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Feasibility study for co-production of algal biofuels and tertiary wastewater treatment using mixotrophic freshwater algae

Richard Smith

r.t.smith@sheffield.ac.uk

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PhD. E-Futures DTC

Abstract

The use of mixotrophic algal species for synchronous phycoremediation and biofuel production is a relatively new concept which may overcome many of the problems limiting commercialisation. Digestate is a potential cheap organic carbon source for mixotrophic growth. This study evaluated the growth potential of a local strain of microalgae in unsterilised digestate and under mixotrophic conditions. *Chlorella* sp. was able to survived and grow in unsterilised digestate, however the potential of digestate as a organic carbon source for mixotrophic growth remains unanswered. *Chlorella sp.* grown under mixotrophic conditions resulted in up to 5 times more lipid production relative to autotrophy, indicating great potential for use in biofuel production.

1. Introduction

The concept of utilising microalgae for the removal of high concentrations of nutrients in wastewater (phyco-remediation) has been development for decades (Oswald et al. 1957, de la Noüe et al. 1992). Phycoremediation studies have repeatedly shown high efficiencies (70-99.8%) of nitrogen, phosphorus and heavy metal removal (Oswald 1988, Muttamara et al. 1995, Hoffmann 1998, Chinnasamy et al. 2010, Wang et al. 2010). The algal process is an effective and low technology process which offers inherent cost savings and provides a more appropriate method of water treatment for developing countries (Pittman et al. 2011). One of the major advantages of algal processes over conventional treatment is the ability to recycle the nutrients forming high value products, such fertilizers, pharmaceutical products, food additives and biofuels (Benemann et al. 1977, Mata et al. 2010, Rawat et al. 2011).

While algal biofuels have been argued to have a number of advantages over terrestrial biofuels, most significantly the fact that they do not directly or indirectly compete with food production, a significant disadvantage is the high fertilizer demand (Lardon et al. 2009, Brennan and Owende 2010). Clarens et al. (2010) carried out a comprehensive life cycle analysis comparison of algal and terrestrial biofuels. The study concluded that due to the high nutrient demand of algal growth, algal biodiesel would consume significantly more energy for production than conventional biofuel crops such as corn and canola (Clarens et al. 2010). Phycoremediation of wastewater with biofuel production, or biofuel co-production is a significantly less energy and capital intensive method of production, creating a far more sustainable fuel (Parmar et al. 2011, Rawat et al. 2011, Park *et al.* 2011, Sturm and Lamer 2011) Clarens *et al.* (2010) estimated a 92% reduction in energy demand compared to conventional algal biofuels production in high rate algal ponds (HRAPs). This reduction is partly due to the reduce fertilizer use (30-50%) but also due to the energy offset from transferring from an energy intensive chemical wastewater treatment system to a significantly lower phycoremediation treatment (50-70%).

Currently, water treatment is an energy intensive process, accounting for almost 1% of the daily electricity consumption in England and Wales (POST 2007). Consequently, UK water treatment companies are becoming increasingly keen to reduce their energy consumption (and associated carbon emissions), while meeting stricter discharge limits. Biofuel co-production could provide a revenue source while offsetting energy demand. Additionally by integrating the biological processes into the conventional wastewater system, overall energy consumption and operating costs could be reduced substantially (Mata et al. 2010, Park *et al.* 2011). Carbon dioxide, produced by activated sludge and biogas combustion, could be supplied to phycoremediating algae to promote growth. The algae in turn can supply oxygen produced during growth back to the aerated activated sludge (Uttley et al. 2011).

In both biofuels production and phycoremediation it is desirable to obtain a high cell density within an algae culture in order to enhance biomass and lipid yield (reducing culture space), increase nutrient removal, and reduce harvesting and downstream processing costs (Yu et al. 2009). Achieving high density algal culture is technically difficult when using autotrophic algae. Due to autotrophic algae's reliance on solar energy for growth, when the culture reaches a certain cell density or when grown in dark coloured wastewater, light penetration and therefore biomass productivity is

limited (Chen and Johns 1995, Liang et al.2009, Bhatnagar et al. 2011).

Some algae species are facultatively heterotrophic and will utilise organic sources of carbon over photosynthetic inorganic carbon assimilation (Bhatnagar et al. 2011). While this eliminates the problem of light limitation, the need for large amounts of high quality organic carbon would make this approach more expensive than photoautotrophy (Wan *et al*. 2011). Alternatively, mixotrophic species can simultaneously uptake both inorganic (through photosynthesis) and organic carbon, leading to a synergistic effect which enhances growth (Wang *et al.* 2011). Additionally in latitudes such as the UK where there is a large seasonal variation in photoperiod, heterotrophic growth during the night will maximise productivity.

Mixotrophy is also thought to significantly increase both the lipid content and lipid productivity (a function of lipid content and biomass productivity) in some species (Bhatnagar *et al.* 2011). In a multi-species study Wan *et al.* (2011) found a 6 fold increase in lipid yield (mg l^{-1}) with the addition of 1g/l glucose to a *Chlorella sorokiniana* culture. Similarly the lipid productivity (mg l^{-1} day^{-1}) of *Chlorella vulgaris* has been reported to be increased by a factor of 13.5 when supplemented with 1g/l of glucose (Liang *et al.* 2009)

Das *et al.* (2011) investigated how two-phase growth (phototrophic for 7 days and mixotrophic for 3 days) affects biomass and lipid productivities. The group found that fatty acid methyl esters (FAME) productivity increased by 270% over 3 days after the addition of 2% w/v glycerol (Das *et al.* 2011). It was speculated that the addition of organic substrate at beginning of the stationary stage of the algae growth, overcame the light limitation (due to cell density) aiding growth and lipid production (Das *et al.* 2011).

While increasing biomass and lipid productivities the two-phase growth system has the additional benefit of providing a passive contamination control method. Mixotrophic algal cultures in an open system (with an organic carbon source) would be vulnerable to contamination from other heterotrophic microorganism such as bacteria and fungi. In a two-phase growth system microalgae establish in the culture autotrophically. With no organic carbon source the culture is less vulnerable to heterotrophic contamination. Once the culture has reached dominance in the medium, it is more resistance to invasion from heterotrophic contaminants after the addition of an organic carbon source for mixotrophic growth (Das *et al.* 2011).

Although mixotrophy could reduce the cost of algal production, it would require a cheap and sustainable organic carbon source for economic feasibility (Bhatnagar *et al.* 2011). Municipal waste water (after secondary treatment) does not typical contain a high enough organic carbon content to maintain mixotrophy (Perez-Garcia *et al.* 2011). A possible solution is to use digestate solids produced as a waste from the anaerobic digestion (AD) process, a process used extensively in the wastewater treatment industry (Rigby and Smith 2011).

Anaerobic digestion is a process in which anaerobic microorganisms are utilised to decompose biodegradable organic material. The process produces methane biogas, which is typically burnt to generate carbon neutral electricity, and digestate sludge (Rigby and Smith 2011). Although digestate is nutrient rich, it has a high moisture content (>90%) which makes it difficult to store, transport and utilise as a fertilizer. Consequently the digestate is sold (or given away) as a low value fertilizer for agriculture. The UK government is backing a rapid expansion of AD to utilise the huge resource of over 100Mt of biodegradable organic material produced each year in the UK (Rigby and Smith 2011). Further markets are needed to ensure the increasing supply of digestate is utilised optimally (Rigby and Smith 2011).

Digestate has a high content of volatile fatty acids (VFA, see Appendix 1), produced from incomplete digestion. Previous studies have shown that algae are able to assimilate these VFA's in digestate while removing nutrients with efficiencies of up to 100 and 75% for TKN and TP respectively (Wang *et al.* 2010). By processing the digestate through algae, a higher value and more manageable algal fertilizer can be produced while increasing the productivity (through mixotrophic growth) of other high value by products. In addition, it is worthy to note, that the use of algal fertilizers has been found to reduce leaching and gaseous emissions of ammonia, decreasing the energy and environmental impact of agriculture (Mulbry and Wilkie 2001, Wilkie and Mulbry 2002, Mulbry *et al.* 2005).

Sterilisation of digestate, needed to prevent contamination, is problematic; using thermal processes may drive off VFA's, while filter sterilisation may remove undissolved organics. In addition both methods would be impractical energetically on a commercial scale. An important aspect of the present study was to assess the feasibility of growing algae on unsterilised digestate. It was hypothesised that the algae would be unable to grow in unsterilised digestate due to contamination from microorganisms within the digestate.

A secondary objective of this study was to determine the optimum trophic conditions for biomass and lipid accumulation on a selected algal strain. The nutrient and

organic carbon composition of the digestate could not be analysed within the project timeframe, therefore in order to reduce unknown variability a synthetic digestate was used in the second series of experiments.

2. Methodology

2.1 Algae selection

A preliminary study (Appendix 2) assessed the mixotrophic capabilities of two algal strains; *Chlamydomonas reinhardtii* and an unidentified *Chlorella* sp. sourced from a pond in Weston Park, Sheffield. The results (Appendix 2) indicated the *Chlorella* sp. responded significantly to an addition of organic carbon, and therefore was used in further experiments.

2.2 Culture conditions.

2.2.1 Digestate growth

To assess the growth of *Chlorella* sp in digestate, 1ml of unsterilised whole digestate and centrifuged digestate supernatant were added to triplicates of 100ml of distilled water inoculated with 1ml of *Chlorella* sp. culture. The digestate was sourced from the outflow of an anaerobic digester at a local wastewater treatment facility. The cultures were grown in 250ml conical flasks, sealed with sponge bungs. Aseptic conditions were used when transferring medium to minimise contamination. The flasks were then incubated for 14 days under a light intensity of 90±11 µmol photons $m^{-2}s^{-1}$ and continuous illumination in a temperature control growth room (25°C).

2.2.2 Autotrophic, Heterotrophic and Mixotrophic growth.

To assess the effect of different trophic conditions on *Chlorella sp.* growth and lipid production, 1 ml aliquots of algae culture were added to triplicates of 100ml of Bold Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V) medium. The medium contained the following chemicals: $NaNO_3$ (0.75g/l), $CaCl_2.2H_2O$ (25mg/l), $MgSO_4.7H_2O$ (75mg/l), $K_2HPO_4.\ 3H_2O$ (75mg/l), KH_2PO_4 (0.175g/l), NaCl (25mg/l), Vitamin B_1 (1.2mg/l), Vitamin B_2 (1µg/l), and a trace element solution (6ml/l), consisting of Na_2EDTA (0.75g/l), $FeCl_3.6H_2O$ (97mg/l), $MnCl_2.4H_2O$ (41mg/l), $ZnCl_2$ (5mg/l), $CoCl_2.6H_2O$ (2mg/l), $NaMoO_4.2H_2O$ (4mg/l). The treatments included autotrophic (no addition), mixotrophic (1.2g/l of sodium acetate, NaOAc, added), heterotrophic (1.2g/l of NaOAc added, wrapped in foil), and a control (no addition, wrapped in foil). The cultures were grown in 250ml conical flasks, sealed with sponge bungs. Aseptic conditions were used when transferring medium to minimise contamination. The flasks were then incubated under standard conditions as described in Section 2.2.1. Additional biomass was needed to analyse the biomass and lipid productivity of the mixotrophic *Chlorella sp.* Six repetitions of mixotrophic culture (see 2.2.2), where grown for 5 days before harvesting under the same conditions as above.

2.2.3 Two-phase growth

To test the effect of two-phase growth, as described in Das *et al.* (2011), on the *Chlorella* sp., triplicates of autotrophic cultures were made using the method described in Section 2.2.2. After 7 days NaOAc (1.2g/l) was added to three cultures, leaving a triplicate of autotrophic control cultures.

2.2.4 Crash control

A pattern of cell density decline (population crash) at the midpoint (7-9 days) of the growth period in mixotrophic cultures was observed. It was speculated that the crash was caused by the depletion of organic sources and the transition to autotrophic growth. To test this hypothesis an experiment was carried out using the same method as described in Section 2.2.2, with two triplicates of mixotrophic cultures. After 7 days a further 1.2g/l of NaOAc was added to one triplicate.

2.2.4 Photoperiod assessment

After noticing a sudden increase in growth rate of a culture left under natural light cycle conditions (Appendix 3), an experiment to assess any growth limitation of the 24 hour light cycle used in previous cycles was carried out. Twelve mixotrophic cultures were prepared as described in described in Section 2.2.2. Six cultures were incubated in the growth conditions as described in Section 2.2.1 (24 hour light cycle). Six cultures were incubated over 14 days under a light intensity of 106±11 µmol photons $m^{-2}s^{-1}$, with a Light:Dark (L/D) cycle of 16:8 hours in a temperature control growth cabinet (25°C).

2.3 Growth rate

The growth was estimated using a using a UV-Vis Unicam Helios α spectrophotometer to quantify the optical density of the culture 600nm (OD_{600nm}). Each cuvette was agitated before a reading was taken to ensure homogeneous cell dispersal. Readings were taken after 5, 7, 9, 12 and 14 days, except on occasions when access was limited.

2.4 Biomass and Lipid productivity.

Biomass and lipid mass per ml of culture were determined using the gravimetric measurement of lipid content described in Chiu et al. (2009).

3. Results

3.1 Digestate growth.

Chlorella sp. was able to survive and grow successfully in unsterilised digestate. Algal growth as indicated by OD_{600nm} (Figure 1), suggests that *Chlorella* sp. had stronger growth in whole digestate rather than the lower turbidity centrifuged digestate, although only significantly on the 7^{th} and 14^{th} day ($t=6.27$, $d.f.=2$, $p=0.018$). The highest OD_{600nm} (0.731) came from a whole digestate culture, which experienced a rapid population crash and recovery on the 12^{th} and 14^{th} day.

The averaged growth curve gives a false indication that the whole digestate treatment reaches its stationary phase after 5 days. However, as the large standard deviation suggests, there was a large inter-treatment variability. In contrast, the growth curve more accurately indicates that the centrifuged digestate (with low standard deviation) reaches its stationary phase of growth after around 5 days of growth.

3.2 Autotrophic, Heterotrophic and Mixotrophic effect

The cultures grown in mixotrophic conditions, illustrated in Figure 2, had significantly higher OD_{600nm} throughout the growing period than other trophic conditions ($P<0.01$), the only exception being on the 7^{th} day between heterotrophic cultures ($P=0.09$, Tukey analyses). The average OD_{600nm} declined for the mixotrophic cultures on the 7^{th} day, before continuing growth.

Heterotrophic cultures (Dark – NaOAc) had a higher average OD_{600nm} than autotrophic (Light) cultures, although there was no significant different ($P>0.05$, Tukey analyses). Autotrophic growth had the lowest optical density (other than the control) peaking at an OD_{600nm} of 0.328 in one culture after 14 days growth.

The growth curve indicates that the stationary phase of growth was reached by the middle and end of the growth period for heterotrophic and mixotrophic treatments respectively. The growth rate of the autotrophic treatment did not decline at any point during the growth period, which suggests it did not reach its stationary phase within 14 days.

3.3 Two-phase treatment effect

Shown in Figure 3 is the effect of two-phase trophic conditions (compared to autotrophic) on growth (indicated by OD_{600nm}). The OD_{600nm} more than doubled in the two-phase treatment 2 days after the addition of NaOAc, while over the same period the autotrophic treatment OD_{600nm} increased by only 36%. This trend continued with a significant difference between the treatments up to the end of the growth period ($t=4.73$, $d.f.=21.4$, $p=<0.001$). The two-phase treatment reached a peak mean OD_{600nm} of 0.942, before declining on the 14^{th} day of the growth period.

3.4 Crash control

The effect of an addition spike of NaOAc at the midpoint of the mixotrophic growth period on growth is shown in Figure 4. There was no significant difference in growth between treatments ($P>0.05$). Both treatments reached similar optical densities to mixotrophic cultures in previous experiments, with the final mean OD_{600nm} measured at 0.98 and 1.03 for the control and treatment with additional NaOAc respectively.

3.6 Photoperiod

The optical density measurements indicate that cultures grown under 16 hours illumination grew at a significantly faster rate than under continuous illumination ($t=9.02$, $d.f.=46$, $p>0.001$, Figure 5). Both treatments showed continued growth over the growth period reaching a mean peak OD_{600nm} of 0.873 and 1.399 for 24 hour and 16 hours illumination respectively.

3.6 Biomass and Lipid productivities

Due to significant biomass loss during the freeze-drying stage of the gravimetric method, the measurements presented in Table 1 are minimum values. The biomass loss is likely to have also affected the lipid content measurements. Due to the inaccuracies added to an already crude analysis results should be analysed as relative values rather than absolute. Lipid contents ranched from 11.59 to 12.70 with no significant differences between treatments ($P>0.05$ by one-way ANOVA and Tukey analyses). In contrast due to significant differences between autotrophic and mixotrophic treatments in biomass productivity, lipid productivities of mixotrophic treatments were increased by a factor 3.45 to 5.40 compared to autotrophic growth. The highest lipid productivity (1.78 m gl^{-1} day^{-1}) was found in mixotrophic cultures grown for 14 days under a 16:8 L/D cycle.

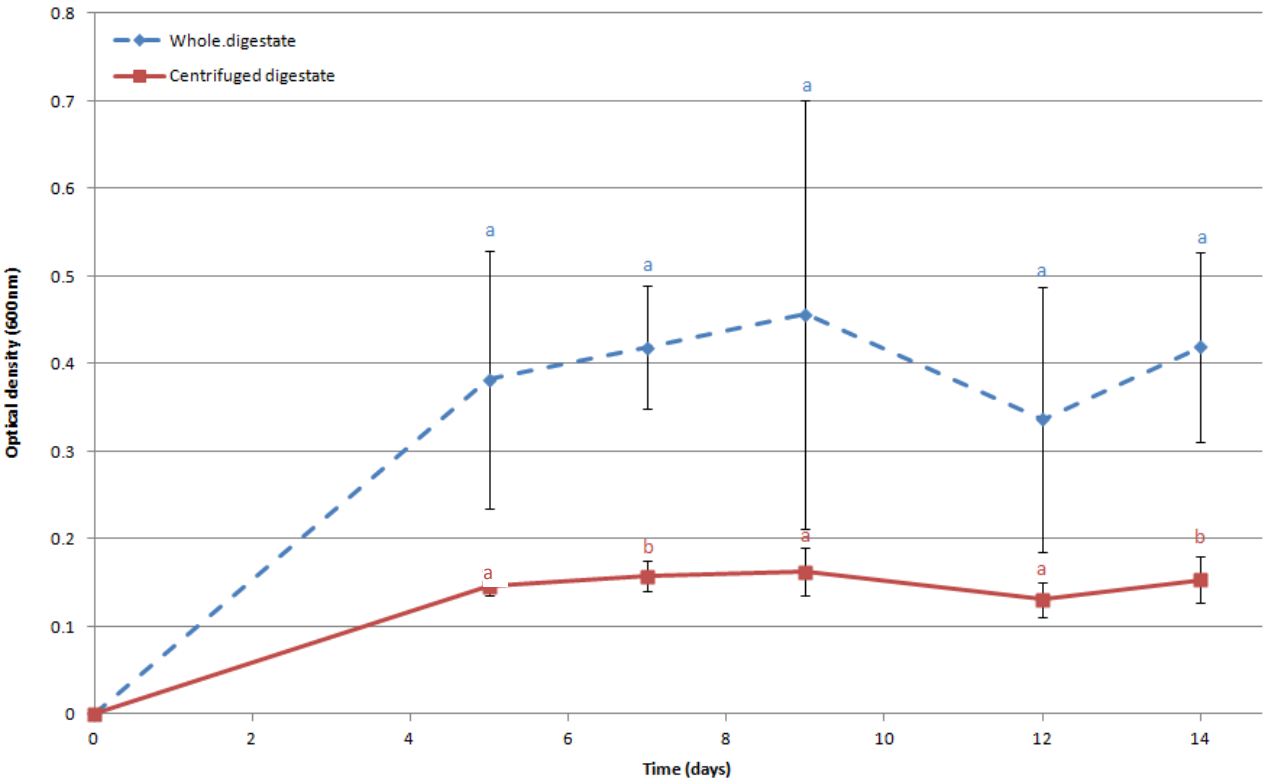

Figure 1. Growth curves (OD_{600nm}) of *Chlorella* sp. growing in a whole and centrifuged digestate supplemented medium. Values denoted by a different letter at each data point differ significantly (P <0.05 by t-test). Bars represent standard deviation.

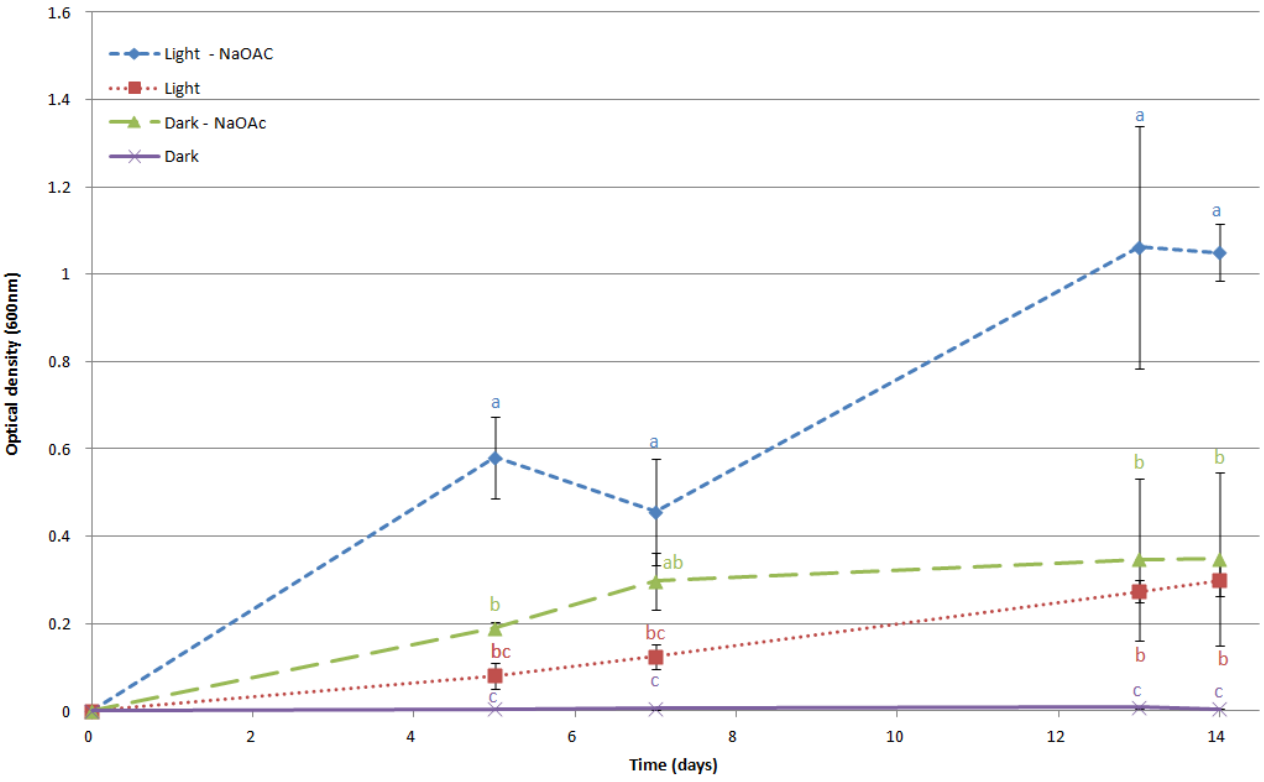

Figure 2. Growth curves (OD_{600nm}) of *Chlorella* sp. growing under mixotrophic (Light – NaOAc), autotrophic (Light), heterotrophic (Dark – NaOAc) and control conditions. Values denoted by a different letter at each data point differ significantly (P <0.05 by t-test). Bars represent standard deviation.

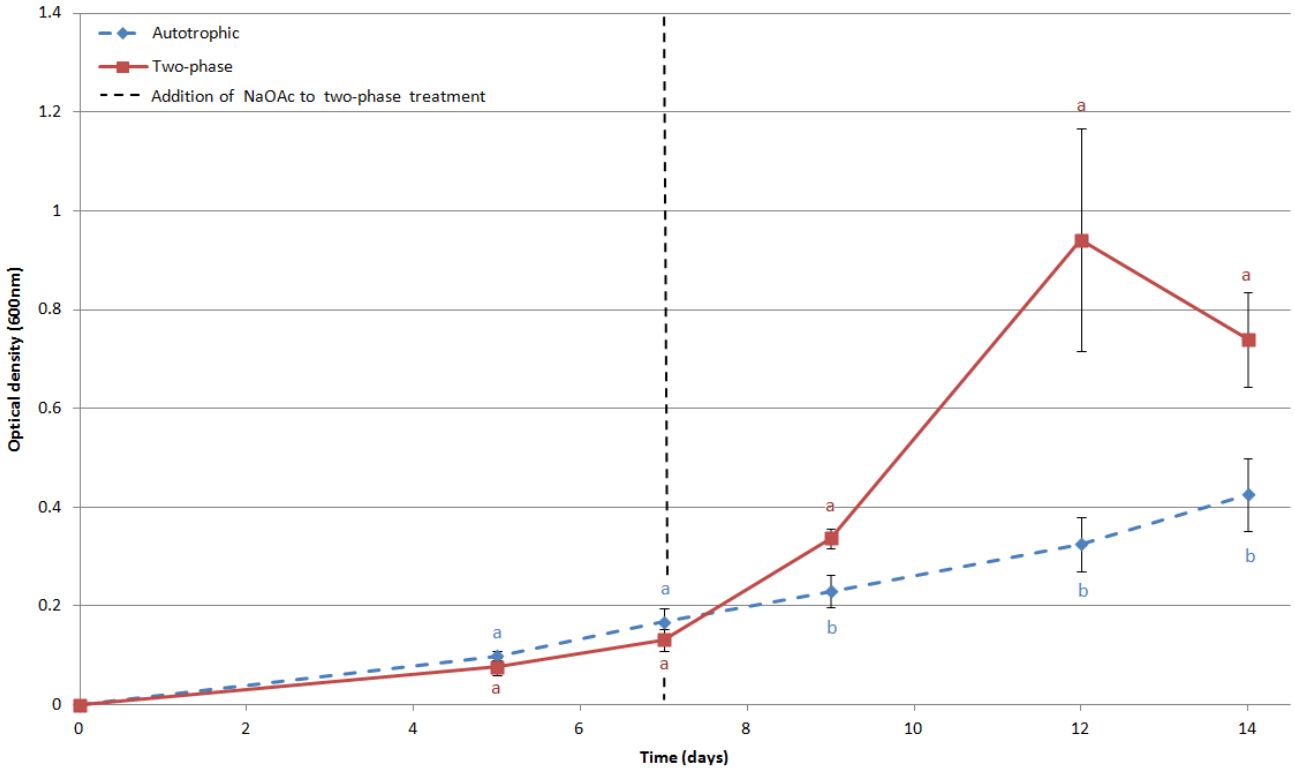

Figure 3. Growth curves (OD_{600nm}) of *Chlorella* sp. growing under autotrophic and two-phase (7 days autotrophic, 7 days mixotrophic) trophic conditions. Values denoted by a different letter at each data point differ significantly (P <0.01 by t-test). Bars represent standard deviation.

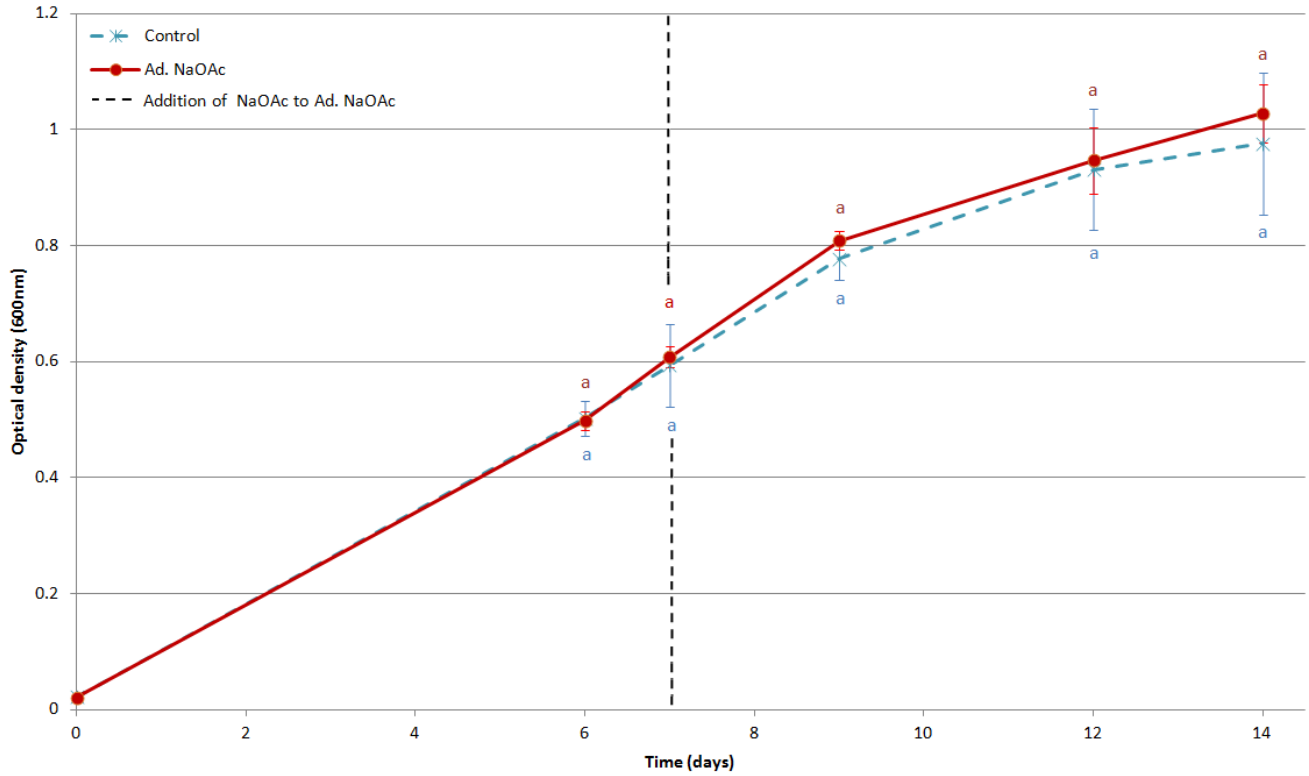

Figure 4. Growth curves (OD_{600nm}) of *Chlorella* sp. growing in mixotrophic conditions (Control) and in mixotrophy conditions with an additional 1.2g/l after 7 days growth (Ad. NaOAc). Values denoted by a different letter at each data point differ significantly (P <0.05 by t-test). Bars represent standard deviation.

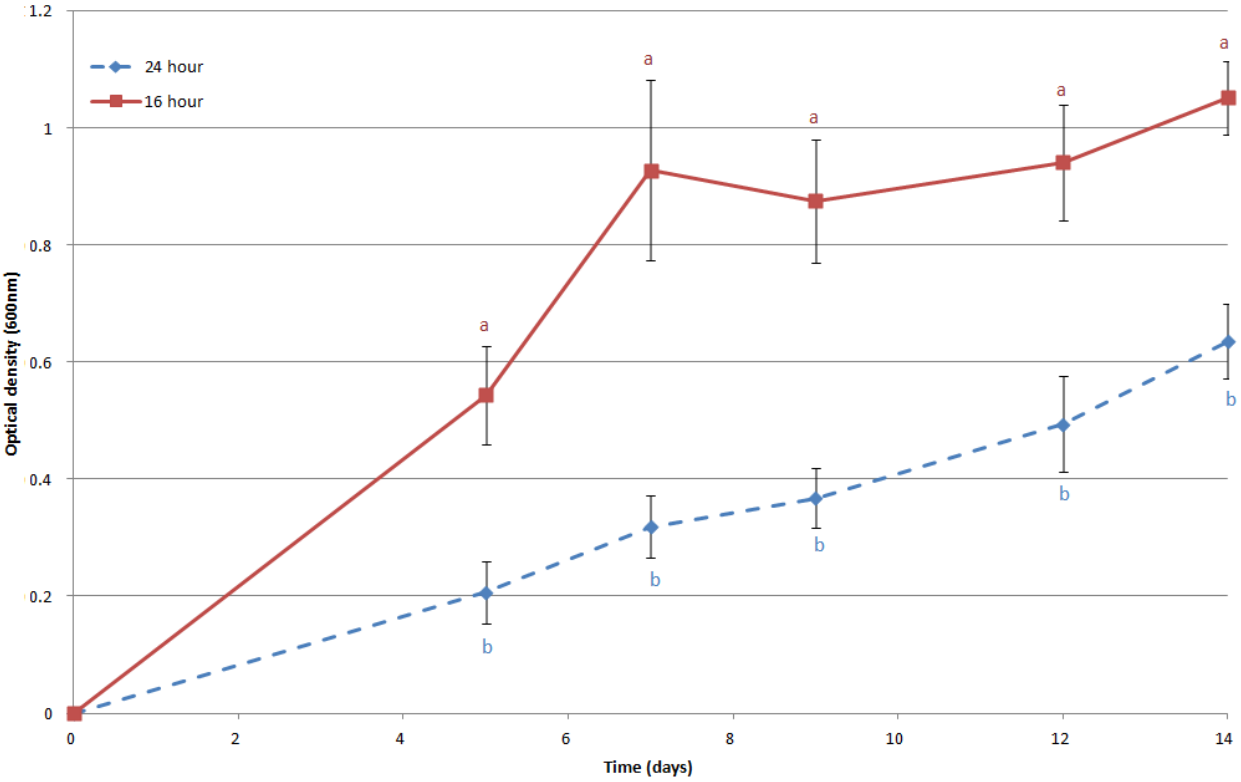

Figure 5. Growth curves (OD_{600nm}) of *Chlorella* sp. growing in mixotrophic conditions under continuous illumination (24 hour) and a 16:8 hour L/D cycle (16 hour). Values denoted by a different letter at each data point differ significantly (P <0.01 by t-test). Bars represent standard deviation.

Table 1. Biomass and lipid productivities of *Chlorella* sp. grown under different conditions

Culture Conditions	Lipid content (%)	Min biomass yield (mg l^{-1})	Min. biomass productivity (mg l^{-1} day^{-1})	Min. lipid productivity (mg l^{-1} day^{-1})
Autotrophic 14 days growth	11.72 ± 0	39.07 ± 0	2.79 ± 0	0.33 ± 0
Mixotrophic 5 days growth	12.25 ± 0.35	46.54 ± 3.54	9.31 ± 0.71	1.14 ± 0.19
Mixotrophic 14 days growth	12.70 ± 3.15	181.63 ± 11.28	12.97 ± 0.81	1.65 ± 0.97
Mixotrophic 16:8 L/D cycle 14 days growth	11.63 ± 4.19	213.62 ± 3.71	15.25 ± 0.26	1.78 ± 0.95
Two-phase growth 14 days growth	11.59 ± 0.9	170.04 ± 5.51	12.14 ± 0.39	1.41 ± 0.31

4. Discussion

Digestate could potentially be used as a cheap source of organic carbon to boost biofuel co-production productivity via mixotrophic growth. In this study, a wild-isolated *Chlorella* sp. was able to both survive and grow in unsterilised whole digestate and centrifuged digestate supernatant (Figure 1). If sterilisation of digestate does not impact growth rates, a significant energy and capital cost could be removed from the commercial scale up of a digestate integrated biofuels co-production system.

Wang *et al.* (2010) studied the effect of different dilutions of filter-sterilised dairy manure digestate on the growth rate and nutrient removal efficiency of algae from the *Chlorella* genus. The study found an inverse linear relationship (R= 0.982) between turbidity and specific growth rate (Wang *et al.* 2010). In contrast, in the present study the higher turbidity whole digestate supported a higher growth rate. It is possible that the particulates removed in the centrifuged digestate contained organic carbon which was able to support mixotrophic growth in the whole digestate, overcoming any light limitation caused by a higher turbidity. However Wang *et al.* (2010) noted that there was no significant difference between the most diluted (20x and 25x) treatment. As the present study used a dilution of 100x, turbidity and the associated light limitation probably had a limited affect on the cultures. It is more likely that the higher growth rate in the whole digestate was supported by a higher concentration of nutrients associated with the particulates.

Wang *et al.* (2010) measured COD removal rates of between 27.9 and 38.4%, with higher removal rates within less diluted (and higher turbidity) digestate medium. This suggests that some algae in the *Chlorella* genus have the ability to growth mixotrophically due to either higher quantities of available organic carbon or due to light limitation. An in-depth gas chromotographical analysis of the digestate before and after algal growth is needed to provide further insight into the potential of digestate as an organic carbon source.

While the effectiveness of digestate as an organic source for mixotrophic growth remains unanswered, this experiment strongly indicates that mixotrophic growth dramatically increases both growth rate (thereby biomass productivity) and lipid productivity for the selected strain of *Chlorella* sp. (Figure 2, Table 1). This study found that under mixotrophic conditions with 0.12% w/v NaOAc (continuous illumination, 14 day growth) a 5 fold increase in lipid productivity can be achieved compared to autotrophic conditions. In a similar study, Liang *et al*. (2009) found *Chlorella vulgaris* lipid productivity to increase by 6.75 fold under mixotrophic conditions with 1% w/v acetate, over a 12 day growth period and under continuous illumination. Higher lipid productivities (and biomass productivities) were achieved when using glucose (1%, 13.5 fold increase) and glycogen (2%, 7.75 fold increase) over a 6 day growth period in continuous illumination (Liang *et al.* 2009). Conversely Perez-Garcia *et al.* 2011a,b found that organic carbon boost (heterotrophic) growth rate in declining order of: Na-acetate (NaOAc), (D-)glucose, (D-)fructose = fulvic acid, Na-citrate = lactic acidic = acidic acid, malic acid and L-arabinose. However, it is possible that this hierarchy may not be maintained when concerning lipid contents (and therefore productivities) or under mixotrophic growth. The difference in growth rates of algae grown in NaOAc and acetic acid (VFA in digestate) supplemented medium found in Perez *et al*'s (2011a) study may indicate that the synthetic digestate employed in this study may overestimate any growth benefit from VFA's in digestate.

It has been suggested that the mixotrophic growth rate (in light with acetate) is approximately the sum of growth rates of in autotrophic and heterotrophic cultures, with each growth process active noncompetitively (Endo *et al.* 1977). In this study the mixotrophic growth rate (estimated from OD_{600nm}) was around 60% higher than the sum of heterotrophic and autotrophic growth. Bhatnagar *et al.* (2011) also observed this phenomenon. It is thought that synchronous activity of autotrophy and heterotrophy generates a synergistic boost to growth, with higher local CO_2 concentrations (due to heterotrophic respiration) aiding photosynthetic efficiency by decreasing photorespiration (Bhatnagar *et al.* 2011).

Das *et al.* (2011) found that the lipid content (FAME) of *Nannochloropsis* sp. increased by 30% in the presence of glycerol compared to autotrophic growth in a two-phase growth experiment. Das *et al.* (2011) found no significant increase with the addition of sucrose or glucose. In this study the addition of acetate to an autotrophic culture reduced the lipid content although by an insignificant amount (Table 1). However, as in Das *et al*'s (2011) study, due to higher biomass productivity, the addition of acetate increased the lipid productivity by a factor of 4.3 (Figure 3, Table 1). While there was a drop in lipid productivities with the two-phase growth compared to 14 day mixotrophic growth, this small drop could be acceptable if a two-phase system reduces the risk of contamination in open systems. Further study is needed to assess the effectiveness of two-phase growth as a contamination control in freshwater mixotrophic cultures.

The noticeable dip in optical density 7 days after the addition of acetate in the mixotrophic culture (Figure 2) and two-phase culture (Figure 3), and an early transition into a stationary phase in the heterotrophic culture (Figure 2), led to the speculation that depletion of acetate after around 7 days of growth caused decline in growth rate. Additional acetate was added after the 7th day to test this hypothesis (Figure 4). There was no significant effect of this acetate addition to growth rate, which indicates that organic carbon depletion was not the cause for the decline in growth rates. It is possible that other causes such as contamination or inconsistencies in the method of OD measurements lead to the drop in optical density.

Due to the similarity in growth rates between the two different NaOAc concentrations it is not possible to determine the optimal concentration for growth. Previous studies have found that the optimal concentration of organic carbon varies considerable with type of organic carbon source and species (Liang *et al.* 2009, Wan *et al.* 2011).

The highest biomass productivity and lipid productivity was achieved in mixotrophic conditions at a 16:8 hour L/D cycle. Although continuous illumination has higher potential biomass productivity through continued autotrophic growth, a period of dark respiration may allow repair and maintenance of photosynthetic apparatus damaged by photoinhibition (Shyam *et al. 1993,* Singh *et al.* 1996). Photoinhibition (or photo-oxidation) is the phenomenon of a decrease in photosynthetic capacity caused by damage to the electron transfer chain in photosystem II when exposed to high irradiance (Han *et al.* 2000). The dark period of maintenance may be more important in open conditions where natural peak light levels exceed 2000 µmol photons $m^{-2}s^{-1}$, 10 orders of magnitude higher than the saturation irradiance of most algae, thereby causing more photolimitation damage (Molina Grima *et al.* 1999, Park *et al.* 2011).

Further investigation needs to be carried out to assess the effect of a dark period on autotrophic growth. It is possible that the biomass gains from the L/D cycle come from heterotrophic growth during the dark period. Continuous illumination is used as a standard in experimentation of unicellular green algae in order to avoid experiment error caused by synchronisation of cell divisions when cells acclimatise to a regular L/D cycle (Krupinska and Humbeck 1994). In the present study measurements were not taken at the same time of day, therefore cells where likely to be at different physiological states which may have affected measurements. It is unlikely that the experimental error associated with the synchronoisation of cell divisions in the L/D treatment could fully explain the increase growth rate, but to avoid error in further investigations measurements should be taken at the same time of day.

5. Conclusion and future work

This study shows that *Chlorella* sp. isolated from a local pond was able to grow in unsterilised digestate. The species grew at higher rate in higher turbidity whole digestate than centrifuged digestate supernatant, which may have been due to higher nutrient levels or mixotrophic growth. Further investigation into the composition of digestate is needed to assess its potential as an organic source for mixotrophic growth. *Chlorella* sp. grew at a faster rate in mixotrophic conditions than heterotrophic and autotrophic growth combined, indicating a synergistic boost due to an increase in local CO_2 concentration. The significantly higher lipid productivities achieved in mixotrophic growth suggests that *Chlorella* sp. has significant potential for biofuels-coproduction. Further lab-scale and field trials experiments should be carried out to assess the species potential for phycoremediation in open mixotrophic conditions. This study showed that mixotrophic *Chlorella* sp. benefited from a dark period, which ideal for commercialisation in natural photoperiods. Further investigation is needed to understand the mechanisms causing the boost in growth in addition to assessing the optimal L/D cycle for maximum growth.

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Appendix

Appendix 1. Mean digestate chemical and physical properties analyses: standard errors in brackets Taylor *et al.* (2011b)

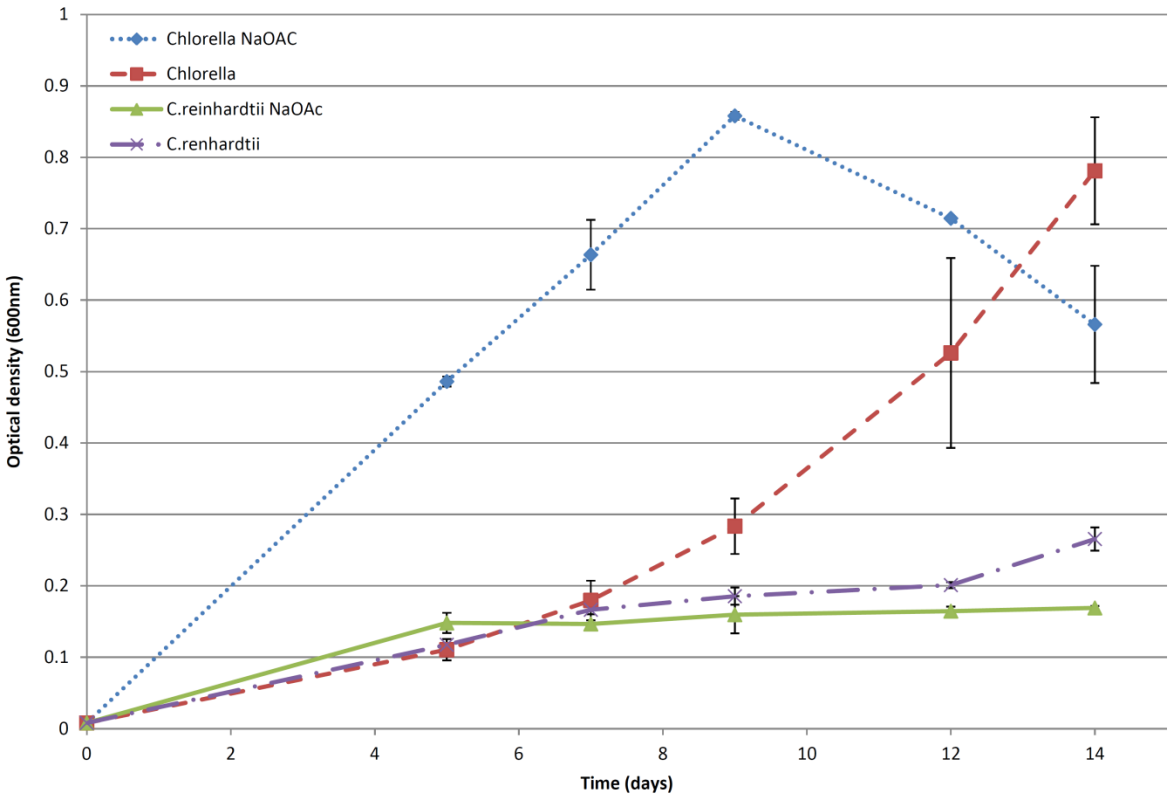
Determinand	Units*/**	Food-based digestate 1 (n=6)	Food-based digestate 2 (n=6)	Manure-based digestate 1 (n=6)
pH		8.51 (0.07)	8.31 (0.02)	8.22 (0.04)
Volatile Fatty Acids:				
acetic acid	mg/kg dm	555 (149)	11027 (2616)	855[3] (84.6)
heptanoic acid	mg/kg dm	1675 (99.5)	1387[5] (312)	302[1]
propionic acid	mg/kg dm	396 (37.8)	1937 (425)	218[3] (18.7)
isobutyric acid	mg/kg dm	1638 (563)	3282 (1113)	<1000
isocaproic acid	mg/kg dm	2057 (425)	1395 (272)	960[2] (60.0)
isovaleric acid	mg/kg dm	651 (112)	2331 (630)	205[2] (39.0)
n-butyric acid	mg/kg dm	516 (40.6)	945 (128)	154[2] (14.5)
n-caproic acid	mg/kg dm	1390 (236)	903[4] (208)	659[1]
n-valeric acid	mg/kg dm	914 (215)	622[5] (102)	334[2] (69.0
Volatile Fatty Acids	g COD/g VS	0.03 (0.01)	0.05 (0.01)	0.005 (0.002)
Total Neutralising Value	% dm (as CaO)	54.5 (3.27)	38.5 (1.87)	24.5 (1.13)
Conductivity	µS/cm 20°C	7100 (420)	6350 (280)	4360 (1023)
Biochemical Oxygen Demand	mg/l	5550 (437)	13010 (889)	5430 (642)
Chemical Oxygen Demand	mg/l	29300 (2164)	49180 (2077)	51840 (3952)
Stability (Residual Biogas Potential)	l/g VS	0.114 (0.02)	0.327 (0.05)	0.108 (0.01)

*mg/kg dm = milligrams/kilogram dry matter, fw = fresh weight; VS = volatile solids; l/g VS = litres/gram of volatile solids

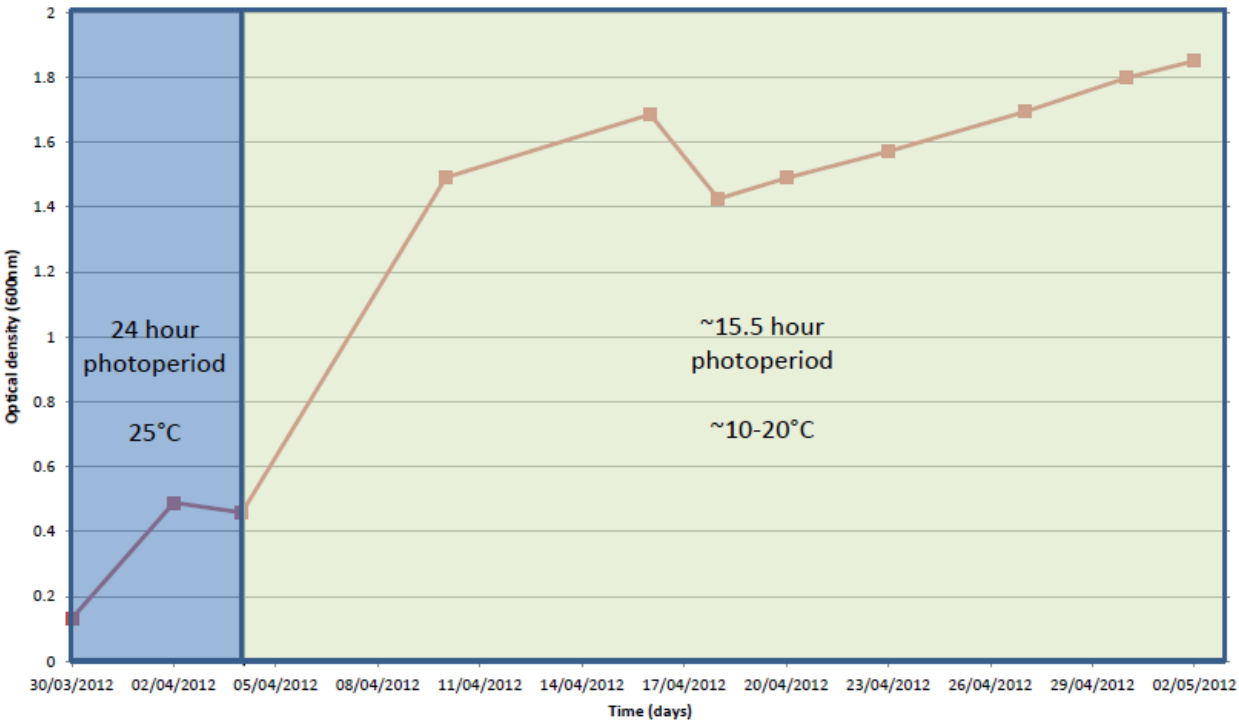
**mean is from one to five replicates as indicated by the superscript number; other replicates below limits of analytical detection

< indicates the reported value is less than the limit of analytical detection-LOD (the minimum amount of a determinand that can be measured); the actual value will be somewhere between zero and the LOD.

NB: standard errors were not calculated where the concentration was from one replicate or where values were all below the limits of analytical detection.



Appendix 2. Growth curves (OD_{600nm}) of *Chlorella* sp. and *Chlamydomonas reinhardtii* growing under mixotrophic (Chlorella NaOAc, C.reinhardtii NaOAc) and autotrophic (Chlorella, C.reinhardtii) conditions. Bars represent standard deviation. Method described in Section 2.2.2, a HS medium (Sueoka 1960) was used for *C.reinhardtii*



Appendix 3. Growth curves (OD_{600nm}) of a single *Chlorella* sp. culture grow under experiment conditions described in Section 2.2.2 (blue section) and then natural light conditions on a laboratory desk (green section).