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Temperature-Dependent Lipid Conversion and Nonlipid Composition of Microalgal Hydrothermal Liquefaction Oils Monitored by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Abstract We illustrate a detailed compositional characterization of hydrothermal liquefaction (HTL) oils derived from two biochemically distinct microalgae, *Nannochloropsis gaditana* and *Chlorella* sp. (DOE 1412), for a range of reaction temperature as observed by high-resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS). The unique capability to unequivocally derive molecular formulae directly from FT-ICR MS-measured mass-to-charge ratio (for several thousand compounds in each oil) shows that lipids are completely reacted/converted for any reaction temperature above 200 °C and reveals the formation of nonlipid reaction products with increasing temperature. Specifically, lipid-rich oil is obtained at low reaction temperature (<225 °C) for both microalgal strains. For positive ion mode, the major lipid components in *Chlorella* sp. and *N. gaditana* HTL oils are betaine lipids and acylglycerols, respectively. Acidic species in the HTL oils (observed by negative ion mode) are dominated by free fatty acids (FFA) regardless of reaction temperature. HTL oils obtained at higher temperatures (≥225 °C) are composed of a variety of basic nitrogen- and oxygen-containing compounds that originate from protein and carbohydrate degradation at elevated temperature. Similar structural features are observed for the abundant nitrogen heterocyclics between the two strains with slightly lower carbon number for *Chlorella* sp., overall.

Keywords *Nannochloropsis* · *Chlorella* · Hydrothermal liquefaction · Lipids · FT-ICR MS · Mass spectrometry

Introduction

The production of liquid fuel and liquid fuel precursors from microalgae is an ongoing interest, and technical challenges remain for the efficient conversion of this feedstock. The transesterification of extracted lipids from microalgae, to produce biodiesel, is a process that suffers numerous challenges and is limited to production species that accumulate high proportions of lipid. Among those challenges (which include reduced biomass productivity upon stress-induced lipid accumulation and high-energy mechanical and/or solvent-based lipid extraction steps), the incomplete utilization of the available biomass is most detrimental to the production economics of the "lipid-only" approach. Consequently, alternative conversion routes have been pursued for the production of liquid fuels from microalgae [1–7], and thermochemical processes which utilize/convert all biomass constituents (e.g., lipids, proteins, carbohydrates and algaenan) to liquid fuel precursors are attractive alternatives. Pyrolysis and gasification require an energy-intensive drying step prior to processing and it is for this reason

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that hydrothermal liquefaction (HTL) appears most promising for the treatment of wet microalgae. Direct HTL processing of wet algal biomass at 5–20 % solids consumes less than 5 % of the energy cost associated with the complete water removal by thermal drying [8]. Additionally, improved cultivation-to-product efficiency can be achieved by hydrothermal liquefaction of robust, rapid growth microalgal strains without the requirement of high lipid content. Hydrothermal liquefaction oil yields of 40–64 % have been reported for microalgal strains [1, 2, 9], and the use of different catalysts to maximize oil yield has also been studied [3, 4, 10].

The chemical composition of a given HTL oil depends on the microalgal feedstock used and the reaction conditions [8, 11]. Here, we present a detailed compositional description of HTL oil produced by a range of HTL reaction temperature (from 180 to 300 °C) for two microalgal strains that have emerged as production candidates from recent US Department of Energy-funded research consortia. *Chlorella* sp. (DOE 1412; hereafter *Chlorella* sp.) and *Nannochloropsis gaditana* (CCMP-1775) contain significantly different protein and lipid fraction ratios. *Chlorella* sp. is a fast-growing freshwater microalga which can tolerate a growth temperature of up to 42 °C [12] and is therefore a potential biofuel candidate for warm-to-hot climate zones where microalgal production benefits from increased solar incidence. The marine microalga, *Nannochloropsis* sp., grows in saline water (which removes challenges with freshwater usage/supply) and these organisms have been well-studied in recent biofuel production research efforts.

With regard to hydrothermal liquefaction oils derived from microalgae, only limited compositional description is provided by standard analytical techniques such as gas chromatography-mass spectrometry (GC-MS) [1, 2], Fourier transform infrared spectroscopy [13], ^{13}C NMR [11], and ^{1}H NMR [3]. Torri et al. [14] studied the hydrothermal liquefaction oil from *Desmodesmus* sp. by pyrolysis-GC-MS coupled with solid-phase microextraction (Py-SPME-GC-MS) with a particular focus on nitrogen-containing compounds. The current report utilizes ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) coupled with electrospray ionization (ESI) to reveal the temperature-dependent conversion of microalgal lipids and the formation of nonlipid HTL products from two microalgae and provides insight for feedstock selection, process optimization, and upgrading strategy designs for improved oil production by HTL for microalgal feedstock.

Methods

Production of Algal Biomass

The freshwater microalgae *Chlorella* sp. (DOE 1412, also known as *Chlorella* strain NAABB2412) was isolated from surface water in Texas by Dr. Juergen Polle (Brooklyn College). Material for HTL oil production was grown in BG-11 medium [15] in a 4000-L Lumian outdoor photobioreactor (Solix Biosystems, CO) composed of multiple 200-L vertical plastic panels suspended in a water basin at New Mexico State University as previously described [16]. Cultures were enriched with 0.8 % CO_2, and the temperature was maintained at ~25 °C. Marine microalgae, *Nannochloropsis gaditana* (CCMP-1775), was obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), and cultures were grown in f/2 medium modified to contain 5 mM NO_3^- and 0.287 mM PO_4^{3-} at a salinity of 20 g/L in the same temperature-controlled 4000-L outdoor photobioreactor at New Mexico State University. *Chlorella* sp. was harvested under nitrogen-replete conditions in linear growth phase at a biomass density of 0.5–0.7 g/L, and *N. gaditana* was harvested in stationary growth phase at a biomass density of 1.5–2.6 g/L. Cells were separated from the media by centrifugation, and the resultant supernatants and biomass were kept frozen until oil production by hydrothermal liquefaction.

Proximate and Biochemical Analyses

Biochemical and proximate composition analyses were performed for the determination of moisture, ash content, crude protein, and lipid content in the feedstock. Crude protein in the biomass was determined by a Leco FP-228 nitrogen determinator as previously described [17]. Lipid content was measured as fatty acid methyl esters (FAMEs) as explained in section "FAME and FFA Quantitation." Moisture and ash content of biomass were determined by thermogravimetric analysis as previously reported [17].

Hydrothermal Liquefaction of Microalgae

Hydrothermal liquefaction was performed in a PARR 4593 stainless steel bench top reactor (Parr Instrument Company, Moline, IL) equipped with a 4843 controller unit. HTL oils were produced from both algal strains at 180, 200, 225, 250, 275, and 300 °C reaction temperature. A 5.0-g portion of dry biomass was loaded to the reactor, and supernatant water collected from biomass centrifugation (i.e., culture media) was added to the reactor to achieve 10 % biomass loading. Residual air was removed from the reactor by three pressurizing/purging cycles with nitrogen (~300 psi). An initial pressure of 200 psi was applied (by filling with nitrogen) to the reactor to achieve rapid boiling of water. The temperature of the reactor was then increased at a rate of 10 °C/min until the desired reaction temperature is reached and maintained at that temperature thereafter. Three replicate runs were performed at each reaction temperature. After the reaction time of 30 minutes had elapsed, the reactor was cooled to room temperature, 30 mL of dichloromethane was added to the reactor and the

contents were mixed. Solid residue was separated via filtration and the remaining reactor contents were transferred to a glass separatory funnel and allowed to phase separate (~15 min). After the aqueous and organic phases had separated, dichloromethane phase was withdrawn and evaporated in a rotary evaporator at 65 °C to recover the HTL oil.

Sample Preparation and Mass Spectrometry

The HTL oils were prepared for mass spectral analysis by dissolution in chloroform/methanol (50:50) as previously described [18]. The concentration of the final dilute oil samples in the electrospray ionization solution was 0.5 mg/mL in both positive- and negative-ionization modes. ESI FT-ICR mass spectrometry was performed with a hybrid linear ion trap 7 T FT-ICR mass spectrometer (LTQ FT, Thermo Fisher, San Jose, CA) equipped with an Advion Triversa Nanomate (Advion Biosystems, Inc.) as recently described [19]. A total of 500 and 400 time domain transients were co-added for each sample in positive- and negative-ion modes, respectively, prior to fast Fourier transformation and frequency to mass-to-charge ratio conversion.

Internal mass calibration of FT-ICR mass spectra was performed using multiple homologous alkylation series of known compounds, where elemental compositions differ by integer multiples of CH_2. Specifically, N_1O_1 compounds with DBE=5 (DBE, double-bond equivalents, number of rings plus double bonds to carbon) that span ~150–650 Da were used for the positive-ion mode mass spectral calibration for all samples. As low-temperature HTL oils exhibit an extended molecular weight distribution, N_1O_7 compounds with DBE=5 (which are identified as diacylglyceryl-*N*,*N*,*N*-trimethylhomoserines) and O_6Na_1 compounds with DBE=4 (identified as triacylglycerols) were also included in the mass calibration for *Chlorella* sp. and *N. gaditana*, respectively, to achieve a broadband internal calibration. For the negative-ion mode mass spectral calibration, O_2 compounds (fatty acids) with DBE=1 and O_4S_1 species (O_4 sulfate lipids) with DBE=1 were used for internal calibration of all oil samples.

High-resolution FT-ICR mass spectra confirm that all observed ions are singly charged as evidenced by the 1 Da spacing between $^{12}C_c$ and $^{13}C_1{}^{12}C_{c-1}$ peaks for the species with the same molecular formula. Peak lists were generated from m/z values in the range of 100–900 Da with peak magnitudes greater than 10 times the standard deviation of the baseline noise. IUPAC measured masses (CH_2=14.01565 Da) were converted to the Kendrick mass scale (CH_2=14.0000 Kendrick mass units) as previously described [20] and sorted by Kendrick mass defect to facilitate identification of homologous series (i.e., compounds with the same heteroatom composition and the same double-bond equivalents but differing in the degree of alkylation). Elemental compositions could be assigned to >96 and >80 % of the total mass spectral signal for *Chlorella* sp. and *N. gaditana* HTL oils, respectively, in both ionization modes. A variety of nonlipid reaction products from HTL processing at different reaction temperatures were identified by their heteroatom class. Residual lipids in the oils were identified as a given lipid class by matching the assigned elemental compositions to an in-house assembled lipid database derived from Lipid Maps (Nature, Lipidomics Gateway) as previously described [21]. Tandem mass measurement was performed for selected species in each abundant compound class for structural identification where possible. For this process, ions of interest were first isolated and signals from isobars within 1 Da of the selected ion mass were determined. If no significant/high-abundance interfering peaks were observed, MS^n fragmentation was performed in the linear ion trap by collision-induced dissociation (CID) with high-resolution/accurate mass measurement FT-ICR MS detection of the fragment ions. The MS^n spectra were evaluated by manual inspection and elemental composition of fragment ions was determined directly from sub-part-per-million mass measurement accuracy. These data represent a detailed qualitative analysis where comparison of individual compound signals is possible between samples, but compound-to-compound signal magnitudes are not comparable due to a lack of available analytical standards for each of the 1,000+ compounds observed.

FAME and FFA Quantitation

Acid- and base-catalyzed FAME analyses were performed for *Chlorella* sp. and *N. gaditana* biomass and their HTL oils produced at reaction temperatures range from 180 to 300 °C. For acid-catalyzed FAME analysis, 5 mL of 0.4 % sulfuric acid was added to 50 mg of sample and vortexed for 30 s. Then, the sample was placed in a hot water bath at 75 °C for 1 h and vortexed in 20-min intervals. One milliliter of deionized water was added to quench the reaction, and the sample was back extracted with 2 mL of hexane containing methyl tricosanoate C23:0 (50 μg/mL), as the internal standard.

Base-catalyzed FAME analysis was performed according to Reddy et al. [22]. Briefly, 5 mL of 0.2 N sodium hydroxide was added to 50 mg of sample and vortexed for 30 s. Then, the sample was placed in a hot water bath at 65 °C for 30 min and vortexed in 10-min intervals. The reaction was quenched by adding 1 mL of 1 M glacial acetic acid, and the sample was back extracted with 2 mL of hexane containing methyl tricosanoate (50 μg/mL). Samples from both acid- and base-catalyzed reactions were diluted 100-fold with hexane prior to analysis by gas chromatography/mass spectrometry.

Final dilute hexane extracts were analyzed with a Hewlett Packard 5890 Series 2 plus GC equipped with an HP 7673 autosampler and a 5972 mass spectrometric detector as previously described [23]. Briefly, 2-μL injections were loaded onto a J & W DB 23 column (30-m length × 0.25-mm

diameter×0.25-μm film thickness (Agilent, Santa Clara, CA)). Helium served as the carrier gas with a 1-mL/min flow rate. Initial column temperature was 80 °C, and then, the temperature was ramped to 220 °C at 20 °C/min and held for 6 min. The inlet and the detector were held at 230 and 250 °C, respectively. Chromatographic signals were matched to a Supelco 37 component FAME mix (Sigma-Aldrich, St Louis, MO), and FAME quantitation was performed by internal calibration using C23:0 as the internal standard.

Results

Results from proximate analysis of the two microalgal strains are listed in Table 1. Oil yields (dry weight basis) from hydrothermal liquefaction of *N. gaditana* and *Chlorella* sp. show a continuous increase with HTL reaction temperature (Fig. 1). *Chlorella* sp. produced 9 % oil yield at 180 °C and that increased to 33 % at 300 °C. *N. gaditana* oil yield increased from 17 to 28 % for reaction temperature between 180 and 275 °C. A dramatic increase in oil yield was observed above 275 °C with the highest oil yield of 48 % observed at 300 °C. HTL oil yields reported for higher temperatures (>200 °C) exceed the lipid content of the biomass, which indicates conversion of other cellular constituents (e.g., protein, carbohydrate, and algaenan) by HTL reaction processes.

Figure 2 shows broadband positive- and negative-ion ESI FT-ICR mass spectra of *Chlorella* sp. HTL oils produced at 180 and 300 °C. Not surprisingly, high-temperature (300 °C) HTL oil is more compositionally complex than the low-temperature (180 °C) oil for both ionization modes. Broadband FT-ICR mass spectra provide molecular weight distribution information and illustrate the compositional complexity of these materials. These data are sorted in several ways to provide visual and accessible interpretation of composition with regard to HTL reaction temperature. Molecular elemental composition assignment combined with Kendrick mass sorting provide three layers of information: class (heteroatom content), type (molecular rings/and or double bonds, DBE), and carbon number distribution (extent of alkylation). Three-dimensional mass spectral images of DBE versus carbon number with relative abundance in the third dimension (color) provide compact visualization of the molecular features for interclass compositional analysis.

Table 1 Proximate and biochemical analyses of *N. gaditana* and *Chlorella* sp. HTL feedstock.

Strain	Moisture (%)	Ash (%)	Lipid (%)	Crude protein (%)
N. gaditana	79.6	7.0	21.7	14.3
Chlorella sp.	74.0	2.5	10.7	44.6

Data reported on dry weight basis

Class Analysis

Heteroatom class analysis for the observed nonlipid constituents of the HTL oils is achieved by grouping the assigned elemental compositions to compound classes with the same heteroatom content. Similarly, lipids are grouped by lipid class and plotted with the observed heteroatom classes (Fig. 3). Relative abundance for each class is calculated by summing the peak magnitudes of all observed ions for each class and dividing by the summed peak magnitude for all assigned monoisotopic masses.

The positive-ion ESI FT-ICR MS class distribution for *Chlorella* sp. HTL oils produced at 180–300 °C is shown in Fig. 3. Predominant species observed at high temperature include a variety of nitrogen-containing compounds with up to 2 oxygen atoms per molecule. A range of lipid classes including monoacylglyceryl-*N*,*N*,*N*-trimethyl-homoserine (MGTS), diacylglyceryl-*N*,*N*,*N*-trimethyl-homoserine (DGTS), diacylglyceryl-carboxyhydroxymethylcholine (DGCC), monoacylglyceryl-carboxyhydroxymethylcholine (MGCC), diacylglycerol (DAG), and free fatty acids are identified for the low-temperature HTL oils. The contribution from nonlipid HTL reaction products to the low-temperature oil is minimal, resulting a lipid-dominated HTL oil at low reaction temperatures, whereas HTL processing at higher temperatures produce oil that mainly comprised nonlipid molecules that include contribution from protein and carbohydrate degradation.

Low-temperature *Chlorella* sp. HTL oil is rich in betaine lipids. Molecular identifications for the reported lipid classes in Fig. 3 were confirmed by tandem mass measurement of the abundant members from each lipid class. Positive ion ESI/MS^n experiments performed for the abundant MGTS and DGTS compounds showed characteristic structural fragmentation of betaine lipids, i.e., a loss of 87 Da representing -CH_2-CH_2-$N^+(CH_3)_3$, a loss of 73 Da denoting -CH_2-$N^+(CH_3)_3$ and a loss of 59 Da indicating trimethylamine [-$N^+(CH_3)_3$]. Depending on the loss of fatty acyl moieties from the parent ion, three most abundant MGTS species with DBE=2, 3, and 7 are identified as 16:0 MGTS, 16:1 MGTS, and 20:5 MGTS, respectively. Online Resource 1 shows ESI MS^2 and MS^3 tandem mass spectra for the identified 16:0 MGTS lipid. Similarly, predominant DGTS species with DBE=4, 5, and 8 are identified as 16:0/16:1 DGTS, 16:1/16:1 DGTS, and 16:0/20:5 DGTS, respectively, by the manual inspection of MS^2 spectra. Online Resource 2 shows ESI MS^2 spectra for the DGTS lipids with 16:0/20:5 and 16:0/16:1 fatty acid distribution. The assignments of MGCC and DGCC lipid classes are

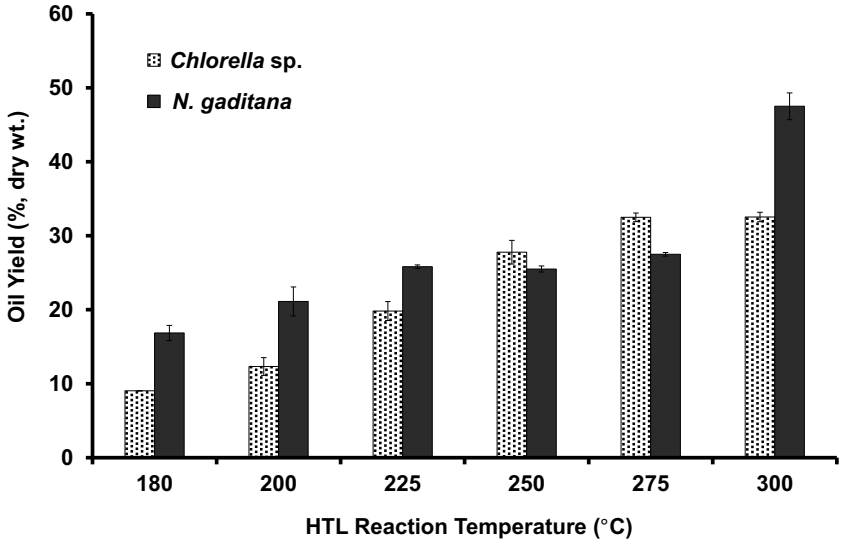

Fig. 1 HTL oil yields

also confirmed by the observation of characteristic structural fragments of betaine, which arise from the loss of trimethylamine, $-CH_2-N^+(CH_3)_3$ and $-CH_2-CH_2-N^+(CH_3)_3$ moieties (spectra not shown).

Protein and carbohydrate degradation and secondary reactions at elevated temperature produce basic-nitrogen compounds that are selectively ionized by positive-ion ESI [14]. Mono-, di-, and tri-nitrogen species observed for *Chlorella* sp.

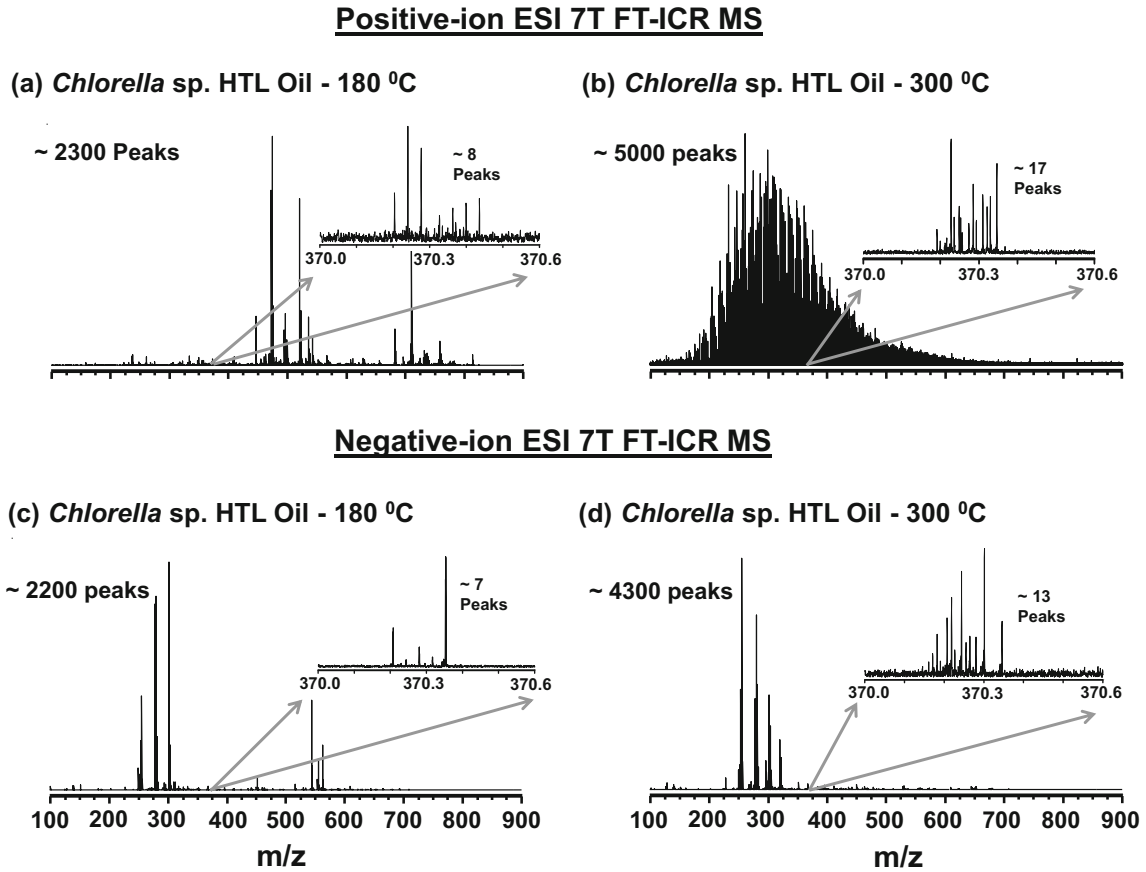

Fig. 2 Broadband positive- and negative-ion ESI FT-ICR mass spectra of *Chlorella* sp. hydrothermal liquefaction oils produced at 180 and 300 °C. Each mass spectrum is obtained from the sum of 500 (positive ion mode) and 400 (negative ion mode) coadded time-domain transients. *Insets* show mass scale expansions at 370 m/z

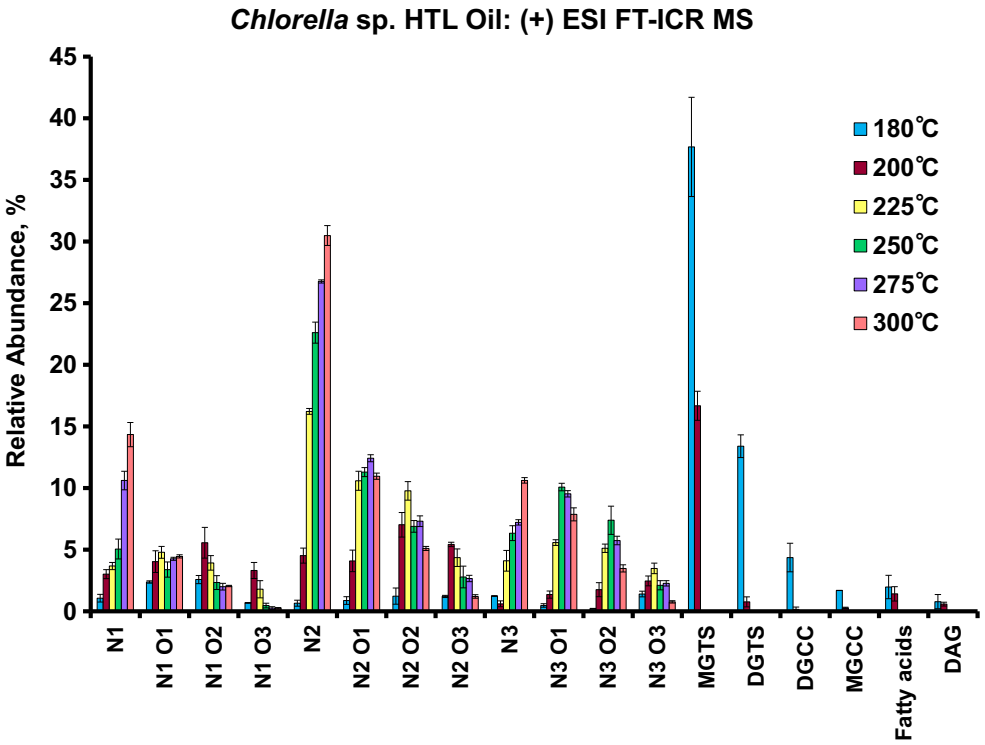

Fig. 3 Class distribution for *Chlorella* sp. HTL oils derived from positive-ion ESI FT-ICR MS. *Error bars* represent standard deviation derived from analysis of three independent HTL oil samples

HTL oils show a continuous increase with HTL reaction temperature. Di-nitrogen (N_2) compounds show a ~46-fold increase in abundance when HTL reaction temperature increased from 180 to 300 °C. The heteroatom classes N_1O_1, N_2O_1, N_2O_2, N_3O_1, and N_3O_2 also show a 10-fold average increase between the lowest and the highest HTL reaction temperature. The increase in nitrogenous compounds with HTL reaction temperature corresponds directly to the observed increase in the HTL oil yield with temperature, as well as high nitrogen and oxygen content in the higher reaction temperature oils (a characteristic of microalgae-derived HTL oils).

The positive-ion ESI FT-ICR MS class distribution for *N. gaditana* HTL oils produced by variable reaction temperature (180–300 °C) is shown in Fig. 4. The distribution of nonlipid materials and their transition with reaction temperature are similar to *Chlorella* sp. HTL oil discussed above. Low-temperature HTL oil is dominated by acylglycerol lipids (observed as sodium ion adducts, $[M+Na]^+$). Again, these lipid class assignments were verified by tandem mass spectrometry for the abundant species in each lipid class. Online Resource 3 shows MS^2 spectra for the predominant DAG and triacylglycerol (TAG) lipids observed in the low-temperature HTL oil. Linear ion trap MS/MS fragmentation with FT-ICR MS detection for the predominant DAG and TAG lipids observed for low-temperature *N. gaditana* HTL oil showed fragments resulting from the loss of fatty acids from the parent ion, as well as loss of carboxylate ion fragments. Two most abundant compounds in DAG lipid class with DBE=2 and 3 are identified as 16:0/16:0 DAG and 16:0/16:1 DAG, respectively, and primary triacylglycerols are identified as 16:0/16:0/16:1 TAG, 16:0/16:1/18:1 TAG, and 16:0/16:1/16:1 TAG, respectively.

The primary difference in HTL oil composition between oils produced from the two microalgal strains (as observed by positive-ion ESI FT-ICR MS) is the variation in lipid class composition for the low-temperature oil. Lipid accumulation in microalgae varies by species and harvest relative to growth period. Our *N. gaditana* material was harvested in the late stationary phase and the produced HTL oil is rich in energy storage lipids: DAG and TAG. A possible explanation for the observation of higher amount of DAG compared to TAG can be due to the thermal degradation of TAG and its transformation into DAG at moderately high temperatures. In contrast, *Chlorella* sp. was harvested before it reaches the stationary phase and betaine lipids (i.e., membrane structural constituents) are observed as the dominant lipid component in the low-temperature oil in positive ion mode.

Positive-ion ESI abundance-contoured plots of DBE versus carbon number for the abundant nitrogen-containing heteroatom classes observed for *N. gaditana* and *Chlorella* sp. HTL oils produced at 300 °C are shown in Fig. 5. Tandem mass measurements for these observed nitrogen-containing species were not possible because they could not be isolated from nearby mass isobars for fragmentation. The N_1 compounds in both algal strains span a similar compositional space with a slight shift to lower carbon number for the abundant species

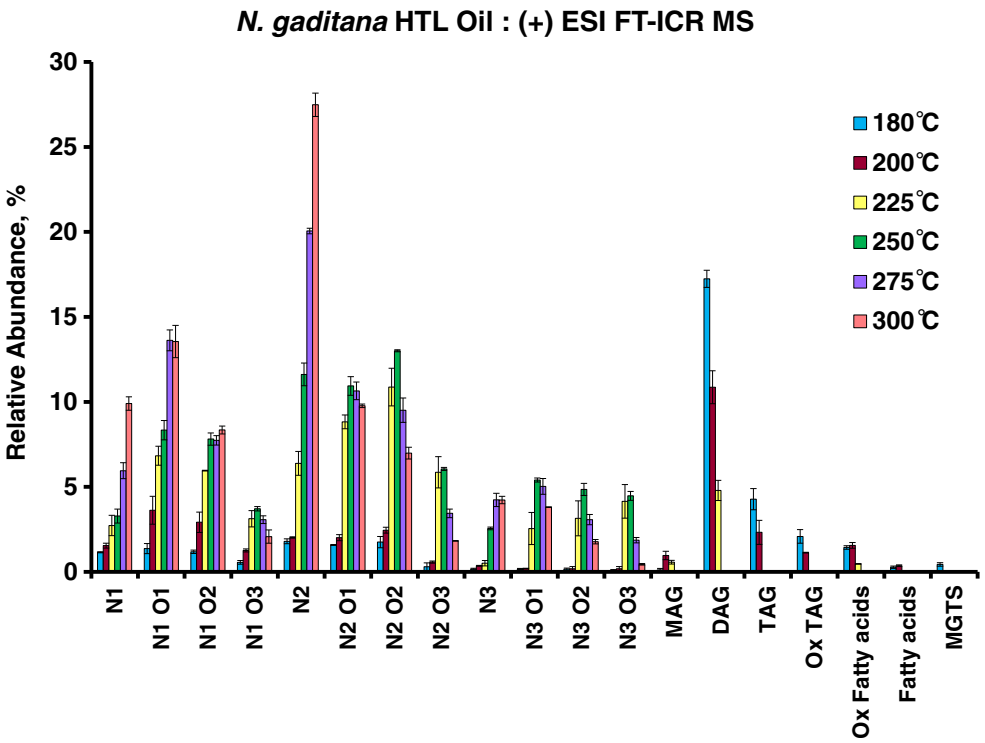

Fig. 4 Class distribution for *N. gaditana* HTL oils derived from positive-ion ESI FT-ICR MS. *Error bars* represent standard deviation derived from analysis of three independent HTL oil samples

in *Chlorella* sp. HTL oil. High carbon content/low DBE observed for the predominant N_1 compounds in both algal strains suggest low aromaticity. The N_1O_1 species with 1–4 DBE and ~15–22 carbons observed for both *Chlorella* sp. and *N. gaditana* are presumably fatty acid amides formed by the condensation of fatty acids and ammonia from protein

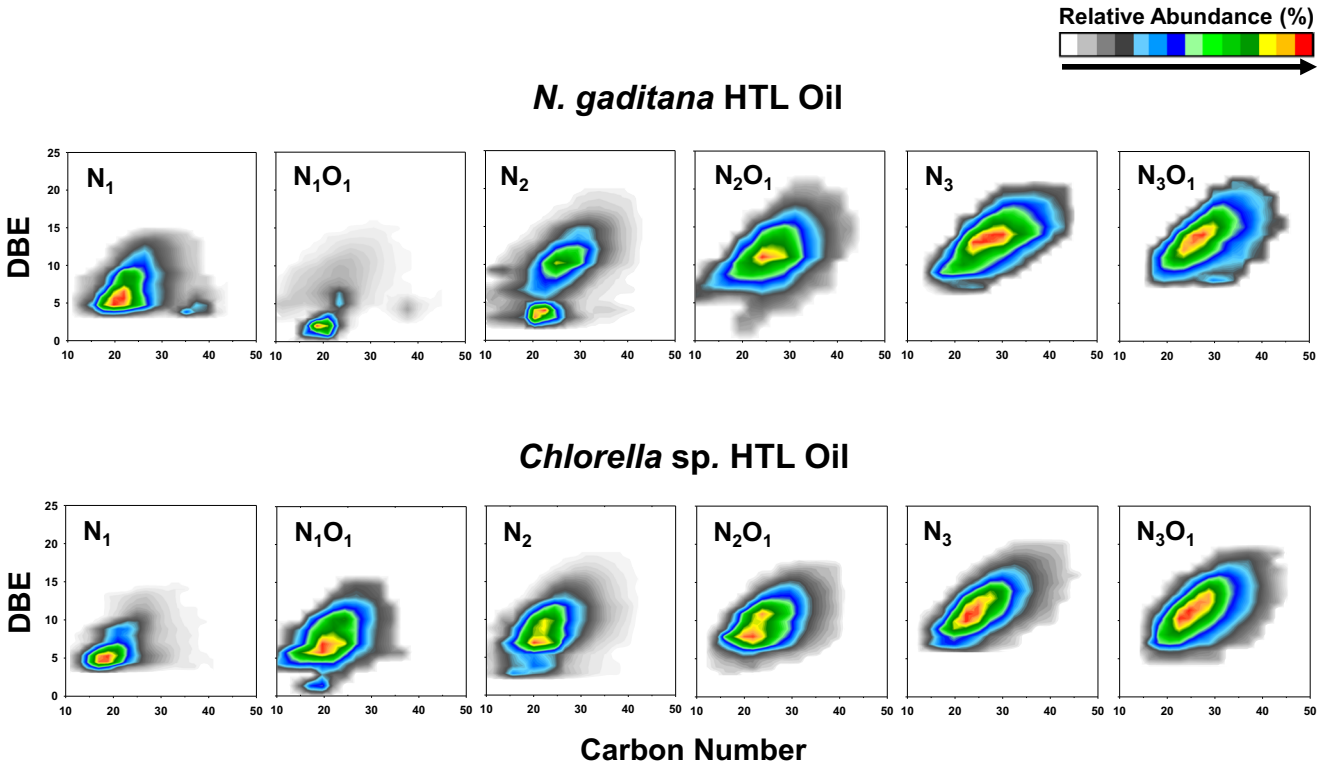

Fig. 5 Positive-ion ESI FT-ICR MS abundance-contoured plots of DBE versus carbon number for N_1, N_1O_1, N_2, N_2O_1, N_3, and N_3O_1 classes observed for *N. gaditana* (*top*) and *Chlorella* sp. (*bottom*) HTL oils produced at 300 °C

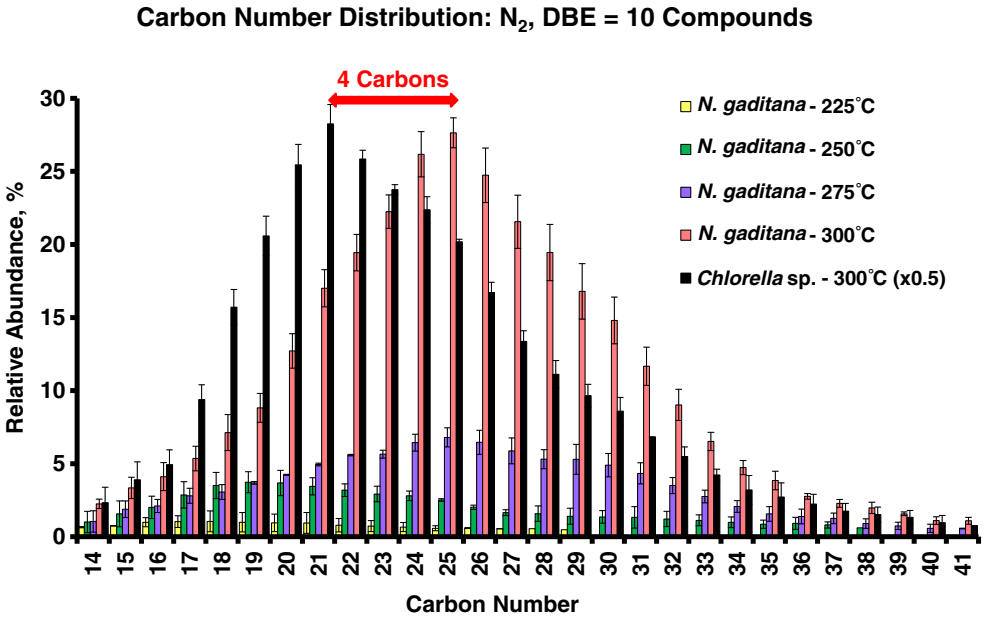

Fig. 6 Carbon number distributions of N_2, DBE=10 compounds for *N. gaditana* HTL oils produced at 225, 250, 275, 300 °C and *Chlorella* sp. HTL oil produced at 300 °C. *Error bars* represent standard deviation derived from analysis of three independent HTL oil samples

decomposition. The N_1O_1 compounds in both strains that span 4–15 DBE show similar carbon number and DBE distribution as N_1 compounds of the corresponding strain. Interestingly, N_1 species observed at 4–5 DBE and ~35–40 carbons for *N. gaditana* HTL oil are also observed for N_1O_1 class at the same DBE and carbon number.

The N_2 compounds for both *Chlorella* sp. and *N. gaditana* HTL oils show DBE=2–20 and carbon content of 10–40 atoms. For *N. gaditana*, relative abundance maxima are observed at DBE=3, 4, and 10. Compounds with DBE=3 and DBE=4 contain 20–23 carbon atoms per molecule and indicate highly alkylated species with low aromaticity. ESI MS^n measurements for structure elucidation were not available for these ions due to the high complexity of the sample. The observed compounds likely correspond to long-chain diamines, alkyl-substituted imidazoles (DBE=3), or alkylated pyrazines (DBE=4) as reported for *N. salina* HTL oil in previous work [18].

Predominant N_2 compounds observed for *N. gaditana* and *Chlorella* sp. HTL oil show similar carbon number distributions with shifted double bond equivalents relative to N_2O_1 species of the corresponding strain. For example, the relative abundance maximum observed for N_2 compounds in *Chlorella* sp. oil at DBE=7 and carbon number=20–22 (Fig. 5, bottom) is also observed for the N_2O_1 class at the same carbon number but with shifted DBE by +1 DBE (i.e., DBE=8). Similarly, *N. gaditana* HTL oil shows "hotspots" at DBE=10 and DBE=11 for N_2 and N_2O_1 classes, respectively, at the same carbon number. Therefore, the observed N_2O_1 species are likely oxygenated versions of observed N_2 compounds, and the DBE increase suggests that the oxygen atom is attached as a carbonyl oxygen.

Primary compounds observed in N_1, N_2, N_2O_1, N_3, and N_3O_1 classes for *N. gaditana* HTL oil from (+) ESI FT-ICR MS show a slight shift/extension to high carbon number and DBE compared to *Chlorella* sp. oil. Figure 6 illustrates this shift in the carbon number for N_2, DBE=10 compounds. Although both algal strains contain N_2, DBE=10 compounds with 14–41 carbon atoms per molecule, the maximum abundance shifts to higher carbon number by 4 carbon atoms per molecule for *N. gaditana* HTL oil. Interestingly, N_2, DBE=10 species observed for *N. gaditana* shows a shift to higher carbon number with increasing temperature, indicating the formation of high molecular weight compounds at higher reaction temperatures.

Figure 7 shows the negative-ion ESI FT-ICR MS class distributions for *Chlorella* sp. (top) and *N. gaditana* (bottom) HTL oils produced at each reaction temperature. Acidic compounds in both strains are dominated by free fatty acids (FFA) that originate from the lipid portion of the microalgae. To verify the observed trends in the relative abundance of free fatty acids with temperature between the two algal strains, FAME and FFA quantitation was performed by gas chromatography/mass spectrometry (Fig. 8). The increase in the FFAs and the respective decrease in the lipid-bound fatty acids with temperature, observed for *N. gaditana* HTL oils, illustrate the hydrolysis of lipids into its component fatty acids at higher temperatures. However, free fatty acids observed for *Chlorella* sp. HTL oils decrease significantly above 225 °C which can be explained by their fatty acid distribution. FAME profiles generated by GC/MS show that *Chlorella* sp. biomass is rich in polyunsaturated fatty acids (PUFA) compared to *N. gaditana* which primarily contains saturated and mono-unsaturated fatty acids (Online Resource 4). PUFAs are highly sensitive to temperature and susceptible to oxidation due to their high degree of unsaturation [24]. Therefore, increasing reaction temperature can generate nonvolatile

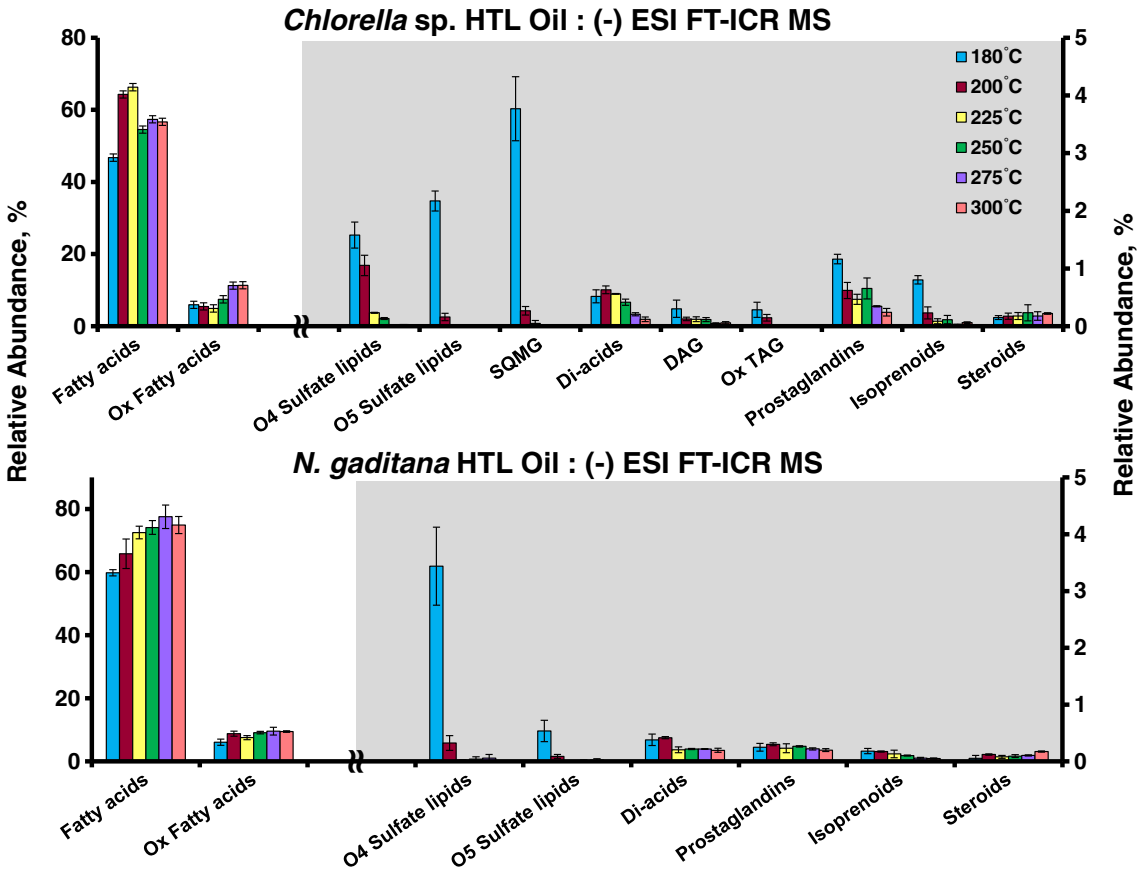

Fig. 7 Class distribution for *Chlorella* sp. (*top*) and *N. gaditana* (*bottom*) HTL oils derived from negative-ion ESI FT-ICR MS. *Error bars* represent standard deviation derived from analysis of three independent HTL oil samples

degradation products of PUFAs as well as facilitate oxidation, transforming the PUFAs to their corresponding oxidation products and decreasing the detectable FFA content by GC/MS and FFA ion abundance in (-) ESI FT-ICR MS (an important consideration when considering lipid quantitation by GC/MS for any feedstock/oil).

Abundance-contoured plots of DBE versus carbon number for oxidized fatty acids observed for *Chlorella* sp. and

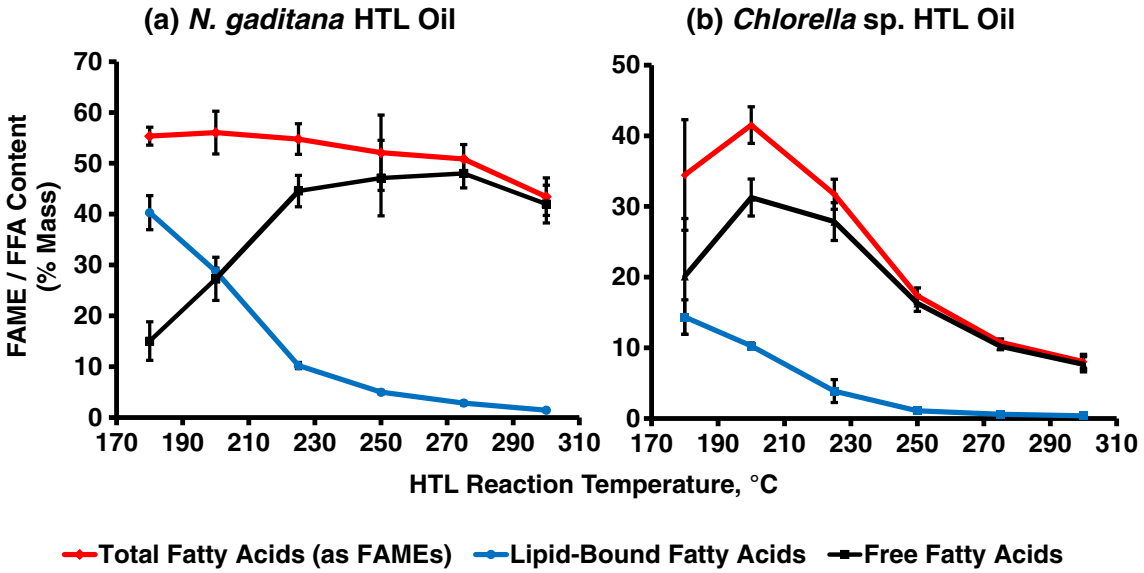

Fig. 8 FAME and FFA quantitation for **a** *N. gaditana* and **b** *Chlorella* sp. HTL oils produced at different reaction temperatures (180–300 °C)

N. gaditana HTL oils produced at 300 °C are shown in Online Resource 5. Major free fatty acids in *Chlorella* sp. biomass include palmitic (16:0), α-linolenic (18:3), eicosapentaenoic (20:5), and palmitoleic (16:1) acids as observed by GC/MS. Interestingly, three-dimensional mass spectral image for *Chlorella* sp. HTL oil shows relative abundance maxima at DBE=3, carbon number=18 and DBE=5, carbon number=20 that correspond to hydroxy-α-linolenic acid and hydroxy-eicosapentaenoic acid, respectively, and a less abundant "hotspot" at DBE=1, carbon number=16 that corresponds to hydroxy-palmitoleic acid. In addition, consistent with the GC/MS FAME profile of the *N. gaditana* biomass, the most abundant oxidized free fatty acid observed for high-temperature *N. gaditana* HTL oil is hydroxy-palmitoleic acid. Three-dimensional mass spectral images shown in Online Resource 5 confirm the conversion of the predominant mono- and polyunsaturated fatty acids into their corresponding oxidation products at elevated temperatures and the subsequent decrease in FFA class abundance above 225 °C and increase in the oxidized fatty acid class abundance with temperature for *Chlorella* sp. HTL oil, as shown in Fig. 7.

The O_4 and O_5 sulfate lipids observed for *Chlorella* sp. and *N. gaditana* low-temperature HTL oils (Fig. 7) correspond to alkyl sulfate lipids and hydroxyl alkyl sulfate lipids, respectively. Predominant compounds in each of these lipid classes contain a 30–32 carbon aliphatic chain and a sulfate head group as identified/confirmed by tandem mass spectrometry. Online Resource 6 shows ESI MS/MS spectra for the most abundant O_4 sulfate lipid and O_5 sulfate lipid observed for low-temperature *N. gaditana* HTL oil. Long-chain saturated and mono-unsaturated alcohols and alkyl diols with similar carbon number distributions (C_{30}–C_{32}) as the sulfate lipids observed here, have been previously identified in marine environments [25, 26], lacustrine sediments [27] as well as in marine and freshwater microalgae [28, 29]. Holguin et al. [21] have recently reported similar sulfate lipids from the lipid extracts of *Nannochloropsis salina* and *Scenedesmus obliquus*. The O_4 sulfate lipid (i.e., $C_{32}H_{63}O_4S_1$) shown in Online Resource 6 likely originates from the dehydration of the corresponding hydroxyl alkyl sulfate lipid (i.e., $C_{32}H_{65}O_5S_1$). The location of the double bond in $C_{32}H_{63}O_4S_1$ can be at C-14 or C-15. A similar mechanism for the transformation of diols to n-alkenols through dehydration by living organisms in a freshwater environment has been proposed by Xu et al. [27].

Sulfoquinovosyl monoacylglycerol (SQMG) lipid assignments for low-temperature *Chlorella* sp. HTL oil are also confirmed by performing tandem mass measurements for the predominant compounds in that class. ESI MS^2 spectra showed that the most abundant SQMG lipid ($C_{25}H_{48}O_{11}S_1$) contains C16:0 fatty acid (Online Resource 7). These SQMG lipids are also observed for *N. gaditana* low-temperature HTL oil, but with <1 % relative abundance.

Conclusion

We have provided a detailed chemical characterization of hydrothermal liquefaction oils produced from two biochemically distinct microalgal strains at reaction temperatures between 180 and 300 °C. These results indicate that reaction temperature may be used as a means to manipulate oil composition. Our results indicate that relatively low HTL processing temperatures (<225 °C) allow extraction of lipids and that the chemical composition of the oil is affected by the microalgal species as well as harvesting period.

For microalgae with low lipid content, HTL processing temperature can be optimized to maximize oil yield. Reaction temperature as high as 375 °C has been used to obtain the maximum oil yield from *Desmodesmus* sp., a relatively low-lipid microalgae [8]. For reaction temperature above 200 °C, protein and carbohydrate degradation increases the oil yield. Protein decomposition and cross-reactions of protein-carbohydrate-derived materials produce a variety of nitrogen- and oxygen-containing species that increase both nitrogen and oxygen content of the HTL oil. Therefore, even if a high oil yield is obtained from low-lipid microalgae at higher processing temperature, elevated nitrogen and oxygen content will require upgrading for the removal of polar molecules. Among upgrading methods, hydrotreatment is a promising technique for the treatment of these oils, and the efficiency of the process highly depends on the chemical nature of the oil [18, 30]. The detailed compositional description of the high-temperature HTL oil shown here will benefit to devise efficient upgrading strategies to produce a high-quality liquid fuel from HTL of microalgae.

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