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Co-production of bio-oil and propylene through the hydrothermal liquefaction of PHB producing cyanobacteria

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Abstract

In this investigation, PHB producing cyanobacteria were converted through hydrothermal liquefaction (HTL) into propylene and a bio-oil suitable for advanced biofuel production. HTL of model compounds demonstrated that in

contrast to proteins and carbohydrates, which react to produce a range of alternative intermediates, no synergistic effects were detected when converting PHB in the presence of algal biomass. Subsequently, *Synechocystis cf. salina*, which had accumulated 7.5 wt% PHB was converted via HTL (15 % dry weight loading at 340 °C). The reaction gave an overall propylene yield of 2.6 %, higher than that obtained from the analogous model compounds, in addition to a bio-oil with a low total nitrogen content of 4.6 %. No propylene was recovered from the alternative non-PHB producing cyanobacterial strains, *Anabaena, Spirulina or Synechococcus*, suggesting that PHB is the sole source of propylene. PHB producing microorganisms could therefore be used as a feedstock for a biorefinery to produce polypropylene and advanced biofuels, with the level of propylene being directly proportional to the accumulated amount of PHB.

1 Introduction

Over the last five years, research on the direct hydrothermal liquefaction (HTL) of microalgae into fuel range compounds has gained significant momentum (Jazrawi et al., 2013; López Barreiro et al., 2013; Tian et al., 2014). Compared to the alternative process for the production of fuels from microalgae, the extraction of lipids followed by the transesterification into biodiesel, direct HTL offers a number of significant advantages: it is capable of converting the entire biomass fraction, not just lipids, and can therefore be used for the conversion of fast growing protein producing eukaryotic algae and cyanobacteria (hereafter collectively referred to as microalgae); it is conducted in the presence of high water loadings, and therefore only partial drying of the biomass is required; and it produces a wide variety of compounds which may be further upgraded into a

range of fuels and chemicals (Lopez Barreiro et al., 2014; Tian et al., 2014). Despite this, a number of challenges remain: most notably, the upgrading of the resulting crude oils (which cannot be treated in conventional refineries due to their high nitrogen contents) and the utilisation of additional product streams to help subsidise fuel costs (Biller et al., 2013; Chuck et al., 2015).

Previous studies have investigated the relationship between bio-oil yields and the biochemical make-up of the algae (Leow et al., 2015; Li et al., 2014; Vardon et al., 2011). The three most important biochemical constituents of microalgae are proteins, lipids and carbohydrates, and their relative compositions depend both on the individual species and the selected growth conditions. These studies identified a strong relationship between lipid content and bio-oil yields, with lipid-rich algae producing the highest bio-crude yields. Using data from the conversion of model compounds and various microalgae species, Biller et al. estimated that the bio-crude yields from the different biochemical fractions range from 55 % to 80 % for lipids, 11 % to 18 % for proteins and 6 % to 15 % for carbohydrates (Biller & Ross, 2011). Similar yields were obtained by Teri et al. for pure model compounds, however, when converting mixtures of polysaccharides and proteins, enhanced oil yields were obtained at a reaction temperature of 350 °C (Teri et al., 2014). The authors suggested that this could be the result of Maillard reactions between sugars and amino acids formed from the carbohydrate and protein fractions respectively. Yang et al. experienced a similar behaviour using algae extracted crude polysaccharides and crude proteins (Yang et al., 2015). Yields obtained during the co-liquefaction of both

compounds were enhanced compared to the yields of single compound liquefaction, also resulting in the highest energy recovery.

Besides these three main classes of biochemical compounds, some species of microalgae have been found to accumulate significant quantities of other types of compounds, such as algaenans, a series of acid and base-resistant aliphatic biomacromolecules (Versteegh & Blokker, 2004). During the HTL of these species, algaenans and algaenan derivatives were almost fully extracted into the biocrude phase at temperatures exceeding 300 °C, and can therefore help to increase potential yields of a low-nitrogen fuel precursor (Torri et al., 2012).

Some cyanobacteria (prokaryotic microalgae) also accumulate polyhydroxyalkanoates (PHAs), such as polyhydroxybutyrate (PHB), particularly in the presence of excess carbon and under nutrient (nitrogen, phosphorus) limited conditions (Markou & Nerantzis, 2013). The PHA content in the biomass can range from below 1% to over 50% and depends on the strain, the carbon and nutrient source and composition as well as on the cultivation conditions (Drosg et al., 2015; Panda et al., 2006; Wu et al., 2002; Wu et al., 2001).

PHAs are seen as a promising class of bio-polymers as they have equivalent properties to many currently available petroleum-derived plastics, with the added benefit of being biodegradable (Somleva et al., 2013). Despite this, PHA concentrations in fast-growing microalgae are usually too low to enable economic extraction. HTL could therefore represent a more effective method for utilising these compounds.

In this paper we investigate the potential of biopolymer-containing species of microalgae for conversion by HTL. To this end, models representing the four classes of biochemical compounds present in these species (lipids, proteins, carbohydrates and PHAs) were first converted in isolation and their product distribution determined. The results from the conversion of the model compounds were then compared to the yields obtained from the cyanobacteria *Spirulina*, before liquefying *Spirulina* in the presence of varying concentrations of PHB to investigate if there is any interaction between the algal and the biopolymer compounds. Finally, three different species of cyanobacteria, both PHB and non-PHB producing, were liquefied and their product distribution analysed.

2 Experimental

2.1 Materials

General lab solvents were purchased from Sigma–Aldrich and used without further purification. Deuterated chloroform ($CDCl_3$) for ^1H NMR analysis was purchased from Fluorochem. Rapeseed oil was purchased from a local supermarket and contained 62% mono-unsaturates, 30% polyunsaturates and 8% saturated esters.

Dried *Spirulina* powder, soy protein and corn flour were obtained from commercial sources. Values for their biochemical composition were obtained

from their packaging information. Polyhydroxybutyrate biopolymer granules were purchased from Goodfellow Cambridge Ltd.

2.2 Cultivation of microalgae

Stock cultures of *Anabaena sp.* (CCAP 1403/4A), *Synechococcus sp.* (WH7803) and *Synechocystis cf. salina* Wislouch (No. 192) were maintained under batch culture conditions and sub-cultured on a weekly basis. *Anabaena* cultures were maintained in BG11 media in 10 L bubble columns under 100 µmols photons m^2 sec^{-1} irradiance on a 16 h: 8 h light: dark cycle at 20 °C (±1 °C), according to literature precedent (Stanier et al., 1971).

Synechococcus sp. was grown in a saline version of BG11 (3.5 % sea salt). Shear stresses prevented the growth of *Synechococcus sp*. in bubble columns and as such were grown in multiple 500 mL batches under the same irradiance measures and cycles and aerated by manual daily shaking. Strains were grown to mid log phase, and then sub cultured in to either fresh medium, or medium lacking the nitrogen component ($NaNO_3$). Culture density was monitored spectrophotometrically.

Synechocystis cf. salina Wislouch (No. 192) – from the culture collection of autotrophic organisms (CCALA) – was cultivated in nutrient limited (nitrogen and phosphorous) mineral medium based on BG11 (Rippka et al., 1979) in a tubular photobioreactor with a working volume of 200 L for 21 days. The biomass was harvested with a nozzle separator (GEA Westfalia, Typ NA 7-06-067/-576) (disc outer diameter 162 mm), stored at -20 °C and lyophilised.

As the quantities of the individual samples were insufficient for separate HTL analysis, harvested *Synechococcus* and *Anabaena* biomass was combined into one sample grown under normal growth conditions (*Synechococcus* to *Anabaena* ratio of 3.53:1), and one grown under nitrogen starved conditions (*Synechococcus* to *Anabaena* ratio of 5.38:1), which will be referred to as *Synechococcus/Anabaena* and *Synechococcus/Anabaena-N*, respectively.

2.3 Biomass characterization

The CHN content of all feedstocks (except PHB) was determined on a Carlo Erba Flash 2000 elemental analyser. The ash and moisture contents were determined by thermogravimetric analysis (TGA), under air flow, ramp rate of 10 °C min^{-1} to 500 °C and 20 °C min^{-1} to 900 °C.

Sugar (carbohydrate) content was determined according to literature precedent (Rao & Pattabiraman, 1989). Briefly, 3 mL conc. sulfuric acid is added to 1 mL algae sample (1 mg rehydrated in distilled water) (n=5) and the reaction allowed to reach maximum temperature for 5 minutes before cooling to 25 °C. 1 mL 5 % (w/v) phenol solution was added and the tubes stood at 25 °C for 30 minutes. The absorbance at λ = 480 nm was determined and glucose was used for standard curve.

Protein content was determined according to the biuret method (Bellou & Aggelis, 2012). Briefly, 1 mL culture (up to 8 mg dried algae rehydrated in

distilled water) (n=5) added to 0.75 mL KH_2PO4, (0.067M pH4.5) and 3 mL NaOH (20 % w/v) and incubated for 5 minutes. Thereafter 0.125 mL $CuSO_4.5H_2O$ solution (25 % w/v) was added and samples periodically shaken for 10 minutes then centrifuged. The absorbance at λ = 540 nm of the supernatant was determined. Bovine serum albumin was used for standard curve.

Lipid content was determined post conversion to fatty acid methyl esters (FAMEs) using GC-MS (Agilent 7890A GC and 5975C inert MSD, Agilent Technologies Ltd., Edinburgh, UK). Nonadecanoic acid (C19:0) was added as an internal standard to each sample (up to 9.5 mg lyophilised algae) (n=5) and cellular fatty acids were converted directly to FAMEs by adding 1 mL of transesterification mix (95:5 v/v 3 N methanolic HCl; 2,2-dimethoxypropane) followed by incubation at 90 °C for 1 h. After cooling, FAMEs were recovered by addition of 1 % w/v NaCl solution (1 mL) and *n*-hexane (1 mL) followed by vortexing. The upper hexane layer was injected directly onto the GC-MS system as previously described (Beacham et al., 2015). FAMEs were identified using retention times and qualifier ion response and quantified using respective target ion responses. All parameters were derived from calibration curves generated from a FAME standard mix (Supelco, Sigma-Aldrich, Gillingham, Dorset, UK).

The PHB concentration was determined using a modified literature method (Karr et al., 1983), where the dried algae / cyanobacteria biomass was digested with concentrated H_2SO_4 at 90 °C for 30 minutes – in this step PHB degrades to crotonic acid. The sample was diluted for high performance liquid chromatography (HPLC) analysis. HPLC analysis was carried out with an Agilent

1100 system, equipped with an ion-exclusion column ION 300 (Transgenomic CARBOSep COREGEL 87H, part no. CHO-99-9861) heated at 65 °C and a refractive index detector (Agilent 1100/1200 – 55 °C). The flow rate of the mobile phase – 0.005 M H_2SO_4 – was 0.9 mL min^{-1}; the method lasted 25 minutes.

2.4 Reactions

Hydrothermal liquefaction reactions were conducted in a batch reactor according to literature precedent (Biller & Ross, 2011). The reactors had an approximate internal volume of 50 mL, were constructed of Swagelok tubing and fitted with a pressure gauge, vent valve and a thermocouple connected to data logging software (Raikova et al., 2016). Each reaction was conducted with 3 g (*Synechococcus/Anabaena and Synechococcus/Anabaena-N*) or 4 g of biomass and 15 mL or 20 mL, respectively, of D.O. water, giving a solid loading of approximately 16.7 %. The reactor was placed inside a tube furnace, which had been preheated to a temperature significantly above the desired reaction temperature. As soon as the desired reaction temperature was reached, the reactor was removed from the furnace. Pressure was generated internally through the partial vaporisation of water and monitored using a pressure gauge. Total heating times varied from 26 to 71 min, depending on the desired reaction temperature, external temperature and exact position of the reactor within the furnace. The reaction system was thoroughly cleaned between reaction runs, by heating 20 mL of D.O. water to approximately 200 °C and venting the produced steam through the reactor vent valve.

Selected reactions for each model compound were conducted in triplicate, to estimate the standard deviations of the reaction results. Triplicate reactions were conducted for *Synechocystis*, but due to insufficient amounts of biomass for the *Synechococcus/Anabaena and Synechococcus/Anabaena-N* samples, these were only converted in a single HTL run.

2.5 Sample workup

Following hydrothermal liquefaction, the reactor was allowed to cool back to room temperature, before the reaction gases were vented into an inverted, water-filled measuring cylinder to determine the volume of gas produced. Selected gas samples were separated on a gas chromatograph (Agilent 7890A) containing a HP-Plot-Q capillary column using helium as carrier gas and analysed with an external Agilent 5975C MSD detector. The samples were loaded at 35 °C, hold time 7 min, ramped to 150 °C at 20 °C min^{-1}, hold time 0 min, ramped to 250 °C at 15 °C min^{-1}, hold time 16 min. Gas compositions were normalized to exclude nitrogen and chloroform, which were not formed during the reactions, but introduced during the reactor loading and cleaning procedures.

The water phase was recovered by decanting the reactor contents through filter paper, leaving behind the crude oil and solid product phases. The yield of non-volatile water residue was determined by drying water phase aliquots of approximately 5 mL in an oven overnight, and the weight of the recovered residue was scaled to the total recovered water phase volume (by weight). The oil phase was recovered by repeatedly washing the reactor and solid residue with chloroform until the solvent remained clear, before filtering the solvent-oil

mixture and evaporating the chloroform in a rotary evaporator. The solid yield was determined from the mass of retentate collected on the filter paper during the filtration of the water and oil product phases. To minimize errors associated with absorbed atmospheric moisture, the filter paper was dried overnight and weighted immediately after removal from the drying oven before and after use. All product phase yields were calculated on a dry, ash-free basis (daf).

2.6 Product analysis

The CHN contents of the oil and residue phases produced from the HTL of proteins and algae were determined by elemental analysis. Ash and moisture contents were determined by TGA, under air flow, ramp rate of 10 °C min^{-1} to 500 °C and 20 °C min^{-1} to 900 °C. The energy densities of the oils and feedstocks were determined on an IKA® C1 bomb calorimeter. For samples which could not be combusted, the energy density was calculated using the Dulong formula,(Faeth et al., 2013) using the results from CHN and TGA analysis:

$$\text{HHV (MJ kg}^{-1}) = 0.338 \times C + 1.428 \times (H - O/8) + 0.095 \times S \quad \textbf{(1)}$$

The oxygen content was calculated by difference, subtracting the carbon, hydrogen, nitrogen and ash weights from 100. Sulphur content was assumed to be negligible.

The ammonia concentration of the water phase was determined using a Randox Urea analysis test kit. Due to the high concentration of ammonium ions in the

produced water, the samples were diluted with distilled water to a concentration of 1 or 2 % v/v. Subsequently, 10 μL of sample was reacted for 5 min with 1000 μL of a urease reactant, followed by the addition of 200 μL of sodium hypochlorite solution to induce the colour change. Finally the sample absorbance was measured at 600 nm and calibrated using a reagent blank and standard solution.

NMR spectra were obtained on a 300 MHz Bruker Avance spectrometer.

3 Results and Discussion

3.1 Biomass characterization

A total of four different microalgae samples and four different model compounds were tested in this work as HTL feedstocks (Table 1). *Spirulina* was selected for this study as it is amongst the most well studied species of microalgae for HTL. Some strains of *Synechocystis* and *Synechococcus* in turn have been reported to accumulate high levels of PHB of up to 55 % (daf) when cultivated under nitrogen and phosphorus depleted conditions (Drosg et al., 2015; Miyake et al., 1996; Nishioka et al., 2001; Panda et al., 2006; Wu et al., 2002; Wu et al., 2001).

Soy protein, rapeseed oil, corn flour and PHB were selected to represent the expected four main biochemical compound fractions found in the biopolymer producing cyanobacteria, namely protein, lipid, carbohydrates and PHAs. The proximate analysis of the models shows high purity in the desired compound group, and therefore the selected models are an adequate representation of the biochemical composition of microalgae.

As a result of its high carbon and hydrogen content, rapeseed oil possesses by far the highest energy density of all the four model compounds. Consequently, much research has focused on increasing the lipid content in microalgae. Unfortunately, this can reduce microalgae growth rates to an uneconomic level and therefore the focus has shifted towards faster growing protein producers. The energy densities of PHB and soy protein are quite similar, despite displaying large differences in elemental composition. PHB contains more carbon and oxygen, but less hydrogen and no nitrogen. Corn flour possesses the lowest energy density of all model compounds, as a result of its high oxygen and low carbon and hydrogen content.

All four model compounds had much lower ash contents than the microalgae samples, especially rapeseed oil, corn flour and PHB. Amongst the microalgae samples the highest ash content was observed for the two mixed *Synechococcus/Anabaena* samples, particularly those grown in normal growth medium (38.1 %), compared to ash contents for *Spirulina* and *Synechocystis* of 9.3 % and 11.2 %, respectively. A potential explanation for the high ash content of the *Synechococcus/Anabaena* samples is that *Synechococcus* was cultured in a saline growth medium, containing 3.5 % sea salt, resulting in the carry-over of high levels of inorganic solids to the resulting biomass. The differences in ash content will not only impact the overall product yields, but could also have important catalytic effects during the HTL of these compound (Biller & Ross, 2011).

The composition of *Spirulina* is typical for fast-growing microalgae, with a low lipid and high protein concentration. Consequently, it has a high nitrogen content of 12.51 % (daf), a carbon content, nitrogen content and energy density similar to those of soy protein. The *Synechocystis* and mixed *Synechococcus/Anabaena* samples all have significantly lower nitrogen concentrations (3.52 to 7.91 %), linked to a reduced protein content (53.7 to 54.9 %). For HTL, the high nitrogen content of the protein fraction is one of the major challenges, as it results in the formation of nitrogen rich oils which require significant further upgrading. Consequently the reduced nitrogen content in the PHA producers is desirable for increased bio-oil quality. The three *Synechocystis* and *Synechococcus/Anabaena* samples also have a reduced carbon content (42.78 to 48.27 %), compared to *Spirulina* (54.62 %), which may be related to an increased ash content and the high oxygen content of the PHA fraction.

3.2 Conversion of model compounds

3.2.1 Product distribution

The liquefaction of the four model compounds rapeseed oil, corn flour, soy protein and PHB was conducted at four reaction temperatures, ranging from 300 to 360 °C, and the yields of the solid, oil, water residue and gas were recorded (Figure 1a). In accordance with the literature, the product distributions from the different compound classes were found to be vastly different (Biller & Ross, 2011; Teri et al., 2014). Whilst rapeseed oil is almost exclusively converted into an oil product, corn flour favours the formation of solids, soy protein the formation of water phase residue and PHB produces very high gas yields, particularly at the higher reaction temperatures.

With the exception of rapeseed oil, the overall mass balance closures obtained from the conversion of the model compounds were poor, particularly for corn flour. Despite this, the results showed high repeatability, which suggests that the deficit must be attributed to systematic causes, rather than experimental error. The most likely explanation is the loss of volatile organic compounds during the evaporation of water from the aqueous phase. Indeed, many of the products previously identified in the water phase from the hydrothermal liquefaction of carbohydrates (glucose) fulfil this requirement, such as acetone (boiling point of 56 °C), formic acid (BP ~ 101 °C), 5-hydroxymetnylfurfural (BP ~ 114 – 116 °C), acetic acid (BP ~ 118 °C), lactic acid (BP ~ 122 °C) and glucoaldehyde (BP ~ 131 °C) (Srokol et al., 2004). As most previous work calculated the water yield by difference rather than direct measurement, this problem has been rarely encountered in literature (Chen et al., 2014).

Analysis by ^{1}H NMR (included in supplementary information) of the rapeseed oil fraction revealed the complete disappeareance of the peaks corresponding to the glycerol backbone (δ = 4.0 – 4.5) and the bis-allylic sites (δ = 2.5 – 3.0), a reduction in the peaks corresponding to the double bonds (δ = 5.0 – 5.5) but almost no change to the ester peaks as reaction temperatures approached 340 °C. This shows that the triglycerides in rapeseed oil fully decomposed into free fatty acids and glycerol, and no further decarboxylation took place.

Increasing the reaction temperature from 300 to 360 °C had different effects on the yields obtained from the various model compounds. Whilst yields obtained

from the liquefaction of corn flour and rapeseed oil remained more or less constant, reaction temperature had a large effect on the yields obtained from soy protein and PHB. In the case of the former, the highest oil yields of 28.6 % were obtained at the highest reaction temperature of 360 °C, which also resulted in the lowest solid (~ 0 %) and water phase residue (35.9 %) yields. Similar to the literature, the increase in oil yields over the investigated temperature range appears to be correlated to a similar reduction in solid yields (Yoo et al., 2015). In contrast, the decreasing water phase residue yields result in a reduced overall mass balance closure, suggesting that water soluble products are broken down into more volatile organic compounds as the reaction temperature is increased.

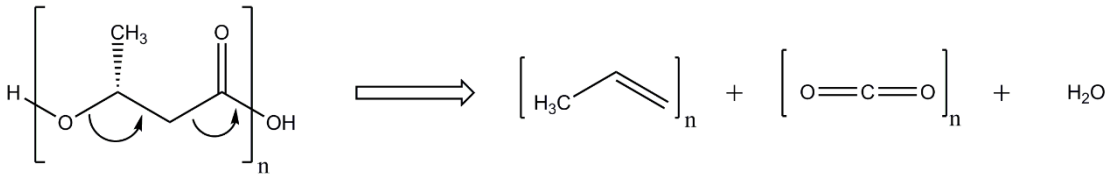
For PHB, over the investigated temperature range, gas yields steadily increased from 33.0 to 71.6 % whereas water residue yields dropped from 35.7 to 14.3 %. Oil yields were reduced from 7.0 % to 2.6 %, and solid yields were low at all reaction temperatures (2.4 % to 1.6 %). In contrast to soy protein, the overall mass balance closure increased as the temperature was raised from 320 to 340 °C, suggesting a successive break-down of solid PHB, first into heavier water soluble compounds, then into lighter organics and finally into gas products.

HTL of corn flour and soy protein resulted in a significant increase in the carbon and hydrogen content of the biocrude compared to the unconverted model compound (see supporting information). In contrast, the carbon and hydrogen content of the rapeseed oil product appeared slightly reduced, whereas for PHB, carbon and hydrogen were reduced at 300 and 320 °C, but increased at the higher reaction temperatures. For soy protein, the nitrogen content in the

biocrude was considerably reduced from an initial value of 13.3 % to a minimum of 6.6 % at 360 °C. Consequently the carbon and hydrogen retention for this feedstock, 37.5 % and 34.0 % respectively, were much higher than the nitrogen retention of only 12.5 %, resulting in an increase in the HHV from 20.8 to 35.4 MJ kg^{-1} (all values for 360 °C). Despite this, the residual nitrogen content remains above the acceptable threshold for fuel use, and therefore further treatment of the oil would be required (Chuck et al., 2015). Increasing the reaction temperature from 300 to 360 °C resulted in a large increase in the HHVs obtained for corn flour (23.3 to 29.5 MJ kg^{-1}) and PHB (15.2 to 27.2 MJ kg^{-1}), likely as a result of an increased degree of deoxygenation, and therefore increased carbon and hydrogen concentration. However, the low oil yields obtained from these two models are unlikely to make a major contribution to the overall oil produced from the HTL of actual, PHB-containing cyanobacteria.

3.2.2 Gas phase analysis

Analysis of representative gas samples from the HTL of soy protein and corn flour revealed that, consistent with the literature, carbon dioxide was by far the dominant gas product, exceeding 90% in both cases (Figure 1b), (Jazrawi et al., 2013). Other compounds included ammonia, 2-butene, 2-methyl-1-propylene and 2-methylfuran. In contrast, the gas product formed from the HTL of PHB was considerably different, consisting of approximately equal amounts of carbon dioxide and propylene, particularly at the higher reaction temperature of 340 °C. Based on these results, a potential decomposition mechanism of PHB under hydrothermal conditions to equal amounts of carbon dioxide and propylene is proposed (Scheme 1). Propylene is an important precursor for the polymer



Scheme 1: PHB decomposition pathway to propene and carbon dioxide

industry, and therefore the conversion of PHB-containing algae with associated gas separation, could represent a viable value-adding product stream. This, in turn, may help to subsidise the cost of the main oil product and make the overall process more cost effective.

3.2.3 Energy Recovery

An important consideration in evaluating the feasibility of HTL towards the conversion of biomass is the overall energy recovery (ER) into the desired reaction product. This can be calculated using the higher heating values of the product and feedstock (HHV_P and HHV_F) and the product yield (Y_P), using equation (2).

$$ER = \frac{HHV_P \times Y_P}{HHV_F} \quad \textbf{(2)}$$

The HHVs of the oil phases were calculated from the elemental composition using the Dulong formula (Figure 1c). The conversion of rapeseed oil resulted in very high energy recoveries to the oil phase of over 90 %, regardless of reaction temperature. For soy protein, the energy recovery increased with increasing reaction temperature, to a maximum value of 42 % at 360 °C, whereas both corn flour and PHB display only marginal energy recovery to the crude oil phase of less than 8 % for corn flour and less than 5 % for PHB. This suggests that HTL of

these compounds is not viable for the formation of a crude oil product. PHB does however produce a significant yield of propylene. Using the HHV of propylene from the literature (48.96 MJ kg^{-1}) and multiplying this value with the mass fraction of propylene in the gas phase, the energy recovery to the gas phase can be calculated. At temperatures above 340 °C, very high energy recoveries of over 64 % could be obtained. This suggests that HTL could represent an energy-efficient method to convert PHB, present in algal biomass, into propylene.

3.3 Effect of biochemical composition on HTL yields

3.3.1 Synergetic effect of biochemical compounds present in *Spirulina*

The previous section focused on the conversion of model compounds in an attempt to understand what contribution they could play in the HTL of whole microalgae. This has revealed some interesting differences in the product distributions and energy recoveries obtained from the different algal components, but the liquefaction behaviour of these components in combination is much more complicated. A number of researchers have attempted to correlate bio-oil yields to algae composition and found that overall yields are not simply a weighted average of the yields obtained from model compounds (Chen et al., 2009; Teri et al., 2014). Whilst reasonable correlations were observed between lipid content and bio-crude yield, much weaker correlations are obtained for protein and carbohydrate contents. In general, the recovered biocrude yields were higher than expected (Biller & Ross, 2011; Teri et al., 2014), and it was suggested that the decomposition products from proteins and carbohydrates – amino acids and sugars – could further react via Maillard type reactions to form nitrogen containing cyclic organic compounds such as pyridines and pyrroles

(Toor et al., 2011). Maillard reactions are complex, and still not fully understood in this complex reaction environment, however nitrogen containing heterocycles are known to be formed through these reactions (Owuso-Apenten, 2004).

To verify these findings, the biochemical composition of the cyanobacteria *Spirulina* was used to calculate theoretical HTL conversion yields (Y_T), and subtracted these from the actual yields obtained (Figure 2a).

$$\text{Theoretical Yield } (Y_T) = A_{CH} \times Y_{CH} + A_P \times Y_P + A_L \times Y_L \quad \textbf{(3)}$$

Where Y_T, denotes the theoretical yield obtained from the conversion of the algae, A_{CH}, A_P and A_L stand for the concentration of carbohydrates, protein and lipid in the algae, on a daf basis; and Y_{CH}, Y_P and Y_L denote the yields obtained from the HTL of carbohydrates, proteins and lipids (daf basis). Carbohydrate, protein and lipid yields were calculated from the conversion of model compounds, however due to the complexity of these calculations, the corresponding equations are shown in the supplementary information.

The results show a remarkable increase in actual biocrude yields and a similar reduction in solid yields, compared to the theoretical yields based on the conversion of model compounds, consistent with the literature findings. Gas yields are slightly increased at higher reaction temperatures, but the water residue yields don't appear to follow a clear trend, potentially due to competing reactions and the high level of uncertainty in determining these values.

As neither the protein nor the lipid models produce notable amounts of solid, the reduction in solid production must be related to lower yields from the carbohydrate fraction present in the algae (20 wt%). This may be a result of catalytic activity from inorganic compounds present in the algal ash phase, such as K, Mg and C. Potassium, in particular, could help to catalyse the decomposition of carbohydrates into lighter reaction products (Jazrawi et al., 2013). As the carbohydrate model, corn flour is virtually ash free (Table 1), the catalytic effect is not present in the conversion of the model compound on its own.

The increase in oil yields could be related to a simple shift in carbohydrate products from the solid to the biocrude phase, however this is unlikely as oil yields obtained from corn flour were very small, and most non-solid products appeared to partition into the water phase. Instead, the increase in oil yields could result from the reaction of protein and carbohydrate breakdown products, as previously suggested. In this case, the nitrogen distribution to the crude oil phase would be expected to increase in comparison with pure protein.

The elemental distribution was calculated from the elemental analysis for the products obtained from the HTL of *Spirulina* (Figure 2b). Both the carbon and hydrogen retention to the bio-oil are significantly higher than the nitrogen retention, with a visible increase as the temperature is increased from 320 to 340 °C. This demonstrates that HTL is effective in concentrating carbon and hydrogen towards the oil phase, increasing the carbon and hydrogen concentration from 46.3 % and 7.4 %, respectively, in the microalgae to a maximum of 71.6 % and 9.4 %, respectively, (at 340 °C) in the HTL oil product.

The nitrogen retention from the HTL of *Spirulina* to the biocrude is approximately twice that of soy protein (21.0 % *vs.* 9.6 % at 340 °C), indicating that increased retention of the protein fraction in the presence of other biochemical products, such as carbohydrates, could indeed play a role in the increase in oil yields. Lastly, energy recoveries to the crude oil phase (not shown) range from 41 % to 49 %, with the highest value obtained at a reaction temperature of 340 °C. This is consistent with the highest oil yield of 36.9 % obtained at this temperature. Consequently this temperature was used for all further HTL reactions.

3.3.2 Liquefaction of *Spirulina*/PHB mixtures

So far, the results have provided further evidence on the occurrence of Maillard type reactions between amino acids and sugars to help increase oil yields, as indicated by an increased nitrogen content in the oil phase. However, the results do not provide any information on the interaction of PHB with other algal compounds, as *Spirulina* does not contain appreciable levels of these biopolymers.

To investigate this interaction, *Spirulina* was liquefied in the presence of varying levels of PHB (5 to 20 % (w/w)), and the mass balance results were compared to those of the pure substances (Figure 3a). The bright red lines represent the theoretical yields based on weighted averages obtained from the yields from the pure substances.

Although some differences can be observed in the actual and expected gas and water residue yields, the maximum differences of 5.8 % for the gas phase and

6.5 % for the water residue phase are much less than the differences obtained from the conversion of biochemical models and *Spirulina* (up to 16.4 %). More importantly, the deviation in the oil and solid yields is almost negligible, and given the high uncertainty in the gas and water residue yield measurements, based on the overall product distribution, it does not appear as if a significant interaction occurs between PHB and other biochemical compounds during the HTL process.

To further investigate any potential interactions between PHB and the biochemical compounds in *Spirulina*, the carbon dioxide and propylene yields were calculated from the sum of overall gas yields and gas composition (Figure 3b). Again, the theoretical yields, calculated on an additive basis from the pure compounds, are displayed as bright red markers. Most of the theoretical values are within the error of the actual gas yields, with the exception of the CO_2 yield for a 5 % PHB loading and the propylene yield at a 20 % loading. There is however a clear increase in propylene yield as the PHB content increases, with a yield of almost 3 % at a PHB loading of 20 %. Given the large uncertainty in gas yields at 0 and 100 %, upon which the theoretical values are based, it can be concluded that the trend observed in this chart suggests that no interaction between PHB and the biochemical compounds present in *Spirulina* occurs during HTL.

3.4 Liquefaction of PHB containing microalgae

To verify the results from the PHB/*Spirulina* conversion studies, the *Synechocystis* and *Synechococcus/Anabaena* samples were converted under

equivalent HTL conditions. All three cyanobacteria samples produced significantly higher solid yields than those obtained for *Spirulina*, with a maximum solid yield of 22.9 % recovered for the *Synechococcus/Anabaena* sample (Figure 4a). TGA analysis revealed that the solid product from the two *Synechococcus/Anabaena* samples contained an organic (combustible) content of 35.4 % and 35.9 %, whereas the solid produced from *Synechocystis* contained 89.7 % of combustibles. These values were consistent with elevated levels of carbon in the solid fraction (12.3 % for *Synechococcus/Anabaena*, 15.8 % for *Synechococcus/Anabaena*–N and 38.9 % for *Synechocystis*). These results suggest a significant partitioning of organic compounds into the solid phase, similar to the results obtained from the carbohydrate model, corn flour.

The carbohydrate content of *Synechocystis* is approximately twice that of *Spirulina,* 40.5 % compared to 20.0 %. This is presumably due to the high solid yield formed from this algae, combined with its high carbon and organic content. In contrast, the carbohydrate content of the *Synechococcus/Anabaena* samples were very similar to *Spirulina*, and despite this, the solid yield was increased significantly. Interestingly, the ash content of the mixed *Synechococcus/Anabaena* samples was approximately 3 to 4 times higher than for *Spirulina,* and for the conversion of *Spirulina* it was found that the catalytic properties of the ash might have helped to convert carbohydrates and reduce the solid content. However, it is possible that in this case increasing the ash content above a certain level has no further beneficial impact, but catalyses coking reactions, or binds some of the heavy oils, preventing them from being extracted with chloroform.

Bio-oil yields from the three cyanobacteria samples were reduced compared to *Spirulina*. However, whilst oil yields from the two *Synechococcus/Anabaena* samples were still in a similar region (31.2 % and 32.0 % compared to 36.9 % from *Spirulina*), oil yields from *Synechocystis* were drastically reduced to only 16.6 %. All oils contained a similar carbon and hydrogen content, ranging from 69.8 % to 71.6 % and 8.6 % to 9.5 %, respectively, whereas the nitrogen content of the cyanobacteria-based oils (4.6 – 6.1 %) was noticeable lower than for *Spirulina* (7.1 %; data included in supplementary information).

The oil with the lowest nitrogen content of 4.6 % was obtained from the HTL of *Synechocystis*, which itself had the lowest N content of all converted cyanobacteria species (3.5 %, see Table 1). For the two *Synechococcus/Anabaena* samples, the one grown under nitrogen depleted conditions, produced an oil with a noticeably lower nitrogen content than that grown under normal growth conditions. These results suggest that cyanobacteria grown under nitrogen-starved conditions could potentially produce higher quality HTL oils than protein producers such as *Spirulina*, albeit with reduced product yield.

Water residue yields from the two *Synechococcus/Anabaena* samples appeared similar to those obtained from *Spirulina*, but the yields from *Synechocystis* were almost 50 % lower. In contrast, the carbon content of the water phase residue was by far the highest for *Synechocystis* (68.4 %), compared to 40.3 % for *Spirulina*, and only 12.4 and 15.8 % for the two *Synechococcus/Anabaena* samples. Nitrogen and hydrogen concentrations were lowest in the residue from

the *Synechococcus/Anabaena* samples, whereas *Synechocystis* produced the lowest concentration of ammonia, correlating to an ammonia yield of only 0.6 %, compared to 5.7 % for *Spirulina*. It is interesting to note that conversion of the *Synechococcus/Anabaena* samples results in a very high degree of mass balance closure (around 95%), whereas the mass balance closure for *Synechocystis* is low at only 70 %. This suggests the presence of high amounts of light organic compounds, soluble in the water phase, which may help explain the low water phase residue yield obtained from this species.

Gas yields from the alternative cyanobacteria were noticeable higher than for *Spirulina*, which is desirable for the production of volatile organic compounds. Compositional analysis shows that all selected cyanobacteria produce significantly higher concentrations of VOCs than *Spirulina* (Figure 4b). However, whilst *Synechocystis* favours the formation of propylene (12.1 %), the major VOC product from *Synechococcus/Anabaena* is ethylene with a contribution of 7.2 % for the nitrogen depleted sample. These findings are consistent with the PHA analysis, which detected a PHB content of 7.5 % in *Synechocystis*, but no PHB in either of the two *Synechococcus/Anabaena* samples.

The findings suggest that propylene is formed from PHB only. Based on gas yields, the overall propylene yield from *Synechocystis* can be calculated at 2.6 %, very similar to those obtained from the PHB/*Spirulina* mix for a 20 wt% PHB loading. The discrepancy between the measured PHB content (7.5 %), and the higher than expected propylene yield could have multiple causes, including differences in the catalytic effect of the ash present in *Spirulina* and

Synechocystis, or differences in the polymeric composition and chain length between model PHB and PHB present within the algae. Therefore, PHB containing microalgae represent a viable feedstock for the production of a low nitrogen oil together with a valuable gas by-product.

4 Conclusion

The potential of using HTL for the conversion of cyanobacteria into a crude oil with reduced nitrogen content, and a high value gas by-product was demonstrated. In contrast to cross-reactions between protein and carbohydrate intermediates, which impact on product quality, no synergistic effects were detected when converting PHB in the presence of algal biomass.

Out of the three potential PHA producers, only *Synechocystis* sp. was found to accumulate significant levels of PHB. In agreement with the model compound experiments, the conversion of this species resulted in the formation of elevated amounts of propylene, a valuable precursor for the polymer industry.

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Figure Captions

Table 1: Proximate and ultimate analysis and energy content of model compounds and cyanobacteria used in this study

Scheme 1: PHB decomposition pathway to propene and carbon dioxide

Figure 1: Results from HTL of model compounds representing biochemical compound classes present in cyanobacteria; A) Product distribution; B) Gas compositions, C) Energy recoveries

Figure 2: Results from the HTL of *Spirulina*; A) Difference between actual yields and theoretical yields, calculated based on yields from model compounds and composition of *Spirulina*; B) Elemental distribution to the different product phases

Figure 3: Results from the HTL of *Spirulina* / PHB mixtures; A) Product distribution; B) Absolute yields of major gas compounds. The red markers indicate theoretical yields, based on a weighted average of the yields obtained from the conversion of the pure substances.

Figure 4: Results from the HTL of PHA-containing cyanobacteria; A) Product distribution; B) Gas composition of the VOC phase, where SA – *Synechococcus/Anabaena*; SA-N – *Synechococcus/Anabaena-N*; SYN – *Synechocystis*