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1 **Hydrothermal Co-liquefaction of Biomasses – Quantitative Analysis of Bio-crude** 2 **and Aqueous Phase Composition**

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6 **Keywords**

- 7 • Hydrothermal liquefaction
- 8 • Biomass mixtures
- 9 • Quantitative analysis
- 10 • Principal component analysis
- 11 • Reaction pathways

12 **Abstract**

13 Hydrothermal liquefaction (HTL) is a promising technology for conversion of wet biomasses to liquid fuels,
14 but considerable amounts of oxygen and nitrogen remain in the bio-crude, while large amounts of water-
15 soluble organics are displaced to the aqueous phase (AqP).

16 In this study the bio-crude and AqP from HTL of 11 different feedstocks of lignocellulosics, residues,
17 macroalgae, microalgae, and their mixtures were analyzed for elemental composition, total acid number,
18 total organic carbon (TOC), total nitrogen, and pH. Quantitative analysis of major compound classes present
19 in both bio-crudes and AqPs was achieved using gas chromatography coupled to mass spectrometry
20 employing prior derivatization of authentic standards.

21 A wide range of biochemical content was obtained through mixing of biomasses and quantitative analysis
22 showed particular interaction between carbohydrates and proteins with extended effect on lipids. The
23 ability of ammonia and amines to form Schiff bases was the key factor affecting elemental distribution and
24 the direction of reaction pathways involved in the formation of cyclic oxygenates, hydroxypyridines,

oxygenated aromatics, diols, and fatty acids in bio-crudes. Similarly, Schiff base formation accounts for increased formation of nitrogen-containing compounds in the AqP, leading to a decrease in TOC and total nitrogen in products from HTL of mixed biomasses.

This work highlights the quantitative differences in bio-crude and AqP composition from HTL of varying biomasses and provides new knowledge of the effect of mixing biomasses on elemental distribution and composition of product fractions. The results provide valuable information for optimizing the feedstocks used for HTL based on biochemical composition.

1. Introduction

As concern for the environmental impact of fossil fuels is increasing, governmental policies aim at becoming less dependent on fossil fuels, and the pursuit for sustainable bio-fuels and chemicals is receiving more attention, a variety of biomasses and residues available in high volumes are being researched with the aim of providing drop-in bio-fuels. Biomasses and residues have high natural moisture contents, making wet processing methods desirable in order to avoid the energy penalty of drying. Hydrothermal liquefaction (HTL) is carried out in aqueous media at medium temperature (e.g. 350 °C) and high pressure (e.g. 250 bar) with typical solid loadings of 5-30 wt%, converting biopolymers to a bio-crude which can be further upgraded to a drop-in fuel comparable to gasoline, diesel, and kerosene.¹⁻³

Since any organic feedstock is amenable to HTL, a vast number of feedstocks have been investigated for their conversion efficiency in HTL. This includes lignocellulosics, macroalgae, microalgae, and a variety of residues.⁴⁻⁷ The HTL process generates four different phases; a desired bio-crude, an aqueous phase (AqP), as well as minor fractions of solid residue and gas. Yields of 5-10 % gas, 20-40 % bio-crude, 30-60 % AqP, and 5-25 % solid residue are typically reported for batch experiments.⁸ These values depend on reactor type, biochemical composition of the biomass, process conditions, and product workup. The gas phase consists predominantly of carbon dioxide with minor contributions of hydrogen and volatile organic compounds.^{9, 10} The substantial amount of organics lost to the AqP has been recognized as an important aspect for making HTL economically feasible especially when processing lignocellulosics.¹¹ The bio-crude

has predominantly been further processed through hydrotreatment to a potential drop-in fuel while a few attempts have been made at blending with conventional fossil based fuels.^{2, 12}

Bio-crude from HTL has been extensively analyzed for general characteristics such as total acid number (TAN), elemental distribution (CHNS-O), higher heating value, water content, viscosity, and boiling point distribution. More advanced techniques such as nuclear magnetic resonance (NMR) and gas chromatography coupled to mass spectrometry (GC-MS) has been employed to obtain molecular information.^{4, 13-15} However, validated quantitative GC-MS analyses applying authentic standards are seldom reported, and interpretations are typically based on relative peak areas, which are highly dependent on sample type, relative response of compounds, sample preparation, instrumental settings, and data processing. The use of relative peak areas thus only allows meaningful comparison between samples from a single feedstock, processed in the same HTL system and analyzed with the same instrumental settings and data processing methods, which makes comparison of literature values difficult at best.

The vast majority of compounds reported from HTL studies contain one or more heteroatoms. Especially the presence of nitrogen is problematic due to the formation of NO_x gases upon combustion and difficulties with hydro-denitrogenation during upgrading.¹⁶ The presence of oxygen has several negative effects as well, such as lower heating values, decreased stability of the bio-crude, and higher hydrogen demand during upgrading. Being able to relate elemental distribution to bio-crude composition could provide increased knowledge of the pathways for formation of oxygenated and nitrogenated compounds. Limited analytical focus has been applied to the aqueous phase and measurements typically include total organic carbon (TOC), total nitrogen (TN), total ammonia, total phosphate, mineral content, and pH.¹⁷

Recently a few studies have developed methods for quantitative GC-MS and GCxGC-MS analysis of the aqueous phase.^{18, 19} The complexity of the product fractions imply that micro-emulsions are likely occurring in both bio-crude and aqueous phase.²⁰ Hence, relating compositional information of these very related product fractions could lead to added knowledge of both reaction pathways and dispersion of compounds.

Recently, the effect of mixing different strains of microalgae feedstocks for HTL was investigated showing that the nitrogen distribution could be estimated from the protein content.²¹ Another recent study investigated the effect of mixing microalgae, wood, and sugar beet feedstocks showing that the nitrogen content of the bio-crude increased when microalgae was mixed.²² Other studies on co-liquefaction of biomass have related the elemental distribution to the composition of bio-crude using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) and GC-MS based on relative peak areas.^{23, 24} In this work we applied one set of processing conditions to a range of 11 feedstocks and eight mixtures of them spanning biochemical composition from high carbohydrate to high lipid or protein, as well as lignin. The biomasses included lignocellulosic (softwood, hardwood, and perennial grasses), aquatic (microalgae and macroalgae), and residues (household waste and DDGS). The study is limited by the use of batch reactors requiring a solvent-based work up, which may alter the composition of especially the bio-crude. Bio-crude and aqueous phase was characterized with quantitative analytical methods to ensure comparable results. The bio-crude was characterized with CHNS-O, TAN, and GC-MS while the aqueous phase was characterized with total organic carbon, total nitrogen, pH, and GC-MS. The analytical GC-MS methods were in both cases validated prior to use and included authentic standards. The link between elemental distribution and specific compound classes was studied through measured and calculated values for mixtures of biomass. However, it should be noticed that oxygen content of bio-crudes was measured by difference leaving a degree of uncertainty in evaluating elemental distribution. A network of reaction pathways was subsequently constructed based on presented results.

2. Materials and methods

2.1 Chemicals and reagents

Potassium carbonate, chloroform (HPLC grade), dichloromethane (HPLC grade), methyl chloroformate (MCF), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), and pyridine (HPLC grade) were purchased from SigmaAldrich. Standards were obtained from SigmaAldrich, Acros Organics, or Fluka (see Table 2 and Table S3 for a full list). Household waste was obtained in 12 wt% aqueous solution. *Miscanthus x giganteus*

100 (*M. x giganteus*), willow wood, and poplar wood was supplied by Department of Agroecology, Aarhus
101 University (Denmark). Timothy white clover (Clover) was harvested in Scotland. Dried Distillers Grains with
102 Solubles (DDGS) was delivered from Lantmännen Agroetanol AB, Norrköping, Sweden. Microalgae included
103 *Spirulina* and *Chlorella vulgaris* (*C. vulgaris*) acquired from commercial sources, and *Nannochloropsis*
104 *gadicola* (*N. gaditana*) from Lgem, The Netherlands, macroalgae included *Laminaria hyperborea* (*L.*
105 *hyperborea*) from the University of Leeds (UK), and *Laminaria digitata* (*L. digitata*) from the Danish
106 Technological Institute, Denmark.

107 **2.2 Hydrothermal liquefaction**

108 Biomass slurries were prepared by mixing 10 wt% biomass, 2 wt.% potassium carbonate, and 88 wt.%
109 demineralized water. Feedstocks consisted of 11 biomasses and eight mixtures; poplar, *spirulina*, and *C.*
110 *vulgaris* were mixed in binary and ternary mixtures. DDGS, *M. x giganteus*, and *N. gaditana* were also
111 prepared as separate binary and ternary mixtures. The biochemical composition of biomasses and mixtures
112 is presented in Table 1 on dry and ash free basis. HTL experiments were performed in 20 ml batch reactors
113 from Swagelok. Experiments were initiated by loading 10 ml of 10 wt% biomass slurry into the reactor.
114 Reactors were sealed and lowered into an Omega Engineering FSB-4 fluidized sand bath (FSB) preheated to
115 350 °C. A reaction time of 20 min was applied, after which the reactors were cooled to ambient
116 temperature in a water bath. The reactors were vented and the AqP was decanted into a centrifuge tube
117 which was centrifuged at 6500 rpm for 5 min before the AqP was transferred with a glass pipette to a
118 preparative glass. The AqP was then stored at 5 °C until further analysis. The centrifuge tube was washed
119 with 2 ml of dichloromethane and the reactor was extracted with 4 x 3 ml of dichloromethane which were
120 combined. The dichloromethane phase was vacuum filtrated, and the residue washed with
121 dichloromethane until the filtrate appeared clear. Dichloromethane was evaporated under a stream of
122 nitrogen until constant weight. Each experiment was performed in duplicate and average values are
123 reported. These conditions have been used during several experiments and the repeatability in recovering
124 both the bio-crude and AqP has previously been presented.^{25, 26} Standard deviations are provided, which

are therefore based on the entire HTL process and analytical uncertainty including sampling of the highly viscous bio-crude.

2.3 Analytical methods

Quantitative GC-MS analysis of bio-crude was performed with multicomponent standard solutions prepared in dichloromethane. Samples and standard solutions were mixed with 100 μ l of MSTFA and made up to a total volume of 1.00 ml with dichloromethane containing internal standard (*p*-bromotoluene 20 μ g ml⁻¹). Dichloromethane contained additional internal standards (bromoacetic acid and 4-bromophenol) used to monitor the silylation efficiency. Samples were vortexed and placed on a shaker board for one hour. Analysis was performed using an Agilent 7890B GC coupled to a quadrupole mass filter MS (Agilent, 5977A). The GC injection port was operated at 280 °C in 20:1 split mode with the carrier gas, helium, at 1 ml min⁻¹, and the injection volume of 1 μ l. The column was a VF-5ms (60m x 0.25mm x 0.25 μ m, 5m EZ-guard, Agilent) with a 5% phenyl, 95% dimethylpolysiloxane stationary phase. The column oven program started at 40 °C which was held for 5 min, progressing at 10 °C min⁻¹ to 100 °C, at 4 °C min⁻¹ to 280 °C (hold time 3 min), and at 10 °C min⁻¹ to 320 °C (hold time 4 min) giving a total run time of 64 min.

Quantitative GC-MS analysis of AqP was performed with multicomponent standard solutions prepared in either water or methanol. Samples and standard solutions were derivatized with methyl chloroformate (MCF) as described in detail elsewhere.²⁵ The same GC-MS and column used for bio-crude analysis was used for AqP analysis. The GC injection port was operated at 280 °C in 20:1 split mode with the carrier gas, helium, at 1 ml min⁻¹, and the injection volume of 1 μ l. The column oven program started at 60 °C which was held for 2 min, progressing at 5 °C min⁻¹ to 200 °C, and at 20 °C min⁻¹ to 320 °C (hold time 3 min), giving a total run time of 39 min.

In both cases ionization was performed using an electron impact (EI) source in positive ion mode with electron energy of 70 eV. Data was acquired in scan mode (35-500 m/z). The MS was tuned using perfluorotributylamine. Data acquisition was performed using Masshunter software. Analyte responses were normalized with the response of the internal standard before construction of calibration curves.

Replicate batch experiments showed only minor differences and the average concentration determined for each experiment is reported and was used for chemometric analysis. Analytes for which a standard was not available were quantified with the calibration curve of an analyte with a similar mass spectrum.

Total organic carbon (TOC) and total nitrogen (TN) was determined with a Hach Lange DR2800 spectrophotometer with TOC-kit LCK387 and TN-kit LCK338, respectively.

Total acid number was determined by dissolving approximately 20 mg of bio-crude in 50 mL of acetone along with thymol blue (thymolsulphonephthalein). The solution was titrated with 0.1 M potassium hydroxide until persistent color change. The total acid number was calculated as the amount of potassium hydroxide in mg needed to neutralize one gram of bio-crude.

Elemental composition of biomass and bio-crude was determined using a CHNS-O Elementar Vario MACRO cube analyzer (Elementar Analysensysteme). Oxygen content was determined by difference.

Total carbohydrate content was determined using the reaction of phenol and sulfuric acid with carbohydrate followed by colorimetric determination at 420 nm.²⁷ Protein content was calculated by applying a factor of 6.25 to the nitrogen content determined from CHNS. Total lipid content was determined gravimetrically after extraction in 2:1 chloroform and methanol aided by sonication. The total lignin content was determined with the acetyl bromide method upon extensive washing to remove proteins.²⁸ The biochemical composition of feedstocks, on dry and ash free basis is presented in Table 1 (mixtures were calculated from the pure feedstocks). The methods for determination of carbohydrate, protein, and lipid contents were adapted from analysis of microalgae⁶ and some degree of matrix effects are expected, especially regarding carbohydrate determination of lignocellulosics and macroalgae which were overestimated and underestimated, respectively. Therefore, carbohydrate contents of lignocellulosics and macroalgae were determined by difference.

Table 1 Elemental distribution and biochemical composition of feedstocks presented in percentage.

	C	H	N	S	O ^a	Carbohydrate	Protein	Lipid	Lignin
<i>M. x giganteus</i>	46.69	6.24	0.49	0.09	43.73	73.8 ^b	3.0	5.4	17.8

Willow	47.20	6.28	0.22	0.04	45.67	75.5 ^b	1.4	4.5	18.5
Poplar	47.65	6.27	0.22	0.01	45.06	80.9 ^b	1.3	5.2	12.7
Timothy white clover	47.61	6.32	1.41	0.07	38.23	74.5 ^b	8.8	8.2	8.5
<i>L. digitate</i>	35.71	5.89	0.74	0.46	43.20	91.4 ^b	4.6	3.2	0.9
<i>L. hyperborea</i>	31.29	5.10	1.62	0.82	34.30	82.7 ^b	10.1	6.0	1.3
<i>C. vulgaris</i>	47.86	6.94	7.85	0.51	25.44	26.5	49.1	16.3	0.0
<i>Spirulina</i>	48.09	6.51	10.91	0.89	15.02	14.0	68.2	20.0	0.0
<i>N. gaditana</i>	49.95	7.36	7.40	0.55	26.18	14.4	46.3	37.6	0.0
DDGS	44.98	6.92	5.23	0.85	36.59	35.0	32.7	22.4	2.8
House hold waste	42.98	5.59	2.10	0.27	31.48	42.2	13.1	40.0	3.2
Nanno/DDGS	47.47	7.14	6.32	0.70	31.39	24.7	39.5	30.0	1.4
DDGS/Misc	45.84	6.58	2.86	0.47	40.16	54.4	17.9	13.9	10.3
Misc/Nanno	48.32	6.80	3.95	0.32	34.96	44.1	24.7	21.5	8.9
Misc/Nanno/DDGS	47.21	6.84	4.37	0.50	35.50	41.1	27.3	21.8	6.9
Pop/Vulg	47.76	6.61	4.04	0.26	35.25	53.7	25.2	10.8	6.4
Spir/Vulg	47.98	6.73	9.38	0.70	20.23	20.3	58.7	18.2	0.0
Spir/Pop	47.87	6.39	5.57	0.45	30.04	47.5	34.8	12.6	6.4
Spir/Pop/Vulg	47.87	6.57	6.33	0.47	28.51	40.5	39.5	13.8	4.2

^{a-b} calculated by difference.

2.4 Principal component analysis

Principal component analysis (PCA) is often used as an exploratory method to gain an overview of multivariable data in the form of either groupings, correlations, or outliers.²⁹ The data is transformed into a set of scores and loadings along with residuals, to maximize the covariation between samples. This leads to a set of principal components (PC) situated along the maximum variation of the data. The scores represent the projection of each sample onto each PC while the loadings represent the weight of each variable on each PC. Hence, similarity or dissimilarity of samples can be evaluated based on score plots, and the weight of each variable can be assessed through loading plots.

Fold changes are often observed for raw data and without scaling samples with high concentrations will tend to present the largest weight and dominate the data analysis. Discrete data, such as quantitative results, are typically autoscaled, this means that for each sample the mean value of each variable is subtracted and it is divided by the standard deviation for each variable. Evaluating PCA after scaling means that samples with high scores on a given PC do not necessarily contain high concentrations of a variable. However, it will have a higher concentration than other samples with a lower score.

3. Results and discussion

190 3.1 Analysis of bio-crudes

191 In this section we present quantitative analysis of elemental composition, total acid number, and single
192 compounds from different compound classes detected in the bio-crudes. Measured values are provided for
193 bio-crudes of biomasses and mixtures of biomasses. Expected values for bio-crudes of mixtures of
194 biomasses are calculated from the average values measured from bio-crude of neat biomasses. Effects
195 from mixing biomasses are explained by comparing measured and calculated values. First observations
196 from elemental analysis and TAN are presented which are then explained from GC-MS analysis.

197 3.1.1 Elemental analysis and total acid number

198 The elemental composition of the bio-crudes was determined to evaluate the overall distribution of carbon,
199 oxygen, nitrogen, and sulfur, which are shown in Figure 1. The carbon content of bio-crudes varied from
200 72.9% to 79.5% and was in all cases significantly higher than the carbon content of the biomasses (Figure
201 1A and Table 1). The lowest carbon content was observed for household waste which is the only feedstock
202 with both carbohydrate and lipid contents > 40%. Low carbon contents (~ 74%) were also observed for
203 biomasses with high protein content (> 49%) and medium carbohydrate and lipid content (< 27%) which is
204 the case for *Spirulina* and *C. vulgaris* and mixtures containing both these biomasses. The remaining bio-
205 crudes made from neat biomasses had carbon contents of approximately 76%, while mixtures of biomasses
206 (apart from mixtures containing both *Spirulina* and *C. vulgaris*) generally had measured carbon contents
207 approximately 1% higher than the calculated carbon contents.

208 The oxygen content of bio-crudes varied from 6-17%, which is significantly lower than the biomasses which
209 contained from 15% for *Spirulina* and up to 46% for willow (Figure 1B). The oxygen content in bio-crudes
210 followed the order of lignocellulosics > macroalgae > microalgae, while residues showed oxygen contents in
211 between. Generally, measured oxygen content of bio-crudes from mixtures was lower than calculated
212 oxygen content. The effect on carbon and oxygen content in bio-crude when mixing biomasses is proposed
213 to be caused by a change in reaction pathway explained in section 3.1.2.

Figure 1. A) Carbon content, B) Nitrogen content, C) Oxygen content, and D) Sulfur content of bio-crudes. Green – lignocellulosics, yellow – macroalgae, blue – microalgae, orange – residue, grey – mixtures. Red circles show calculated values and black triangles show values of the feedstock. HHW is household waste.

The nitrogen content of bio-crudes ranged from 0.3-5.6% (Figure 1C). Generally it was observed that the nitrogen content is increased in bio-crudes from neat biomasses and mixtures with < 3% feedstock nitrogen while it is decreased in bio-crudes made from biomasses with > 3% feedstock nitrogen, with a few exceptions. Bio-crudes from neat lignocellulosics, macroalgae and household waste (<3% feedstock nitrogen) had twice the nitrogen content of the feedstock, while the nitrogen content in bio-crudes from microalgae, DDGS, and mixtures (>3% feedstock nitrogen) decreased, dependent on the nitrogen content

225 of the feedstock. It is especially noteworthy that the nitrogen in bio-crudes from mixtures of lignocellulosics
226 and microalgae (which exhibit a combined high protein and carbohydrate content) were comparable to
227 those in bio-crudes made from microalgae with significantly higher protein contents. Furthermore, the
228 measured values of nitrogen content in these mixtures were generally higher than the calculated values.
229 Thus, the reaction between degradation products from carbohydrates and proteins lead to formation of
230 products soluble in the bio-crude retaining more of the nitrogen. To further investigate this trend, mixtures
231 of model compounds (cellulose, soy protein, and rapeseed oil) were subjected to HTL. The nitrogen content
232 of bio-crude from pure protein was 5.8%, when mixing protein with rapeseed oil (1:1) it was 2.7, while it
233 was 5.6 when mixing 1:1 with carbohydrate (Supplementary I).

234 The sulfur content of the bio-crudes was between 0.04% and 1.03% (Figure 1D). The sulfur content
235 increased slightly for bio-crudes of lignocellulosics, *Spirulina*, and *C. vulgaris* while it decreased in all other
236 bio-crudes. The significant amounts of nitrogen and sulfur will lead to formation of NO_x and SO_x emission
237 during combustion and further upgrading of the bio-crude is necessary to obtain a cleaner bio-fuel.

238 The TAN is an important indicator of potential corrosion effects by the bio-crude. In the petroleum industry
239 high TANs are associated with naphthenic acids and corrosion is generally higher for crude oils with TAN
240 values higher than 0.5.³⁰ However, TAN only indicates the potential for corrosion since the identity of the
241 acids is equally important. In bio-crudes the TAN varied from 91 mg g⁻¹ for *C. vulgaris* to 166 mg g⁻¹ for *N.*
242 *gaditana*. Bio-crudes of lignocellulosics, macroalgae, *Spirulina*, and household waste generally had TANs of
243 100-130 mg g⁻¹. No obvious trend for bio-crudes of mixtures could be observed and no apparent correlation
244 to GC-MS analysis could be made (Figure 2).

Figure 2. Total acid number for bio-crudes from HTL of biomass and mixtures. Green – lignocellulosics, yellow – macroalgae, blue – microalgae, orange – residue, grey – mixtures. Red circles show calculated values. HHW is household waste.

3.1.2 GC-MS analysis

High resolution Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry has revealed the presence of > 6000 unique compounds in bio-crude from microalgae.³¹ The difference in response factors means that a multitude of reference standards would be required to quantify even a minor part of the bio-crude. Therefore, 200 compounds were selected based on peak area, and characteristic ions were determined for each compound to avoid integration of co-eluting peaks. Peak areas of extracted ion chromatograms were subjected to PCA with prior auto-scaling (Figure 3A-B). Compounds were grouped into 12 different classes; 1) carboxylic acids, 2) alcohols, 3) cyclic oxygenates, 4) amines, 5) cyclic oxygenates, 6) phenolics, 7) benzenediols, 8) hydrocarbons, 9) fatty acids, 10) monoglycerides, 11) phenones, 12) unknowns.

Bio-crude from household waste was distinctively different from the remaining samples and was not included in any of the mixtures. Therefore, the sample was removed from the PCA. PC1 explained 51.1% of the total variance and separated bio-crudes of microalgae and residue from mixtures and macroalgae,

263 which were further separated from bio-crudes of lignocellulosics. PC2 explained 13.8% of the total variance
264 and separated bio-crudes with increasing scores in the order wood, microalgae, macroalgae, mixtures,
265 grass, and DDGS. Small organic acids (C₂-C₄) in the form of acetic acid, propionic acid, butyric acid, glycolic
266 acid, and lactic acid were characteristic for bio-crudes of grass while formic acid and C₅ acids were
267 characteristic of DDGS and > C₆ acids were found in bio-crudes of microalgae. Alcohols were less well-
268 grouped but smaller alcohols of ethylene glycol, propylene glycol, and butane-2,3-diol were found in higher
269 concentrations in lignocellulosics, while monoalcohols were most prevalent for microalgae, and glycerol
270 was found in highest concentrations in DDGS bio-crude.

271 Cyclic oxygenates were highly characteristic of lignocellulosics, which has previously been attributed to
272 their formation from degradation of carbohydrates.³² Especially ketones of C₅ ring structures are abundant,
273 as previously reported.³³ In this work cyclopentanones had positive values and cyclopent-2-enones had
274 negative values on PC2 indicating that higher concentrations of cyclopentanones are obtained from grass
275 feedstocks compared to wood feedstocks and vice versa for cyclopent-2-enones.

276 Cyclic nitrogenates were characteristic of microalgae and DDGS. The main cyclic nitrogenates of DDGS were
277 hydroxypyridines and pyrrolidine, while microalgae showed higher concentrations of piperidines and
278 indoles.

279 Phenolics and benzenediols are mainly produced from degradation of lignin and to some extent from
280 carbohydrates.³² Generally it was observed that alkylated phenols and catechols had positive loading on
281 PC1-3 separating *M. x giganteus* from clover and wood. Alkylated phenols were also characteristic of DDGS.

282 Microalgae were generally characterized by higher concentrations of C₁₆₋₁₇ fatty acids and hydrocarbons
283 from degradation of pigments, while DDGS were characterized by higher concentrations of C₁₈ fatty acids.

284 Despite a significantly lower lipid content in macroalgae and lignocellulosics these bio-crudes were
285 characterized by higher concentrations of monoglycerides which could originate from partial breakdown of
286 triglycerides. Bio-crudes from grass were further characterized by higher concentrations of 2-
287 monopalmitoyl glycerol and 2-monostearin glycerol while macroalgae and wood bio-crudes had higher

288 concentrations of 1-monopalmitoyl glycerol and 1-monostearin glycerol. Furthermore, it is interesting to
289 note that neither monopalmitoleoyl glycerol nor monooleyl glycerol were detected.

290
291 *Figure 3. Biplots of scores and loadings from analysis of bio-crudes – A) PC1 and PC2, B) PC1 and PC3.*
292 Selected abundant compounds from each compound class were quantitated in order to evaluate the
293 quantitative effects of the different feedstocks and their mixtures (Table 2). Results of bio-crude from
294 household waste have been included for comparison, despite the apparent outlier nature of this sample.

295 Table 2. Quantitative results from GC-MS analysis of selected compounds in bio-crude. HHW is household waste

	<i>M. x giganteus</i> mg g ⁻¹	Willow mg g ⁻¹	Poplar mg g ⁻¹	Clover mg g ⁻¹	<i>L. digitata</i> mg g ⁻¹	<i>L. hyperborea</i> mg g ⁻¹	<i>C. vulgaris</i> mg g ⁻¹	<i>Spirulina</i> mg g ⁻¹	<i>N. gaditana</i> mg g ⁻¹	DDGS mg g ⁻¹	HHW mg g ⁻¹
3-methylcyclopentanone	0.46 ± 0.02	0.35 ± 0.01	0.04 ± 0.02	0.36 ± 0.05	0.14 ± 0.01	0.052 ± 0.02	0.005 ± 0.002	ND	0.007 ± 0.001	0.023 ± 0.003	0.18 ± 0.06
2,3-dimethylcyclopent-2-enone	3.71 ± 0.18	3.23 ± 0.09	2.93 ± 0.27	2.90 ± 0.15	1.42 ± 0.07	1.06 ± 0.12	0.47 ± 0.02	0.15 ± 0.02	0.27 ± 0.02	0.67 ± 0.19	1.70 ± 0.18
3-hydroxypyridine	0.094 ± 0.022	0.054 ± 0.005	0.059 ± 0.07	0.34 ± 0.01	0.18 ± 0.02	0.16 ± 0.00	0.21 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.40 ± 0.06	0.34 ± 0.08
palmitic acid	6.54 ± 0.59	5.02 ± 0.37	8.33 ± 0.05	10.52 ± 0.07	14.31 ± 1.12	20.15 ± 2.75	27.85 ± 1.97	41.52 ± 0.14	31.36 ± 0.07	28.91 ± 4.99	17.61 ± 1.12
Phenol	5.81 ± 0.22	1.36 ± 0.02	5.17 ± 0.15	3.97 ± 0.28	0.53 ± 0.01	0.81 ± 0.05	1.85 ± 0.00	2.83 ± 0.12	1.36 ± 0.02	1.95 ± 0.20	1.15 ± 0.10
2-methoxyphenol	2.73 ± 0.51	2.28 ± 0.05	2.54 ± 0.08	1.03 ± 0.04	ND	ND	ND	ND	ND	0.022 ± 0.004	0.24 ± 0.01
4-ethylphenol	5.71 ± 0.66	0.21 ± 0.02	0.45 ± 0.01	2.01 ± 0.07	0.045 ± 0.000	0.54 ± 0.07	0.65 ± 0.03	1.71 ± 0.31	0.62 ± 0.04	1.02 ± 0.05	0.41 ± 0.05
ethyleneglycol	0.50 ± 0.09	0.37 ± 0.02	0.32 ± 0.02	0.40 ± 0.28	0.32 ± 0.02	0.08 ± 0.01	0.035 ± 0.003	0.014 ± 0.003	0.05 ± 0.01	0.07 ± 0.01	0.48 ± 0.05
propyleneglycol	0.42 ± 0.04	0.31 ± 0.01	0.28 ± 0.01	0.34 ± 0.15	0.27 ± 0.03	0.17 ± 0.01	0.14 ± 0.03	0.11 ± 0.01	0.12 ± 0.01	0.17 ± 0.02	0.39 ± 0.04
Glycerol	0.22 ± 0.03	0.18 ± 0.02	0.19 ± 0.01	0.26 ± 0.10	0.27 ± 0.01	0.24 ± 0.01	0.36 ± 0.11	0.43 ± 0.13	0.56 ± 0.15	0.93 ± 0.27	1.67 ± 0.35
formic acid	0.37 ± 0.04	0.20 ± 0.01	0.13 ± 0.01	0.39 ± 0.05	0.32 ± 0.04	0.44 ± 0.04	0.32 ± 0.06	0.36 ± 0.03	0.28 ± 0.01	1.00 ± 0.01	0.42 ± 0.00
acetic acid	2.43 ± 1.43	1.94 ± 0.21	0.31 ± 0.03	2.40 ± 1.01	1.54 ± 0.25	1.04 ± 0.09	0.79 ± 0.57	0.88 ± 0.04	0.61 ± 0.26	1.55 ± 0.01	4.10 ± 0.43
glycolic acid	0.81 ± 0.47	0.21 ± 0.02	0.14 ± 0.01	0.92 ± 0.48	0.41 ± 0.12	0.25 ± 0.02	0.030 ± 0.014	0.11 ± 0.02	0.086 ± 0.024	0.29 ± 0.09	0.67 ± 0.06
lactic acid	2.84 ± 1.55	0.85 ± 0.01	0.61 ± 0.01	4.28 ± 2.14	5.13 ± 0.98	2.33 ± 0.03	0.45 ± 0.23	0.98 ± 0.17	1.04 ± 0.43	2.22 ± 0.62	6.76 ± 0.69

302 The highest concentrations of 3-methylcyclopentanone and 2,3-dimethylcyclopent-2-enone were found in
303 bio-crude from *M. x giganteus* with 0.46 mg g⁻¹ and 3.71 mg g⁻¹, respectively. These compounds are
304 primarily produced from carbohydrates and the different carbohydrate structures of macroalgae are likely
305 the reason for the lower concentrations of these cyclic oxygenates in bio-crudes from *L. digitate* and *L.*
306 *hyperborea*.³⁴ The concentration of cyclic oxygenates was significantly lower for bio-crudes of microalgae
307 and DDGS while the concentration of 3-hydroxypyridine was highest for bio-crude of DDGS (0.40 mg g⁻¹).
308 Previous studies have suggested that 3-hydroxypyridines are formed from reaction between ammonia and
309 furfurals and 2-acetylfurans which are formed from degradation of especially cellulose.^{35, 36} Several studies
310 have identified the formation of Schiff bases, in what is often referred to as Maillard type reactions, as a
311 key reaction for formation of nitrogen containing compounds during HTL. In this work, the formation of
312 Schiff bases from degradation products of carbohydrates and protein is proposed to account for the
313 decreased oxygen content and increased nitrogen content observed for the bio-crudes of mixtures of
314 biomasses. Figure 3 shows the concentrations of single compounds in bio-crudes from mixtures of biomass.
315 Bio-crudes from mixtures of microalgae and DDGS had concentrations of 2,3-dimethylcyclopent-2-enone
316 and 3-hydroxypyridine, which could be calculated from the averages detected in bio-crudes from the
317 respective feedstocks (Figure 4A-B). In contrast, the measured concentrations were approximately 0.5
318 times for 2,3-dimethylcyclopent-2-enone and 2 times for 3-hydroxypyridine compared to the calculated
319 concentrations when mixing biomasses of lignocellulosics and microalgae. Hence, the degradation products
320 which lead to the formation of cyclic oxygenates are converted into nitrogen-containing ring structures
321 which include hydroxypyridines, pyrazines (further discussed in section 3.2.2), and quinolines (Submitted
322 for publication). Pyrazines were not present in the bio-crude due to the work up method employed but are
323 known to be highly prevalent from DDGS.²⁰

324 Palmitic acid was the most abundant compound in most bio-crudes with up to 41.5 mg g⁻¹ from *Spirulina*
325 while the lowest concentration was detected from willow with 5.0 mg g⁻¹. The measured concentration of
326 palmitic acid in bio-crudes from mixtures of microalgae and DDGS was in agreement with the calculated

concentration, while measured concentrations in mixtures of lignocellulosics (high carbohydrate) and microalgae (high protein) were higher than the calculated concentrations (Figure 4C). This could have two reasons; 1) carbohydrate and protein degrades significantly faster than triglycerides during the HTL process leading to reaction of ammonia with products from carbohydrates; 2) the ammonia reacts significantly faster with products from carbohydrates (aldehydes and ketones) than with free fatty acids. Consequently, more fatty acids and less fatty amides are formed. The combined effect of increased fatty acid and Schiff base formation with less formation of small alcohols and organic acids could partly explain the increased carbon content observed for bio-crudes from mixtures of lignocellulosics and microalgae, which was not present for microalgae and DDGS mixtures. Further palmitic acid and stearic acid was found to be present in the form of monoglycerides, which had an estimated concentration of 1-10 mg g⁻¹. Assuming a linear dependency, the concentration of 1-palmitoyl glycerol and 1-stearoyl glycerol were approximately two times higher in bio-crude from *M. x giganteus* and wood compared to algae and DDGS, while 2-palmitoyl glycerol and 2-stearoyl glycerol were approximately three times higher in macroalgae compared to microalgae and DDGS with lignocellulosics having concentrations in between (Supplementary II). Furthermore, it was observed that roughly 10 times more 1-palmitoyl glycerol and 1-stearoyl glycerol were found compared to 2-palmitoyl glycerol and 2-stearoyl glycerol.

Oxygenated aromatics are highly abundant in bio-crude from lignocellulosics due to degradation of lignin in particular and to some extent from carbohydrates and protein. Protein and lignin are the main contributors to 4-ethylphenol, and its highest concentrations were found in bio-crude from *M. x giganteus*, Clover, and *Spirulina* with 5.71 mg g⁻¹, 2.01 mg g⁻¹, and 1.71 mg g⁻¹, respectively, while 10-20 times lower concentrations were observed in bio-crudes from wood. The measured and calculated concentrations of 4-ethylphenol in bio-crudes from mixtures of biomass were in accordance showing that it is formed from degradation of biochemical constituents for which intermediates are not involved in other pathways (Figure 4D). Similar observations could be made for phenol, which can be formed from degradation of cellulose, indicating that the formation of phenol and cyclopent-2-enones constitute two separate reaction pathways.

352 A phenolic compound produced solely from lignin is 2-methoxyphenol which was abundant in bio-crudes of
 353 lignocellulosics such as *M. x giganteus* (2.73 mg g^{-1}) while being absent in algae bio-crudes. The measured
 354 concentration of 2-methoxyphenol in bio-crudes from mixtures was between 1.6 and 15 times lower than
 355 the calculated concentrations (Figure 4E). This indicates that the formation of 2-methoxyphenol from lignin
 356 occurs through reactive intermediates that are most likely aldehydes or ketones involved in Schiff base
 357 formation.

358 Another interesting observation is that the measured concentration of ethylene glycol (and butan-2,3-diol)
 359 in bio-crudes from mixtures of biomass shows the exact same trend compared to the calculated
 360 concentrations as observed for 2,3-dimethylcyclopent-2-enone and 2-methoxyphenol (Figure 4F). Previous
 361 studies have proposed that monosaccharides are degraded through either a retro-aldol reaction or
 362 dehydration. The retro-aldol reaction produces small oxygenated compounds which can further
 363 deoxygenate to diols, while dehydration produces amongst others furans, furfurals, and γ -keto acids which
 364 can ultimately produce hydroxypyridines and pyrrolidin-2-ones in the presence of ammonia. Hence, it is
 365 proposed that the retro-aldol and dehydration reaction occur in equilibrium and the addition of nitrogen
 366 pulls the equilibrium towards dehydration as furans, furfurals, and γ -keto acids are converted.

367 Glycerol is generally the most abundant alcohol observed in bio-crudes and up to 0.93 mg g^{-1} and 1.67 mg g^{-1}
 368 ¹ was found from DDGS and household waste, respectively. Bio-crudes from microalgae, macroalgae, and
 369 lignocellulosics contained approximately 0.40 mg g^{-1} , 0.25 mg g^{-1} , and 0.20 mg g^{-1} of glycerol, respectively.
 370 Experiments with model compounds have shown that only minor amounts of glycerol are obtained in bio-
 371 crudes from cellulose, hemicellulose, and protein meaning that glycerol is almost exclusively obtained from
 372 triglycerides (data not shown). The high glycerol contents in bio-crude from DDGS and household waste
 373 also shows the highest content of triglycerides is found in these feedstocks. The significant amount of
 374 glycerol found in bio-crudes of macroalgae and lignocellulosics is likely due to the presence of other
 375 compounds creating micro-emulsions similar to what is observed for fatty acids of the aqueous phase. The
 376 solubility of glycerol is likely linked to the presence of small organic acids of which acetic acid and lactic acid

377 were particularly abundant. Bio-crudes of household waste, *L. digitate*, and Clover contained some of the
378 highest amounts of acetic acid (4.10 mg g^{-1} , 1.54 mg g^{-1} , and 2.4 mg g^{-1} , respectively) and lactic acid (6.76
379 mg g^{-1} , 5.13 mg g^{-1} , and 4.28 mg g^{-1} , respectively) while concentrations were significantly lower for bio-
380 crudes of microalgae. The concentration of glycerol and lactic acid in bio-crudes from mixtures showed
381 similar tendencies where the measured concentration of these compounds was lower than the calculated
382 concentration when mixing biomasses of high lipid content together (DDGS and *N. gaditana*). Meanwhile,
383 the measured concentration was higher or similar to the calculated concentration for the remaining
384 mixtures (Figure 4G-H).

385

386 Figure 4. Concentration of A) 2,3-dimethylcyclopent-2-enone, B) 3-hydroxypyridine, C) palmitic acid, D) 4-

387 ethylphenol, E) 2-methoxyphenol, F) ethylene glycol, G) glycerol, and H) lactic acid in bio-crudes from

388 mixtures. Black circles are calculated values.

3.2 Analysis of aqueous phase

The AqP has been recognized as an important fraction for making the HTL process economically feasible due to the distribution of significant amounts of carbon. In this section we report TOC, TN, pH, and quantitative GC-MS analysis of compound classes similar to the bio-crude.

3.2.1 TOC, TN, and pH

Table 3 shows the values for TOC, TN, and pH of HTL AqPs. A general trend can be observed showing that all values increased in the order of lignocellulosics < macroalgae < residue < microalgae. The TN of the AqPs was highly dependent on the protein content of the feedstock. We have previously reported that TN and pH values of the AqP can be predicted based on the protein content of the feedstock.³⁷ Other studies have identified ammonium as the main nitrogen-containing compound of the AqP leading to increasing pH values for HTL AqP from protein rich biomass.¹⁷

The TOC values were lowest for AqP from feedstock of lignocellulosics and macroalgae, which were approximately 21,000 mg L⁻¹. However, the TOC value increased substantially when feedstocks with > 30% protein were used (microalgae and residues), which also lead to pH > 8.00. Furthermore, the TOC value increases steadily with increasing protein content and thereby increasing pH values. It is proposed that the increase in TOC value could be due enhanced deprotonation of phenolics (phenol pK_a 10) and protonation of nitrogenated aromatics (pyridine pK_b 8.8) leading to exponential increase in their ionized form and thus higher solubility in water.

Table 3. Total organic carbon, total nitrogen, pH values of AqP from HTL of biomass and mixtures.

	TOC - mg L ⁻¹	TN - mg L ⁻¹	pH
<i>M. x giganteus</i>	20845 ± 148	231 ± 11	6.52 ± 0.40
Willow	20800 ± 42	83 ± 1	5.71 ± 0.08
Poplar	21095 ± 573	87 ± 4	5.76 ± 0.06
Clover	20010 ± 424	600 ± 29	7.16 ± 0.03
<i>L. digitata</i>	21365 ± 445	542 ± 78	7.35 ± 0.35
<i>L. hyperborea</i>	19550 ± 396	1355 ± 35	7.70 ± 0.08

C. vulgaris	24785 ± 693	6850 ± 375	9.09 ± 0.14
Spirulina	26715 ± 7	10150 ± 71	9.49 ± 0.15
N. gaditana	23660 ± 516	7135 ± 127	9.00 ± 0.08
DDGS	23310 ± 283	5010 ± 693	8.14 ± 0.00
HHW	22705 ± 346	2210 ± 863	7.15 ± 0.12

409

410 Despite the apparent linear dependency of TOC and TN on protein content, measured TOC and TN values

411 for AqPs from mixtures were approximately 10 % lower than calculated values in most cases (Figure 5A-B).

412 It has previously been shown that increasing concentrations of nitrogenated compounds in the AqP leads to

413 further displacement into the bio-crude.²⁶ Hence, the increasing formation of nitrogenated compounds

414 observed in the bio-crude and AqP (section 3.2.2) caused by reaction of ammonium with degradation

415 products leads to less ammonium in the AqP and more nitrogen ending up in the bio-crude. Furthermore,

416 the change in equilibria towards dehydration of monosaccharides means that less small polar compounds

417 (< C₅) soluble in AqP are formed, which lowers the TOC value.

418

419 Figure 5. Concentrations of A) Total organic carbon and B) Total nitrogen in aqueous phase from HTL of

420 mixtures. Black circles are calculated values.

421

422

423

424 3.2.2 GC-MS analysis

425 In this section we present quantification of a set of diverse of compounds with the aim of elaborating on
426 findings from analysis of the bio-crude. The sum of quantitated small organic acids, fatty acids, oxygenated
427 aromatics, cyclic oxygenates, and nitrogenates are presented in Table 4 and the full list of quantitated
428 compounds is provided in Supplementary III.

429 Small organic acids are a major part of the AqP and have a potential for production of value-added
430 chemicals³⁸. They are also the most diverse group of compounds detected in this work. Total
431 concentrations of small organic acids ranged from a minimum of 4446 mg l⁻¹ in AqP from *L. digitata* to a
432 maximum of 8063 mg l⁻¹ for willow. However, Most AqPs maintained concentrations of approximately 6200
433 – 8000 mg l⁻¹.

434 Acetic acid was the most abundant compound detected in the AqP, with the analytical method used in this
435 work. Acetic acid is especially prevalent from lignocellulosics with concentrations of up to 5226 mg l⁻¹ for
436 willow, which is comparable to previously published results.¹⁸ A previous study of model compounds
437 showed the largest formation of acetic acid in the AqP from protein feedstock.³⁷ However, the model
438 compounds had been purified with removal of protective groups, probably leading to reduced formation of
439 acetic acid. DDGS is a residue from bio-ethanol production and has also been purified and showed low
440 concentrations of acetic acid while macroalgae had the lowest concentration despite the feedstock having
441 carbohydrate concentrations similar to lignocellulosics. Microalgae led to AqP with intermediate acetic acid
442 concentrations. Hence, acetic acid is predominantly formed from hydrolysis of acetyl groups especially
443 prevalent in lignocellulosics with contributions from degradation of carbohydrates and proteins. This
444 observation was further supported by the observation that measured concentrations of small organic acids
445 (predominantly acetic acid) showed only minor difference from calculated concentrations (Supplementary
446 IV).

447 Addition of potassium carbonate as catalyst entails some degree of saponification, which will affect the
448 fatty acid concentrations. Fatty acids are insoluble in water, and it has been suggested that fatty acids are

449 present as micro-emulsions.²⁰ The concentration of fatty acids in AqP from HTL of grass species and
 450 macroalgae were < 41 mg l⁻¹ while wood species gave 109-127 mg l⁻¹ indicating that triglycerides from wood
 451 is more easily hydrolyzed in HTL than triglycerides from grass and macroalgae. It is noteworthy that fatty
 452 acid concentration of AqP from *N. gaditana* was approximately 2.3 times higher than *C. vulgaris*, which is
 453 similar to the difference in lipid content, while *C. vulgaris* had fatty acid concentrations 4 times higher than
 454 DDGS despite having similar biochemical composition, with *C. vulgaris* even having lower lipid content.
 455 Comparison of the bio-crude from these samples shows similar fatty acid profiles; hence the difference is
 456 unlikely to be explained by the lipid profile of the feedstock. Furthermore, the pH value for AqP of *C.*
 457 *vulgaris* (pH 9.0) was higher than for DDGS (pH 8.1) which could indicate that saponification is significantly
 458 enhanced across this range. The saponification effect is apparent for AqP of mixtures, as measured fatty
 459 acid concentrations are significantly lower than calculated concentrations when mixing microalgae and
 460 lignocellulosics, while it increases for the mixture of DDGS and *N. gaditana* (Supplementary IV). However,
 461 saponification seems to be only part of the explanation as mixtures with lignocellulosics in many cases lead
 462 to values that were significantly lower than for lignocellulosic feedstock alone which had acidic AqP.
 463 Furthermore, a significantly lower concentration of free fatty acids from *Spirulina* with *C. vulgaris* is
 464 observed which is due to the higher protein content leading greater formation of fatty acid amides.³⁹
 465 *Table 4. Concentrations of small organic acids, fatty acids, oxygenated aromatics, cyclic oxygenates, and*
 466 *nitrogenates in AqP from HTL of biomasses and mixtures.*

	Small organic acids mg L ⁻¹	Fatty acids mg L ⁻¹	Oxygenated aromatics mg L ⁻¹	Cyclic oxygenates mg L ⁻¹	Nitrogenates mg L ⁻¹
<i>M. x giganteus</i>	7552	40.5	392.7	1683	155.4
Willow	8063	126.5	97.8	1703	73.9
Poplar	7831	109.0	345.3	1580	78.4
Clover	6200	21.3	167.5	1926	464.9
<i>L. digitata</i>	4446	12.1	42.0	1878	186.4
<i>L. hyperborea</i>	5568	13.3	66.4	1274	268.9
<i>C. vulgaris</i>	7703	975.0	182.8	630.9	1966
<i>Spirulina</i>	6720	256.6	238.7	340.6	796.0
<i>N. gaditana</i>	6651	2401	194.9	497.5	899.0
DDGS	6804	237.0	119.0	1178	1927

Household waste	7388	23.3	112.7	1933	1253
Nanno/DDGS	6484	2101	164.8	870.7	1366
DDGS/Misc	7010	37.2	266.2	1552	1233
Misc/Nanno	7417	105.6	300.6	1144	960.5
Misc/Nanno/DDGS	8024	37.4	255.0	1198	1336
Pop/Chlo	7407	17.5	256.7	1311	1704
Spir/Chlo	7009	208.4	193.9	483.1	1301
Spir/Pop	7874	134.6	298.4	971.5	1404
Spir/Pop/Chlo	8035	267.7	254.9	939.6	1633

467

468 The most commonly reported oxygenated aromatics were quantitated (phenols, cresols, hydroquinones).
469 Concentrations amongst lignocellulosics varied greatly with *M. x giganteus* and poplar having the greatest
470 concentrations of 393 and 345 mg l⁻¹ in their AqPs, respectively, while clover and willow had only 168 and
471 98 mg l⁻¹, respectively. The lowest concentrations were obtained in AqP from macroalgae with less than 70
472 mg l⁻¹, while AqP from microalgae contained approximately 200 mg l⁻¹ supporting that lignin is the main
473 contributor of oxygenated aromatics but also emphasizing that significant amounts are produced from
474 protein. The substantial concentrations found in AqP from microalgae may be attributed to the alkaline pH
475 of the AqP which is nearing the pK_a value of phenol (pK_a ~10) leading to a greater degree of deprotonation
476 and thereby solubility in water leading to increasing TOC values. The formation of oxygenated aromatics
477 seems to be independent of mixing of biomasses as accurate calculation of concentrations in AqP from
478 mixtures could be made (Supplementary IV). Thus, these compounds look to be formed mainly from simple
479 degradation of lignin and amino acids while smaller contributions occurs from degradation and
480 intramolecular reaction of carbohydrates which do not compete with other reaction pathways.
481 In previous work with model compounds a linear dependency on carbohydrate content was observed for
482 formation of cyclic oxygenates in AqP.³⁷ The concentration of cyclic oxygenates indeed decreases in the
483 following order lignocellulosics/macroalgae > DDGS > microalgae. The highest concentration was observed
484 for 3-methylcyclopent-2-enone with 1060 mg L⁻¹ in AqP from clover. It is interesting to note that the
485 measured concentration of cyclic oxygenates from mixtures could be calculated (Supplementary IV) and did

not correlate with observations in the bio-crude. However, it has previously been shown that the concentration of cyclic oxygenates in bio-crude does not necessarily correlate with AqP concentrations.²⁶ Nitrogenated compounds reported from HTL AqP are most often amides, pyrroles, pyrazines, pyrrolidinones, and hydroxypyridines with pyrazines constituting the most varied compound class. The analytical method used in this work allowed quantification of pyrazines, pyrrolidin-2-one, and 3-hydroxypyridine. Generally the total concentration varied more than one order of magnitude (with up to two orders of magnitude for some pyrazines) between AqP from HTL of different feedstocks. Particularly high concentrations were found in the AqP from HTL of DDGS and *C. vulgaris*. Pyrazines are formed via several pathways which include (i) self-condensation of amino acids, (ii) Amadori rearrangement (AR) of carbohydrate products and ammonium/amines, and (iii) Strecker type degradation (SD) of carbohydrate products and amino acids⁴⁰. Previous work on model compounds showed that the formation of pyrazines highly depends on the ratio of carbohydrate to protein of the feedstock.³⁷ In that work, it was found that a linear fit was required when protein to carbohydrate was <1, a 2nd order polynomial was required when the ratio was >1, and an exponential fit was required when the ratio was ~ 1. Similar observations were made in this work which is especially noticed for AqP of mixtures supporting the observations made for TN and TOC values in AqP and elemental distribution in bio-crude. The measured and calculated concentration in AqP of mixtures for nitrogenates, methylpyrazine, pyrrolidin-2-one, and 3-hydroxypyridine are presented along with carbohydrate to protein ratios in the mixtures in Figure 6A-D. Mixing microalgae together or with DDGS leads to excess protein and the measured and calculated values of the compounds are consistent as expected from a linear dependency. Mixing lignocellulosics with microalgae leads to carbohydrate to protein ratios between 1 and 3, and increasing discrepancy between measured and calculated values are observed as ratios approach 1 which is expected from 2nd order polynomial and exponential functions. The largest differences are generally observed with the mixture of *Spirulina* and poplar, where a 10 fold higher concentration of methylpyrazine is observed. The relative difference between measured and calculated concentrations is highest for pyrazines, which could mean that if the bio-crude is not solvent extracted a

511 more significant amount of nitrogen will be found in the bio-crude of feedstocks with carbohydrate to
 512 protein ratios close to 1.

513

514

515 Figure 6. Concentration of A) nitrogenates, B) methylpyrazine, C) pyrrolidin-2-one, and D) 3-hydroxypyridine
 516 in AqP from HTL of mixtures of biomass. Primary axes show concentration and secondary axes show
 517 carbohydrate to protein ratio.

518

519 3.2.3 PCA

520 In order to evaluate the variation in concentration of single compounds, PCA was performed which also
 521 serves to simplify interpretation and provide visual comparison. Figure 4 shows the biplots of scores
 522 (samples) and loadings (products) of PC1-3.

Again AqP from HTL of household waste displayed a highly different composition than the remaining samples and was removed as an outlier. Three principal components explained 69.5 % of the total variance. PC 1 explained 42.4 % of the total variance and separated AqP samples obtained from high carbohydrate feedstocks from samples obtained from high protein and lipid feedstocks (Fig. 7A). Microalgae and most biomass mixtures were clustered together and characterized by higher concentrations of fatty acids, nitrogenates, and numerous small organic acids as well as oxygenated aromatics formed from degradation of amino acids. The major compounds of these compound classes from microalgae were palmitoleic acid (952 mg l⁻¹, *N. gaditana*), methylpyrazine (969 mg l⁻¹, *C. vulgaris*), succinic acid (1568 mg l⁻¹, *C. vulgaris*), and hydrocinnamic acid (88.9 mg l⁻¹, *Spirulina/C. vulgaris*).

PC 2 explained 18.0 % of the total variance and separated samples of macroalgae from the remaining samples. AqPs from macroalgae were distinctly different from the other samples and were characterized by higher concentrations of especially unsaturated small organic acids and several cyclic oxygenates and significantly lower concentration of acetic acid (1171 mg l⁻¹, *L. hyperborea*). The higher concentration of unsaturated small organic acids, mainly C₃₋₅, in AqP from macroalgae is due to the presence of acidic polysaccharides, such as alginate, which decompose and dehydrate. However, the highest concentration was found for methacrylic acid, which was only 124 mg l⁻¹ (*L. hyperborea*).

AqP of lignocellulosics showed negative scores on PC 2 and were characterized by higher concentrations of several small organic acids of which acetic acid is especially prevalent (5226 mg l⁻¹, willow). Thus, PC 2 displays either the difference in carbohydrate composition of macroalgae and lignocellulosics, such as the degree of acetylation, or the difference in their processing due to the higher ash content of macroalgae. The change of reaction pathways for nitrogenate formation is well described by PC 1 and 3 which explains 54.1 % of the total variance. Figure 7B supports our previous findings that when carbohydrate to protein ratios move closer to 1 in the feedstock, the formation of nitrogenates significantly increases and mixtures of samples are grouped close to samples from DDGS and *C. vulgaris*.³⁷ This is especially notable for the formation of methylpyrazine in poplar (4.3 mg l⁻¹), *Spirulina* (139 mg l⁻¹), *C. vulgaris* (969 mg l⁻¹), and the

548 mixture of Poplar/*Spirulina*/*C. vulgaris* (684 mg l⁻¹). Due to the increasing displacement of nitrogenates
549 from AqP to bio-crude, the increasing pyrazine formation in mixtures explains the higher nitrogen contents
550 of the bio-crudes observed in section 3.1.1, making mixtures of high protein biomasses and lignocellulosics
551 less desirable.
552 Additional PCs, encompassing up to 90 % of the total variance, were investigated in order to determine
553 potential matrix effects or additional interactions from mixing of biomasses. This did not reveal any hidden
554 structures in the dataset. However, mixtures containing macroalgae were not prepared and it is likely that
555 mixtures with high protein biomass could yield different nitrogenates, considering the differences in
556 formation of cyclic oxygenates from lignocellulosics and macroalgae.

557

558 *Figure 7. Biplots of scores and loading in AqPs for A) PC1-2 and B) PC1-3.*

559

560 **3.3 Reaction pathways**

561 In this section we propose a reaction network for formation of products from HTL of biomass (Figure 8).

562 Each reaction is assigned a capital letter, which is presented in parenthesis in the description below.

563 High lipid containing biomass has generally been considered the most applicable to HTL due to the ease of

564 hydrolysis (A) and the high resulting bio-crude yields. The current work shows that only partial hydrolysis

565 occurs, which has also been observed in other studies from model compounds.³⁶ Glycerol released from

566 hydrolysis has been proposed to degrade to a number of aldehydes and ketones,⁴¹ which are highly

567 reactive in the presence of especially ammonia and amines (B). Fatty acids are often the most abundant

568 compounds of the biomass and are thermally stable. In the presence of protein they can be converted to a

569 range of fatty amides (C).³⁹ Recent work shows that the conversion to fatty amides is dependent on the

570 ratios of carbohydrate, protein, and lipids and their abundance is often overlooked due to the larger

571 presence of fatty acids.⁴² The ammonia is obtained from deamination of amino acids hydrolyzed from

572 protein, resulting in a large number of small organic acids mainly dispersed to the AqP (D).⁴³ Especially

573 succinic acid is abundant and can react with the released ammonia or amines to form succinimides (E),⁴⁴

574 which in a reductive environment can produce pyrroles (F). Pyrroles have previously been shown to be

575 abundant when bio-crudes are recovered without solvent extraction.²⁰ Amino acids can also condensate

576 with carbonyls in a Knorr pyrrole reaction leading to substituted pyrroles (G). Decarboxylation of amino

577 acids leads to formation of amines, which are highly reactive and capable of the same reactions mentioned

578 for ammonia (H). Amino acids can self-condensate into diketopiperazines (I),⁴⁵ which at elevated

579 temperatures may reduce to pyrazines (J). Pyrazines can also be formed via products from Strecker

580 degradation and Amadori rearrangement (K),⁴⁶ which condensate and reduce to pyrazines (L).

581 It has previously been established that two main pathways for degradation of cellulose and hemicellulose

582 occur in HTL, namely dehydration and retro-aldol reaction.³³ Retro-aldol leads to formation of small organic

583 acids such as formic acid, acetic acid, glycolic acid, and lactic acid found to be highly abundant in the AqP
584 (M).⁴⁷ The formation of these compounds is furthermore found to be highly dependent on the pH of the
585 medium.⁴⁸ These can again perform aldol reactions to form acetone and small dicarboxylic acids (N) of
586 which malonic acids are highly reactive and could be reacting with resorcinols resulting in umbelliferone
587 derivatives (O). Resorcinols are abundant in plant material but are typically not reported in bio-crudes from
588 HTL making the proposed reaction doubtful. The acetone produced from aldol condensation could be part
589 of condensation reaction with for example glycolic acid and lactic acid to form cyclopentanones and
590 cyclopent-2-enones (P),³³ potentially reacting further with ketoacids to form indanones and indenones (Q).
591 Acetone and small organic acids may also degrade further to gaseous products.⁴⁹
592 In the presence of protein, cyclic ketones may form Schiff bases (R) potentially reacting with ketoacids to
593 form indoles (S). Often indoles are reported as *N*-alkylated suggesting that indoles are formed from the
594 reaction of the amine moiety from amino acids, which could suggest the pathway through cyclic ketones.
595 The highly abundant glycolic acid and lactic acid may also reduce to glycols (T) reported in AqP from HTL of
596 wood or they may condensate to produce oxygenated aromatics (U) such as phenol and catechol observed
597 from HTL of cellulose and hemicellulose.
598 Monosaccharides exist as tautomers (V), which can be reduced to different furans, furfurals, and levulinic
599 acid derivatives (W). Furans and furfurals have been suggested to react with ammonia to produce 3-
600 hydroxypyridines (Y),³⁶ while levulinic acid derivatives may react with ammonia to form pyrrolidin-2-ones
601 (Z) which are often reported in the literature.¹⁹ Furfurals may also be an additional source of indanones and
602 indenones.⁵⁰
603 When carbohydrate is mixed with protein we observe decreasing concentrations of lactic acid, glycols and
604 cyclic ketones along with increasing concentrations of pyrazines and 3-hydroxypyridines, which suggests
605 that equilibria shift towards formation of furfurals, Strecker products, and Amadori products.
606 Degradation products of lignin consist predominantly of oxygenated aromatics (X). The decomposition
607 involves the formation of aldehydes and ketones of which vanillin is most often reported. These

compounds are likely involved in either Schiff base formation or nucleophilic attack on the aromatic ring by ammonia. More work is required to elucidate these effects. Lignin is also known to be the source of a number of aromatic oligomers, alkylated benzenes, and small aldehyde, ketones, carboxylic acids, and alcohols, which are not displayed in this scheme.^{42, 51}

The reaction network presented in this work is by no means comprehensive due to the limitation of GC to detect volatile and semi-volatile compounds. Furthermore, compounds formed from pigments, triterpenoids, and waxes were not included for simplicity. Additional characterization is required on the non-volatile fraction with analytical techniques such as NMR, FT-ICR MS, pyrolysis GC-MS, and LC-MS.

Figure 8. Proposed reaction network for HTL of lipid, protein, cellulose, hemicellulose, and lignin.

618

619 **4. Conclusion**

620 Feedstocks of lignocellulosics, macroalgae, microalgae, and residues were subjected to HTL and
621 quantitative analysis of bio-crudes and AqPs was conducted. The oxygen content in bio-crudes decreased in
622 the order lignocellulosics > algae > residues. Nitrogen and sulfur contents increased compared to the
623 biomass when the initial value was < 3% nitrogen while it decreased for initial values of > 3% nitrogen.
624 Mixtures of lignocellulosics and microalgae led to decreasing oxygen contents and increasing carbon,
625 nitrogen, and sulfur contents in bio-crudes when compared to calculated values. These effects were linked
626 to the formation of Schiff bases by ammonia/amines with furans and furfurals in the bio-crude leading to
627 less formation of smaller oxygenated compounds such as diols. Therefore, mixing of microalgae and
628 lignocellulosics leads to a bio-crude of lower than expected quality.
629 Decreasing concentrations of TOC and TN were observed for AqPs of mixtures of lignocellulosics and
630 microalgae when compared to calculated values. This was due to the formation of pyrazines which was
631 significantly increased when carbohydrate to protein ratios were close to 1. The substantially increased
632 pyrazine formation could lead to a lower quality bio-crude without solvent extraction.
633 This work shows that detailed chemical analyses provide important information for elucidating reaction
634 pathways during HTL and for optimizing feedstock composition/blend to obtain a desired bio-crude
635 composition.

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