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Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process

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ABSTRACT

Two major considerations of the emerging algae biofuel industry are the energy intensive dewatering of the algae slurry and nutrient management. The proposed closed loop process which involves nutrient recycling of the aqueous phase from the hydrothermal liquefaction of microalgae offers a solution to both aspects. Hydrothermal liquefaction has been shown to be a low energy process for bio-crude production from microalgae. For the purpose of this research, microalgae strains of *Chlorella vulgaris*, *Scenedesmus dimorphus* and the cyanobacteria *Spirulina platensis* and *Chlorogloeopsis fritschii* were processed in batch reactors at 300 °C and 350 °C. Following liquefaction the product phases were separated and the water phase recovered. The bio-crude yields ranged from 27 to 47 wt.%. The bio-crudes were of low O and N content and high heating value making them suitable for further processing. The water phase was analysed for all major nutrients, TOC and TN to determine the suitability of the recycled aqueous phase for algae cultivation. Growth trials were performed for each algae strain in a standard growth medium and compared to the growth rates in a series of dilutions of the recycled process water phase. Growth was determined by cell count and *chlorophyll a* absorbance. Growth occurred in heavy dilutions where the amount of growth inhibitors was not too high. The results show that the closed loop system using the recovered aqueous phase offers a promising route for sustainable oil production and nutrient management for microalgae.

1. Introduction

Hydrothermal liquefaction (HTL) is a process in which biomass is converted in hot compressed water to a liquid bio-crude. Processing temperatures range from 200 to 350 °C with pressures of around 15–20 MPa; depending on the temperature, as the water has to remain in the sub-critical region to avoid the latent heat of vaporisation. At these conditions complex molecules are broken down and re-polymerise to oily compounds [1]. HTL is an ideal route for the conversion of high moisture content biomass, such as microalgae, as a drying step of the feedstock is not necessary. Numerous studies have shown that a bio-crude with a high heating value can be produced from the HTL of microalgae [2–12], although the oxygen content and nitrogen content are typically still higher than crude oil [5,6,8]. More recently researchers have shown that the amount of nitrogen and oxygen in the bio-crude can be reduced with the use of heterogeneous catalysts [7,13]. An additional benefit of the hydrothermal processing routes is the potential to recycle process water which is rich in nutrients such as nitrogen and phosphorous and elements such as Fe, Ca, Mg, K, as well as other mineral matter and polar organics [6]. One of the challenges previously identified concerning HTL of microalgae is the large amount of organic carbon in the process water [8]. This represents a loss of carbon efficiency and reduces the bio-crude yields. Therefore researchers have suggested the catalytic supercritical gasification of microalgae which produces a syngas and results in considerably lower TOC levels in the process water [14]. An extensive review of the different hydrothermal routes is described by Peterson et al. [1]. It is expected that the organic carbon in the process water of HTL can act as a substrate for mixotrophic growth of microalgae. This can lead to increased biomass yields and higher carbon efficiency. Bhatnagar et al. showed that strains of *Chlamydomonas*, *Chlorella* and *Scenedesmus* were able to grow mixotrophically in various sources of process water high in TOC, which led to a higher biomass yields [15]. The potential for nutrient recycling is thought to be essential for the economic development of large scale microalgae cultivation. Nutrient recycling potential has largely been focussed on conversion by anaerobic digestion [16] and only limited studies have evaluated hydrothermal processing routes. Tsukahara et al. demonstrated the potential for nutrient recycling on *C. vulgaris* by low temperature gasification of microalgae [17] and more recently Jena et al. [18] have shown that it is possible to cultivate microalgae in the process water following HTL of the freshwater microalgae *C. minutissima* [18] although they used a different strain for cultivation than for the HTL experiments. Each of these options, whether anaerobic digestion or hydrothermal processing have their associated problems, but it is recognised that integration of microalgae cultivation with some sort of nutrient recycle is essential. This investigation reports

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the potential of recycling the process water from HTL of four different algae strains and two different HTL processing temperatures.

It is envisioned that a final industrial process could be integrated into a closed loop concept with integrated nutrient recycling. Microalgae would be grown, harvested and processed to a slurry of around 10 wt.% with minimum energy requirements. The solids concentration can be altered to the specific requirements and capabilities of the processing facility. When the solids concentration is higher, the amount of water per mass of algae to be heated is less; reducing the energy required for heating but at the same time more energy is required to achieve the high solids concentration. Anastasakis and Ross, and Jena et al. both showed that HTL is possible at a range of solids loading (5–20% and 10–50% solids concentration respectively) [10,19]. Subsequently the slurry is processed in hot compressed water to produce the bio-crude. The product phases are separated producing a gas, process water, solid residue and bio-crude. The gas consisting mainly of CO_2 can be fed to the algae cultivation as the algae require CO_2 for photosynthesis and the growth rates can be increased with additional CO_2. The bio-crude is ready for further refining after separation. The solid residue which is high in nitrogen and minerals but low in carbon can be used as a fertilizer or bio-char. The process water containing high amounts of nitrogen and carbon are fed back to the cultivation system to provide nutrients for microalgae growth.

In this laboratory study the cultivation systems were simple, small scale 500 ml conical flasks and the HTL was performed in batch 75 or 660 ml high pressure reactors. The purpose of the current research is to demonstrate the feasibility of using the process water for nutrient recycling. However, in a commercial system a more sophisticated cultivation and harvesting system would be required. A continuous HTL reactor would also be desirable and the use of solvents to separate the phases would then be avoidable due to self separation of the bio-crude.

2. Materials and methods

Chlorella vulgaris, *Scenedesmus dimorphous* and *Spirulina platensis* were grown and harvested in respective standard growth medium in the University of Leeds, UK laboratory. The cyanobacteria *Chlorogloeopsis fritschii* was cultured and harvested by the Plymouth Marine Laboratories, UK. The microalgae ash contents were determined by heating the samples to 550 °C for 5 h using a carbolite muffle furnace (Elite Thermal Systems Ltd, Leicestershire, UK). The moisture content was determined in the same manner but at 105 °C for 2 h. The ash and moisture content are referred to as the proximate analysis and was performed in triplicate. The C, H, N and S contents (ultimate analysis) of the microalgae were analysed in duplicate using a series Flash EA 1112 elemental analyser (CE Instruments, UK). The biochemical composition of the microalgae strains was determined colourimetrically; for the protein analysis the J. Waterborge method was used [20] which involves the use of a Folin reagent, subsequent absorbance measurements at 720 nm and comparison to a bovine standard absorbance at the same wavelength. Carbohydrates were determined by a method developed by Sol M. Gerchakov [21]. Lipid extraction was performed using the Bligh and Dyer method employing a 2:1 methanol/chloroform extraction at room temperature [22]. The lipid analysis was carried out in duplicate while the protein and carbohydrate analysis was only carried out once. For the hydrothermal liquefaction experiments at 350 °C approximately 3 g (*Chlorella* and *Scenedesmus*) of microalgae were added to a 75 ml Parr high pressure reactor with 27 ml of distilled water. The reactor was heated to 350 °C at a heating rate of 10 °C/min using an electrical furnace. The top temperature was held for 1 h, after which compressed air was blown onto the reactor to cool it to room temperature at approximately 20 °C/min. The experiments at 300 °C for *Chlorogloeopsis*, *Spirulina* and *Chlorella* were processed at the same ratios (~24 g biomass/220 ml H_2O) and times but in a 660 ml Parr reactor. Both Parr reactors are constructed of 316 Stainless Steel with an elemental composition of 65% Fe, 12% Ni, 17% Cr, 2.5% Mo, 2.0% Mn and 1% Si. The experiments at 350 °C were carried out in duplicate and average values are reported however due to limited availability of microalgae biomass the experiments at 300 °C in the larger reactors were only carried out once. Following HTL the products were phase separated using dichloromethane in a separation funnel and the solid residue was recovered following filtration. A schematic diagram of the HTL procedure and the growth trials is presented in Fig. 1 and additional details on the separation of the product phases is described elsewhere [6]. The bio-crude yields were calculated on a dry ash free (daf) basis. The bio-crude was analysed in duplicate for CHNS content and the pH of the aqueous phase was measured. Using the elemental composition of the bio-crude and the unprocessed biomass samples the higher heating values were estimated by *DuLong*'s formula [23]. The aqueous phase was diluted to 1 l and filtered through a Type 3 Whatman filter and analysed by ion chromatography (DX-100, Dionex, USA) to identify and quantify the main anions and cations present. Total organic carbon (TOC) in the aqueous phase was determined using a TOC analyser (HACH-IL 550 TOC, Hach-Lange, Germany). Ammonium, Total Nitrogen and Phosphate concentration were determined using HACH-LANGE colorimetry test cuvettes (LCK302, LCK338, LCK350, Hach-Lange, Germany). The trace metal concentration in the aqueous phase was measured using an Optima 5300 DV inductively coupled plasma spectrometer (ICP) with optical emission spectrometry (Perkin Elmer, Cambridge, UK). Analyses involving the water phase were only carried out once.

Bulk samples of *C. vulgaris*, *Spirulina* and *Scenedesmus* were cultivated in 10 litre bioreactors using axenic strains obtained from the *Culture Collection of Algae and Protozoa* (SAMS Research Services Ltd, Scottish Marine Institute, Oban, Scotland). *Scenedemus* and *Chlorella* were grown in 3N-BBM + V and *C. fritschii* in BG 11 standard media, *Spirulina* was grown in a media with the following nutrient concentrations: Values in g/l: NaCl 1; $MgSO_47H_2O$ 0.2; $CaCl_2$ 0.04; $FeSO_47H_2O$ 0.01; EDTA 0.08; K_2HPO_4 0.5; $NaNO_3$ 2.5; K_2SO_4 1; $NaHCO_3$ 15. Growth trials of the 300 °C HTL process waters were carried out at the Plymouth Marine Laboratories and the trials of the 350 °C process waters at the University of Leeds. In Leeds, 10 ml of approximately 150 mg/l concentrated media culture was used to inoculate the cultivation trials in 500 ml conical flasks in a media consisting of diluted process water or fresh standard cultivation media. The trials in Plymouth were inoculated at higher concentrations of around 85 mg biomass dry matter in 250 ml conical growth flasks. The process water was diluted (50×, 100×, 200×, 400× and 600×) and the growth rate compared to standard media measured over a 11–12 day period. Each growth trial was carried out in duplicate and the standard deviation is plotted using error bars for each respective growth data point. Ambient air was supplied to the reactors to provide agitation and CO_2 at an approximate concentration of 390 ppm; the reactors were continuously illuminated. The cell count was estimated using a haemocytometer each day and the final biomass produced was separated and dried to obtain a final yield. Biomass accumulation was determined by chlorophyll a absorbance at 660 nm; for each sample, 1.5 ml culture was recovered from each growth flask, the cells were pelleted with a micro centrifuge and the supernatant decanted into a separate tube. 1.5 ml of acetone was added to the pelleted biomass, mixed and incubated overnight in a fridge. Subsequently the biomass was centrifuged again and the supernatant scanned in a UV/Vis spectrophotometer to provide a relative biomass concentration. Relative biomass concentration was estimated by a spectrophotometer scan at 660 nm. The growth trials were carried out in duplicate and average measurements of growth are reported. The remaining spent media was analysed by ion exchange chromatography and photometry to assess the uptake of different nutrients.

3. Results and discussion

3.1. Analysis of feedstock

The ultimate and proximate analysis of the two cyanobacteria and the two microalgae investigated are presented in Table 1. The

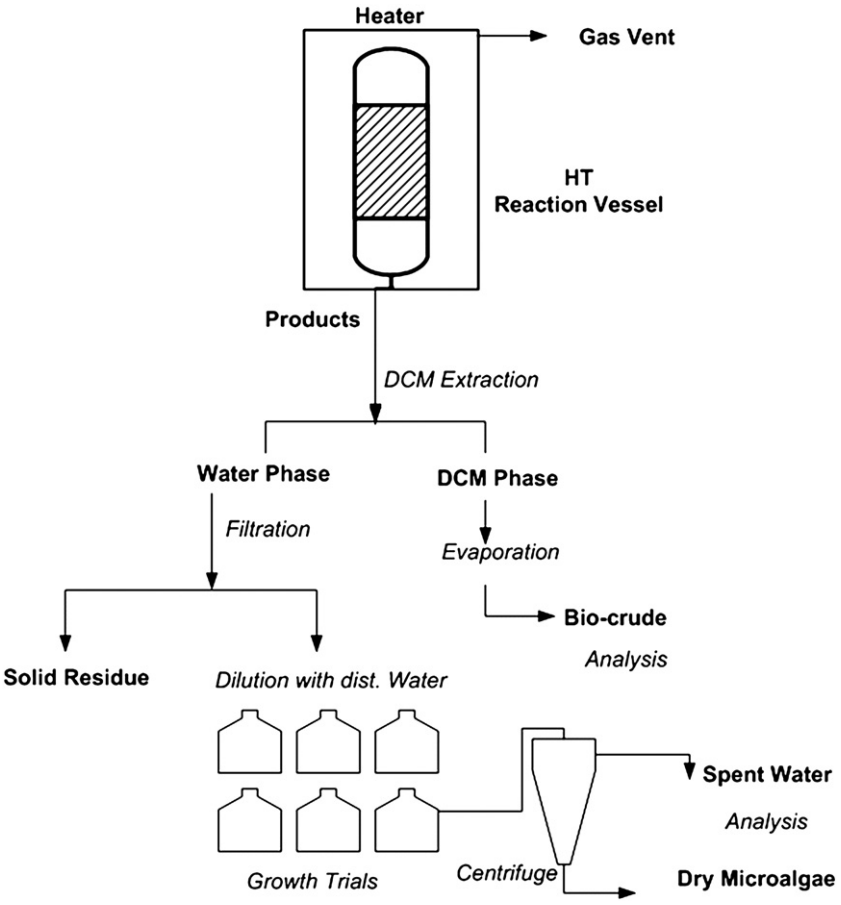

Fig. 1. Schematic layout of the HTL reactions, sample workup and cultivation trials. DCM = *dichloromethane*, *HT = hydrothermal.*

proximate analysis is designed to determine the ash and moisture content of the feedstock so that the organic faction can be calculated while the ultimate analysis identifies the elemental composition of the organic fraction based on C, H, N and S with O calculated by difference. The microalgae were chosen to provide a range of different biochemical contents to study the change in aqueous phase composition and subsequent nutrient recycling. It is known that the biochemical composition affects the HTL behaviour but the nutrient recycling potential has not been investigated. The two cyanobacteria *Chlorogloeopsis* and *Spirulina* have very low lipid content, 10–20% lower than the microalgae strains. *Spirulina* has the highest protein content which corresponds to its highest nitrogen content. There is also a range of carbohydrates present which is especially high for *Chlorogloeopsis* (44%). The oxygen content of all strains was typically around 30%. The oxygen and the nitrogen components are the two elements primarily removed during HTL.

3.2. Hydrothermal liquefaction (HTL) results

Following HTL the bio-crude was weighed and analysed. The elemental composition and the calculated Higher Heating Value (HHV) of the bio-crudes are shown in Table 2. The reactions performed at 300 °C were processed in a 660 ml Parr reactor while the reactions at 350 °C were processed in a smaller 75 ml reactor. The bio-crude yields range from 27% for *Scenedesmus* at 350 °C to 47% for *Chlorella* at 300 °C. The high bio-crude yields of *Chlorella* are due to its higher lipid content. At higher temperatures, more decomposition to polar organics is observed which result in a higher TOC content in the water phase and a reduction in polar organics in the bio-crude. At higher temperatures, the oxygen content of the bio-crude is also lower. The oxygen content varies significantly for the bio-crude derived from the different algae strains, being highest for *Chlorogloeopsis* which corresponds to the lowest HHV. The nitrogen content of the two cyanobacteria *Chlorogloeopsis* and *Spirulina* are highest as a large fraction of the bio-crude originates from the protein fraction. The nitrogen content of the *Chlorella* bio-crude is higher at 350 °C as more protein is broken down than at the lower temperatures.

The product distribution following HTL is shown in Fig. 2. The gas yields are relatively low and consist mainly of CO_2. The data is presented on an as received basis which explains the lower bio-crude yields than the dry ash free yields presented in Table 2. The product

Table 1
Proximate, ultimate and biochemical analysis of the biomass investigated.

	Proximate (%)			Ultimate (% daf)				Biochemical (% daf)		
Strain	Ash	H_2O	C	H	N	S	O*	Protein	Carbohydrate	Lipid
C. fritschii	7.6 ± 0.09	6.8 ± 0.0	54.4 ± 2.1	6.9 ± 0.5	7.3 ± 0.3	ND	31.4	50	44	7 ± 0.3
S. platensis	7.6 ± 0.03	7.8 ± 0.1	55.7 ± 0.4	6.8 ± 0.1	11.2 ± 0.1	0.8 ± 0.1	26.4	65	20	5 ± 0.1
C. vulgaris	7.0 ± 0.06	5.9 ± 0.1	52.6 ± 0.8	7.1 ± 0.1	8.2 ± 0.2	0.5 ± 0.0	32.2	55	9	25 ± 2.2
S. dimorphous	11.8 ± 0.1	1.6 ± 0.1	53.4 ± 0.6	7.8 ± 0.2	7.9 ± 0.1	ND	31.0	43	16	18 ± 1.6

* By difference; daf = dry ash free; ND = not detected; proximate analysis in triplicate, ultimate and lipid analysis in duplicate and single protein and carbohydrate analysis.

Table 2
Influence of temperature and biomass species on bio-crude composition, higher heating value, and yield.

Strain	Temp. (°C)	Elemental composition of bio-crude (% daf)					HHV (MJ/kg)	Bio-crude yield (% daf)
		C	H	N	S	O*		
Chlorogloeopsis	*300*	66.5 ± 0.9	7.2 ± 0.3	6.8 ± 0.1	0.4 ± 0.1	19.0	32.0	38.6
Spirulina	*300*	72.7 ± 0.5	8.8 ± 0.4	6.3 ± 0.1	0.6 ± 0.0	11.5	36.1	35.5
Chlorella	*300*	75.9 ± 1.2	9.0 ± 0.2	5.3 ± 0.2	0.4 ± 0.0	9.3	37.5	46.6
Chlorella	*350*	70.7 ± 1.0	8.6 ± 0.1	5.9 ± 0.1	0.0 ± 0.0	14.8	35.1	35.8 ± 0.3
Scenedemus	*350*	73.0 ± 1.5	8.2 ± 0.2	5.7 ± 0.1	0.5 ± 0.0	12.6	33.6	27.1 ± 0.8

* By difference; daf = dry ash free; HHV = Higher Heating Value; elemental analysis in duplicate, yields in duplicate and single for 300 °C experiments.

yields of bio-crude range from 23 to 41% for *Scenedesmus* and *Chlorella* 300 °C respectively. The solid residue is highest for *Scenedesmus* which exhibits the highest ash content. The solid residue consists mainly of the mineral matter but also small amounts of carbon and nitrogen [6]. The largest fraction is shown to be the process water ranging from 46% for *Chlorella* at 300 °C to 68% for *Spirulina*. This clearly represents a major loss if this fraction was not recovered. The amount of carbon and nitrogen in the product phases is distributed very similarly as shown previously [8]. Up to 40% of carbon and 50% of nitrogen accumulate in the process water, resulting in low carbon recovery efficiencies; this was shown in a previous study by the authors where a mass balance on C and N in the product phases was carried out on the HTL of four microalgae strains at the same conditions [8]. Therefore the feasibility of recycling the process water for algae cultivation is investigated to recover the nitrogen and carbon lost to the water phase. The water phase has also been shown to be rich in PO_4^{3-}, NH_4^+ and K, compounds essential for algal growth [6].

3.3. Analysis of process water

Due to the large fraction of product distributed in the process water, its composition was examined to determine the suitability of using this as a source of nutrients for microalgae cultivation. Table 3 lists the main components identified in the water phase as determined by ion exchange chromatography, photometry and ICP-OES. In addition, the water phase is known to contain nitrogen heterocycles such as pyroles, indoles and phenols from the decomposition of the protein component [6]. The process water was analysed for total phenol content by photometry as these compounds are toxic to certain algae and can inhibit growth. Scragg reported that the growth of *C. vulgaris* was inhibited with concentrations of 400 ppm, but even at concentrations of 100 and 200 ppm the growth rate was reduced [24]. Table 3 shows that for all species except nitrate, concentrations in the process water are much higher than those in the standard growth media 3N-BBM + V. In particular, concentrations of K, NH_4^+, acetate and PO_4^{3-} are very high, orders of magnitude higher than those found in the media. These three nutrients are important to algal growth and are one of the main economic constraints. Acetate is of particular interest because it could potentially act as a substrate for mixotrophic growth, increasing productivity and recycling carbon [15]. In order to reach levels of nutrients similar to the standard media, the process water must be substantially diluted as shown by Jena et al. [18]. It is important to note that the nitrogen source in the media is in the form of nitrate, whereas in the process water the nitrogen is mainly present as ammonium. Microalgae are able to use both sources of nitrogen, and it has been suggested that neither actually provide an advantage to growth [25]. The total amount of nitrogen ranges from 3000 to 8000 ppm, *Scenedesmus* and *Chlorogloeopsis* have the lowest amounts due to the low nitrogen content in the algae, *Spirulina* on the other hand has the highest value corresponding to its high protein and nitrogen content. Concerning the nitrogen concentrations compared to the three fold nitrogen BBM media, the process water should be diluted between 25 and 65× its original volume to achieve the same nitrogen levels. For a standard BBM medium the dilutions would be 75–200 times its original volume. Nickel concentrations are of particular importance due to the inhibitory effects on microalgae, particularly for *C. vulgaris* which was observed to be inhibited by nickel levels as low as 0.85 ppb [26]. Nickel is present in very small amounts in the algae but it is also added to the process water by leaching of the reactor walls during HTL. A previous study by the authors in a Hastelloy 75 ml reactor with only distilled water led to a nickel concentration of 41 ppm and 2.5 ppm Fe at the same conditions [27]. It is expected that the leaching of nickel from 316 Stainless Steel as used in this study is less because of the lower nickel content of this steel alloy. This is preliminary confirmed by the nickel analysis in Table 3 ranging from 0 to 4 ppm. Haiduc et al. realized the significance of the nickel leaching effect in their study on hydrothermal gasification of microalgae [28]. They state that when a continuous system with nutrient recycling is used nickel concentrations would accumulate over time leading to growth inhibition. In the growth trials Haiduc et al. carried out, the media was doped with nickel and all concentrations (1–25 ppm) had adverse effects on algae growth. They came to the conclusion that reactor wall corrosion, metal leaching and nickel concentrations in the

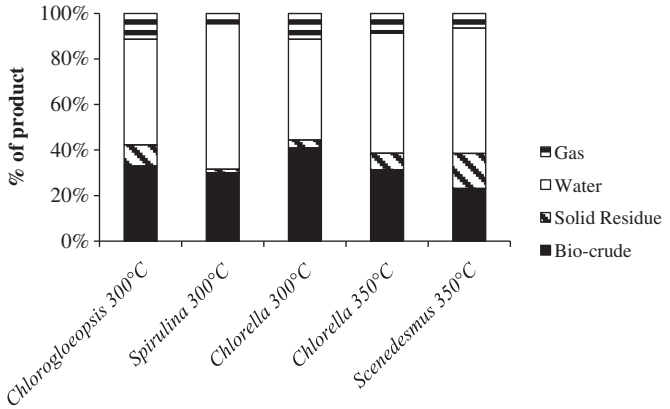

Fig. 2. Product distribution from the hydrothermal liquefaction of the different microalgae strains at 300 °C and 350 °C.

Table 3
Nutrient analysis of the process water compared to standard growth media 3N-BBM + V.

(ppm)	*Chlorogloeopsis 300 °C*	*Spirulina 300 °C*	*Chlorella 300 °C*	*Chlorella 350 °C*	*S. dimorphus 350 °C*	*3N-BBM +V**
pH	8.9	8.9	9	9.2	8.4	6.8
TOC	9060	15,123	11,373	13,764	11,119	–
Total N	5636	8136	6636	6888	3139	124
$NH4^+$	4748	6295	5673	5920	5280	–
PO_4^{3-}	280	2159	3109	1121	1470	153
K	303	1506	1460	1419	1150	63
Acetate	2146	7131	4106	5378	1290	–
NO_3-	508	194	329	237	192	547
Ni	3.8	0	0.1	0.4	0.8	–
Phenols	178	98	108	158	80	–

* Composition of 3N-BBM + V calculated based on prepared media compostion; no replicate of analysis available for process waters.

process water need to be monitored closely. They suggest that levels of 25 ppm should not be exceeded by either additional dilution or removal of the metal toxicant, e.g., by ion exchange.

The amount of phenols in the process water could also pose a problem due to their known inhibition affect on algae growth [24]. Phenols are typically present at concentrations of 100–200 ppm, without dilution of the water phase this would most likely inhibit algal growth. The pH of the process water is more alkaline for all conditions compared to the 3N-BBM media. This is due to the large amounts of ammonium present. The TOC analysis shows that there are significant levels of organic carbon in the process water. The mass balance in Fig. 2 showed large amounts of the product distributed to the process water and in a previous study by the authors it was shown that this fraction contains large amounts of carbon [8]. The highest values of TOC (Spirulina, ~15000 ppm TOC) in the process water correspond to the highest fraction of product in the entire mass balance in Fig. 2. High TOC levels are beneficial if the microalgae are capable of using this carbon for mixotrophic growth although ideally it would be beneficial if the bio-crude yields were higher. The organic content of the process water is largely dependent upon the biochemical content of the microalgae as was shown in previous work by the authors where model proteins, carbohydrates and lipids were processed under the same conditions [8]. In general, the higher the protein content, the higher the phenol and nitrogen heterocycles present in the oil [6]. Phenol and alkyl phenols are also present in the water as well as pyrolidinones, piperidines, pyrroles and indoles [7].

3.4. Cultivation trials

Due to the high concentration of the major nutrients in the process water compared to the media, it was necessary to dilute the process water with distilled water before cultivation. Jena et al. (2010) used similar dilutions for growth trials with the recovered aqueous phase from the HTL of *Spirulina*. They found that a tenfold dilution was too strong and no growth occurred [18]. The growth trials for this study were performed on dilutions of 50×, 100×, and 400× of the original process water. Trials were also performed in the standard media for comparison as well as a distilled water control. Growth was determined by chlorophyll a absorbance as described previously. The cyanobacterium *Chlorogloeopsis* exhibits a very strong cell wall so that the acetone could not extract the chlorophyll a from the cells. Due to this there is no data available for the growth trials over time of *Chlorogloeopsis* but the final growth was determined. Fig. 3(a and b) shows the relative biomass accumulation of *Spirulina* and *Chlorella* grown in the recovered aqueous phase at 300 °C. The chlorophyll a absorbance of *Spirulina* for the distilled water, 50× and 100× dilutions showed that no algae growth occurred at these conditions (Fig. 3 (a)). The 400× dilution of the process water and the standard media have positive chlorophyll a absorbance indicating growth was occurring. The values follow the same trend of steadily increasing growth after day two and show similar growth although the standard media absorbance is slightly higher. It appears that *Spirulina* is not able to grow in the stronger 50× and 100× dilutions, due to inhibitory effects, which may be attributed to the presence of metals such as nickel and organics such as phenols. According to Belkin and Boussiba the ammonia uptake at the observed concentrations should not pose a problem for *Spirulina*, especially at the pH levels measured (8.9 pH) [29]. The growth of *Chlorella* is plotted in Fig. 3(b). The growth in the process water dilutions showed growth in all three dilutions while no growth occurred in the distilled water control. A fourfold increase in biomass concentration was observed in the first 7 days, then a reduction in concentration is observed for all three trials. It appears that there are not sufficient nutrients available past day 7 for further growth. The 100× dilution exhibits a small final increase in absorbance resulting in the highest relative biomass concentration.

The growth trials of the HTL process water from *Chlorella* and *Scenedesmus* at 350 °C was carried out in the same manner but the dilutions chosen were 50×, 200×, 400× and 600× the original volume. The cultivation trials are compared to the growth in respective standard media 3N-BBM + V. The cell concentration of the growth trials are plotted in Fig. 4(a and b) for *Chlorella* and *Scenendesmus* respectively. Both algae showed no growth in the strongest dilution of 50×. This is most likely due to the high concentration of inhibitors such as nickel, phenols and fatty acids. *Chlorella* grew best in the standard media with a final cell concentration of ~6.0×10^{06}/ml. Growth in the 400× and 200× dilutions resulted in a similar final cell concentration of around half

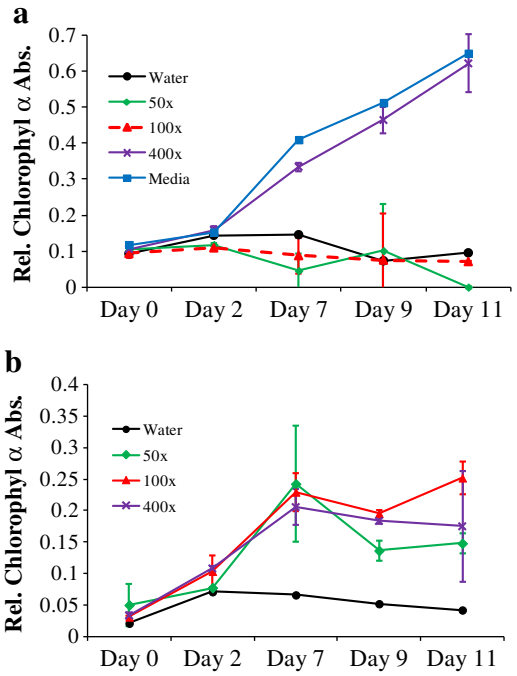

Fig. 3. Growth of algae in process water dilutions of (a) *Spirulina* 300 °C (b) *Chlorella* 300 °C determined by chlorophyll a absorbance.

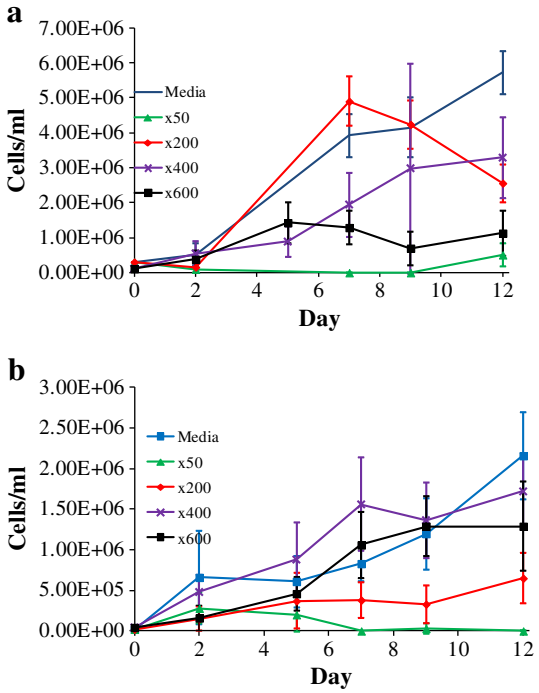

Fig. 4. Growth of algae in process water dilutions of (a) *Chlorella* 350 °C (b) *Scenedesmus* 350 °C determined by cell count.

compared to the media. On day 7 the 200× cell count was very high, higher even than in the media. The 600× dilution showed some growth but the cell count was never above 1.5×10^{06}/ml indicating insufficient nutrient availability for microalgae growth. It appears that the ideal concentration for cultivation of *Chlorella* in recovered process water lies between 200 and 400× dilution; there are sufficient nutrients available to support growth and the concentration of inhibitors is not too high. The results from the *Scenedesmus* growth trials are similar; highest cell count occurred for the media with 2.2×10^{06}/ml. The cell counts for *Scenedesmus* are much lower due to their larger cell size compared to *Chlorella*. The highest cell concentration for *Scenedesmus* is only 20% lower than in the media while the highest results for *Chlorella* is 40% lower. Similarly to *Chlorella* the cell counts on day 7 are higher for the process water dilutions 400× and 600×. Best growth for both algae strains occurred at 400× dilution, but the cell counts of *Scenedesmus* were closer to the media than *Chlorella*. The second highest cell count in process water dilutions for *Scenedesmus* occurred in 600× while it was 200× for *Chlorella*. This suggests that *Scenedesmus* can cope with lower nutrient availability than *Chlorella*. At 200× dilution, *Scenedesmus* struggled to reproduce; an increase in cell count only occurred on the last sampling day indicating that the amount of inhibitors were too large to allow normal cell reproduction.

Cultures were harvested at 11 and 12 days of growth, respectively for the cultures grown with the process waters from HTL conditions at 300 °C and 350 °C, centrifuged, dried in a desiccator and weighed to obtain a final biomass yield. The yield is compared to the yield in the respective standard medium and is presented in Table 4. Again the 300 °C samples were investigated for 50×, 100× and 400× while the 350 °C samples were grown in 50×, 200×, 400× and 600× dilutions of the original process water. The trials at 300 °C led to a significantly higher final biomass concentration due to the higher amount of starting material used to inoculate the growth trials. The purpose of this section is however to compare the process water cultivation to the respective standard media growth. *Chlorogloeopsis* for which no growth data by absorbance is available showed growth in the 100× and 400× dilutions but no growth at the stronger 50× dilution. Again the amount of inhibitors such as nickel, which is high in this case (Table 3), potentially inhibited the cells to reproduce. Table 4 shows that at 100× dilution the total biomass production of *Chlorogloeopsis* is only a third compared to the standard medium. For the 400× dilution on the other hand, the yield is increased by a third compared to the media. This indicates that *Chlorogloeopsis* is indeed able to use the organic carbon in the process water to grow mixotrophically. Mixotrophic growth can lead to higher biomass production compared to exclusively phototrophic growth [15]. It appears that the other cyanobacteria *Spirulina* is more sensitive to the growth inhibitors present, as there is no biomass production at the 100× dilution. The potential amount of growth inhibiting fatty acids could be quite high, as the TOC levels are very high for the *Spirulina* process water (Table 3). It is known that some fatty acids are dissolved in the process water; these are included in the TOC measurement and can act as inhibitors for microalgae growth [30]. Table 4 shows that at 400× dilution the final growth is similar to the standard media at around 700 mg/l. Both these observations are confirmed by the absorbance data in Fig. 3(a). The process water from *Chlorella* processed at 300 °C exhibits growth in all three dilutions investigated, indicating that *Chlorella* is less sensitive to potential growth inhibition. The growth is highest at 100× dilution, being only 15% less than the 3N-BBM + V media. At the lower and higher dilutions the growth is similar, just under half of the media growth. The absorbance data in Fig. 3(b) shows the same trend. The growth trials of *Chlorella* and *Scenedesmus* using process waters from the 350 °C HTL process were performed for 50×, 200×, 400× and 600× dilutions. Both microalgae strains were able to grow in all dilutions above 50×. It appears that the 50× dilutions are too concentrated for algal growth to occur. *Chlorella* was able to grow in the 50× dilution of 300 °C process water but not in the 350 °C process water. This is due to the considerably higher concentrations of TOC, phenols and Ni (Table 3). Additional nickel will leach into the process water from the reactor walls at elevated temperatures. At 200× dilution, *Chlorella* was able to grow only about half the amount of cells compared to the media (Fig. 4(a)) but the final biomass yield was 15 mg/l higher. As shown with *Chlorogloeopsis* this indicates that mixotrophic growth is occurring leading to higher biomass yields. At the higher dilutions the biomass yield is only 60% and 40% compared to the standard media for 400× and 600× respectively. The cell count at 400× is actually higher than at 200× but the biomass yield lower, this is attributed to the fact that the TOC levels at 200× are higher leading to increased mixotrophic growth which in turn leads to increased biomass. The biomass yields of *Scenedesmus*, even though they are high in cell numbers are quite poor with a maximum of 48 mg/l at 400× dilution, compared to 117 mg/l for the standard media. This indicates that *Scenedesmus* is not able to use the organic carbon for mixotrophic growth to the same extent as *Chlorella* and *Chlorogloeopsis*. Additionally the process water of *Scenedesmus* has very low acetate levels which can act as a substrate for mixotrophic growth. The authors acknowledge that the deviance in the data presented in Table 4 is high which is most likely due to the small volumes and resulting mass of algae leading to high estimation of errors. Nevertheless the data presented generally fits with the growth curves in Figs. 3 and 4 and shows that higher biomass yields can be achieved than in the standard media.

Table 4
Dry weights of harvested algae at the end of growth trials in dilutions of process water and standard media (mg/l).

	Process water dilutions for growth media					
	50×	100×	200×	400×	600×	Media*
Chlorogloeopsis 300 °C	*No growth*	124 ± 10	*NA*	498 ± 99	*NA*	*386*
Spirulina 300 °C	*No growth*	*No growth*	*NA*	657 ± 92	*NA*	*706*
Chlorella 300 °C	449 ± 18	877 ± 280	*NA*	459 ± 78	*NA*	*1020*
Chlorella 350 °C	*No growth*	*NA*	94 ± 19	47 ± 3	30 ± 3	79
Scenedemus 350 °C	*No growth*	*NA*	33 ± 2	48 ± 3	28 ± 1	117

NA = not analysed.
* No replicates carried out, process water trials carried out in duplicate.

3.5. Analysis of process water after cultivation

Following cultivation in the process water from HTL, the culture was centrifuged and the supernatant was reanalysed for the same nutrient parameters as before. Because *Spirulina* requires high bicarbonate (~15 g/l) for growth, all *Spirulina* cultures were supplemented with $NaHCO_3$. Due to the large amounts of Na, ion exchange chromatography

Table 5
Nutrient analysis of the culture medium before and after cultivation trials in different dilutions of process water. All units in ppm, no replicates carried out.

	Media	*Spent*	*Media*	*Spent*	*Media*	*Spent*	*Media*	*Spent*
	NH_4^+ *(ppm)*		*K (ppm)*		*Acetate (ppm)*		PO_4^{3-}*(ppm)*	
Chlorogloeopsis 300 °C								
100×	58.7	9.6	13.5	1.6	105.2	0.3	4.4	2.2
400×	30. 4	0.4	4.3	0.2	21.5	0.8	0	0
Chlorella 300 °C								
50×	64.9	5.6	13	0.5	129.9	3.34	119.5	96.4
100×	53.1	0.9	11.8	0.3	32.2	1.49	54.6	45.3
400×	13.4	0	4.4	0.2	0	0	15.3	13
Chlorella 350 °C								
200×	28.4	18.4	7.3	3.3	20.5	0.9	8.3	2.5
400×	23.8	11.7	3.4	1.8	10.3	0	4.1	5.7
600×	15.9	8.2	2.3	1.1	5.7	0.8	7.5	4.1
Scenedesmus 350 °C								
200×	26.4	17	5.8	4.4	6.5	0	14	7.4
400×	13.2	0	2.9	2.5	3.2	0	4.1	3.7
600×	8.8	0	1.9	1.1	2.2	0.7	4.2	2.5

could not quantify the minor nutrients present with certainty. Due to this, the data is not presented for the growth trials of *Spirulina* 300 °C process water. *Chlorogloeopsis* 50×, *Chlorella* 350 °C 50× and *Scenedesmus* 50× data is also not presented as no growth occurred as shown in Section 3.4. Table 5 presents a summary of four nutrients at the point of inoculation and of the supernatant after harvest. The data shows that all strains are able to use NH_4^+ as a source of nitrogen rather than nitrate as it is present in the media. This has previously been reported and is essential if nutrient recycling from HTL is considered, as most of the N is in the form of NH_4^+ [31]. At the 200x dilution, practically all nutrients are consumed by *Chlorogloeopsis* resulting in the high growth observed in Table 4. Growth does not seem to be inhibited by a lack of PO_4^{3-} at this condition. At 100× the nutrient uptake appears to be significant for all four nutrients, especially acetate, which is readily consumed. From this it could have been expected that the final biomass yield would be slightly higher. *Chlorella* 300 °C shows similar trends with NH_4^+ and acetate readily consumed at all dilutions, especially at 400× dilution it is apparent that there were insufficient nutrients available for further growth. This is confirmed by the dip in cell production in Fig. 3(b) after day 7 when the nutrients are all consumed. The data leads to the conclusion that the limiting nutrients for *Chlorella* are nitrogen and K. There is still PO_4^{3-} present after cultivation and acetate is not necessary, as *Chlorella* only consumes this in mixotrophic or heterotrophic growth. The uptake of nutrients for *Chlorella* 350 °C is different to *Chlorella* 300 °C, as there is no apparent limiting source of nutrients. NH_4^+, K and PO_4^{3-} are all still present in ample concentrations after cultivation although around half of the nutrients in each dilution have been consumed. Only acetate is entirely consumed which leads to the higher biomass yields presented in Section 3.4. From the nutrient uptake it cannot be determined why there is a fall in biomass productivity of *Chlorella* at the 200× dilution after day 7 (Fig. 4(a)). *Scenedesmus* process water at 350 °C is relatively low in acetate which could be the reason why a lower biomass yield is observed compared to *Chlorogloeopsis* and *Chlorella* which both readily used acetate as a substrate for mixotrophic growth. At 200× and 400× *Scenedesmus* seems to run out of nitrogen but K and PO_4^{3-} seem to be present in ample concentrations.

4. Conclusions

The results indicate that a bio-crude of good quality and high yield can be produced from microalgae by HTL. The properties and yields of bio-crude are largely dependent on the biochemical composition of the feedstock. The maximum bio-crude yield of 47% and a HHV of 37.5 MJ/kg was produced from HTL of *Chlorella* at 300 °C. The water phase was shown to be high in all required nutrients for algae growth, orders of magnitude higher than in standard growth media. The growth trials in the recycled process water show that heavy dilution of the water phase is necessary to avoid the effects of growth inhibitors such as phenols, fatty acids and nickel. All algae strains were able to grow in the recycled water but different optimum dilutions were observed. All strains were able to use acetate as a substrate for mixotrophic growth and NH_4^+ as a source of nitrogen. *Chlorogloeopsis* at 400× and *Chlorella* 350 °C at 200× achieved higher biomass yields than in their respective media by growing mixotrophically. The analysis of the spent water after cultivation showed that choosing the right dilution for each specific case is necessary to achieve optimum growing conditions. It was shown that the optimum dilution is strain dependent but ranges between 200 and 400×. By recycling the organic carbon in the water phase both the carbon efficiency and the biomass yields can be improved.

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References

[1] A.A. Peterson, F. Vogel, R.P. Lachance, M. Froling, M.J. Antal Jr., J.W. Tester, Thermochemical biofuel production in hydrothermal media: a review of sub- and supercritical water technologies, Energy & Environmental Science 1 (1) (2008) 32–65.
[2] Y. Dote, S. Sawayama, S. Inoue, T. Minowa, S.- Yokoyama, Recovery of liquid fuel from hydrocarbon-rich microalgae by thermochemical liquefaction, Fuel 73 (12) (1994) 1855–1857.
[3] T. Minowa, S.- Yokoyama, M. Kishimoto, T. Okakura, Oil production from algal cells of *Dunaliella tertiolecta* by direct thermochemical liquefaction, Fuel 74 (12) (1995) 1735–1738.
[4] S. Sawayama, T. Minowa, S.Y. Yokoyama, Possibility of renewable energy production and CO2 mitigation by thermochemical liquefaction of microalgae, Biomass and Bioenergy 17 (1) (1999) 33–39.
[5] T.M. Brown, P. Duan, P.E. Savage, Hydrothermal liquefaction and gasification of *Nannochloropsis* sp. Energy & Fuels 24 (6) (2010) 3639–3646.
[6] A.B. Ross, P. Biller, M.L. Kubacki, H. Li, A. Lea-Langton, J.M. Jones, Hydrothermal processing of microalgae using alkali and organic acids, Fuel 89 (9) (2010) 2234–2243.
[7] P. Biller, R. Riley, A.B. Ross, Catalytic hydrothermal processing of microalgae; Decomposition and upgrading of lipids, Bioresource Technology 102 (7) (2011) 4841–4848.
[8] P. Biller, A.B. Ross, Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content, Bioresource Technology 102 (1) (2011) 215–225.
[9] L. Garcia Alba, C. Torri, C. Samorì, J. van der Spek, D. Fabbri, S.R.A. Kersten, D.W.F. Brilman, Hydrothermal treatment (HTT) of microalgae: evaluation of the process as conversion method in an algae biorefinery concept, Energy & Fuels 26 (1) (2012) 642–657.
[10] U. Jena, K.C. Das, J.R. Kastner, Effect of operating conditions of thermochemical liquefaction on biocrude production from *Spirulina platensis*, Bioresource Technology 102 (10) (2011) 6221–6229.
[11] G. Yu, Y. Zhang, L. Schideman, L. Funk T., G. Wang Z., Hydrothermal liquefaction of low lipid content microalgae into bio-crude oil, American Society of Agricultural Engineers, St. Joseph, MI, ETATS-UNIS, 2011, pp. 239–246.
[12] Z. Shuping, W. Yulong, Y. Mingde, I. Kaleem, L. Chun, J. Tong, Production and characterization of bio-oil from hydrothermal liquefaction of microalgae *Dunaliella tertiolecta* cake, Energy 35 (12) (2010) 5406–5411.
[13] P. Duan, P.E. Savage, Hydrothermal liquefaction of a microalga with heterogeneous catalysts, Industrial and Engineering Chemistry Research (2010) (p. null-null).
[14] S. Stucki, F. Vogel, C. Ludwig, A.G. Haiduc, M. Brandenberger, Catalytic gasification of algae in supercritical water for biofuel production and carbon capture, Energy & Environmental Science 2 (5) (2009) 535–541.
[15] A. Bhatnagar, S. Chinnasamy, M. Singh, K.C. Das, Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters, Applied Energy 88 (10) (2011) 3425–3431.
[16] Sialve, B., N. Bernet, and O. Bernard, Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances 27 (4) (2009) 409–416.
[17] K. Tsukahara, T. Kimura, T. Minowa, S. Sawayama, T. Yagishita, S. Inoue, T. Hanaoka, Y. Usui, T. Ogi, Microalgal cultivation in a solution recovered from the low-temperature catalytic gasification of the microalga, Journal of Bioscience and Bioengineering 91 (3) (2001) 311–313.
[18] U. Jena, N. Vaidyanathan, S. Chinnasamy, K.C. Das, Evaluation of microalgae cultivation using recovered aqueous co-product from thermochemical liquefaction of algal biomass, Bioresource Technology 102 (3) (2011) 3380–3387.
[19] K. Anastasakis, A.B. Ross, Hydrothermal liquefaction of the brown macro-alga *Laminaria saccharina*: effect of reaction conditions on product distribution and composition, Bioresource Technology 102 (7) (2011) 4876–4883.
[20] J. Waterborge, *The Lowery Method for protein quantification* in *Th Protein ProtocolsHandbook*, in: J.M. Walker (Ed.), Human Press, New Jersey, 2002.
[21] S.M. Gerchakov, P.G. Hatcher, Improved technique for analysis of carbohydrates in sediments, Limnology and Oceanography 17 (6) (1972) 938–943.
[22] E.G. Bligh, W.J. Dyer, A rapid method for total lipid extraction and purification, Journal of Biochemistry and Physiology 37 (1959) 911–917.
[23] R.A. Corbitt, Standard Handbook of Environmental Engineering, McGraw-Hill Inc., New York, 1998.
[24] A.H. Scragg, The effect of phenol on the growth of *Chlorella vulgaris* and Chlorella VT-1, Enzyme and Microbial Technology 39 (4) (2006) 796–799.
[25] Chen, M., H. Tang, H. Ma, T.C. Holland, K.Y.S. Ng, and S.O. Salley, Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology 102 (2) (2011) 1649–1655.
[26] D.F. Spencer, L.H. Nichols, Free nickel ion inhibits growth of two species of green algae, Environmental Pollution Series A, Ecological and Biological 31 (2) (1983) 97–104.
[27] A.B. Ross, P. Biller, C. Hall, Catalytic hydrothermal processing of microalgae with integrated nutrient recycling, 18th European Biomass Conference and Exhibition, 2011, Berlin, Germany.
[28] A. Haiduc, M. Brandenberger, S. Suquet, F. Vogel, R. Bernier-Latmani, C. Ludwig, SunCHem: an integrated process for the hydrothermal production of methane from microalgae and CO₂ mitigation, Journal of Applied Phycology 21 (5) (2009) 529–541.
[29] S. Belkin, S. Boussiba, High internal pH conveys ammonia resistance in spirulina platensis, Bioresource Technology 38 (2–3) (1991) 167–169.
[30] R. Bosma, K. Miazek, S.M. Willemsen, M.H. Vermuë, R.H. Wijffels, Growth inhibition of *Monodus subterraneus* by free fatty acids, Biotechnology and Bioengineering 101 (5) (2008) 1108–1114.
[31] M. Chen, H. Tang, H. Ma, T.C. Holland, K.Y.S. Ng, S.O. Salley, Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*, Bioresource Technology 102 (2) (2011) 1649–1655.