

Subcritical Hydrothermal Liquefaction of Microalgae Residues as a Green Route to Alternative Road Binders

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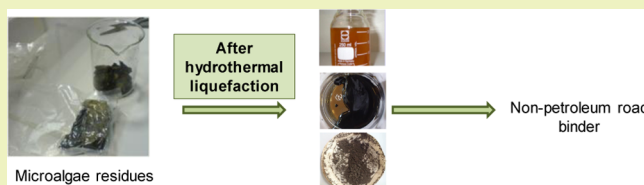
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Supporting Information

ABSTRACT: The valorization of *Scenedesmus* sp. microalgae byproducts was investigated, as a potential route for the production of road binders from renewable sources. Under hydrothermal liquefaction conditions, a water-insoluble viscous material was obtained in a ca. 55% yield, which consists of an oily fatty acid-based fraction mixed with organic and inorganic solid residues (up to 22%). Although the chemical composition of the obtained materials completely differs from that of petroleum-based bitumen, similar viscoelastic properties were observed in some cases, depending on the hydrothermal liquefaction experimental conditions. A rheological simple material could thus be obtained, which compared well with a bitumen reference.

KEYWORDS: Microalgae, *Scenedesmus* sp., Hydrothermal liquefaction, Biomass, Green binder, Rheological properties



INTRODUCTION

Microalgae are being more and more considered of great potential for energy production to reduce dependence on petroleum. The conversion of this biomass feedstock into biodiesel has been investigated for decades, due to significant benefits over other biomass sources (i.e., cellulosic), including low competition for arable land, high per hectare biomass yields, large harvesting turnovers, and the opportunity to recycle wastewater and carbon dioxide as a contribution to sustainable development.

In that context, hydrothermal liquefaction (HTL)^{1–3} has recently been under intense investigation as a route for efficient oil production from microalgae.^{4,5} However, many R&D challenges have still to be addressed before large-scale microalgae production and HTL-mediated conversion to third-generation biofuels. Moreover, the development of this technology is very likely to result in the production of significant amounts of waste (i.e., the nonvalorized solid residues of microalgae), and the present study proposes to explore potential solutions to this issue that might be critical for the commercial viability of the HTL technology. Indeed, in the past recent years, tensions have appeared on the asphalt supply for road construction, because cracking of heavy petroleum

distillation residues to produce gasoline has become economically competitive due to the high level of the crude oil market. Thus, valorization of microalgae residues for the production of biosourced road binders might be attractive, following a concept of integrated biorefinery that could mirror the case of petroleum refinery plants.

Bitumen is a sticky viscoelastic material mostly used to bind aggregate in pavement mixture, and to a lesser extent for waterproofing. During the past decade, sustainable processes to produce nonpetroleum based bitumen have been developed, using various vegetable matter. Biobitumen generally consist of a vegetable oil (i.e., rapeseed) mixed with natural or modified resin and a synthetic polymer, in order to tune their rheological properties.^{6–8} However, the presence of edible oil in the composition of these alternative binders is a major drawback, from a social acceptability viewpoint.

By contrast, the use of microalgae as a biomass source to manufacture bioasphalt is a novel option that was investigated in the present study. The major scientific issue is to obtain a

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material capable to mimic the particular properties of bitumen: (i) liquid above 100 °C, it can coat mineral aggregates; (ii) viscoelastic between -20 and +60 °C, it allows to ensure the cohesion of the granular structure, support mechanical loading and relax stresses, at the same time. Although many reports on the HTL of microalgae can be found in the literature, the latter are mostly focused on the potential of this method for transportation biofuel production.^{9–12} The present paper demonstrates the high potential of these HTL products as road binders, showing that the rheological behavior of the isolated hydrophobic material is strongly influenced by the HTL operating conditions. Viscoelastic properties similar to that of petroleum-based bitumen can thus be reached using an appropriate parameters setup, giving evidence that the amount and nature of the solid residues in the isolated crude oil play a critical role on the rheological behavior.

MATERIALS AND METHODS

Feedstock. *Scenedesmus* sp. microalgae for which the major part of water-soluble proteins was removed by centrifugation were purchased from Alphabiotec, Asserac, France. This biomass, containing about 80 wt % of water, was frozen for storage, and then freeze-dried for 1 week at -90 °C before use.

Soxhlet Extraction of the Biomass. Lipids present in the biomass were extracted by a Soxhlet extraction. In a typical procedure, 15 g of the freeze-dried microalgae residues was placed in a Soxhlet extraction thimble and the solvent (90 mL of a mixture of chloroform and methanol (2:1, v/v)) was placed in the round-bottom flask. The continuous extraction process was performed for 24 h. The solvent was then evaporated under reduced pressure and the yield of extraction was then measured from the mass of the evaporated oily residue. Sonication of this residue in CHCl₃ led to the separation of a solid fraction insoluble in CHCl₃ that was isolated by filtration.

HTL Experiments. The HTL experiments were performed using two types of equipment, for which no stirring system was available: (i) A 50 mL batch reactor (Model 4792, Parr Instrument, Equilabo Company, Caluire et Cuire, France) that consisted of a stainless steel cylinder, a thermocouple temperature probe, and a flexible aluminum heating mantle with a maximum operating temperature of 350 °C and a maximum pressure of 20 MPa. The heating rate was approximately 5 °C min⁻¹. (ii) A tailor-made 1 L batch reactor (Sacmo Company, Couëron, France) that consisted of a stainless steel cylinder, with heating elements included inside the walls, a maximum operating temperature of 400 °C and a maximum pressure of 19 MPa. The heating rate was approximately 15 °C/min. Before reaction, both reactors were flushed with nitrogen to purge the residual air.

In a typical experiment using the 50 mL reactor, 41.5 g of freeze-dried microalgae residues was suspended in deionized water (4 mL/g), with selected experimental conditions: reaction temperature (250–300 °C), holding time at temperature setpoint (0–60 min), N₂ initial pressure (0.8–50 bar).

After reaction, the system was allowed to cool down, the gaseous products were purged, and the remaining content was poured on a Büchner funnel. The residue was then washed with CH₂Cl₂ until the washing solution was colorless. Similar washings were then performed with water, and the remaining solid was finally dried in an oven at 80 °C. The aqueous and organic layers were separated by decantation, and the organic layer was dried over MgSO₄ and evaporated under reduced pressure. The volume of the aqueous layer was reduced by 75% under reduced pressure, and the sample was then freeze-dried.

In a typical experiment using the 1 L reactor, microalgae (130 g) were suspended in deionized water (4 mL/g) and charged in the autoclave. The autoclave was heated for 1 h at the selected reaction temperature (260, 280, or 300 °C), and then allowed to cool. The gas phase was collected at 40 °C for gas chromatography with thermal conductivity detection (GC-TCD) analysis while the aqueous phase was pumped out at room temperature. The water-insoluble fraction was then removed from the reactor using a spatula, the remaining

residues were collected by rinsing the autoclave with toluene, and the washings were combined with the viscous solid phase. The resulting mixture was finally concentrated under reduced pressure.

Analysis of Lipids by GC-FID and Gas Chromatography–Mass Spectrometry (GC–MS). The total lipid extracts were esterified using an adapted procedure.¹³ 12 mL of boron trifluoride in methanol (14 wt %) were added to 200 mg of the lipid extract. The mixture was heated in a sealed tube at 90 °C overnight. The resulting fatty acid methyl esters (FAMES) were extracted using hexane, washed with water, and dried over MgSO₄. The solvent was removed under vacuum at 40 °C, and FAMES were dissolved in isopropyl alcohol for their analysis by gas chromatography.

GC analyses were performed on an Agilent 6890 apparatus equipped with a flame ionization detector and a BPX70 capillary column (70% cyanopropyl dimethylpolysiloxane, 30 m × 0.32 mm ID, 0.25 μm film thickness). Helium was used as carrier gas at 1.3 mL/min. The oven temperature was 120 °C, held for 4 min, raised to 220 °C at a 6 °C/min rate, and held at 220 °C for 5 min. It was then raised to 250 °C at a 15 °C/min rate, and held at 250 °C for 18 min. The injector and detector temperatures were set at 250 °C. The identification of fatty acids was performed by comparison with the retention time of standards.

GC–MS analysis¹⁴ was performed on a ThermoScientific DSQ II Single Quadrupole GC/MS in electron impact ionization mode (EI), equipped with an auto-sampler (AS3000, ThermoScientific) and with a Gaz chromatograph (TRACE-GC Ultra, ThermoScientific). A TR-SMS nonpolar capillary column (30 m length, 0.25 mm I.D., 0.25 μm film thickness) separated the constituents. A volume of 2 μL was injected for each sample. The injector temperature and split ratio were 240 °C and 1:15, respectively. The oven temperature was programmed to hold from 80 to 300 °C, with a ramp at 3 °C/min and hold at 300 °C for 10 min. The flow rate of He carrier gas was 1 mL/min. The samples for GC–MS analysis were prepared as solutions in dichloromethane. Compounds were identified by comparison of their mass spectra with the NIST library.

Fourier Transform infrared (FT-IR) and NMR Analysis. FT-IR spectra were recorded on a Bruker IR-TF Vector 22. Oily samples were dissolved in CCl₄ and placed between two KBr plates for the acquisition. A KBr pellet was used for the analysis of solid samples (1 wt % in KBr).

Liquid-state ¹H and ¹³C spectra were recorded in deuterated solvents on a Bruker Avance apparatus operating at 300 or 400 MHz.

Solid-state ¹H and ¹³C magic angle spinning (MAS) NMR experiments were performed on a Bruker Avance III spectrometer operating at 20.0 T (¹H and ¹³C Larmor frequencies of 850 and 213.8 MHz, respectively) using a 3.2 mm triple-resonance MAS probe. The ¹³C quantitative MAS spectra were recorded with π/8 flip angle (pulse length of 1.2 μs) and a recycle delay of 9 s. ¹³C-{¹H} cross-polarization (CP) MAS spectra were recorded with a contact time of 2 ms and a recycle delay of 1 s.¹⁵ The ¹³C-{¹H} refocused INEPT MAS experiments were performed using echo delays of 2 ms and a recycle delay of 1 s. All ¹³C spectra were acquired with ¹H SPINAL-64 decoupling¹⁶ (¹H nutation frequency of 70 kHz) at a spinning frequency of 15 kHz.

Elementary Analysis. The C, H, N, and S content of the samples were measured using a ThermoScientific FLASH 2000 Series CHNS/O Analyzer.

DSC. DSC analyses were performed on a Netzsch DSC 204 device. Aluminum sample pans were used for the classic analysis, while 10 μm gold coated sealed pans (217 MPa, 400 °C) from the TÜV SÜD Company were used to mimic the conditions of the hydrothermal liquefaction. In the first case, around 20 mg of the material were placed into the sample pan. The oven was heated from -25 to +125 °C, at a 2 °C/min speed. In the second case, 3 mg of microalgae and 12 μL of water were placed into the sample pan that was then sealed and heated from 25 to 300 °C at a 2 °C/min rate. Then, the temperature was held at 300 °C for 1 h. After the sample was cooled at 25 °C, the same heating procedure was repeated.

Rheological Properties. The complex modulus and phase angle of the samples were measured with a Metravid viscoanalyser DMA

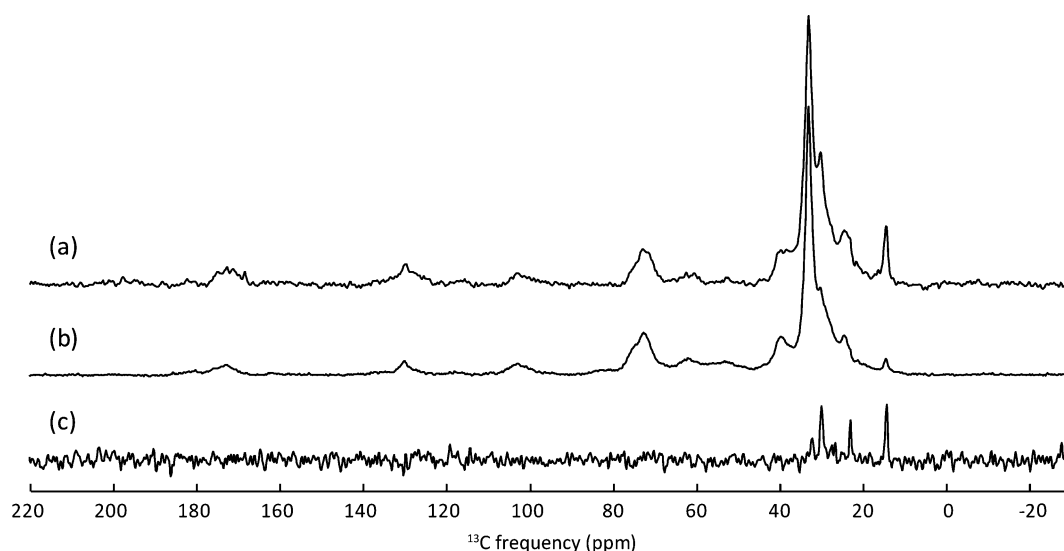


Figure 1. (a) ^{13}C MAS, (b) $^{13}\text{C}\{-^1\text{H}\}$ CP-MAS, and (c) $^{13}\text{C}\{-^1\text{H}\}$ refocused INEPT MAS NMR spectra of the chloroform insoluble fraction of the Soxhlet extract of *Scenedesmus* sp. residues.

+450, in the shear mode, within a 1–125 Hz frequency range and a 10–80 °C temperature range.

RESULTS AND DISCUSSION

To investigate the potential of microalgae residues to produce bioasphalt, an industrial source of waste based on *Scenedesmus* sp. was supplied by the Alphabiotec Company. The biomass used in this study consisted of a wet paste, containing about 80 wt % of water, obtained after extraction of water-soluble proteins from freshly harvested microalgae, and subsequent separation from the solid residues, by centrifugation. Using classical methods from the literature, the amount of polysaccharides¹⁷ and proteins¹⁸ (ca. 30 and 21 wt %, respectively) in the freeze-dried biomass were measured, showing that only part of the proteins is removed during the industrial extraction procedure.

Analysis of the Biomass Treated by Soxhlet Extraction. At first, given that petroleum-based road binders are viscoelastic hydrophobic materials, the content of hydrophobic compounds in the microalgae residues was assessed via a classical Soxhlet extraction of the freeze-dried biomass.¹⁹ After evaporation of the solvents, a black and viscous oil was obtained in a 24 ± 2 wt % yield, based on the dry biomass. Attempts at recording ^1H NMR data of that oil dissolved in deuterated chloroform showed that part of the sample was not soluble. After removal of the insoluble part by filtration, the ^1H NMR spectrum showed peaks specific to free fatty acids (Figure S1 in the Supporting Information).

Surprisingly, the absence of resonance (i) in the 4–4.5 ppm range in the ^1H NMR spectrum (ii) in the ^{31}P NMR spectrum gave evidence that no triglyceride nor phospholipid were present in the sample. Given that lipids in microalgae are known to accumulate as triglycerides, the presence of free fatty acids very likely results from the hydrolysis of triglycerides by lipases. The activity of these enzymes might have been preserved after centrifugation and freezing, thus promoting the cleavage of triglyceride ester groups, because Ryckebosch et al. have reported that only addition of isopropanol or freeze-drying are able to deactivate lipases.²⁰

Six saturated fatty acids from C10:0 to C20:0 were detected by GC-FID, with a predominance of palmitic acid (C16:0),

along with nine unsaturated fatty acids from C16:1 to C22:1, with a predominance of oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). The presence of other fatty acids in smaller quantities, from C24 to C28 (saturated or unsaturated), was also evidenced by GC–MS. The fatty acids identified in the Soxhlet extract (Table S1 in the Supporting Information) were in good agreement with the literature on *Scenedesmus* sp.^{21–23}

As mentioned previously, after evaporation of the clear Soxhlet extraction solution, part of the resulting oil did not resolubilize in chloroform or any common polar solvent. The insoluble fraction was thus isolated by filtration and dried, and this component was found to represent about 23 wt % of the extracted bio-oil (i.e., 5.5 wt % of the dry initial biomass), meaning that the content of lipids was 18.5 wt % of the dry initial biomass. Analysis of this solid phase by FT-IR spectroscopy showed two absorption bands characteristic of an aliphatic-rich compound (2916 and 2850 cm^{-1}) in addition to the presence of carbonyl groups (intense broad absorption band in the 1720–1640 cm^{-1} range) (Figure S2 in the Supporting Information).

The FT-IR data suggest that this solid component is a high molecular weight biopolymer, based on an aliphatic polyester/polyether network and referred to as algaenan in the literature.^{24–33}

This assumption was in good agreement with the quantitative ^{13}C MAS NMR spectrum, which showed intense and broad resonances centered around 30 ppm assigned to $(\text{CH}_2)_n$ chains present in large amounts in the biopolymer backbone (with terminal methyl groups observed at 14.5 ppm) as well as carbon atoms in α to an oxygen (ca. 70 ppm), carbon–carbon saturations (130–140 ppm), and carbonyl functions (ca. 170 ppm) (Figure 1). ^{13}C CP-MAS and refocused INEPT experiments were also performed to identify the signals of the rigid and mobile molecular segments of the biopolymer, respectively.^{34,35} Indeed, all the above-mentioned broad ^{13}C resonances are observed in the CP-MAS spectrum whereas the refocused INEPT MAS NMR spectrum only show weak signals in the 14–32 ppm range, giving evidence of a highly cross-linked rigid polymer.

Table 1. Experimental Data Related to the HTL of *Scenedesmus* sp. Residues in a 1 L Autoclave

entry	amount of biomass loaded	130 g of microalgae suspended in 520 mL of water		
1	sample name	IF260	IF280	IF300
2	reaction temperature (°C)	260	280	300
3	time to reach temperature setpoint (min)	12	12	16
4	internal pressure (bar) at temperature setpoint	55	68	98
5	internal pressure (bar) at room temperature at the end of the HTL reaction	18.8	18.7	18.2
6	main components in the gas fraction	CO ₂ (90.3%) CO (1.5%)	CO ₂ (91.7%) CO (2.0%)	CO ₂ (89.2%) CO (1.5%)
7	yield for the gas fraction (%)	8.2	8.2	8.0
8	yield for the water-soluble fraction (%)	21	19	17
9	yield for the water-insoluble fraction (dichloromethane soluble fraction + insoluble solid residues) (%)	50	53	61
10	% of solid residues insoluble in dichloromethane, in the water-insoluble fraction	22	12.3	5.4
11	% of solid residues insoluble in both dichloromethane and HCl, in the water-insoluble fraction	7.3	2.1	1.1
12	elemental analysis of the dichloromethane soluble fraction (wt %)	C (68.4), H (8.2), N (4.5)	C (67.5), H (8.3), N (4.3)	C (72.6), H (9.3), N (4.6)

To have more information about structural features of this very stable algaenan, various chemical hydrolysis have been performed in the literature using for example hydrogen iodide and sodium thiomethoxide,^{30,31} or boron trifluoride etherate.^{36,37} Ruthenium tetroxide-mediated oxidative degradation has also been proposed,^{30,31,38,39} as well as pyrolysis at high temperatures (between 400 and 600 °C).^{29–31} In our study, ruthenium tetroxide was found to be one of the most powerful option, allowing the cleavage of 35% of the algaenan fraction to form various carboxylic acid fragments that were subsequently derivatized and analyzed by GC–MS. A complex mixture of esterified fatty monoacids (based on linear and poly-methylated saturated chains made of 11 to 20 carbon atoms) and α,ω -diacids was thus identified (for some examples, see Table S2 in the Supporting Information). These fragments can result from the cleavage of alkene, ether, and/or ester functions present in the algaenan backbone. It is very likely, however, that only one part of the algaenan component is extracted using the Soxhlet experiment, due to its low solubility, and therefore that the algaenan content in the crude biomass is higher.

Given these preliminary promising results, subcritical hydrothermal liquefaction (HTL)^{40,41} was considered to produce biobinders because this process offers significant advantages: (i) A wet biomass can be directly used, (ii) water acts as a solvent and reagent, (iii) the microalgae microstructure is expected to be degraded to release chemical building blocks that can react under HTL conditions, and (iv) the hydrophilic fraction can undergo chemical reactions, including oxidation/decarboxylation and dehydration, which results in the deoxygenation of that fraction, thus increasing the biocrude oil yield.

Although HTL has been largely studied to form biofuels from different species of microalgae,^{42–47} to the best of our knowledge, the potential of this process for the production of biobitumen has never been investigated. In addition, this process will allow to produce water-insoluble liquefaction products in sufficient amount for a detailed investigation of how their rheological properties compare with the different grades of bitumen used as aggregates binder for paving.

Subcritical Hydrothermal Liquefaction of the *Scenedesmus* sp. Biomass. Influence of Experimental Conditions on the Distribution of Liquefaction Products. To select the experimental conditions for hydrothermal liquefaction, preliminary tests were performed using differential scanning calorimetry (DSC).

The DSC plot (Figure S3 in the Supporting Information) in the 25–300 °C range showed a nonreversible exothermic peak around 220 °C, likely due to the hydrolysis of polysaccharides. Then, hydrothermal liquefaction of the biomass was first investigated using a 50 mL autoclave. Freeze-dried microalgae were suspended in water, while varying the reaction temperature (250–300 °C), the holding time at temperature setpoint (0–60 min), and the N₂ initial pressure (0.8–50 bar). The use of a central composite experimental design,⁴⁸ generated thanks to the Statgraphics Centurion XVI software, allowed us to reduce the number of needed experiments from 125 to 20, performed in a random way (Table S3 in the Supporting Information). For each experiment, after evacuation of the gas phase, the yield of the water-soluble fraction, the dichloromethane soluble fraction, and the insoluble solid residues were measured, showing average values of 18 ± 1 , 41 ± 1.7 , and $13 \pm 1.7\%$, respectively, for the set of center points ($T = 275$ °C, initial N₂ pressure = 10 bars, hold time = 30 min, 6 replicates). This corresponds to an average total isolated yield of 72% and the difference in the mass balance is likely due to gas formation during the HTL in addition to evaporation of volatile compounds and losses during isolation of the reaction products (extraction, filtration).

The hydrophobic oily component (bio-oil), which corresponds to the soluble fraction in dichloromethane, could be obtained in a maximum yield of 45%, at a temperature of 275 °C held for 30 min, under a 50 bar N₂ initial pressure. Statistical analysis of the results gave evidence that both N₂ initial pressure (that leads to higher values for the reaction pressure at a given temperature, see Table S3 in the Supporting Information) and reaction temperature had a significant positive effect on the yields of the dichloromethane soluble fraction, at a 95% confidence level. On the contrary, the hold time (0–60 min) at temperature setpoint (250–300 °C) had no significant influence, but it is important to note that ca. 20 min are necessary for a 200 to 300 °C temperature variation of the reaction medium in the autoclave during the heating period and 15 min for a 300 to 200 °C temperature variation during the cooling period.

Interestingly, the yield of products insoluble in water (dichloromethane soluble fraction + insoluble solid residues) was found to be constant ($54 \pm 3.6\%$) with a linear relationship between these two fractions, i.e., a decrease of the amount of isolated solid residues results in a proportional increase of the

formation of the dichloromethane soluble fraction (Figure S4 in the Supporting Information).

A scale-up of the process was then investigated using a 1 L autoclave equipped with an internal heating system, with a reaction time fixed to 60 min. The equipment did not allow us to apply any initial nitrogen pressure. Three reaction temperatures (260, 280, and 300 °C) were studied, and the autoclave was loaded with freeze-dried microalgae in water. Satisfactory repeatability of the experiments was obtained when using different batches of microalgal residues. Analysis by GC-TCD of the composition of the gas phase at the end of the HTL process showed the presence of carbon dioxide as the major compound (ca. 90%, see Table 1, entry 6), with small amounts of carbon monoxide (1.5–2%) and short saturated and nonsaturated hydrocarbons (C1 to C4, typically 0.1–0.3%). The yield of the gas fraction was assessed from the residual gas pressure in the autoclave, after reaction (ca. 8%, entry 7). It is noteworthy that the collected hydrophobic fraction was isolated with yields similar to those observed in the case of the 50 mL autoclave (ca. 55%, see Table 1, entry 9). Finally, the yield of the water-soluble fraction was ca. 20% (Table 1, entry 8). The estimated cumulative yield is thus ranging between 80 and 85%, and the difference in the mass balance is likely due to evaporation of volatile compounds when concentrating the water-soluble and insoluble fractions, in addition to losses during isolation of the reaction products (extraction, filtration).

Chemical Analysis of the Liquefaction Products. The crude HTL fraction insoluble in water was treated with dichloromethane, showing the presence of insoluble solid residues that were isolated by filtration. Energy dispersive X-ray (EDX) analysis of the solid showed calcium as the main inorganic element present, likely as calcium carbonate and calcium hydroxide (Figure S5 in the Supporting Information). Indeed, calcium carbonate is used to control the pH of the culture medium of the microalgae, and part of it is trapped in the harvested microalgae during the isolation process, in a ca. 9 wt % (relative to the freeze-dried biomass) measured by calcimetry. This was also confirmed by FT-IR spectroscopy showing characteristic absorption bands of calcium carbonate at 1430 and 875 cm^{-1} , respectively (Figure S6 in the Supporting Information).

These calcium species were thus removed by treatment of the solid residues with 6 M HCl overnight, leading to a carbon-rich brown powder (Figure S5 in the Supporting Information), the IR spectrum of which was similar to that of algaenan isolated from the Soxhlet extraction of *Scenedesmus* sp. residues (Figure S6 in the Supporting Information). This was also confirmed by ^{13}C MAS and CP-MAS NMR spectra (Figure S7 in the Supporting Information) showing resonances characteristic of aliphatic chains (ca. 15, 30, and 33 ppm) along with broad signals in the 100–140 ppm range and at about 170 ppm assigned to insaturations present in the polymer backbone and carbonyl functions, respectively, as observed for algaenan. Interestingly, it was found that the relative amount of algaenan in the solid residues significantly decreased while increasing the HTL temperature, as a result of a probable cracking of the biopolymer (Table 1, entry 11).

On the other hand, the dichloromethane solutions mentioned above were evaporated, leading to black oils. Whatever the volume of the autoclave used for the HTL process, the IR spectra were found to be similar for all experiments and consistent with those reported in the literature

for oils obtained by HTL of other microalgae species.^{49,50} The main observations were intense absorption bands at 2900 and 2850 cm^{-1} that denoted the presence of a highly aliphatic component, in addition to a significant absorption band at 1678 cm^{-1} related to carbonyl-containing compounds. This observation was consistent with the elemental analysis of these oils (Table 1, entry 12).

Qualitative GC–MS analysis of these oils (see Figure S8 in the Supporting Information) gave evidence of a complex mixture with two main families of products: (i) Aromatic compounds, mainly based on phenol and indole derivatives; (ii) Highly aliphatic compounds, including (a) long chain alkenes likely resulting from the decarboxylation of fatty acids (b) free fatty acids and their amide derivatives. As mentioned previously, ca. 21 wt % of proteins are present in the biomass used in this study. The HTL process results in the hydrolysis of those proteins, releasing ammonia, amines and amino acids that form condensation products with the lipid fraction.

In summary, the global composition of the water-insoluble fraction obtained by HTL in this study was quite similar to that reported in the literature for other microalgae.^{14,49,51–53}

Finally, GC–MS qualitative investigation of the composition of the water-soluble fraction extracted with *n*-butanol indicated the presence of a complex mixture, containing volatile compounds (mainly substituted cyclic compounds, including ketones, pyrroles, pyridines, and pyrazines), lipids (mainly containing 14 to 22 carbon atoms) and a large variety of cyclic amino acid dimers.

Rheological Properties of the Insoluble Liquefaction Products. To assess the potential of water-insoluble HTL fractions as a substitute of petroleum-based bitumen classically used as a binder for aggregates in pavements, their rheological properties were investigated. In the case of standard petroleum bitumen, these properties strongly vary as a function of the temperature and loading time: a relatively high stiffness at low temperature and a Newtonian behavior at high temperature. Between 0 and 60 °C, standard bitumen shows a behavior between a liquid and a solid, commonly called the viscoelastic state. This physical state allows the material to support mechanical loading and relax stress, at the same time.

The linear viscoelastic properties of water-insoluble HTL fractions prepared in the 1L autoclave (IF260, IF280 and IF300, Table 1) were evaluated by measuring the complex modulus $E^* = E_1 + iE_2 = |E^*|e^{i\delta}$, which consists in recording the sinusoidal stress response to a sinusoidal strain. The ratio between the two amplitudes is the norm of the complex modulus $|E^*|$, the phase lag between both sinusoids corresponds to the phase angle δ . For a liquid, $\delta = 90^\circ$, while for a perfect elastic body, $\delta = 0^\circ$. Between these extreme values, the material is in the viscoelastic-state.

Tests were performed within temperature and frequency ranges that are in agreement with actual road binders loading conditions. Figure 2 shows the norm of the complex modulus (at 1 Hz) versus temperature, for the water-insoluble fractions IF260, IF280, and IF300 compared to standard bitumen. For all the materials, the stiffness decreased dramatically with temperature, while the HTL fractions showed a lower modulus than bitumen in the cold temperature range ($T \sim 5^\circ\text{C}$). However, in the high temperature range, IF260 and IF300 exhibit higher or similar modulus. In fact, the water-insoluble HTL fractions appear to be less sensitive to temperature than bitumen.

The phase angle at 1 Hz for the IF260, IF280, and IF 300 samples in comparison with a standard bitumen is shown in

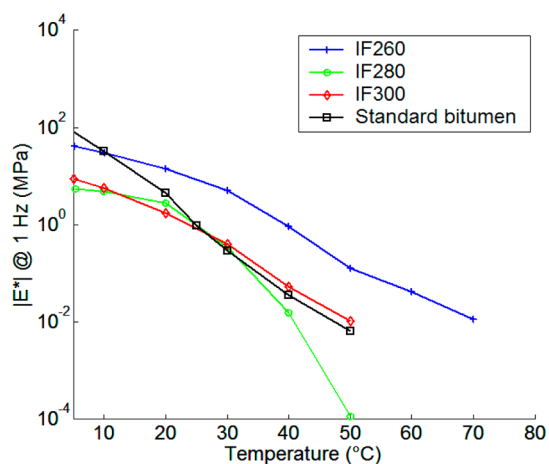


Figure 2. Norm of the complex modulus versus temperature at 1 Hz, for the water-insoluble HTL fractions (IF260, IF280, IF300) compared to a standard bitumen.

Figure S9 in the Supporting Information. In the cases of IF280 and IF300, the phase angles at 1 Hz remained below 40° , whatever the temperature. At this frequency, these materials behave more like an elastic solid than a liquid, showing a poor ability to relax stress. On the contrary, the phase angle of IF260 resembles that of a standard bitumen. It increases from 20° to 90° , from a quasi-elastic state to a totally liquid state, even though the temperature to reach 90° are different: 40°C for a standard bitumen and 70°C for the IF260 sample.

Figure 3 shows the viscoelastic data of the water-insoluble HTL fractions compared to a standard bitumen, plotted in a

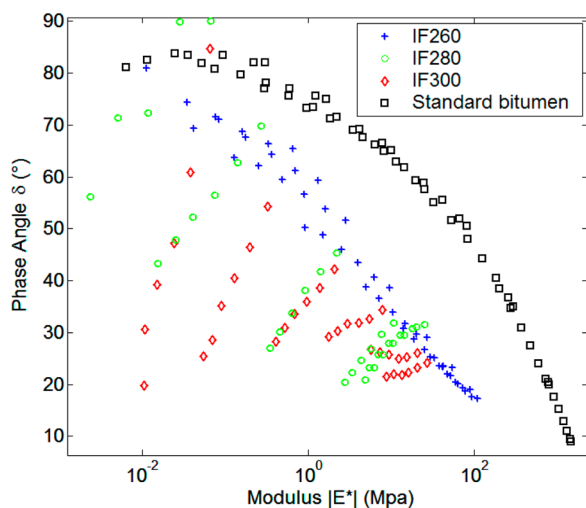


Figure 3. Complex modulus of the water-insoluble HTL fractions (IF260, IF280, IF300) compared to a standard bitumen, plotted in the Black space.

Black diagram (i.e., phase angle versus norm of the complex modulus measured at several temperatures and frequencies). In the Black diagram, the overall viscoelastic behavior is described in one graph and corresponds to the rheological signature of the materials in the linear domain. The rheological behavior of both IF280 and IF300 samples markedly differs from that of the IF260 sample, which is close to a simple rheological state, as observed for a standard bitumen. In fact, for these two materials, the isotherms overlap in the Black space showing a

continuity of the curve which means a kind of equivalency between temperature and frequency (i.e., a same viscoelastic state can be found for different temperature/frequency couples). As a consequence, the molecular structure of the IF260 sample appears to be stable with temperature, like a standard bitumen. On the contrary, the IF280 and IF300 samples show isotherms that do not overlap in the Black diagram, giving evidence of molecular rearrangements occurring as a function of temperature.

As mentioned previously, increasing the liquefaction temperature results in a significant decrease of the algaenan content in the HTL water-insoluble fractions (Table 1, entry 11, from ca. 7 to 1 wt %). It is very likely that fractionation of algaenan takes place, leading to a decrease of the mean molecular weight of this biopolymer. This feature seems to be critical since the solid residues probably structure the oily phase to lead to a rheological simple material, with a role similar to asphaltene in standard bitumen. However, assessment of the viscoelastic properties of the solid residues separated from the dichloromethane soluble fraction using dynamic shear rheometry (DSR) were unsuccessful, since algaenan did not melt in the $60\text{--}100^\circ\text{C}$ temperature range. We were also unable to measure its molecular weight distribution as a function of the liquefaction temperature, due to the insolubility of the material in usual solvents.

CONCLUSION

This proof of concept study has demonstrated that the hydrothermal liquefaction of *Scenedesmus* sp. microalgae residues allows us to produce a water-insoluble material, made of a mixture of an oily component and solid residues. Over the temperature range tested ($260\text{--}300^\circ\text{C}$), the HTL experimental conditions notably influence the nature and the amount of solid residues in the final mixture. In the case of HTL run at 260°C , these solid residues seemed to structure the oily phase, leading to a rheological simple material and viscoelastic properties similar to that of petroleum-based bitumen. This is not the case when conducting HTL at higher temperature, since in these conditions the amount of solid residues in the HTL product is significantly decreased.

To confirm that hydrothermal liquefaction can be a general route for the production of biobitumen from microalgal sources, complementary studies are underway to investigate whether diverse biomass species and their residues issued from different kinds of valorization processes can similarly lead to water-insoluble liquefaction products, with suitable rheological properties for their use as road binders. The development of a continuous HTL process will also be considered.

ASSOCIATED CONTENT

Supporting Information

^1H NMR spectrum, FT-IR spectrum and GC-MS identification of fatty acids isolated by Soxhlet extraction from the *Scenedesmus* sp. microalgae residues; example of identified fragments after RuO_4 -mediated oxidative degradation of the chloroform insoluble fraction of the Soxhlet extract of *Scenedesmus* sp. residues; DSC plot of a water/microalgae mixture (4 mL g^{-1}); results from the central composite experimental design used for the HTL of the *Scenedesmus* sp. microalgae residues in a 50 mL autoclave; plotting of the solid residues yield versus the dichloromethane soluble fraction yield; energy dispersive X-ray analysis, FT-IR spectra and ^{13}C MAS NMR spectra of the *Scenedesmus* sp. HTL fraction insoluble in

both water and dichloromethane; GC–MS identification of products identified in the water-insoluble HTL fraction; phase angle versus temperature plot for the water-insoluble HTL fractions compared to a standard bitumen. This material is available free of charge via the Internet at <http://pubs.acs.org/>

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The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Carr, A. G.; Mammucari, R.; Foster, N. R. A review of subcritical water as a solvent and its utilisation for the processing of hydrophobic organic compounds. *Chem. Eng. J.* **2011**, *172* (1), 1–17.
- (2) Kruse, A.; Dinjus, E. Hot compressed water as reaction medium and reactant - Properties and synthesis reactions. *J. Supercrit. Fluids* **2007**, *39* (3), 362–380.
- (3) Toor, S. S.; Rosendahl, L.; Rudolf, A. Hydrothermal liquefaction of biomass: A review of subcritical water technologies. *Energy* **2011**, *36* (5), 2328–2342.
- (4) Chen, W.-T.; Zhang, Y.; Zhang, J.; Yu, G.; Schideman, L. C.; Zhang, P.; Minarick, M. Hydrothermal liquefaction of mixed-culture algal biomass from wastewater treatment system into bio-crude oil. *Bioresour. Technol.* **2014**, *152* (0), 130–139.
- (5) Li, H.; Liu, Z.; Zhang, Y.; Li, B.; Lu, H.; Duan, N.; Liu, M.; Zhu, Z.; Si, B. Conversion efficiency and oil quality of low-lipid high-protein and high-lipid low-protein microalgae via hydrothermal liquefaction. *Bioresour. Technol.* **2014**, *154* (0), 322–329.
- (6) Colas, S. A. Utilisation d'un liant à base de biopolymère dans des applications routières, pararoutières ou liées au génie civil. French Patent FR 2932797 A1, 2009.
- (7) Eiffage. Composition comprenant une fraction organique pour la réalisation d'une couche et/ou d'un revêtement de voie ou de bâtiment. French Patent FR 2915204 B1, 2007.
- (8) Eurovia. Liant synthétique essentiellement à base de matières issues de ressources renouvelables, en particulier d'origine végétale, et ses applications en technique routière. French Patent FR 2955586 A1, 2010.
- (9) Chisti, Y.; Yan, J. Energy from algae: Current status and future trends Algal biofuels - A status report. *Appl. Energy* **2011**, *88* (10), 3277–3279.
- (10) Davis, R.; Aden, A.; Pienkos, P. T. Techno-economic analysis of autotrophic microalgae for fuel production. *Appl. Energy* **2011**, *88* (10), 3524–3531.
- (11) Dragone, G. Fernandes, B.; Vicente, A. A.; Teixeira, J. A. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*; Mendez-Vilas, A., Ed.; Formatex Research Center: Badajoz, Spain, 2010; Vol. 2.
- (12) Pienkos, P. T.; Darzins, A. The promise and challenges of microalgal-derived biofuels. *Biofuels, Bioprod. Biorefin.* **2009**, *3* (4), 431–440.
- (13) Morrison, W. R.; Smith, L. M. Preparation of fatty acid methyl esters + dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* **1964**, *5* (4), 600–608.
- (14) Brown, T. M.; Duan, P. G.; Savage, P. E. Hydrothermal liquefaction and gasification of *Nannochloropsis* sp. *Energy Fuels* **2010**, *24*, 3639–3646.
- (15) Metz, G.; Wu, X. L.; Smith, S. O. Ramped-amplitude cross-polarization in magic-angle-spinning NMR. *J. Magn. Reson., Ser. A* **1994**, *110* (2), 219–227.
- (16) Fung, B. M.; Khittrin, A. K.; Ermolaev, K. An improved broadband decoupling sequence for liquid crystals and solids. *J. Magn. Reson.* **2000**, *142* (1), 97–101.
- (17) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28* (3), 350–356.
- (18) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193* (1), 265–275.
- (19) Boyd, A. R.; Champagne, P.; McGinn, P. J.; MacDougall, K. M.; Melanson, J. E.; Jessop, P. G. Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production. *Bioresour. Technol.* **2012**, *118*, 628–632.
- (20) Ryckeboosch, E.; Muylaert, K.; Foubert, I. Optimization of an analytical procedure for extraction of lipids from microalgae. *J. Am. Oil Chem. Soc.* **2012**, *89* (2), 189–198.
- (21) Lee, J.-Y.; Yoo, C.; Jun, S.-Y.; Ahn, C.-Y.; Oh, H.-M. Comparison of several methods for effective lipid extraction from microalgae. *Bioresour. Technol.* **2010**, *101*, S75–S77.
- (22) Tan, Y.; Lin, J. Biomass production and fatty acid profile of a *Scenedesmus rubescens*-like microalga. *Bioresour. Technol.* **2011**, *102* (21), 10131–10135.
- (23) Tang, S.; Qin, C.; Wang, H.; Li, S.; Tian, S. Study on supercritical extraction of lipids and enrichment of DHA from oil-rich microalgae. *J. Supercrit. Fluids* **2011**, *57* (1), 44–49.
- (24) Allard, B.; Rager, M. N.; Templier, J. Occurrence of high molecular weight lipids (C80+) in the trilaminar outer cell walls of some freshwater microalgae. A reappraisal of algaenan structure. *Org. Geochem.* **2002**, *33* (7), 789–801.
- (25) Allard, B.; Templier, J. Comparison of neutral lipid profile of various trilaminar outer cell wall (TLS)-containing microalgae with emphasis on algaenan occurrence. *Phytochemistry* **2000**, *54* (4), 369–380.
- (26) Allard, B.; Templier, J. High molecular weight lipids from the trilaminar outer wall (TLS)-containing microalgae *Chlorella emersonii*, *Scenedesmus communis* and *Tetraedron minimum*. *Phytochem.* **2001**, *57* (3), 459–467.
- (27) Allard, B.; Templier, J.; Largeau, C. An improved method for the isolation of artifact-free algaenans from microalgae. *Org. Geochem.* **1998**, *28* (9–10), 543–548.
- (28) Bertheas, O.; Metzger, P.; Largeau, C. A high molecular weight complex lipid, aliphatic polyaldehyde tetraterpenediol polyacetal from *Botryococcus braunii* (L race). *Phytochem.* **1999**, *50* (1), 85–96.
- (29) Blokker, P.; Schouten, S.; de Leeuw, J. W.; Damste, J. S. S.; van den Ende, H. Molecular structure of the resistant biopolymer in zygosporic cell walls of *Chlamydomonas monoica*. *Planta* **1999**, *207* (4), 539–543.
- (30) Blokker, P.; Schouten, S.; Van den Ende, H.; De Leeuw, J. W.; Hatcher, P. G.; Damste, J. S. S. Chemical structure of algaenans from the fresh water algae *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum*. *Org. Geochem.* **1998**, *29* (5–7), 1453–1468.
- (31) Gelin, F.; Boogers, I.; Noordeloos, A. A. M.; Damste, J. S. S.; Hatcher, P. G.; deLeeuw, J. W. Novel, resistant microalgal polyethers: An important sink of organic carbon in the marine environment? *Geochim. Cosmochim. Acta* **1996**, *60* (7), 1275–1280.
- (32) Gelin, F.; DeLeeuw, J. W.; Damste, J. S. S.; Derenne, S.; Largeau, C.; Metzger, P. Scope and limitations of flash pyrolysis-gas chromatography mass-spectrometry as revealed by the thermal-behavior of high-molecular-weight lipids derived from the green microalga *Botryococcus braunii*. *J. Anal. Appl. Pyrolysis* **1994**, *28* (2), 183–204.
- (33) Rodriguez, M. C.; Cerezo, A. S. The resistant “biopolymer” in cell walls of *Coelastrum sphaericum*. *Phytochem.* **1996**, *43* (4), 731–734.

- (34) Alonso, B.; Massiot, D. Mufti-scale NMR characterisation of mesostructured materials using ^1H - ^{13}C through-bond polarisation transfer, fast MAS, and ^1H spin diffusion. *J. Magn. Reson.* **2003**, *163* (2), 347–352.
- (35) Sackewitz, M.; Scheidt, H. A.; Lodderstedt, G.; Schierhorn, A.; Schwarz, E.; Huster, D. Structural and dynamical characterization of fibrils from a disease-associated alanine expansion domain using proteolysis and solid-state NMR spectroscopy. *J. Am. Chem. Soc.* **2008**, *130* (23), 7172–7173.
- (36) Dessort, D.; Connan, J.; Derenne, S.; Largeau, C. Comparative studies of the kinetic parameters of various algaenans and kerogens via open-system pyrolyses. *Org. Geochem.* **1997**, *26* (11–12), 705–720.
- (37) Hold, I. M.; Schouten, S.; Van Kaam-Peters, H. M. E.; Damste, J. S. S. Recognition of n-alkyl and isoprenoid algaenans in marine sediments by stable carbon isotopic analysis of pyrolysis products of kerogens. *Org. Geochem.* **1998**, *28* (3–4), 179–194.
- (38) Blokker, P.; van den Ende, H.; de Leeuw, J. W.; Versteegh, G. J. M.; Damste, J. S. S. Chemical fingerprinting of algaenans using RuO_4 degradation. *Org. Geochem.* **2006**, *37* (8), 871–881.
- (39) Schouten, S.; Moerkerken, P.; Gelin, F.; Baas, M.; de Leeuw, J. W.; Damste, J. S. S. Structural characterization of aliphatic, non-hydrolyzable biopolymers in freshwater algae and a leaf cuticle using ruthenium tetroxide degradation. *Phytochemistry* **1998**, *49* (4), 987–993.
- (40) Peterson, A. A.; Vogel, F.; Lachance, R. P.; Froeling, M.; Antal, M. J., Jr.; Tester, J. W. Thermochemical biofuel production in hydrothermal media: A review of sub- and supercritical water technologies. *Energy Environ. Sci.* **2008**, *1* (1), 32–65.
- (41) Vardon, D. R.; Sharma, B. K.; Blazina, G. V.; Rajagopalan, K.; Strathmann, T. J. Thermochemical conversion of raw and defatted algal biomass via hydrothermal liquefaction and slow pyrolysis. *Bioresour. Technol.* **2012**, *109*, 178–187.
- (42) Barbier, J.; Charon, N.; Dupassieux, N.; Loppinet-Serani, A.; Mahe, L.; Ponthus, J.; Courtiade, M.; Ducrozet, A.; Quoineaud, A.-A.; Cansell, F. Hydrothermal conversion of lignin compounds. A detailed study of fragmentation and condensation reaction pathways. *Biomass Bioenergy* **2012**, *46*, 479–491.
- (43) Chu, F.-F.; Chu, P.-N.; Shen, X.-F.; Lam, P. K. S.; Zeng, R. J. Effect of phosphorus on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. *Bioresour. Technol.* **2014**, *152* (0), 241–246.
- (44) Feng, P.; Deng, Z.; Hu, Z.; Wang, Z.; Fan, L. Characterization of *Chlorococcum pamirum* as a potential biodiesel feedstock. *Bioresour. Technol.* **2014**, *162* (0), 115–122.
- (45) Im, H.; Lee, H.; Park, M. S.; Yang, J.-W.; Lee, J. W. Concurrent extraction and reaction for the production of biodiesel from wet microalgae. *Bioresour. Technol.* **2014**, *152* (0), 534–537.
- (46) Jin, B.; Duan, P.; Xu, Y.; Wang, F.; Fan, Y. Co-liquefaction of micro- and macroalgae in subcritical water. *Bioresour. Technol.* **2013**, *149* (0), 103–110.
- (47) Wu, P.-F.; Teng, J.-C.; Lin, Y.-H.; Hwang, S.-C. J. Increasing algal biofuel production using *Nannochloropsis oculata* cultivated with anaerobically and aerobically treated swine wastewater. *Bioresour. Technol.* **2013**, *133* (0), 102–108.
- (48) Box, G. E. P.; Hunter, J. S. Multi-factor experimental-designs for exploring response surfaces. *Ann. Math. Stat.* **1957**, *28* (1), 195–241.
- (49) Zou, S. P.; Wu, Y. L.; Yang, M. D.; Kaleem, I.; Chun, L.; Tong, J. M. Production and characterization of bio-oil from hydrothermal liquefaction of microalgae *Dunaliella tertiolecta* cake. *Energy* **2010**, *35* (12), 5406–5411.
- (50) Zou, S. P.; Wu, Y. L.; Yang, M. D.; Li, C.; Tong, J. M. Bio-oil production from sub- and supercritical water liquefaction of microalgae *Dunaliella tertiolecta* and related properties. *Energy Environ. Sci.* **2010**, *3* (8), 1073–1078.
- (51) Biller, P.; Ross, A. B. Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content. *Bioresour. Technol.* **2011**, *102* (1), 215–225.
- (52) Ross, A. B.; Biller, P.; Kubacki, M. L.; Li, H.; Lea-Langton, A.; Jones, J. M. Hydrothermal processing of microalgae using alkali and organic acids. *Fuel* **2010**, *89* (9), 2234–2243.
- (53) Vardon, D. R.; Sharma, B. K.; Scott, J.; Yu, G.; Wang, Z. C.; Schideman, L.; Zhang, Y. H.; Strathmann, T. J. Chemical properties of biocrude oil from the hydrothermal liquefaction of *Spirulina* algae, swine manure, and digested anaerobic sludge. *Bioresour. Technol.* **2011**, *102* (17), 8295–8303.