2 (e.g. a power station) in order to successfully cultivate microalgae. The evidence from the bubble column experiments (Chapter 5.2) shows that supply of concentrated CO_2 was the only method capable of achieving a significant positive energy return: At air-CO_2 concentrations there was insufficient CO_2 in the sparging gas to permit microalgal biomass production to offset the energy consumption. Thus cultivation of microalgae in gas-sparged photobioreactors is dependent upon a supply of CO_2 rich gases, making fossil fuel fired power plant particularly valuable in this respect. One potential problem is the type of power station employed, as not all power plant is run as baseload (continuously). Since microalgae are living organisms, the systems cannot simply be switched on and off like chemical CO_2 mitigation technologies, and this may be a major disadvantage in using microalgal processes. It may also limit the number of power plant suitable for microalgal cultivation.

In Chapter 1, it was demonstrated that microalgae could provide both CO_2 mitigation and production of bio-energy. However, it may not be possible to capitalize (via claims of environmental benefits and/or financial benefits) on both CO_2 emissions savings (i.e. CO_2 'captured') *and* renewable fuel production from the same process. To clarify, the benefit of using algae to remove CO_2 and produce a fuel is actually derived from using the biomass to displace fossil fuels that would otherwise be combusted (Campbell et al., 2011). As a result, the environmental benefits of CO_2 mitigation and bio-energy production using microalgae are only equivalent to the net life-cycle calorific value and CO_2 fossil-equivalent emissions savings. Other interpretations may be amoral and environmentally damaging. As a result, where algal technologies are compared to chemical-engineering carbon capture methods (e.g.

Amine CCS), their performance must be based on their *net* life-cycle CO_2 emissions savings and not on the amount of CO_2 they remove from flue gas.

Aside from the environmental impacts, it is essential that microalgal technologies can be implemented in a cost-effective manner. For example, mitigation of CO_2 emissions from power plant using algal or chemical engineering methods will invariably incur a loss of efficiency. Such effects inevitably lead to increased production costs which, for the power generation industry, affects the market price of electricity. From a purely financial perspective, the life-cycle burdens of current algal cultivation technologies mean that no current production system is capable of producing algal fuels competitive with conventional liquid transport fuels (Jorquera et al., 2010). The same authors estimated that microalgae cultivated in flat plate and raceway systems would produce competitive liquid fuels from microalgae when petroleum rose to 165 and 89 US dollars per barrel respectively. Singh et al (2010) report that liquid fuel from algae currently costs in the region of 50 Euros/ L^{-1}. Whatever the absolute figures, it seems that in order for microalgal technologies to become viable, it will require significant improvements in cultivation efficiency and a relatively high and stable price for CO_2 emissions/ liquid transport fuels.

8.7. Concluding remarks

In terms of improving the performance of microalgal technologies, many aspects of microalgal cultivation are currently under investigation. At one extreme, advances in molecular engineering (e.g. of the photosynthetic antennae structure) may help improve photosynthetic efficiency in strong sunlight (Melis, 2009; Tredici, 2010). However, considering the number of very significant life-cycle burdens involved with producing microalgae (Chapters 4 to 6) the greatest improvements in performance

will likely derive from managing and improving the energy and global-warming impacts of microalgal production. Future work should therefore be targeted at these issues first and foremost.

Some companies manufacturing and supplying photobioreactors have made claims that their systems can produce extremely large quantities of biomass/ lipid. However as observed by Tredici (2010), many of these claims are thermodynamically impossible. It would be to great scientific and environmental detriment if photobioreactor systems fail to live up to manufacturer claims or expectations because, despite the potential issues surrounding yields and life-cycle burdens, microalgae offer considerable environmental and energy security benefits. As a result, the use of microalgal technologies for CO_2 mitigation and bio-energy will require an approach which is realistic and acknowledges their capabilities.

At present, there is a wealth of data concerning the growth of microalgae in photobioreactors, mostly at a small scale. A number of life-cycle assessments have examined overall efficiency for certain modelled designs (Stephenson et al., 2010; Jorquera et al., 2010). What is missing, and urgently required, is experimental work specifically targeting the optimisation of microalgal photobioreactors in terms of life-cycle and energy balance using scalable systems. Such data is essential to feed into future assessments. Without this, there is the risk that data from non-optimised, or sub-optimal systems, could be used to justify rejection of closed photobioreactors as CO_2 mitigation/ bio-energy technologies, where actually there may be significant potential.

In the past, microalgal research programs were cut in response to falling oil prices (Wijffels & Barbosa, 2010). However, depletion of fossil fuel reserves indicates that oil prices will continue to rise steadily (Stephens et al., 2010), making

alternative renewable fuels more competitive in the marketplace. In particular, the recent implementation of carbon trading and renewable energy policy (Chapter 1) means that CO_2 mitigation technologies will play an important role in market economics (Giovanni & Richards, 2010). Considering the demand for energy and the targets set by EU policy for the next few decades, the area of land required for conventional biofuel crops in EU countries will be considerable (Krasuska et al., 2010). Due to their high areal yields, microalgae seem to be amongst the most suitable candidates for future development. However, it is clear that considerable research is still required to produce economically and environmentally sound microalgal technologies.

9. References for Chapters 1, 2 and 8.

Aust, H., 2007. Air filtration and power generation: Flue gas desulphurization. Filtration & Separation. Dec, 36-37.

Biersmith, A., Benner, R., 1998. Carbohydrates in phytoplankton and freshly produced organic matter. Marine Chemistry. 63, 131–144.

Blankenship, R., 2002. Molecular mechanisms of photosynthesis. Blackwell Publishing.

Bligh, E., Dyer, W., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Physiology and Pharmacology. 37, 911-917.

Bouillon, P., Hennes, S., Mahieux, C., 2009. ECO_2: Post-combustion or Oxyfuel– A comparison between coal power plants with integrated CO_2 capture. Energy Procedia. 1, 4015-4022.

Brown, T., Duan, P., Savage, P., 2010. Hydrothermal liquefaction and gasification of *Nannochloropsis* sp. Energy and Fuels. 24, 3639-3646.

Campbell, P., Beer, T., Batten, D., 2011. Life-cycle assessment of biodiesel production of microalgae in ponds. Bioresource Technology. 102, 50-56.

Carlson, C., 2002. Production and removal processes. In: Carlson, C., Hansell, D. (Eds.), Biogeochemistry of Marine Dissolved Organic Matter. Academic Press, London, pp. 91–139.

Chen, C., Yeh, K., Aisyah, R., Lee, D., Chang, J., 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. Bioresource Technology. 102, 71-81.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnology Advances. 25, 294-306.

Chisti, Y., 1989. Airlift Bioreactors. Elsevier, NewYork.

Chiu, S., Kao, C., Chen, C., Kuan, T., Ong, S., Lin, C., 2008. Reduction of CO_2 by a high-density culture of *Chlorella sp.* in a semicontinuous photobioreactor. Bioresource Technology. 99, 3389–3396.

Chow, T., 2010. A review on photovoltaic/thermal hybrid solar technology. Applied Energy. 87, 365-379.

Clarens, A., Resurrecion, E., White, M., Colosi, M., 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environmental Science and Technology. 44, 1813-1819.

Collet, P., Helias, A., Lardon, L., Ras, M., Goy, R., Steyer, J., 2011. Life-cycle assessment of microalgae culture coupled to biogas production. Bioresource Technology. 102, 207-214.

Converti, A., Casazza, A., Ortiz, E., Perego, P., Borghi, M., 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chemical Engineering and Processing: Process Intensification. 48, 1146-1151.

Dalgaard, T., Halberg, N., Porter, J., 2001. A model for fossil energy use in Danish agriculture used to compare organic and conventional farming. Agriculture, Ecosystems and Environment. 87, 51-65.

Damstedt, B., Pederson, J., Hansen, D., Knighton, T., Jones, J., Christensen, C., Baxter, L., Tree, D., 2007. Biomass cofiring impacts on flame structure and emissions. Proceedings of the Combustion Institute. 31, 2813-2820.

Dean, A., Sigee, D., Estrada, B., Pittma, J., 2010. Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. Bioresource Technology. 101, 4499-4507.

De, S., Assadi, M., 2009. Impact of cofiring biomass with coal in power plants– A techno-economic assessment. Biomass & Bioenergy. 33, 283-293.

Demirbas, A., 2003. Sustainable cofiring of biomass with coal. Energy Conversion and Management. 44, 1465-1479.

Demirbas, A., 2010. Use of algae as biofuel sources. Energy Conversion and Management. 51, 2738-2749.

Degrenne, B., Pruvost, J., Christophe, G., Cornet, J., Cogne, G., Legrand, J., 2010. Investigation of the combined effects of acetate and photobioreactor illuminated fraction in the induction of anoxia for hydrogen production by *Chlamydomonas reinhardtii*. International Journal of Hydrogen Energy. 35, 10741-10749.

Dismukes, G., Carrieri, D., Bennette, N., Ananyev, G., Posewitz, M., 2008. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current Opinion in Biotechnology. 19, 235–240.

Douskova, I., Doucha, J., Livansky, K., Machat, J., Novak, P., Umysova, D., Zachleder, V., Vitova, M., 2009. Simultaneous flue gas bioremediation and reduction of microalgal biomass production costs. Applied Microbiology and Biotechnology. 82, 179-185.

Egenhofer, C., 2007. The making of the EU Emissions Trading Scheme: Status, prospects and implications for business. European Management Journal. 25, 453-463.

Falkowski, P., Raven, J., 2007. Aquatic Photosynthesis. Princeton University Press, New Jersey.

Fernandez, E., Boyd, P., Holligan, P., 1993. Production of organic and inorganic carbon within a large-scale coccolithophore bloom in the northeast Atlantic-Ocean. Marine Ecology-Progress Series. 97, 271-285.

Fernandez, F., Camacho, F., Perez, J., Sevilla, J., Molina Grima, E., 1997. A model for light distribution and average solar irradiance inside outdoor tubular photobioreactors for the microalgal mass culture. Biotechnology & Bioengineering. 55, 701-704.

Fulke, A., Mudliar, S., Yadav, R., Shekh, A., Srinivasan, N., Ramanan, R., Krishnamurthi, K., Devi, S., Chakrabarti, T., 2010. Bio-mitigation of CO_2, calcite formation and simultaneous biodiesel precursors production using *Chlorella sp.* Bioresource Technology. 101, 8473-8476.

Georgopoulou, E., Sarafidis, Y., Mirasgedis, S., Lalas, D., 2006. Next allocation phase of the EU emissions trading scheme: How tough will it be? Energy Policy. 34, 4002-4023.

Gibbins, J., Chalmers, H., 2008. Carbon capture and storage. Energy Policy. 36, 4317-4322.

Giovanni, E., Richards, K., 2010. Determinants of the costs of carbon capture and sequestration for expanding electricity generation capacity. Energy Policy. 38, 6026-6035.

Graham, L., Wilcox, L., 2000. Algae. Prentice Hall.

Grasshoff, K., Ehrhardt, M., Kremling, K., 1983. Methods of seawater analysis, 2nd Edition. Verlag Chemie, Wienheim, Germany, pp. 419.

Grierson, S., Strezov, V., Ellem, G., Mcgregor, R., Herbertson, J., 2009. Thermal characterization of microalgae under slow pyrolysis conditions. Journal of Analytical and Applied Pyrolysis. 85, 118-123.

Hales, B., van Greer, A., Takahashi, T., 2004. High-frequency measurements of seawater chemistry: Flow-injection analysis of macronutrients. Limnology and Oceanography: Methods. 2, 91-101.

Harun, R., Davidson, M., Doyle, M., Gopiraj, R., Danquah, M., Forde, G., 2010. Technoeconomic analysis of an integrated microalgae photobioreactor, biodiesel and biogas production facility. Biomass & Bioenergy. 35, 741-747.

Hase, R., Oikawa, H., Sasao, C., Morita, M., Watanabe, Y., 2000. Photosynthetic production of microalgal biomass in a raceway system under greenhouse conditions in Sendai city. Journal of Bioscience and Bioengineering. 89, 157-163.

Heinzel, T., Siegle, V., Spliethoff, H., Hein, K., 1998. Investigation of slagging in a pulverized fuel co-combustion of biomass and coal at a pilot-scale test facility. Fuel Processing Technology. 54, 109-125.

Herzog, H., 2010. Scaling up carbon dioxide capture and storage: From megatons to gigatons. Energy Economics. doi: 101016/j.eneco.2010.11.004.

Hill, J., Nelson, E., Tilman, D., Polasky, S., Tiffany, D., 2006. Environmental,

economic, and energetic costs and benefits of biodiesel and ethanol fuels. Proceedings of the National Academy of Sciences of the United States of America. 25, 11206-11210.

Ho, S., Chen, W., Chang, J., 2010. *Scenedesmus obliquus* CNW-N as a potential candidate for CO_2 mitigation and biodiesel production. Bioresource Technology. 101, 8725-8730.

Holmes, R., Aminot, R., Kerouel, B., Hocker, A., Peterson, B., 1999. A simple and precise method for measuring ammonium in marine and fresh water. Canadian Journal of Fish and Aquatic Science. 56, 1801–1808.

Hsieh, C., Wu, W., 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresource Technology. 100, 3921-3926.

Hulatt, C., Thomas, D., Bowers, D., Norman, L., Zhang, C., 2009. Exudation and decomposition of chromophoric dissolved organic matter (CDOM) from some temperate macroalgae. Estuarine, Coastal and Shelf Science. 84, 147-153.

Ibrahim, A., Othman, M., Ruslan, M., Mat, S., Sopian, K., 2011. Recent advances in flat plate photovoltaic/thermal (PV/T) solar collectors. Renewable and Sustainable Energy Reviews. 15, 352-365.

Illman, A., Scragg, A., Shales, S., 2000. Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzyme and Microbial Technology. 27, 631-635.

Jenkins, B., Baxter, L., Miles, T., Miles, T., 1998. Combustion properties of biomass. Fuel Processing Technology. 54, 17-46.

Jorquera, O., Kiperstock, A., Sales, E., Embirucu, M., Ghirardi, M., 2010. Comparative energy life-cycle analysis of microalgal biomass production in open ponds and photobioreactors. Bioresource Technology. 101, 1406-1413.

Kadam, K., 1997. Power plant flue gas as a source of CO_2 for microalgae cultivation: Economic impact of different process options. Energy Conversion and Management. 38, 505-510.

Kadam, K., 2002. Environmental implications of power generation via coal-microalgae cofiring. Energy. 27, 905-922.

Kalyanasundaram, K., Graetzel, M., 2010. Artificial photosynthesis: biomimetic approaches to solar energy conversion and storage. Current Opinion in Biotechnology. 21, 298-310.

Kirat, D., Ahamada, I., 2011. The impact of the European Union Emission Trading Scheme on the electricity-generation sector. Energy Economics. doi: 10.1016/j.eneco.2011.01.012.

Klessman, C., Lamers, P., Ragwitz, M., Resch, G., 2010. Design options for cooperation mechanisms under the new European renewable energy directive. Energy Policy. 38, 4679-4691.

Krasuska, E., Cadorniga, C., Tenorio, J., Testa, G., Scordia, D., 2010. Potential land availability for energy crops production in Europe. Biofuels, Bioproducts & Biorefining. 4, 658-673.

Kroon, H., 1993. Determination of nitrogen in water: comparison of continuous flow method with on-line UV digestion with the original Kjedahl method. Analytica Chimica Acta. 276, 287–293.

Kurose, R., Makino, H., Suzuki, A., 2004. Numerical analysis of pulverized coal combustion characteristics using advanced low-NO_x burner. Fuel. 83, 693-703.

Lardon, L., Helias, A., Sialve, B., Steyer, J., Bernard, O., 2009. Life-Cycle Assessment of Biodiesel Production from Microalgae. Environmental Science and Technology. 43, 6475–6481.

Lee, J., Kim, D., Lee, J., Park, S., Koh, J., Cho, H., Kim, S., 2002. Effects of SO_2 and NO on growth of *Chlorella* sp. KR-1. Bioresource Technology. 82, 1-4.

Lopez, C., Garcia, M., Fernandez, F., Bustos, C., Chisti, Y., Sevilla, J., 2010. Protein measurements of microalgal and cyanobacterial biomass. Bioresource Technology. 101, 7587-7591.

MacIntyre, H., Kana, T., Anning, T., Geider, R., 2002. Photoacclimation of photosynthesis irradiance response curves and photosynthetic pigments in microalgae and cyanobacteria. Journal of Phycology. 38, 17-38.

Mairet, F., Bernanrd, O., Masci, P., Lacour, T., Sciandra, A., 2011. Modelling neutral lipid production by the microalga *Isochrysis* aff. *galbana* under nitrogen limitation. Bioresource Technology. 102, 142-149.

Malinsky-Rushansky, N., Legrand, C., 1996. Excretion of dissolved organic carbon by phytoplankton of different sizes and subsequent bacterial uptake. Marine Ecology-Progress Series. 132, 249–255.

Markewitz, P., Schreiber, A., Vogele, S., Zapp, P., 2009. Environemtnal impacts of German CCS strategy. Energy Procedia. 1, 3763-3770.

Melis, A., 2009. Solar energy conversion efficiencies in photosynthesis: Minimising the chlorophyll antennae to maximize efficiency. Plant Science. 177, 272-280.

Merchuk, J., Gluz, M., Mukmenev, I., 2000. Comparison of photobioreactors for cultivation of the red microalga *Porphyridium* sp. Journal of Chemical Technology and Biotechnology. 75, 1119–1126.

Miles, T., Miles, T., Baxter, L., Bryers, R., Jenkins, B., Oden, L., 1996. Boiler deposits from firing biomass fuels. Biomass & Bioenergy. 10, 125-138.

Moheimani, N., Borowitzka, M., 2006. The long-term culture of the coccolithophore Pleurochrysis carterae (Haptophyta) in outdoor raceway ponds. Journal of Applied Phycology. 6, 703-712.

Molina Grima, E., Fernandez, J., Acien, F., Chisti, Y., 2001. Tubular photobioreactor design for algal cultures. Journal of Biotechnology. 92, 113-131.

Molina Grima, E., Fernandez, A., Camacho, F., Chisti, Y., 1999. Photobioreactors: light regime, mass transfer, and scaleup. Journal of Biotechnology. 70, 231-247.

Molina Grima, E., Sevilla, J., Perez, J., Camacho, F., 1996. A study on simultaneous photolimitation and photoinhibition in dense microalgal cultures taking into account incident and averaged irradiances. Journal of Biotechnology. 45, 59-69.

Mulvenna, P., Savidge, G., 1992. A modified manual method for the determination of urea in seawater using diacetylmonoxime reagent. Estuarine, Coastal and Shelf Science. 34, 429-438.

Nagaraja, R., 1984. Photosynthetically active components of global solar radiation: Measurements and model computations. Meteorology and Atmospheric Physics. 34, 354-364.

Nagase, H., Yoshihara, K., Eguchi, K., Yokota, Y., Matsui, R., Hirata, K., Miyamoto, K., 1997. Characteristics of biological NO_x removal from flue gas in a *Dunaliella tertiolecta* culture system. Journal of Fermentation and Bioengineering. 83, 461-465.

Obernosterer, I., Herndl, G., 1995. Phytoplankton extracellular release and bacterial growth: dependence on the inorganic N:P ratio. Marine Ecology-Progress Series. 116, 247–257.

Oh, S., Kim, J., Premier, G., Lee, T., Kim, C., Sloan, W., 2010. Sustainable wastewater treatment: How might microbial fuel cells contribute. Biotechnology Advances. 28, 871-881.

Oswald, W., Golueke, C., 1960. Biological transformations of solar energy. Advances in Applied Microbiology. 2, 223-262.

Pan, P., Hu, C., Yang, W., Li, Y., Dong, L., Zhu, L., Tong, D., Qing, R., Fan, Y., 2010. The direct pyrolysis and catalytic pyrolysis of *Nannochloropsis* sp. residue for renewable bio-oils. Bioresource Technology. 101, 4593-4599.

Park, J., Jin, H., Lim, B., Park, K., Lee, K., 2010. Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. Bioresource Technology. 101, 8649-8657.

Park, J., Craggs, R., Shilton, A., 2011. Wastewater treatment high rate algal ponds for biofuel production. Bioresource Technology. 102, 35-42.

Perez-garcia, O., Escalante, F., de-Bashan, L., Bashan, Y., 2010. Heterotrophic cultures of microalgae: Metabolism and potential products. Water Research, doi: 10.1016/j.watres.2010.08.037.

Pete, R., Davidson, K., Hart, M., Gutierrez, T., Miller, A., 2010. Diatom derived organic matter as a driver of bacterial productivity: The role of nutrient limitation. Journal of Experimental Marine Biology and Ecology. 391, 20-26.

Pienkos, P., Darzins, A., 2009. The promise and challenges of microalgal-derived biofuels. Biofuels, Bioproducts and Biorefining. 3, 431-440.

Powell, E., Mapiour, M., Evitts, R., Hill, G., 2009. Growth kinetics of *Chlorella vulgaris* and its use as a cathodic half cell. Bioresource Technology. 100, 269-274.

Pinto, J., Matthews, J., Sarno, G., 2010. Stealth technology for wind turbines. IET Radar Sonar Navigation. 4, 126-133.

Pruvost, J., Vooren, G., Gouic, B., Couzinet-Mossion, A., Legrand, J., 2011. Systematic investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel application. Bioresource Technology. 102, 150-158.

Qian, J., Mopper, K., 1996. An automated, high performance, high temperature combustion dissolved organic carbon analyzer. Analytical Chemistry. 68, 3090–3097.

Ralph, P., Gademann, R., 2005. Rapid light curves: A powerful tool to assess photosynthetic activity. Aquatic Botany. 82, 222-237.

Ramirez, C. Worrell, E., 2006. Feeding fossil fuels to the soil: An analysis of energy embedded and technological learning in the fertilizer industry. Energy Conservation & Recycling. 46, 75-93.

Ras, M., Lardon, L., Bruno, S., Bernet, N., Steyer, J., 2011. Experiemntal study on a coupled process of production and anaerobic digestion of *Chlorella vulgaris*. Bioresource Technology. 102, 200-206.

Rodrigues, M., Ferreira, L., Converti, A., Sato, S., Carvalho, J., 2010. Fed-batch cultivation of *Arthrospira* (Spirulina) *platensis*: Potassium nitrate and ammonium chloride as simultaneous nitrogen sources. Bioresource Technology. 101, 4491-4498.

Rojan, J., Anisha, G., Nampoothiri, K., Pandey, A., 2011. Micro and macroalgal biomass: A renewable source for bioethanol. Bioresource Technology. 102, 186-193.

Rosebaum, M., He, Z., Angenent, L., 2010. Light energy to bioelectrivity: Photosynthetic microbial fuel cells. Current Opinion in Biotechnology. 21, 259-264.

Schiermeier, Q., 2006. Carbon market survives gas leaks. Nature. 441, 405.

Scott, S. Davey, M., Dennis, J., Horst, I., Howe, C., Lea-Smith, D., Smith, A., 2010. Biodiesel from algae: challenges and prospects. Current Opinion in Biotechnology. 21, 277-286.

Scragg, A., Illman, A., Carden, A., Shales, S., 2002. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass Bioenergy. 23, 67–73.

Serodio, J., Vieira, S., Cruz, S., 2008. Photosynthetic activity, photoprotection and photoinhibition in intertidal microphytobenthos as studied in situ using variable chlorophyll fluorescence. Continental Shelf Research. 28, 1363-1375.

Servais, P., Garnier, J., Demarteau, N., Brion, N., Billen, G., 1999. Supply of organic matter and bacteria to aquatic ecosystems through waste water effluents. Water Research. 33, 3521–3531.

Shang, H., Scott, J., Shepherd, S., Ross, G., 2010. A dynamic thermal model for heating microalgae incubator ponds using off-gas. Chemical Engineering Science. 65, 4591-4597.

Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances. 27, 409-416.

Sierra, E., Acien, F., Fernandez, J., Garcia, J., Gonzalez, C., Molina, E., 2008. Characterization of a flat plate photobioreactor for the production of microalgae. Chemical Engineering Journal. 138, 136-147.

Singh, J., Gu, S., 2010. Commercialization potential of microalgae for biofuels production. Renewable and Sustainable Energy Reviews. 14, 2596-2610.

Singh, A., Nigam, P., Murphy, J., 2011. Mechanism and challenges in commercialisation of algal biofuels. Bioresource Technology. 102, 26-34.

Slegers, P., Wijffels, R., Straten, G., Boxtel, A., 2011. Design scenarios for flat panel photobioreactors. Applied Energy. doi:10.1016/j.apenergy.2010.12.037.

Stephens, E., Ross, I., Musgnung, J., Wagner, L., Borowitzka, M., Posten, C., Kruse, O., Henkamer, B., 2010. Future prospects of microalgal biofuel production systems. Trends in Plant Science. 15, 554-564.

Stephenson, A., Kazamia, E., Dennis, J., Howes, C., Scott, S., Smith, A., 2010. Life-cycle assessment of potential algal biodiesel production in the United Kingdom: A comparison of raceways and air-lift tubular bioreactors. Energy Fuels. 24, 4062-4077.

Stephenson, A., Dennis, J., Howe, C., Scott, S., Smith, A., 2010b. Influence of nitrogen-limitation regime on the production by *Chlorella vulgaris* of lipids for biodiesel feedstocks. Biofuels. 1, 47-58.

Sobrino, F., Monroy, C., 2009. Critical analysis of the European Union directive which regulates the use of biofuels: An approach to the Spanish case. Renewable and Sustainable Energy Reviews. 13, 2675-2681.

Tang, D., Han, W., Penglin, L, Miao, X., Zhong, J., 2011. CO_2 biofixation and fatty acid composition of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in response to different CO_2 levels. Bioresource Technology. 102, 3071-3076.

Travieso, L., Hall, D., Rao, K., Benitez, F., Sanchez, E., Borja, R., 2001. A helical tubular photobioreactor producing *Spirulina* in a semicontinuous mode. International Biodeterioration & Biodegradation. 47, 151-155.

Tredici, M., 2010. Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. Biofuels. 1, 143-162.

Ugwu, C., Aoyagi, H., Uchiyama, H., 2008. Photobioreactors for mass cultivation of algae. Bioresource Technology. 99, 4021–4028.

Velasquez-Orta, S., Curtis, T., Logan, B., 2009. Energy from algae using microbial fuel cells. Biotechnology and Bioengineering. 103, 1068-1076.

Vunjak-Novakovic, G., Kim, Y., Wu, X., Berzin, I., Merchuk, J., 2005. Air-lift bioreactors for algal growth on flue gas: mathematical modeling and pilot-plant studies. Industrial Engineering and Chemical Research. 44, 6154–6163.

Walker, D., 2009. Biofuels, facts, fantasy and feasibility. Journal of Applied Phycology. 21, 509-517.

Watanabe, Y., Saiki, H., 1997. Development of a photobioreactor incorporating *Chlorella* sp. for removal of CO_2 in stack gas. Energy Conversion and Management. 38, 499-503.

Westerhoff, P., Hu, Q., Esparza-Soto, M., Vermaas, W., 2010. Growth parameters of microalgae tolerant to high levels of carbon dioxide in batch and continuous-flow photobioreactors. Environmental Technology. 31, 523-532.

Weyer, K., Bush, D., Darzins, A., Willson, B., 2009. Theoretical maximum algal oil production. Bioenergy Research. doi: 10.1007/s12155-009-9046-x.

Williams, P., Laurens, L., 2010. Microalgae as biodiesel and biomass feedstocks: review and analysis of the biochemistry, energetics and economics. Energy and Environemtnal Science. doi:10.1039/b924978h.

Wijffels,, R., Barbosa, M., 2008. An outlook on microalgal biofuels. Science. 330, 913.

Xin, L., Hong-ying, H., Ying-xue, S., 2010. Effects of different nitrogen and phosphorus concentrations on the nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresource Technology. 101, 5494-5500.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y., 2011. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresource Technology. 102, 159-165.

Yen, H., Brune, D., 2007. Anaerobic co-digestion of algal sludge and waste-paper to produce methane. Bioresource Technology. 98, 130-134.

Zhang, K., Miyachi, S., Kurano, N., 2001. Photosynthetic performance of a cyanobacterium in a vertical flat-plate photobioreactor for outdoor microalgal production and fixation of CO_2. Biotechnology Letters. 23, 21-26.

Zijffers, J., Schippers, K., Zheng, K., Jansen, M., Tramper, J., Wijffels, R., 2010. Maximum photosynthetic yield of green microalgae in photobioreactors. Marine Biotechnology. 12, 708-718.

Zou, S., Wu, Y., Yang, M., Li, C., Tong, J., 2009. Thermochemical catalytic liquefaction of the marine microalga *Dunaliella tertiolecta* and characterization of bio-oils. Energy & Fuels. 23, 3753-3758.

Appendix 1.

Manuscript 'Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass', submitted to *Biotechnology for Biofuels*.

Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass

Aino-Maija Lakaniemi[1]*, Christopher J. Hulatt[2], David N. Thomas[2], Olli H. Tuovinen[1,3], Jaakko A. Puhakka[1]

[1]Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland

[2]School of Ocean Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, United Kingdom

[3]Department of Microbiology, Ohio State University, Columbus, OH 43210, U.S.A.

*Corresponding author. Tel.: +358 3 3115 2776; fax +358 3 3115 2869; E-mail address: aino-maija.lakaniemi@tut.fi

Abstract

Microalgae are a promising feedstock for biofuel and bioenergy production due to their high photosynthetic efficiencies, high growth rates and no need for organic carbon. In this study, utilization of *Chlorella vulgaris* (a fresh water alga) and *Dunaliella tertiolecta* (a marine alga) biomass was tested as a feedstock for anaerobic H_2 and CH_4 production. Anaerobic serum bottle assays were conducted at 37 °C with enrichment cultures from a municipal anaerobic digester. Low levels of H_2 were produced by anaerobic enrichment cultures but H_2 was subsequently consumed even in presence of 2-bromoethanesulfonic acid, an inhibitor of methanogens. Without inoculation, algal biomass still produced H_2 due to the activities of satellite bacteria associated with algal cultures. CH_4 was produced from both types of microalgal biomass with the anaerobic enrichments. PCR-DGGE profiling showed both H_2 producing and H_2 consuming bacteria in anaerobic enrichment cultures and the presence of H_2 producing bacteria among the satellite bacteria of both algal biomasses. H_2 production by the satellite bacteria was higher from *D. tertiolecta* (12.6 mL H_2 g-VS^{-1}) than from *C. vulgaris* (10.8 mL H_2 g-VS^{-1}), whereas CH_4 production was significantly higher from *C. vulgaris* (286 mL g-VS^{-1}) than from *D. tertiolecta* (24 mL g-VS^{-1}). High Na^+ and Cl^- concentrations in *D. tertiolecta* slurry likely decreased the CH_4 production.

Keywords

Chlorella vulgaris, *Dunaliella tertiolecta,* hydrogen, methane, fermentation, anaerobic digestion

1. Introduction

Photosynthetic biomass-based fuels are considered as sustainable alternatives to fossil fuels. Currently major share of biofuels and other forms of bioenergy are produced from terrestrial plants (Schenk et al. 2008). Microalgae may prove an alternative to terrestrial crops because they have higher photosynthetic efficiencies, higher yields and growth rates, and fewer requirements for cultivation land and they may be grown in saline waters and in arid land areas (Posten and Schaub 2009, Schenk et al. 2008, Velasquez-Orta et al. 2009). Microalgal biomass is potent for anaerobic conversion as it has high content of lipids, starch and proteins, and does not contain recalcitrant lignin (Fan et al. 1981, Schenk et al. 2008, Posten and Schaub 2009). However, robust cell walls of some microalgal species may limit digestibility (Chen and Oswald 1998, Carver et al. 2011).

Anaerobic digestion of microalgal biomass for CH_4 production has been studied in various temperatures and with pretreatments and co-substrates (Samson and LeDuy 1983a and b, Chen and Oswald 1998, Yen and Brune 2007, De Schamphelaire and Verstraete 2009). Chen and Oswald (1998) reported that pretreatment of algal biomass at 100 °C for 8 h increased digestibility up to 33%, but the energy lost in pretreatment was higher than the enhancement gained in CH_4 production (Yen and Brune 2007).

Some green algae, such as *Chlamydomonas reinhardtii* (Kosourov et al. 2002) and *Chlorella salina* (Chader et al. 2009) produce hydrogen under anaerobic conditions via direct photolysis (Melis 2002). However, despite extensive research this process has low yields and is rather feeble and filled with metabolic and technical obstacles (Benemann 2000) and remains as

58 unlikely source of sustainable energy. Indirect photolysis of microalgal biomass by first
59 hydrolyzing the biomass with lactic acid bacteria followed by photosynthetic H_2 production,
60 resulted in H_2 yields up to 8 mol H_2 mol-starch-glucose^{-1} from *C. reinhardtii* (66% starch
61 conversion efficiency) (Ike et al. 1997). Carver et al. (2011) reported fermentative H_2 production
62 from *Chlorella vulgaris* and *Dunaliella tertiolecta* at 60 °C by an anaerobic microbial
63 consortium. Further, Gfeller and Gibbs (1984), Miura et al. (1986) and Ueno et al. (1998)
64 reported hydrogen fermentation in microalgal cells under dark, anaerobic conditions.
65
66 The aim of this study was to examine the formation of f H_2 and CH_4 from microalgal biomass.
67 Two green microalga, *Chlorella vulgaris* (a freshwater species) and *Dunaliella tertiolecta* (a
68 marine species) were used as feedstock in this study. Experiments were carried out in batch
69 bottles at 37 °C without pretreatment of the algal biomass and the microbial communities were
70 characterized by PCR-DGGE profiling.
71
72 **2. Materials and methods**
73 *2.1. Microalgal biomass production and harvest*
74 *Chlorella vulgaris* (Culture Collection of Algae and Protozoa, UK strain 211/11B) and
75 *Dunaliella tertiolecta* (Sammlung von Algenkulturen Göttingen, Germany, strain SAG 13.86)
76 were grown photoautotrophically in 20 L column (Ø 0.16m) photobioreactors with 0.5 vvm air
77 sparging and photon flux density averaging 225 μmol photons PAR m^2 s^{-1}. *C. vulgaris* was
78 grown in milliQ-water based Jaworski's medium (http://www.ccap.ac.uk/media/recipes/JM.htm)
79 and *D. tertiolecta* in natural seawater from the Menai Strait, UK, treated by filtration (0.2 μm)

and UV irradiation, with nutrients supplied according to Walne's medium (www.ccap.ac.uk/media/documents/Walnes.pdf).

Algal biomass was harvested by flocculation followed by centrifugation. *C. vulgaris* was harvested by adding a chitosan stock solution (4 g chitosan, 50 mL acetic acid, 950 mL water) to the culture at approximately 2% of the total volume and adjusting pH to 7 by adding 3 M NaOH to initiate the flocculation. *D. tertiolecta* was flocculated by adding 50-100 mL of 3 M NaOH to raise the pH to approximately pH 9.5 (Horiuchi et al. 2003). The biomass of both species was then collected and centrifuged at 1000×g for 10 min to produce a thick paste. The pH of *C. vulgaris* and *D. tertiolecta* biomass was adjusted to 7.0±0.2 with HCl and the biomass slurries were stored at -20°C until used in the gas production experiments.

2.2. Experimental conditions

Anaerobic inocula were enriched from an anaerobic digester treating municipal wastewater sludge (City of Tampere, Finland). Serum bottle enrichments were prepared as series of batch incubations at 37 °C with 5 g-VS L^{-1} of substrate. In the first three phases substrate consisted of 25% algal biomass and 75% of activated sludge, followed by 50% of algal biomass and 50% of activated sludge, and finally 75% of algal biomass and 25% of activated sludge. In the following enrichment phases, 100% of algal biomass was used. Four different cultures were enriched. Two H_2-fermenting cultures, one with *C. vulgaris* biomass, B-C, and one with *D. tertiolecta* biomass as the substrate, B-D, and two CH_4-producing cultures, one utilizing *C. vulgaris* biomass, U-C, and one *D. tertiolecta* biomass, U-D. Methanogenesis was suppressed in the H_2-fermenting

cultures by 20 mM BESA. The medium was prepared according to Zehnder et al. (1980) with modifications by Karlsson et al. (1999) and Ejlertsson et al. (1996).

Gas production potential from *C. vulgaris* and *D. tertiolecta* was studied after nine passages of the corresponding enrichment culture at 37 °C in 120 mL anaerobic serum bottles with 50 mL of medium and 10% (v/v) inoculum. The incubations included two types of negative controls, with inoculum but no substrate and with 5 g-VS L^{-1} algal biomass but without anaerobic enrichment inoculum. Three types of positive controls were prepared containing enriched anaerobic inoculum and either 5 g L^{-1} glucose, 5 g L^{-1} cellulose or 5 g L^{-1} chitosan.

2.3. Chemical analyses

Volatile solids (VS) of the biomass samples were measured according to the Finnish standard SFS 3008 (SFS 1990). C and N were measured with Thermo-Electron Flash EA 1112 after drying the samples at 80°C for 72 hours. The elemental analyzer was calibrated using the standards sulphanilamide, 2,5-bis(5′-tert-butyl-benzoxazolyl)thiophene and L-cystine. DL-methionine was used as a reference material. Gas production was measured according to Owen et al. (1979). The headspace gas composition (H_2, CH_4 and CO_2) was measured using Shimadzu gas chromatograph GC-2014 equipped with Porapak N column (80/100 mesh) and a thermal conductivity detector. The temperatures of the oven, injector and detector were 80, 110 and 110 °C, respectively. N_2 was used as carrier gas at a flow rate of 20 mL min^{-1}. The formation of organic acids and alcohols (lactate, formate, acetate, propionate, butyrate and ethanol) were analyzed with a Shimadzu HPLC with a Shodex Sugar SH1011 column (Showa Denko K.K., Japan) and a refractive index detector (Shimadzu, Kyoto, Japan). Mobile phase was 5 mM H_2SO_4 and flow rate 0.9 mL min^{-1}. The chemical oxygen demand (COD) was analyzed before (total COD, COD_{tot}) and after filtration (COD of soluble

126 compounds, COD_s) trough 0.45 µm polyester syringe filter (Macherey-Nagel, Düren, Germany) with
127 dichromate method according to standard SFS 5504 (SFS 1988).
128
129 *2.4. Microbial community analyses*
130 Duplicate samples of 1.5 mL were taken from the original digester sludge and from batch bottles
131 at the end of 49-day incubation and stored at -20 °C. Prior to DNA extraction samples were
132 pelleted by centrifugation (10 000×g, 5 min) and the supernatant was removed. DNA was extracted
133 from the pellets with PowerSoil™ DNA isolation kit (Mo Bio laboratories, Inc., Carlsbad, CA). The
134 extracted DNA sample was used as a template for the PCR. Partial bacterial 16S rRNA genes of the
135 community DNA were amplified by using primer pair GC-BacV3f (Muyzer et al. 1993) and 907r
136 (Muyzer et al. 1996) as described by Koskinen et al. (2007). Denaturing gradient gel electrophoresis
137 (DGGE) was performed with INGENYphorU2×2-system (Ingeny International BV, GP Goes, The
138 Netherlands) using 8% polyacrylamide gels with denaturing gradient from 30% to 70% (100%
139 denaturing solution contains 7 M of urea and 40% formamide). Gels were run at 60 °C in 1×TAE
140 (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.3) with 100 V for 22 h and stained with SYBR®
141 Gold (Molecular Probes Invitrogen, Eugene, OR). The dominant bands were excised from the gels,
142 eluted in 20 µL of sterile water at +4 °C overnight, stored in -20 °C and reamplified for sequencing.
143 Sequencing was conducted at Macrogen Inc. (Taiwan). Sequence data were analyzed with BioEdit-
144 software and compared with sequences in GenBank.
145
146 *2.5. Calculations*
147 Cumulative H_2 and CH_4 production were calculated according to Logan et al. (2002). The data
148 were fitted to a modified Gompertz equation (Chen et al. 2002) by minimizing the square of the
149 measurement's and the estimate's subtraction to give lag times and H_2/CH_4 production rates. The

150 calorific yields from maximum H_2 and CH_4 yields were calculated from the lower heating
151 values, 120 MJ kg^{-1} for H_2 and 50 MJ kg^{-1} for CH_4.
152
153 **3. Results**
154 *3.1. Enrichment cultures*
155 During the first five enrichment phases no H_2 was produced in any of the cultures, while in
156 enrichment phases 6 to 9 low levels of H_2 were detected in B-C and B-D enrichments during the
157 first few days, but usually by day 5 H_2 level decreased below detection limit (results not shown).
158 No CH_4 was produced in the cultures amended with BESA (Figure 1).
159
160 With U-C and U-D CH_4 production was higher from *D. tertiolecta* biomass than from *C.*
161 *vulgaris* biomass in the first enrichment phase with 25% algal biomass and 75% activated sludge
162 (Figure 1A). From phase 2 onwards, when the proportion of algal biomass in the substrate was
163 increased to 50% or higher, CH_4 production from *C. vulgaris* surpassed that from *D. tertiolecta*
164 (Figure 1B-F). With 100% *C. vulgaris* and *D. tertiolecta* biomass CH_4 production rate ranged
165 between 3.4-6.5 and 1.2-4.9 mL d^{-1} and lag time between 2.6-5.1 and 5.3-10 d, respectively. CH_4
166 yield and CH_4 production rate decreased and lag time increased from *D. tertiolecta* as the
167 enrichment proceeded. CH_4 yield from *C. vulgaris* remained more or less constant after
168 enrichment phase 4 (Figure 1).
169
170 *3.2. H_2 and CH_4 production potential*
171 Gas production potential from *C. vulgaris* and *D. tertiolecta* was studied using the enrichment
172 cultures after nine passages. Some CO_2 was produced in all bottles indicating degradation in all

173 cultures, including all controls with no anaerobic inoculum (Table 1, Figures 2B and 3B). CO_2
174 production was higher from *C. vulgaris* as compared to *D. tertiolecta*.
175
176 H_2 was produced in all bottles including the controls on day 1. With glucose especially, high
177 levels of H_2 were produced during first few days. Over the time course H_2 did not decrease to
178 undetectable levels in cultures with algal biomass, but no inoculum and in cultures with glucose
179 and B-D. In all other cultures H_2 was consumed to undetectable levels due to interspecies H_2
180 transfer, and cumulative H_2 production from algal biomass with the anaerobic inocula was
181 negligible (Table 1). With no anaerobic inoculum, H_2 production was higher from *D. tertiolecta*
182 biomass, 8.4 and 12.6 mL H_2 g-VS^{-1}, than from *C. vulgaris* biomass, 7.9 and 10.8 H_2 g-VS^{-1},
183 with and without BESA, respectively. Further enhancement of H_2 production was attempted by
184 using these cultures as inoculum in batch bottle incubations, but after four enrichment steps no
185 increase in H_2 production was detected.
186
187 No CH_4 was produced in the cultures amended with BESA (Figure 2). Significant amount of
188 CH_4 was produced only with *C. vulgaris* and U-C, glucose and U-C, and glucose and U-D (Table
189 1). Some CH_4 was also produced with *D. tertiolecta* and U-D as well as with chitosan and U-C
190 (Table 1). CH_4 production from cellulose was negligible. CH_4 production from chitosan was
191 significantly lower than that from microalgal biomass. Gas production in controls with no
192 substrate but inoculum was very low, and was taken into account in calculation of the gas
193 production yields (Table 2). Thus, CH_4 was produced from both *C. vulgaris* and *D. tertiolecta*
194 biomass, while the yield was substantially lower with *D. tertiolecta* than with *C. vulgaris* (Table
195 2). With *C. vulgaris* biomass 30.6% of carbon was released as CH_4 and 13.6% as CO_2, while

196 with *D. tertiolecta* biomass the corresponding values were 5.2 and 2.6%, respectively. CH_4
197 production from *C. vulgaris* biomass was higher than in glucose controls, while CH_4 production
198 from *D. tertiolecta* remained far below that of glucose controls. With glucose, cellulose or
199 chitosan H_2 production was generally higher with B-D enrichment than with B-C enrichment, but
200 CH_4 production was generally higher with U-C enrichment than with U-D enrichment (Table 1).
201
202 Initially the pH of the medium was not adjusted in the beginning of the anaerobic incubation and
203 it was pH 8 in the cultures with algal biomass and pH 8.5 with the other substrates and cultures
204 with no substrate. With no substrate, cellulose and chitosan pH changes were minimal, pH
205 ranging from 8 to 8.5 during the incubation. With algal biomass, but no inoculum the pH varied
206 between 7.5 and 8. With *C. vulgaris* and U-C the pH was 7.5-8, with *C. vulgaris* and B-C 7-8,
207 with *D. tertiolecta* and U-D 8-8.5, and with *D. tertiolecta* and B-D 7.5-8. In cultures with
208 glucose the pH varied between 6 and 8.5 during the cultivation.
209
210 Organic acids accumulated in the cultures with B-C and B-D enrichments as well in the cultures
211 with no inoculum. In the cultures inoculated with U-C and U-D organic acid accumulated only in
212 the beginning of the incubation and were reduced later on to CH_4 (Figure 2C and D). In some
213 cultures, such as with *C. vulgaris* and U-C, VFA and ethanol concentrations were actually lower
214 on day 49 than on day 0 (Table 1). Total concentrations of the soluble degradation products were
215 lower with *D. tertiolecta* than with *C. vulgaris* (Table 1). Main VFAs with the anaerobic inocula
216 were acetate and propionate (Figure 2C and D), and acetate and formate in the cultures with no
217 inoculum (Figure 3C and D).
218

219 The initial COD_{tot} values were significantly higher in cultures with *C. vulgaris* than with *D.*
220 *tertiolecta* in spite of the same initial algal VS in all of the cultures. The addition of BESA also
221 increased the initial COD concentration. The COD_{tot} concentrations decreased in all cultures
222 between days 0 and 49, except in the case of no substrate and in cultures with *C. vulgaris*, BESA
223 and no inoculum. Reduction of COD_{tot} was higher in bottles with U-C and U-D than in the
224 bottles with B-C and B-D as inoculum, respectively (Figure 4A). COD_{tot} reduction was 52.0%
225 with *C. vulgaris* and U-C, and 57.1% *D. tertiolecta* and U-D, but only 21.3% with *C. vulgaris*
226 and B-C, and 15.0% with *D. tertiolecta* and B-D, respectively. The ratio of COD_s to COD_{tot}
227 decreased with CH_4 production, but increased in the other cultures (Figure 4B). The COD results
228 were in line with the VFA and alcohol results.
229
230 *3.3. Microbial community composition*
231 Based on bacterial DGGE, initial anaerobic inoculum contained bacteria belonging to phyla
232 Firmicutes, Bacteroidetes, Proteobacteria and Chloroflexi (Table I supplementary material). No
233 species level matches for these bacterial sequences could be attained from the GenBank.
234
235 The bacterial community became enriched during the ten serial batch incubations. Bacterial
236 DGGE profiles were different with the two algal biomass types. The addition of BESA also
237 affected the bacterial community composition (Figure 5). For example, bands B13 and B29 were
238 only clear with *C. vulgaris* and B-C, but not with *C. vulgaris* and U-C. Further, bands B18 and
239 B21 were only clear in *C. vulgaris* and U-C, but not with *C. vulgaris* and B-C. In addition, bands
240 B30-B33 were present in cultures with *D. tertiolecta*, but no corresponding bands were seen in
241 cultures with *C. vulgaris* (Figure 5).

242

243 Most of the bacterial 16S rDNA sequences amplified from the anaerobic enrichments matched
244 uncultured bacteria with no species-level information (Table II supplementary material). The
245 matches in the enrichments were *Petrimonas* sp. (band B14), *Bacteroides* sp. (B15), *Bilophila*
246 *wadsworthia* (B26), *Wolinella succinogenes* (B34), *Oceanibulbus indolifex* (B35), and
247 *Syntrophobacter sp.* (B39). The *Petrimonas* sp. was present in all cultures with *C. vulgaris* and
248 anaerobic inoculum, *B. wadsworthia* in *C. vulgaris* and B-C and *Bacteroides* sp. in *C. vulgaris*
249 and B-C as well as in the duplicates of *D. tertiolecta* and U-D. *W. succinogenes*, *O. indolifex* and
250 *Syntrophobacter* sp. were present in all cultures with *D. tertiolecta* and anaerobic inoculum.
251
252 High diversity of bacteria was also present in cultures with no anaerobic inoculum (Figure 4,
253 Table III supplementary material). These bacteria included *Acidobacterium* sp. (band B44),
254 *Clostridium* sp. (B45, B46, B47, B61), *Clostridium celerecrescens* (B48, B63), *Brevundimonas*
255 sp. (B49), *Hafnia alvei* (B50, B54), *Hafnia alvei* or *Obesumbacterium proteus* (B51), *Gordonia*
256 *terrae* (B56), *Clostridium sulfidigenes* (B57, B58, B59, B60), *Oceanibulbus indolifex* (B62),
257 *Roseobacter* sp. (B65), *Exiguobacterium* sp. (B66), *Bacillus thermoamylovorans* (B67) and four
258 unknown species (B52, B53, B55, B64).
259
260 The DGGE profiles of associated bacteria in *C. vulgaris* and *D. tertiolecta* were different. For
261 example, *H. alvei* was seen only with *C. vulgaris*, whereas *C. sulfidigenes* and B.
262 *thermoamylovorans* only with *D. tertiolecta*. In cultures with *C. vulgaris,* addition of BESA
263 resulted in negligible changes in the bacterial DGGE profile. The only detectable difference was
264 B54 that was identified from the cultures with BESA, but not in the cultures without BESA. In

265 cultures with *D. tertiolecta*, bands B57, B58 and B67 were only visible in cultures without BESA
266 and B63 was significantly brighter with BESA in the medium.
267
268 **4. Discussion**
269 This work demonstrated CH_4 production from *C. vulgaris* and *D. tertiolecta* biomass with
270 municipal anaerobic digester sludge enrichments. Biogenic H_2 was also produced, but it was
271 subsequently consumed without CH_4 production. H_2 was produced also in the cultures with algal
272 biomass but no anaerobic inoculum.
273
274 H_2 was produced from both *C. vulgaris* and *D. tertiolecta* biomass by the H_2 enrichment cultures
275 (containing BESA), but it was subsequently consumed by non-methanogenic microorganisms.
276 The pH was relatively high in these assays. In the cultures with added anaerobic inoculum, H_2
277 production was most sustained in the positive controls with glucose, where the pH was also the
278 lowest. Karadag and Puhakka (2010) showed that the pH significantly affected H_2 production
279 due to pH mediated shifts in fermentation pathways and the microbial community composition.
280 They reported pH 5.0 optimal for H_2 production.
281
282 In the present work, several bacteria were identified from the anaerobic inoculum and algal
283 biomass. These included *Petrimonas* sp. that has been shown to produce H_2 (Grabowski et al.
284 2005). *Syntrophobacter* spp. have been shown to convert propionate to acetate, H_2 and CO_2, but
285 only when co-cultivated with H_2-consuming organisms (Boone and Bryant 1980, de Bok et al.
286 2004). *B. wadsworthia* and *W. succinogenes* utilize H_2 as their electron donor (da Silva et al.
287 2008, Gross et al. 1998). According to Chassard et al. (2005) *Bacteroidetes* spp. can suppress H_2

288 production from cellulosic material in a mixed culture, because they are non-H_2-producing
289 bacteria with a relatively high cellulolytic activity. *O. indolifex* is an ogligately aerobic marine
290 bacterium (Wagner-Döbler et al. 2004) with no activity under anaerobic conditions and thus it
291 originated from the algal biomass slurry.
292
293 H_2 accumulated in the cultures supplemented only with algal biomass. These cultures formed
294 CO_2 and accumulated organic acids and alcohols. Gfeller and Gibbs (1984), Miura et al. (1986)
295 and Ueno et al. (1998) reported hydrogen fermentation in microalgal cells under dark and
296 anaerobic conditions, with H_2 yields up to 2 mmol H_2 g-dw^{-1} (Miura et al. 1986). In this study,
297 H_2 yields were approximately 25% of that in the cultures with no added anaerobic inoculum
298 (Table 2). However, the DGGE profiles had matches with several H_2-producing bacteria such as
299 *Clostridium* spp. (Koskinen et al. 2007, Hung et al. 2010) and *Hafnia alvei* (Podesta et al. 1997),
300 which are known H_2-producers. Some *Bacillus* spp., such as *B. cereus*, *B. thuringiensis* (Porwal
301 et al. 2008) and *B. megaterium* (Jeong et al. 2008) also produce H_2, but Combet-Blanc et al.
302 (1995) reported that *B. thermoamylovorans* is not able to produce H_2. *O. proteus* is typical in
303 brewery environments and known to cause beer spoilage (Prest et al. 1994). Some
304 *Exiguobacterium* spp. such as *E. profundum* are facultatively anaerobic and produce lactate as
305 the main fermentation product (Crapart et al. 2007).
306
307 Carver et al. (2011) reported H_2 production without anaerobic inoculum by heterotrophs
308 associated with *C. vulgaris* biomass, but negligible H_2 production with heterotrophs associated
309 with *D. tertiolecta*. In this study, the *D. tertiolecta*-associated bacteria produced somewhat more
310 H_2, but approx. 4.5 times less VFAs and alcohols and approx. 3 times less CO_2 than the *C.*

311 *vulgaris*-associated bacteria. The higher H_2 production from *D. tertiolecta* was likely due to the
312 lack of proper cell wall in *D. tertiolecta* and different associated bacteria. However, the H_2 yields
313 reported in this study were low. For comparison, Park et al. (2009) reported the production of 28
314 mL H_2 per g dry weight of seaweed *Laminaria japonica* pretreated by ball milling and heat
315 treatment at 120 °C for 30 min using anaerobic sewage sludge as an inoculum. Carver et al. (2011)
316 reported production of ~3mmol H_2 g-VS^{-1} from *C. vulgaris* by only *C. vulgaris* associated bacteria
317 and no added anaerobic inoculum, and ~2mmol H_2 g-VS^{-1} from *D. tertiolecta* by a thermophilic
318 consortium at 60 °C.
319
320 In the cultures with no added anaerobic inoculum, H_2 production was somewhat lower with
321 BESA in the medium. This indicates that BESA is inhibitory to some bacteria involved in
322 fermentation. Bacteria present in cultures with no added anaerobic inoculum were associated
323 with the algal culture or were introduced during handling of the biomass.
324
325 CH_4 was produced from both *C. vulgaris* and *D. tertiolecta* biomass, while the yields were not
326 comparable. CH_4 production was approximately 12 times higher from *C. vulgaris* than from *D.*
327 *tertiolecta* per added VS but only approximately 3 times higher per added or removed COD_{tot}
328 (Table 2). The large difference in CH_4 production between the two algal biomasses was likely
329 due to inhibition by Na^+ and/or Cl^- or ions in the salt water alga *D. tertiolecta* slurry flocculated
330 with NaOH (Samson and LeDuy 1983a). Another reason for low CH_4 production from *D.*
331 *tertiolecta* biomass may be that *W. succinogenes* was identified from cultures with *D. tertiolecta*
332 and U-D, but not from cultures with *C. vulgaris* and U-C. Co-existence of *W. succinogenes* has
333 been reported to markedly reduce CH_4 production (Iwamoto et al. 2002). Chen and Oswald
334 (1998) reported 320 mL CH_4 g-VS^{-1} from biomass of a mixed microalgal culture from high-rate

335 sewage stabilization ponds heat treated at 100 °C for 8 h. Yen and Brune (2007) reported 143
336 mL CH_4 g-VS^{-1} from an algal mixture including *Scenedesmus* spp. and *Chlorella* spp without
337 pretreatment. *C. vulgaris* biomass contained also some chitosan, as chitosan was used in
338 flocculation of the biomass. Co-digestion of algal biomass (N-rich material) with C-rich material
339 such as cellulose or chitosan may enhance digestibility (Yen and Brune 2007). However, the
340 anaerobic enrichments used in this study were not able to utilize chitosan very efficiently and
341 thus it is assumed that the co-digestion effect was negligible and that CH_4 was mainly produced
342 from the algal biomass.
343
344 The calorific yields calculated for the maximum H_2 and CH_4 yields were 0.14 kJ g-VS^{-1} for H_2
345 production from *D. tertiolecta* without added anaerobic inoculum and 10 kJ g-VS^{-1} for CH_4
346 production from *C. vulgaris* with enriched digester sludge without BESA. *C. vulgaris* biomass is
347 amenable to methanogenic digestion without pretreatment, but high Na^+ and Cl^- content of
348 flocculated microalgal biomass may lower the CH_4 yields. Hydrolytic pretreatment of algal
349 slurries may substantially improve H_2 production from a complex substrate as was shown by
350 Lakaniemi et al. (2011) with reed canary grass. Pretreatment may enhance CH_4 production from
351 the biomass of thick cell walled algae, such as *C. vulgaris*, but the energy cost of pretreatment
352 need to be considered.
353
354 **5. Conclusions**
355 CH_4 was produced from *C. vulgaris* and *D. tertiolecta* biomass by mesophilic municipal
356 anaerobic digester sludge enrichments. H_2 was also produced with the anaerobic enrichments but
357 was concurrently consumed by non-methanogenic micro-organisms. H_2 was produced by

satellite bacteria associated with algal biomass. PCR-DGGE profiling demonstrated the presence of H_2 producing and H_2 consuming bacteria in the anaerobic enrichments and H_2 producing bacteria among the satellite bacteria of both microalgal biomasses. H_2 production by the satellite bacteria was higher from *D. tertiolecta* than from *C. vulgaris*, but CH_4 production by the anaerobic enrichments was significantly higher from *C. vulgaris* than from *D. tertiolecta*. The different CH_4 yields may reflect inhibition by high Na^+ and Cl^- concentrations in the *D. tertiolecta* slurry.

Acknowledgements

This research was funded by the Finnish Funding Agency for Technology and Innovation (Finland Distinguished Professor Programme, 402/06).

369
370

371 **References**

372 Benemann, J. R. 2000. Hydrogen production by microalgae. Journal of Applied Phycology 12:
373 291-300.

374

375 Boone, D. R., Bryant, M. P. 1980. Propionate-degrading bacterium, *Syntrobacter wolinii* sp. nov.
376 gen. nov., from methanogenic ecosystems. Applied and Environmental Microbiology 40: 626-
377 632.

378

379 Carver, S. M., Hulatt, C. J., Thomas, D. N., Tuovinen, O. H. 2011 Thermophilic, anaerobic co-
380 digestion of microalgal biomass and cellulose for H_2 production. Biodegradation, doi:
381 10.1007/s10532-010-9419-z.

382

383 Chader, S., Hacene, H., Agathos, S. N. 2009. Study of hydrogen production by three strains of
384 *Chlorella* isolated from the soil in the Algerian Sahara. International Journal of Hydrogen
385 Energy 34: 4941-4946.

386

387 Chassard, C., Gaillard-Martinie, B., Bernalier-Donadille, A. 2005. Interaction between H_2-
388 producing and non-H_2-producing cellulolytic bacteria from the human colon. FEMS
389 Microbiology Letters 242: 339-344.

390

391 Chen, C-C., Li, C-Y., Lin, M-C. 2002. Acid-base enrichment enhances anaerobic hydrogen
392 production process. Applied Microbiology and Biotechnology 58: 224-228.

Chen, P., H., Oswald, W. J. 1998. Thermochemical treatment for algal fermentation. Environment International 24: 889-897.

Combet-Blanc, Y., Ollivier, B., Streicher, C., Patel, B. K. C., Dwivedi, P. P., Pot, B., Prensier, G., Garcia, J-L. 1995. *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. International Journal of Systematic Bacteriology 45: 9-16.

Crapart, S., Fardeau, M-L., Cayol, J-L., Thomas, P., Sery, C., Ollivier, B., Combet-Blanc, Y. 2007. *Exiguobacterium profundum* sp. nov., a moderately thermophilic, lactic acid-producing bacterium isolated from a deep-sea hydrothermal vent. International Journal of Systematic and Evolutionary Microbiology 57: 287-292.

da Silva, S. M., Venceslau, S. S., Fernandes, C. L. V., Valente, F. M. A., Pereira, I. A. C. 2008. Hydrogen as an energy source for the human pathogen *Bilophila wadsworthia*. Antonie van Leeuwenhoek 93: 381-390.

de Bok, F. A. M., Plugge, C. M., Stams, A. J. M. 2004. Interspecies electron transfer in methanogenic propionate degrading consortia. Water Research 38: 1368-1375.

De Schamphelaire, L., Verstraete, W. 2009. Revival of the biological sunlight-to-biogas energy conversion system. Biotechnology and Bioengineering 103: 296-304.

Ejlertsson J., Johansson E., Karlsson A., Meyerson U., Svensson B. H. 1996. Anaerobic degradation of xenobiotics by organisms from municipal solid waste under landfilling conditions Antonie van Leeuwenhoek 69:67-74.

Fan, L. T., Gharpuray, M. M., Lee, Y-H. 1981. Evaluation and pretreatments for enzymatic conversion of agricultural residues. Biotechnology and Bioengineering Symposium. 11: 29-45.

Gfeller, R. P., Gibbs, M. 1984. Fermentative metabolism of *Chlamydomonas reinhardtii*. Plant Physiology 75: 212-218.

Grabowski, A., Tindall, B. J., Bardin, V., Blanchet, D., Jeanthon, C. 2005. *Petrimonas sulfuriphila* gen. nov., sp. nov., a mesophilic fermentative bacterium isolated from a biodegraded oil reservoir. International Journal of Systematic and Evolutionary Microbiology 55: 1113-1121.

Gross, R., Simon, J., Theis, F., Kröger, A. 1998. Two membrane anchors of *Wolinella succinogenes* hydrogenase and their function in fumarate and polysulfide respiration. Arch Microbiol 170: 50-58.

Horiuchi, J., Ohba, I., Tada, K., Kobayashi, M., Kanno, T., Kishimoto, M. 2003. Effective cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control. Journal of Bioscience and Bioengineering. 95: 412-415.

438 Hung, C-H., Cheng, C-H., Guan, D.-W., Wang, S.-T., Hsu, S-C., Liang, C-M., Lin, C-Y. 2010.
439 Interactions between *Clostridium* sp. and other facultative anaerobes in a self-formed granular
440 sludge hydrogen-producing bioreactor. International Journal of Hydrogen Energy,
441 doi:10.1016/j.ijhydene.2010.06.010.
442
443 Ike, A., Toda, N., Tsuji, N., Hirata, K., Miyamoto, K. 1997. Hydrogen photoproduction form
444 CO_2-fixing microalgal biomass: application of halotolerant photosynthetic bacteria. Journal of
445 Fermentation and Bioengineering 84: 606-609.
446
447 Iwamoto, M., Asanuma, N., Hino, T. 2002. Ability of *Selenomonas ruminantium*, *Veillonella*
448 *parvula* and *Wolinella succinogenes* to reduce nitrate and nitrite with special reference to the
449 suppression of ruminal methanogenesis. Anaerobe 8: 209-215.
450
451 Jeong, T-Y., Cha, G-C., Yeom, S. H., Choi, S. S. 2008. Comparison of hydrogen production by
452 four representative hydrogen-producing bacteria. Journal of Industrial and Engineering
453 Chemistry 14: 333-337.
454
455 Karadag, D., Puhakka, J. A. 2010. Direction of glucose fermentation towards hydrogen or
456 ethanol production through on-line pH control. International journal of Hydrogen Energy 35:
457 10245-10251.
458
459 Karlsson A., Ejlertsson J., Nezirevic D., Svensson B. H. 1999. Degradation of phenol under
460 meso- and thermophilic, anaerobic conditions. Anaerobe 5: 25-35.

461

462 Koskinen P. E. P., Kaksonen A. H., Puhakka J. A. 2007. The relationship between instability of
463 H_2 production and compositions of bacterial communities within a dark fermentation fluidized-
464 bed bioreactor. Biotechnology and Bioengineering 97: 742- 758.

465

466 Kosourov, S., Tsygankov, A., Seiber, M., Ghirardi, M. L. 2002. Sustained hydrogen production
467 by *Chlamydomonas reinhardtii*: Effects of culture parameters. Biotechnology and
468 Bioengineering 78: 731-740.

469

470 Lakaniemi, A-M., Koskinen, P. E. P., Nevatalo, L. M., Kaksonen, A. H., Puhakka, J. A. 2011.
471 Biogenic hydrogen and methane production from reed canary grass. Biomass and Bioenergy 35:
472 773-780.

473

474 Logan, B. E., Oh, S-E., Kim, I. S., van Ginkel, S. 2002. Biological hydrogen production
475 measured in batch anaerobic respirometers. Environmental Science and Technology 36: 2530-
476 2535.

477

478 Melis, A. 2002. Green alga hydrogen production: progress, challenges and prospects.
479 International Journal of Hydrogen Energy 27: 1217-1228.

480

481 Miura, Y., Ohta, S., Mano, M., Miyamoto, K. 1986. Isolation and characterization of a
482 unicellular marine green alga exhibiting high activity in dark hydrogen production. Agricultural
483 and Biological Chemistry 50: 2837-2844.

Muyzer, G., De Waal, E. C., Uitterlinden, A. G. 1993. Profiling complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59: 695-700.

Muyzer, G., Hottenträger, S., Teske, A., Waver, C. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA - A new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans, A. D. L., van Elsas, J. D., De Bruijn, F. (editors) Molecular microbial ecology manual. Dordrecht: Kluwer Academic Publishers. p. 3.4.4/1-23.

Owen W. F., Stuckey D. C., Healy J. B., Jr., Young L. Y., McCarty P. L. 1979. Bioassay for monitoring methane potential and anaerobic toxicity. Water Research 13:485-492.

Park, J-I., Lee, J., Sim, S. J., Lee, J-H. 2009. Production of hydrogen from marine macro-algae biomass using anaerobic sewage sludge microflora. Biotechnology and Bioprocess Engineering 14: 307-315.

Podesta, J. J., Gutiérrez-Navarro, A. M., Estrella, C. N., Esteso, M. A. 1997. Electrochemical measurement of trace concentrations of biological hydrogen produced by *Enterobacteriaceae*. Res. Microbiol. 148: 87-93.

505 Porwal, S., Kumar, T., Lal, S., Rani, A., Kumar, S., Cheema, S., Purohit, H. J., Sharma, R., Patel,
506 S. K. S., Kalia, V. C. 2008. Hydrogen and polyhydroxybutyrate producing abilities of microbes
507 from diverse habitats by dark fermentative process. Bioresource Technology 99: 5444-5451.
508
509 Posten, C., Schaub, G. 2009. Microalgae and terrestrial biomass as source for fuels – A process
510 view. Journal of Biotechnology 142: 64-69.
511
512 Prest, A. G., Hammond, J. R. M., Stewart, G. S. A. B. 1994. Biochemical and molecular
513 characterization of *Obesumbacterium proteus*, a common contaminant of brewing yeasts.
514 Applied and Environmental Microbiology 60: 1635-1640.
515
516 Samson, R., LeDuy, A. 1983a. Influence of mechanical and thermochemical pretreatments on
517 anaerobic digestion of *Spirulina maxima* algal biomass. Biotechnology Letters 5: 671-676.
518
519 Samson, R., LeDuy, A. 1983b. Improved performance of anaerobic digestion of *Spirulina*
520 *maxima* algal biomass by addition of carbon-rich wastes. Biotechnology letters 5: 677-682.
521
522 Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C.,
523 Kruse, O., Hankamer, B. 2008. Second generation biofuels: High-efficiency microalgae for
524 biodiesel production. Bioenergy Research 1:20-43.
525

526 SFS 1988. SFS 5504: Determination of chemical oxygen demand (COD Cr) in water with the
527 closed tube method. Oxidation with dichromate. Finnish Standards Association (SFS). 4pp.
528 (http://sales.sfs.fi/index.jsp?setLang=1).
529
530 SFS 1990. SFS 3008: Determination of total residue and total fixed residue in water, sludge and
531 sediment. Finnish Standards Association (SFS). 3pp. (http://sales.sfs.fi/index.jsp?setLang=1).
532
533 Ueno, Y., Kurano, N., Miyachi, S. 1998. Ethanol production by dark fermentation in the marine
534 green alga, Chlorococcum littorale. Journal of Fermentation and Bioengineering 86: 38-43.
535
536 Velasquez-Orta, S. B, Curtis, T. P, Logan, B. E. 2009. Energy from algae using microbial fuel
537 cells. Biotechnology and Bioengineering 103: 1068-1076.
538
539 Wagner-Döbler, I., Rheims, H., Felske, A., El-Ghezal, A., Flade-Schröder, D., Laatsch, H.,
540 Lang, S., Pukall, R., Tindall, B. J. 2004. *Oceanibulbus indolifex* gen. nov., sp. nov., a North Sea
541 alphaproteobacterium that produces bioactive metabolites. International Journal of Systematic
542 and Evolutionary Microbiology 54: 1177-1184.
543
544 Yen, H-W., Brune, D. E. 2007. Anaerobic co-digestion of algal sludge and waste paper to
545 produce methane. Bioresource Technology 98: 130-134.
546
547 Zehnder, A. J. B., Huser, B. A., Brock, T. D., Wuhrmann, K. 1980. Characterization of an
548 acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. Archives of Microbiology

549 124:1-11.

Figure captions

Figure 1 Methane production (±SE) in enrichment phase 1 (A), 2 (B), 3 (C), 4 (D), 5 (E) and 6 (F) with ● *C. vulgaris* and U-C,■ *C. vulgaris* and B-C, ▲ *D. tertiolecta* and U-D, and × *D. tertiolecta* and B-D.

Figure 2 CH_4 (A), CO_2 (B) and the main fermentation products acetate (C) and propionate (D) with ● *C. vulgaris* and U-C,■ *C. vulgaris* and B-C, ▲ *D. tertiolecta* and U-D, and × *D. tertiolecta* and B-D.

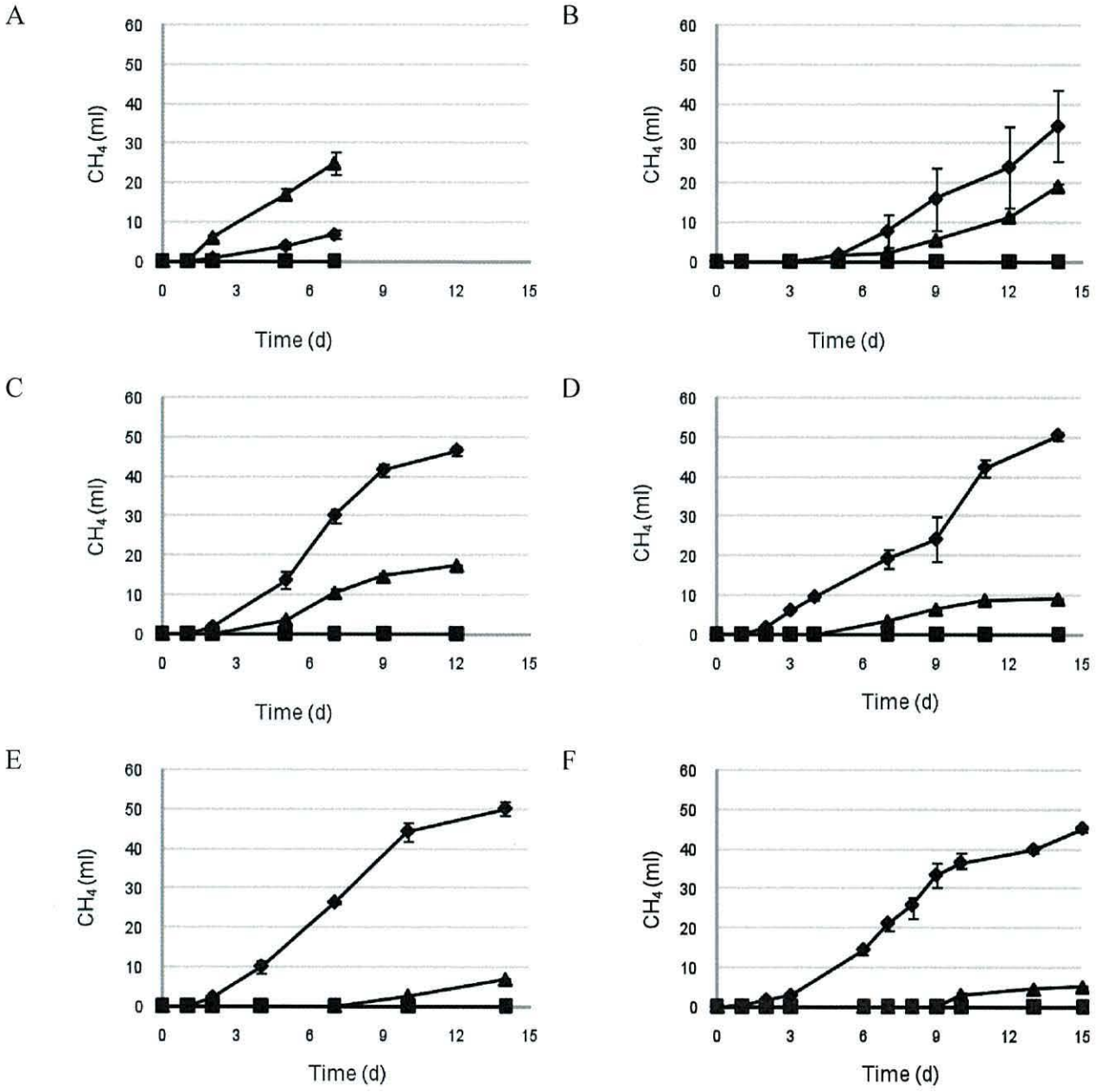
Figure 3 H_2 (A), CO_2 (B) and the main fermentation products acetate (C) and formate (D) with ● *C. vulgaris* and no inoculum,■ *C. vulgaris*, BESA and no inoculum, ▲ *D. tertiolecta* and no inoculum, and × *D. tertiolecta*, BESA and no inoculum.

Figure 4 Total COD (COD_{tot}) concentrations and the persentage of CODs at the beginning and end of the cultivation in the cultures with *C. vulgaris* and *D. tertiolecta* biomass.

Figure 5 Bacterial community profiles from the original anaerobic digester sludge (A), *C. vulgaris* enrichment cultures (B), *D. tertiolecta* enrichment cultures (C), *C. vulgaris* associated bacteria (D) and *D. tertiolecta* associated bacteria (E). See Tables I, II and III in the supplementary material for the labeled bands.

572

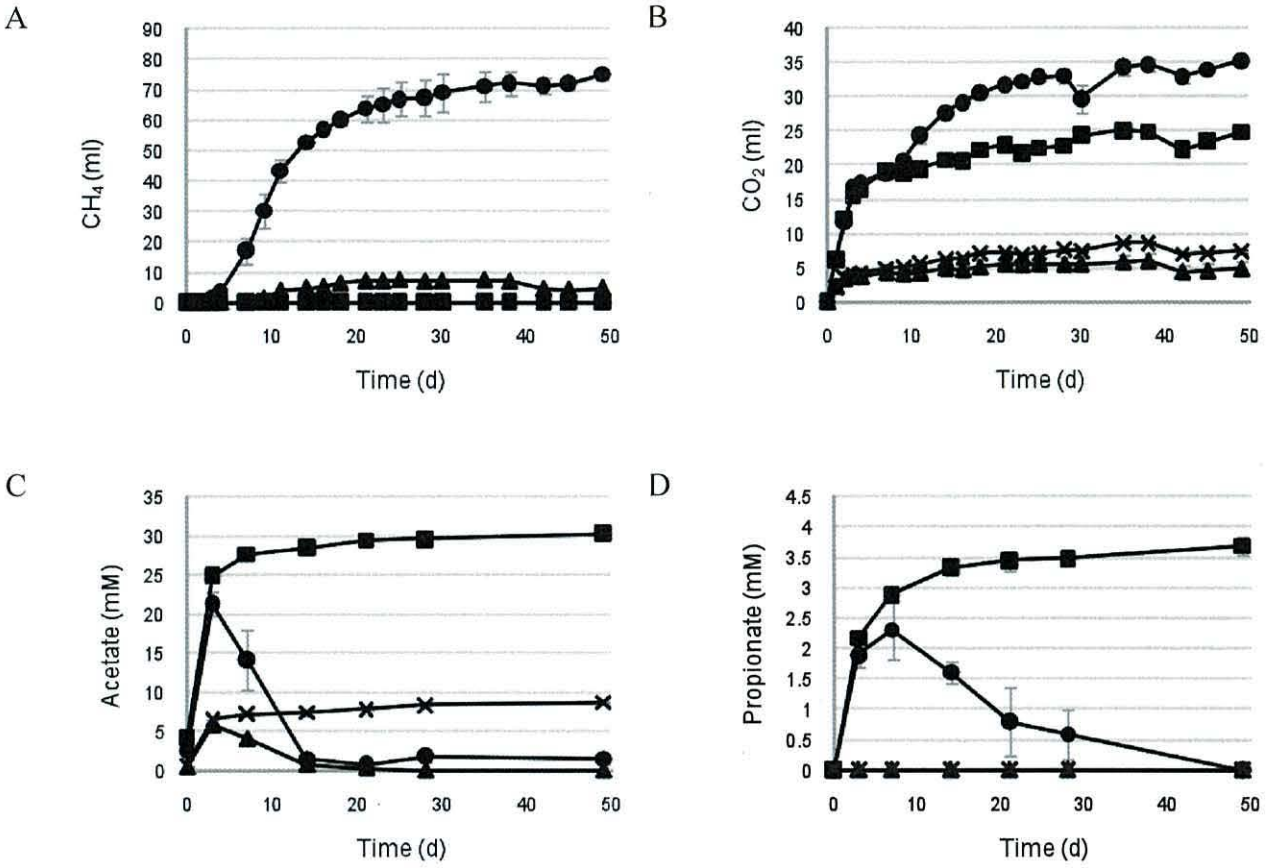
573



574 Figure 1

575

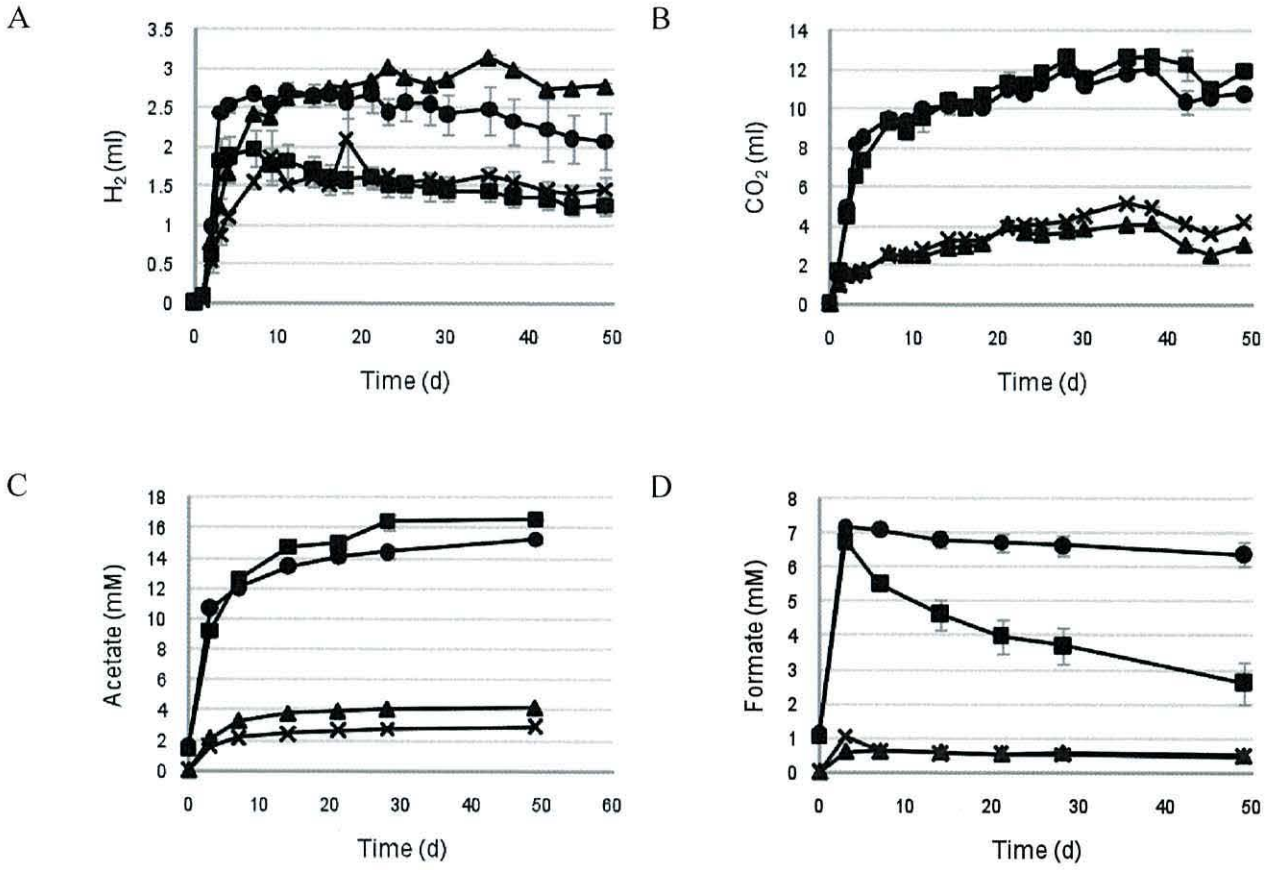
575



576 Figure 2

577

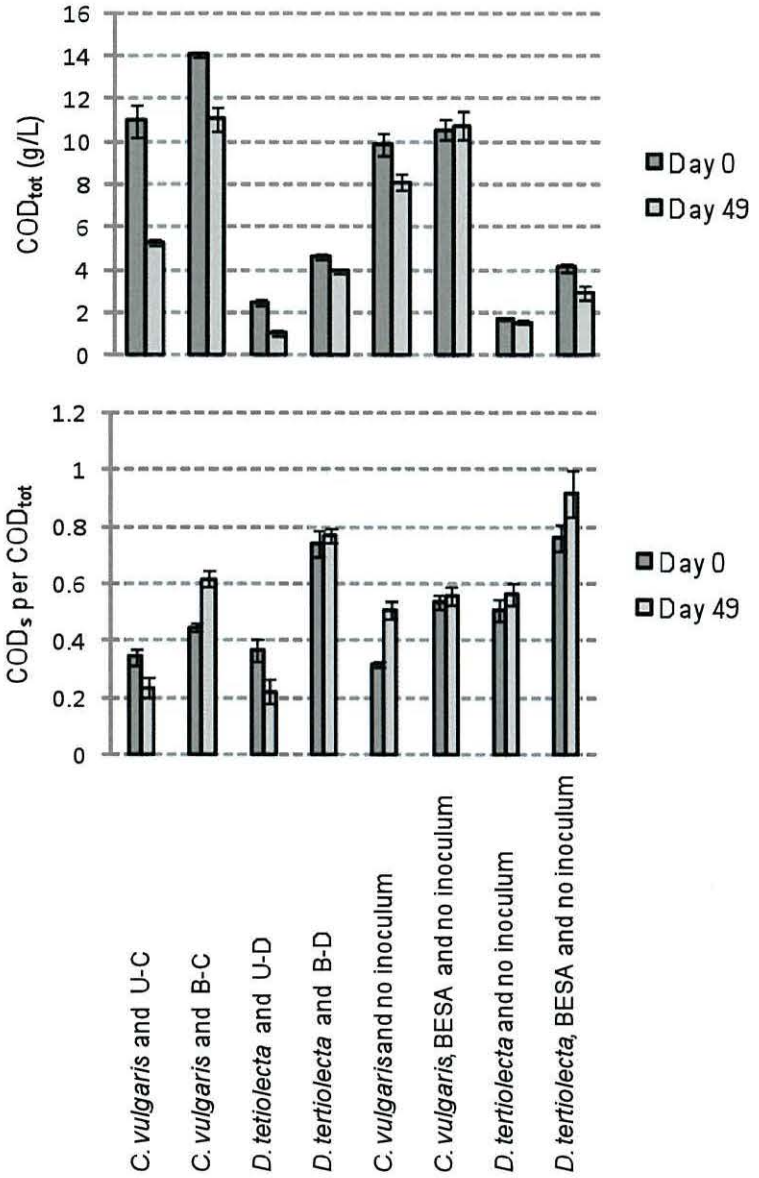
577



578 Figure 3

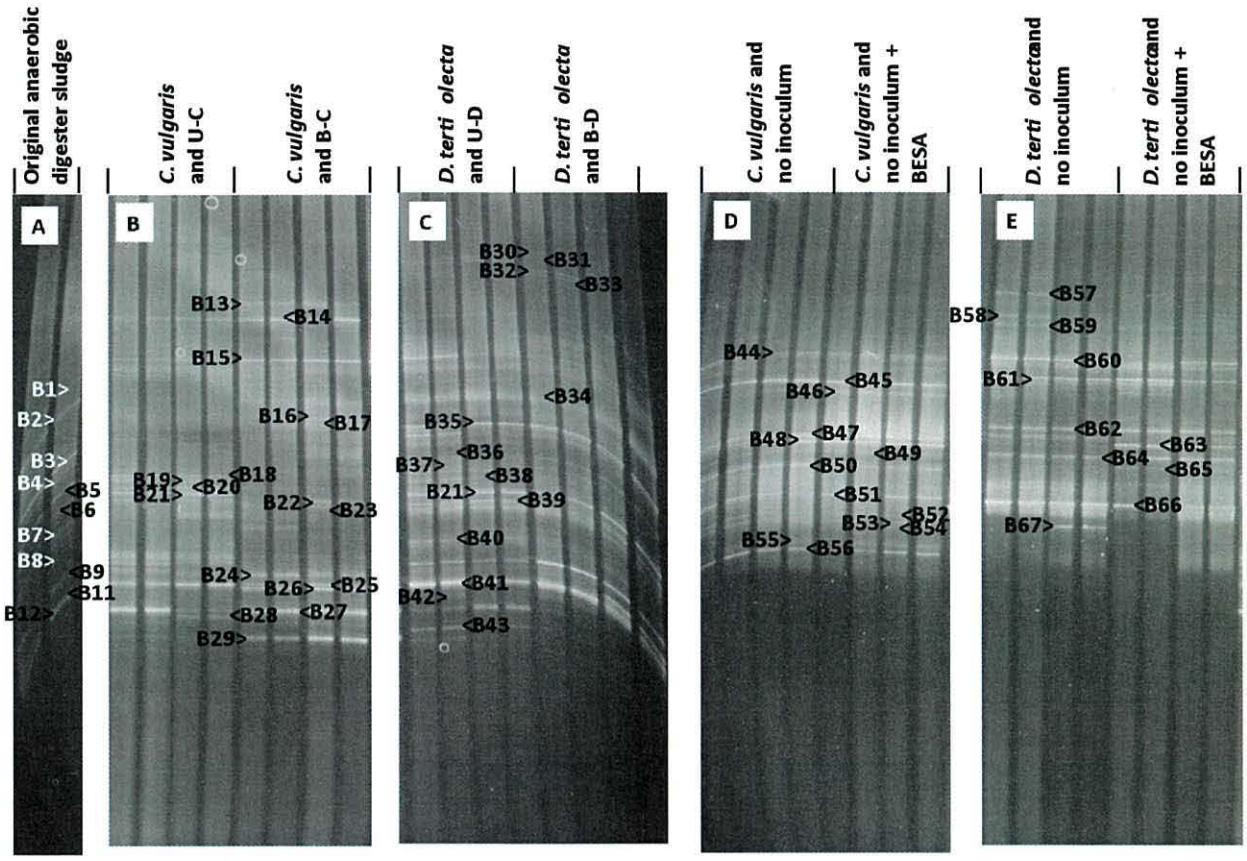
579

579



580
581 Figure 4
582

582



583

584 Figure 5

585

585 Table 1 Cumulative gas production and accumulation of degradation products during the 49 day incubation of the
586 batch bottles. The values include standard errors.

Substrate	Inoculum	H_2 (mL)	CH_4 (mL)	CO_2 (mL)	Sum of VFAs and alcohols (mM)
None	U-C	0.0 ± 0.0	0.2 ± 0.3	2.3 ± 0.4	-0.6 ± 0.7
None	B-C	0.0 ± 0.0	0.0 ± 0.0	4.7 ± 0.2	5.1 ± 0.5
None	U-D	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 0.0	-0.5 ± 0.1
None	B-D	0.0 ± 0.0	0.0 ± 0.0	4.5 ± 0.2	3.7 ± 0.4
C. vulgaris.	None	2.1 ± 0.7	0.0 ± 0.0	10.8 ± 0.6	22.1 ± 1.8
C. vulgaris and BESA	None	1.3 ± 0.2	0.0 ± 0.0	12.0 ± 0.7	19.1 ± 5.1
D. tertiolecta	None	2.8 ± 0.1	0.0 ± 0.0	3.0 ± 0.4	5.1 ± 0.1
D. tertiolecta and BESA	None	1.5 ± 0.3	0.0 ± 0.0	4.2 ± 0.2	4.0 ± 0.3
C. vulgaris	U-C	0.0 ± 0.0	74.9 ± 3.6	35.2 ± 0.3	-3.8 ± 1.0
C. vulgaris	B-C	0.1 ± 0.0	0.0 ± 0.0	24.8 ± 0.0	31.2 ± 0.7
D. tertiolecta	U-D	0.0 ± 0.0	4.7 ± 0.2	4.9 ± 0.0	0.4 ± 0.1
D. tertiolecta	B-D	0.0 ± 0.0	0.0 ± 0.0	7.4 ± 0.8	8.9 ± 0.4
Glucose	U-C	4.9 ± 0.4	56.4 ± 0.1	62.5 ± 0.4	2.9 ± 0.7
Glucose	B-C	7.1 ± 0.4	0.0 ± 0.0	57.9 ± 0.9	46.1 ± 0.4
Glucose	U-D	5.2 ± 0.9	38.5 ± 14.0	56.2 ± 7.0	13.3 ± 14.9
Glucose	B-D	14.6 ± 2.3	0.0 ± 0.0	60.8 ± 0.5	44.4 ± 7.1
Cellulose	U-C	0.0 ± 0.0	0.3 ± 0.4	0.9 ± 0.1	0.7 ± 1.4
Cellulose	B-C	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 0.0	7.6 ± 0.4
Cellulose	U-D	0.0 ± 0.0	0.1 ± 0.1	1.8 ± 0.1	0.0 ± 0.1
Cellulose	B-D	0.0 ± 0.0	0.0 ± 0.0	4.6 ± 0.3	5.4 ± 1.3
Chitosan	U-C	0.0 ± 0.0	3.6 ± 4.1	2.4 ± 2.5	0.4 ± 1.5
Chitosan	B-C	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 0.3	6.9 ± 0.5
Chitosan	U-D	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	0.1 ± 0.1
Chitosan	B-D	0.0 ± 0.0	0.0 ± 0.0	3.2 ± 0.1	5.6 ± 0.8

587 *Note:* minus sign in front of sum of VFAs and alcohols indicates that the sum of VFAs and alcohols was higher on
588 day 0 than on day 49.
589

589
590 Table 2 The H_2 and CH_4 production yields from *C. vulgaris* and D. *tertiolecta* biomass.

	mmol CH_4 per L	mmol CH_4 per g VS	mmol CH_4 per g added COD_{tot}	mmol CH_4 per g removed COD_{tot}
C. vulgaris and U-C	59.6	11.9	5.8	11.3
C. vulgaris and B-C	0	0	0	0
D. tertiolecta and U-D	5.1	1.0	2.1	3.6
D. tertiolecta and B-D	0	0	0	0
	mmol H_2 per L	mmol H_2 per g VS	mmol H_2 per g added COD_{tot}	mmol H_2 per g removed COD_{tot}
C. vulgaris and no inoculum	2.3	0.45	0.23	1.7
C. vulgaris, BESA and no inoculum	1.6	0.33	0.15	-[1)]
D. tertiolecta and no inoculum	2.6	0.52	1.6	21.1
D. tertiolecta, BESA and no inoculum	1.7	0.35	0.42	4.0

591 *Note:* [1)]H_2 yield per g removed COD_{tot} could not be calculated as no COD_{tot} reduction was detected.
592

592
593
594 Table I Selected band identities and affiliations of the initial anaerobic digester sludge inoculum from DGGE
595 conducted with samples amplified with the bacterial specific PCR primers.

Band label[a]	SL[b]	Sim (%)[c]	Affiliation (acc)[d]	Phylum / Family	Origin of the sample with the closest match
B1	386	91.4	Uncultured Firmicutes bacterium (CU926869)	Firmicutes / unknown	A full-scale mesophilic anaerobic digester
B2	392	100	Uncultured Bacteroidetes bacterium (CU918722)	Bacteroidetes / unknown	A full-scale mesophilic anaerobic digester
B3	464	100	Uncultured Deltaproteobacteria bacterium (CU926802)	Proteobacteria / unknown	A full-scale mesophilic anaerobic digester
B4	393	99.2			
B5	434	99.5	Uncultured bacterium (EU542511)	Unknown / unknown	Dechlorinating microbial community from anoxic estuarine sediment
B6	410	91.0	Uncultured Chloroflexi bacterium (CU918692)	Chloroflexi / unknown	A full-scale mesophilic anaerobic digester
B7	359	79.9			
B8	399	89.7	Uncultured Chloroflexi bacterium (CU918692)	Chloroflexi / unknown	A full-scale mesophilic anaerobic digester
B9	408	85.8	Uncultured bacterium (GQ487786)	Unknown / unknown	Microbial community in a groundwater/ surface water redox transition zone
B10	460	99.6	Uncultured bacterium (DQ088231)	Unknown / unknown	A sulfidogenic bioreactor
B11	447	97.4			
B12	431	98.1	Uncultured Firmicutes bacterium (CU922533)	Firmicutes / unknown	A full-scale mesophilic anaerobic digester

596

596
597 Table II Selected band identities and affiliations of the batch bottles with algal biomass and enriched anaerobic
598 inocula from DGGE conducted with samples amplified with the bacterial specific PCR primers.

Band label[a]	SL[b]	Sim (%)[c]	Affiliation (acc)[d]	Phylum / Family	Origin of the sample with the closest match
B13	351	94.0	Uncultured bacterium (CT574432)	Unknown / unknown	A municipal anaerobic sludge digester
B14	488	97.3	*Petrimonas* sp. (GU583826)	Bacteroidetes / *Porphyromonadaceae*	Chinese luzhou-flavor liquor cellar mud
B15	494	94.3	*Bacteroides* sp. (AY554420)	Bacteroidetes / *Bacteroidaceae*	A landfill leachate bioreactor
B16	340	91.8	Uncultured bacterium (GQ203639)	Unknown / unknown	An anaerobic baffled reactor
B17	363	97.0			
B18	463	97.4			
B19	329	99.4	Thermotogales bacterium (HM003101)	Thermotogae / unknown	An environmental sample from temperate climate
B20	382	91.9			
B21	381	86.9			
B22	452	96.0	Uncultured bacterium (GQ203639)	Unknown / unknown	An anaerobic baffled reactor
B23	458	97.8			
B24	429	96.3	Uncultured bacterium (GQ324637)	Unknown / unknown	A sulfidogenic wastewater biofilm
B25	345	95.9	Uncultured Spirochaetes bacterium (CU922720)	Spirochaetes / unknown	A full-scale mesophilic anaerobic digester
B26	348	95.1	*Bilophila wadsworthia* (AB117562)	Deltaproteobacteria / *Desulfovibrionaceae*	Bile metabolism
B27	483	100	Uncultured bacterium (CT574327)	Unknown / unknown	A municipal anaerobic sludge digester
B28	450	97.8	Uncultured bacterium (AB248641)	Unknown / unknown	A mesophilic anaerobic chemostat fed with butyrate
B29	488	98.0	Uncultured spirochete clone (AY648566)	Spirochaetes / unknown	An anaerobic bioreactor processing sulfate-rich waste streams
B30	406	79.8	Uncultured Bacteroidetes bacterium (AB478930)	Bacteroidetes / unknown	A cassette-electrode microbial fuel cell
B31	450	84.0	Uncultured bacterium (FN563280)	Unknown / unknown	A mesophilic and fuzzy logic controlled 2-phase biogas reactor
B32	458	86.9	Uncultured Bacteroidetes bacterium (CU922564)	Bacteroidetes / unknown	A full-scale mesophilic anaerobic digester
B33	437	96.3			
B34	472	100	*Wolinella succinogenes* (NR_025942)	Proteobacteria / *Helicobacteraceae*	Laboratory culture of *Wolinella succinogenes*
B35	456	100	*Oceanibulbus indolifex* (DQ915614)	Proteobacteria / *Rhodobacteraceae*	Not given
B36	462	84.4	Uncultured bacterium (FJ901102)	Unknown / unknown	Reservoir with a high water cut stage
B37	446	96.0	Alpha proteobacterium (GU061126)	Proteobacteria / unknown	The Yellow Sea
B38	414	73.2	Uncultured Thermotogae bacterium (EU722197)	Thermotogae / unknown	Production water from an Alaskan mesothermic petroleum reservoir
B39	487	91.2	*Syntrophobacter* sp. (EU888828)	Proteobacteria / *Syntrophobacteraceae*	An upflow anaerobic sludge blanket reactor degrading propionate
B40	403	76.4	Uncultured bacterium (EF559198)	Unknown / unknown	A mesophilic anaerobic solid waste digester
B41	489	100	Uncultured bacterium (AY667253)	Unknown / unknown	A dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer
B42	456	100			
B43	448	99.8	Uncultured bacterium (FJ645714)	Unknown / unknown	Microbial population treating anaerobically PCP-contaminated waste streams at low-temperature

599

Table III Selected band identities and affiliations of the batch bottles with algal biomass and no anaerobic inoculum from DGGE conducted with samples amplified with the bacterial specific PCR primers.

Band label[a]	SL[b]	Sim (%)[c]	Affiliation (acc)[d]	Class / Family	Origin of the sample with the closest match
B44	418	93.8	Uncultured *Acidobacterium* sp. (AB257652)	Acidobacteria / *Acidobacteriaceae*	Endolithic microorganisms from the pores in exposed dolomite rocks in the Piora Valley
B45	461	100	*Clostridium* sp. (DQ479415)	Firmicutes / *Clostridiaceae*	Bacterial communities involved in sulfur cycle in metalliferous organic soils
B46	337	97.0	*Clostridium* sp. (GU195653)	Firmicutes / *Clostridiaceae*	A bifenthrin degrading bacterium isolated from waste water
B47	474	100	*Clostridium* sp. (FJ384378)	Firmicutes / *Clostridiaceae*	A mesophilic anaerobic digestor
B48	434	99.8	*Clostridium celerecrescens* (FM994938)	Firmicutes / *Clostridiaceae*	A H_2 producing anaerobic sequencing batch reactor
B49	306	88.2	*Brevundimonas* sp. (HM777012)	Proteobacteria/ *Caulobacteraceae*	Not given
B50	433	100	*Hafnia alvei* (AB244475)	Proteobacteria/ *Enterobacteriaceae*	Crop of the antlion species *Myrmeleon bore*
B51	388	100	*Hafnia alvei* (AB519795) / *Obesumbacterium proteus* (FJ492810)	Proteobacteria/ *Enterobacteriaceae*	Not given / bacteria associated with brewery yeasts
B52	405	90.1	Uncultured bacterium (EF154421)	Unknown / unknown	Digestive tracts of ground beetles
B53	421	90.0	Uncultured beta proteobacterium (FJ975852)	Unknown / unknown	Human gut microbiome
B54	415	96.6	*Hafnia alvei* (AB519795)	Proteobacteria/ *Enterobacteriaceae*	Not given
B55	421	94.5	Uncultured bacterium (EU803296)	Unknown / unknown	Ocean sample
B56	467	96.6	*Gordonia terrae* (AY771337)	Actinobacteria / *Gordoniaceae*	Laboratory culture collection
B57	474	100	*Clostridium sulfidigenes* (HM163536)	Firmicutes / *Clostridiaceae*	Bacterial communities associated with photosynthetic plants
B58	475	100			
B59	422	97.9			
B60	479	99.8			
B61	488	99.8	*Clostridium* sp. (DQ168187)	Firmicutes / *Clostridiaceae*	Soil from the Florida Everglades
B62	456	100	*Oceanibulbus indolifex* (DQ915614)	Proteobacteria / Rhodobacteraceae	Not given
B63	460	100	*Clostridium celerecrescens* (FM994938)	Firmicutes / *Clostridiaceae*	A H_2 producing anaerobic sequencing batch reactor
B64	363	97.8	Uncultured bacterium (FJ203216)	Unknown / unknown	Bacteria associated with the coral *Montastraea faveolata*
B65	445	99.6	*Roseobacter* sp. (EF512125)	Proteobacteria / *Rhodobacteraceae*	Symbiotic microorganisms in *Isochrysis galbana* culture
B66	461	99.6	*Exiguobacterium* sp. (FN435981)	Firmicutes / *Bacillaceae*	Microbial community inhabiting deteriorated stones
B67	454	97.8	*Bacillus thermoamylovorans* (AB360808)	Firmicutes / *Bacillaceae*	Not given