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Lipid Analysis of *Neochloris oleoabundans* by Liquid State NMR

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ABSTRACT: This study is an evaluation of liquid state NMR as a tool for analyzing the lipid composition of algal cultures used for biodiesel production. To demonstrate the viability of this approach, ^{13}C NMR was used to analyze the lipid composition of intact cells of the algal species, *Neochloris oleoabundans* (UTEX #1185). Two cultures were used in this study. One culture was "healthy" and grown in conventional media, whereas the other culture was "nitrogen-starved" and grown in media that lacked nitrate. Triglyceride was determined to be present in both cultures by comparing the algal NMR spectra with published chemical shifts for a wide range of lipids and with a spectrum obtained from a triglyceride standard (glyceryl trioleate). In addition, it is shown that (1) the signal-to-noise ratio of the ~29.5 ppm methylene peak is indicative of the lipid content and (2) the nitrogen-starved culture contained a greater lipid content than the healthy culture, as expected. Furthermore, the nitrogen-starved culture produced spectra that primarily contained the characteristic peaks of triglyceride (at ~61.8 and ~68.9 ppm), whereas the healthy culture produced spectra that contained several additional peaks in the glycerol region, likely resulting from the presence of monoglyceride and diglyceride. Finally, potential interferences are evaluated (including the analysis of phospholipids via ^{31}P NMR) to assess the specificity of the acquired spectra to triglyceride. These results indicate that NMR is a useful diagnostic tool for selectively identifying lipids in algae, with particular relevance to biodiesel production.

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KEYWORDS: NMR; algae; lipid; triglyceride; composition analysis; biodiesel

Introduction

Biodiesel produced from algae is a widely studied potential alternative to petroleum-based transportation fuels. Algal biodiesel is produced by upgrading algal lipids into standard biodiesel composition, which primarily contains fatty acid methyl esters. There are multiple production pathways capable of producing biodiesel from algae and each of these pathways involves multiple processing steps (Beal et al., in preparation; Benemann and Oswald, 1996; Huang et al., in press; Li et al., 2007; Schenk et al., 2008; Sheehan et al., 1998). Researchers require analytical tools to evaluate the effectiveness of each production step and the benefits associated with various algal growth scenarios. However, as a relatively young field, the availability of standardized methods and instrumentation for characterizing the intermediate products in the algal biodiesel production pathways is limited.

There are many analysis tools that can be adapted to evaluate algal cultures, algal lipids, or fatty acid methyl esters, and each tool provides a unique set of information. Two of the most common algal lipid analysis methods are solvent extraction for gravimetric lipid quantification (Bligh and Dyer, 1959; Iverson et al., 2001; Lee et al., 2010) and fluorescence microscopy using a lipid stain such as Nile Red or BODIPY (Chen et al., 2009; Cooksey et al., 1987; Elsey et al., 2007; Walther and Farese, in press). Chromatography has been used to separate, identify, and in some cases quantify lipid extracts. Specifically, high-pressure liquid chromatography (HPLC) can be used to quantify different types of lipids in algal extracts (e.g., mono-, di-, and triglyceride) (Gillan and Johns, 1983; Kato et al., 1996; Medina et al., 1998; Nordbäck et al., 1998; Poenie, 2009); gas chromatography coupled with mass spectrometry (GC-MS) can be used to separate, identify, and quantify volatile compounds in a sample, such as fatty acid methyl esters produced from algal

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lipids (Cheng et al., 2009; Li et al., 2007; Meher et al., 2006; Meireles et al., 2003; Mühling et al., 2005); and thin layer chromatography (TLC) can be used to identify (and possibly quantify) extracted algal lipid classes (Choi et al., 1987; Vieler et al., 2007; Yongmanitchai and Ward, 1992). Several forms of spectroscopy have also been applied to analyze algal lipids. Specifically, UV–Vis spectroscopy can be used to determine the algal concentration of a culture (Chiu et al., 2008; Li and Qin, 2005; Liu et al., 2008); Raman spectroscopy can be used to identify compounds of interest in algae (Heraud et al., 2007; Huang et al., 2009; Largeau et al., 1980); and Fourier transform infrared (FTIR) spectroscopy can be used to analyze algal nutrient status and identify algal species (Domenighini and Giordano, 2009; Heraud et al., 2005; Hirschmugl et al., 2006; Kansiz et al., 1999).

Liquid state NMR has the potential to be included in this analysis toolbox but has not been widely studied for algal biodiesel applications. The advantages of liquid state NMR include the ability to analyze intact algae (thus avoiding lengthy extraction procedures, which have the potential of not extracting all of a desired compound or altering the sample), analyzing bulk algal samples (i.e., many cells at once), and the ability to obtain detailed chemical structure information (e.g., distinguishing between types of lipids and types of fatty acids in lipids).

Liquid state NMR has been used in many studies to analyze lipids (Gunstone, 1991a,b, 1993, 1994; Lie Ken Jie and Mustafa, 1997; Mannina et al., 1999; Meneses and Glonek, 1988; Ng, 1985; Sacchi et al., 1997; Schiller et al., 2007; Sotirhos et al., 1986; Vlahov, 1996; Vlahov and Angelo, 1996; Vlahov et al., 2002). There have also been studies published for the application of liquid state NMR to evaluate algal lipid extracts (Boyle-Roden et al., 2003; Meneses et al., 1993; Pollesello et al., 1992, 1993) and "bio-oil" produced from the liquefaction of algae (Zou et al., 2009). In a recent advancement, the use of time domain (TD) NMR has been used to quantify total lipid content in algal cultures (Cheng et al., 2009; Gao et al., 2008). Although TD-NMR is more robust than ordinary NMR, it yields less specific information about the chemical composition of the analyzed sample. Finally, solid state NMR has also been used in previous studies to analyze lipids in biological samples (Everts and Davis, 2000; Hedges et al., 2002; Schiller et al., 2007; Zelibor et al., 1988).

To the best of the authors' knowledge, this is the first reported results of using ^{13}C liquid state NMR to identify lipids of intact algae. The compositional measurements suggest that this approach may provide a useful complimentary analysis method to TD-NMR. Thus, this work represents a contribution to the suite of tools available for analyzing algae-based biofuels.

Materials and Methods

Two cultures of *Neochloris oleoabundans* (UTEX #1185) were grown at The Culture Collection of Algae at the University of Texas at Austin (i.e., UTEX). A "healthy" culture was grown in Bold 3N media for 15 days and a "nitrogen-starved" culture was grown in Bold 3N media for 7.5 days and then transferred to modified Bold 3N media that contained no $NaNO_3$ (i.e., nitrogen-starved) for an additional 7.5 days (contents of Bold 3N media include H_2O, $NaNO_3$, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, K_2HPO_4, NaCl, and vitamin B_{12}, cf. UTEX, www.utex.org). In this study, nitrogen starvation was applied to one culture to promote lipid production so that there would be different lipid densities in the two cultures of the same species, a phenomenon that has been demonstrated in many algal species, including *N. oleoabundans* (Gouveia et al., 2009; Hu et al., 2008; Liang et al., 2009; Pruvost et al., 2009; Sheehan et al., 1998; Sriharan et al., 1991; Tornabene et al., 1983; Yamaberi et al., 1998). Both cultures were grown under continuous aeration (using 1.5% CO_2 in air), 15 W/m^2 of continuous lighting (using F32/T8 fluorescent bulbs), and at a temperature of about 21°C.

When harvested, 1.5 L of each culture was centrifuged and rinsed several times to produce dense pastes of algal cells. The starved sample paste was yellow-green in color and less viscous than the dark green healthy sample. To prepare algal samples for ^{13}C NMR measurements, 1 g of algal paste was mixed with 1 mL of deuterium oxide (D_2O, CAS 7789-20-0) and then transferred to a 17.8 cm, 300 MHz, lab glass Wilmad NMR tube. Six ^{13}C NMR samples were prepared from the two algal cultures: three from the healthy culture and three from the nitrogen-starved culture. Finally, a single NMR sample was prepared from chemical grade glyceryl trioleate (Sigma Aldrich T7140, CAS 122-32-7) dissolved in chloroform-d (CAS 865-49-6) and tetramethylsilane (TMS) to serve as a reference compound.

Liquid state ^{13}C NMR was conducted on all samples using the Varian Direct Drive 600 MHz spectrometer in the University of Texas at Austin's Analytical Services NMR Laboratory. All samples were prepared within 3 h of testing and the NMR tubes were thoroughly mixed immediately prior to testing to circumvent the algae settling. All of the samples' ^{13}C NMR spectra are referenced to D_2O (the glyceryl trioleate sample was referenced to TMS, which was externally referenced to D_2O). All ^{13}C NMR tests were conducted with identical acquisition settings (pulse width of 7.9 μs, flip angle of 90°, relaxation delay of 2.3 s, 95,000 acquisition data points, and 2,000 scans) and processed with either 1 or 10 Hz line broadening.

Liquid state ^{31}P NMR was also conducted on a second batch of healthy and nitrogen-starved *N. oleoabundans* cultures to evaluate phospholipid signal contributions. These cultures were grown under the same conditions as the cultures for ^{13}C NMR. The ^{31}P NMR tests were conducted with a pulse width of 13.0 μs, flip angle of 90°, relaxation delay of 4 s, 200,000 acquisition data points, 1,000 scans, and processed with 4 Hz line broadening.

Results and Discussion

Two methods are used to evaluate liquid state NMR as a lipid diagnostic for algal cultures. In the first method, the

peak locations of the glyceryl trioleate spectrum are compared and matched to those of the algal spectra, which indicates the presence of triglyceride in both cultures. In the second approach, the average methylene peak intensity at ~29.5 ppm of the nitrogen-starved culture is compared with that of the healthy culture.

Spectral Analysis

Identification of Triglyceride

The peak locations and shape of the glyceryl trioleate spectrum agree well with the spectra obtained from the algal cultures, indicating that neutral lipids, specifically triglyceride, contribute to the measured algal NMR spectra. Figure 1 displays the seven spectra recorded and Figure 2 presents three regions that contain the most relevant peaks. In Figures 1 and 2, 10 Hz line broadening was used to process the spectra to improve the signal-to-noise ratio. The regions shown in Figure 2, denoted "A," "B," and "C," are labeled above the spectra in Figure 1. The amplitudes of the spectra shown in Figures 1 and 2 were scaled for presentation. Therefore, these figures indicate consistency on peak location but do not indicate relative abundance. Table I lists the averaged, normalized signal intensity for significant peaks for the seven spectra. These data were calculated by normalizing the measured peak heights by the maximum peak intensity of that spectrum (which was produced by the methylene group at ~29.5 ppm for all spectra). This normalization was necessary because the total signal strength varied from sample-to-sample, likely caused by density variations. Then, the normalized heights associated with the indicated chemical shift ranges (and therefore associated with particular functional groups) were averaged across the three spectra for each culture.

Inhomogeneous field line broadening is present in the algal spectra, which is apparent by comparing the peak widths of the glyceryl trioleate spectrum to those of the algal spectra in Figures 2 and 3. The difference is apparent when 10 Hz line broadening was used to process the spectra (Fig. 2), and particularly evident when 1 Hz line broadening was used, which is illustrated in Figure 3. The inhomogeneous field line broadening is likely a result of several factors, including local magnetic field inhomogeneities caused by the solid biomass in the algal samples, physical motion restraints on the molecules within the algal cells (which may prohibit adequate motional narrowing), and effects of the D_2O solvent viscosity. In addition, slight variations in the chemical shifts exist for the peaks in each sample due to

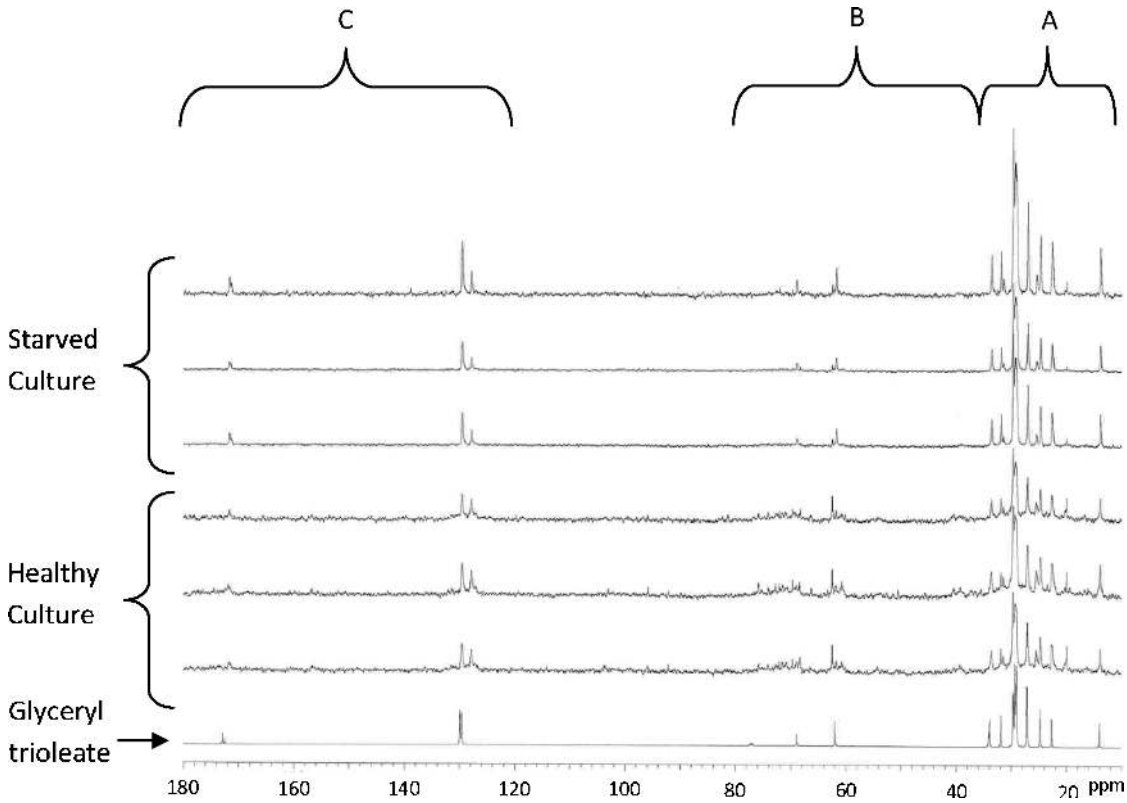

Figure 1. The measured ^{13}C NMR spectra for the nitrogen-starved culture, healthy culture, and glyceryl trioleate are very similar in shape. The regions A, B, and C are shown in more detail in Figure 2 (note: vertical scales vary and 10 Hz line broadening was used).

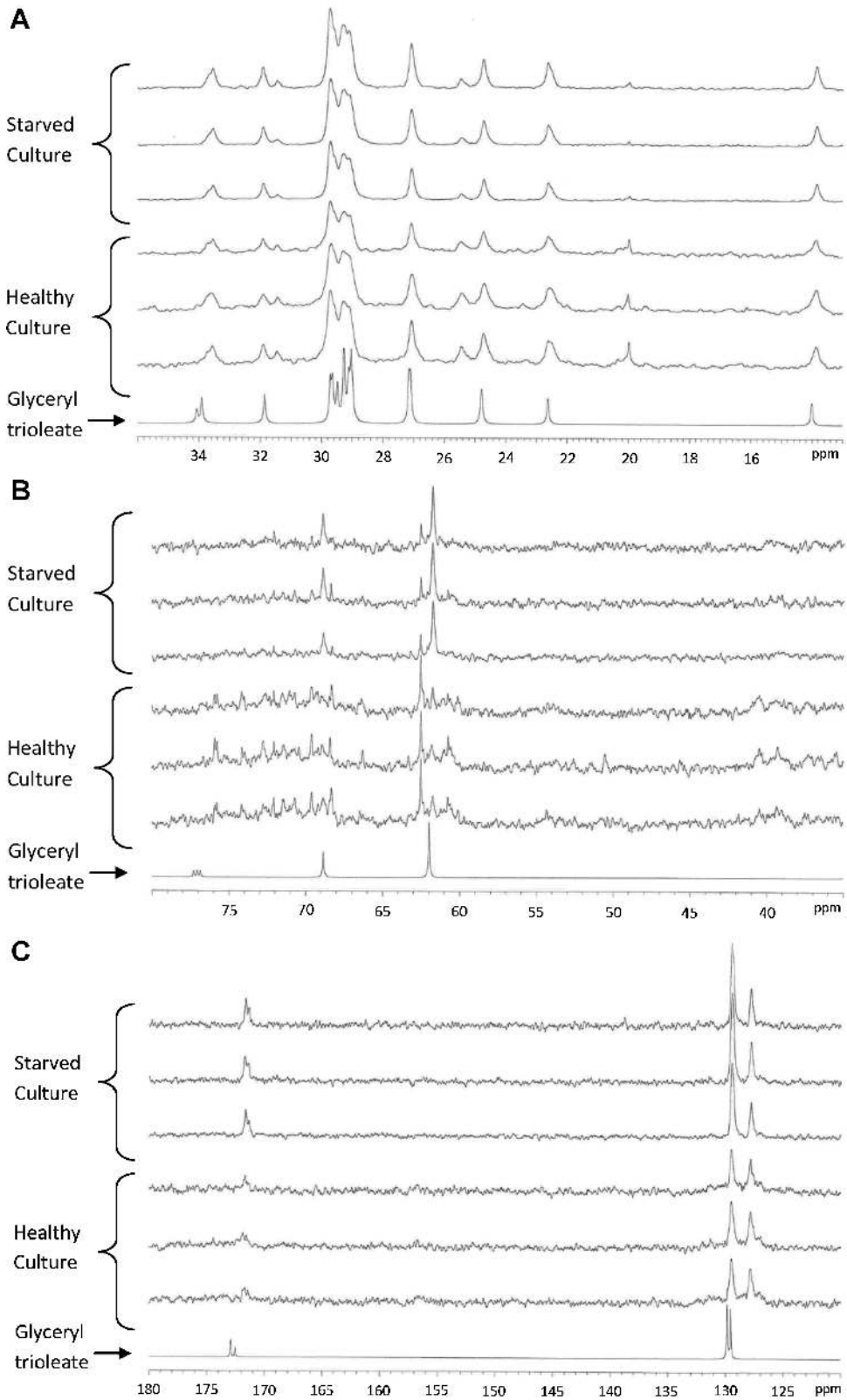

Figure 2. (**A**): 13–35 ppm, (**B**) 35–80 ppm, and (**C**) 120–180 ppm. The measured ^{13}C NMR spectra for the nitrogen-starved culture, healthy culture, and glyceryl trioleate for regions containing the most prevalent peaks. Characteristic triglyceride peaks are present in both algal spectra (note: vertical scales vary and 10 Hz line broadening was used).

Table I. Peak locations and associated average, normalized peak intensities for the healthy culture, nitrogen-starved culture, and glyceryl trioleate sample.

Peak location (ppm)	Glyceryl trioleate (a.u.)	Starved (a.u.)	Healthy (a.u.)	Probable functional group; (carbon atom)[a]
13.852–14.004	0.297	0.296	0.322	Methyl, CH_3 (ω_1)
19.963–19.978	—	0.077	0.256	Unidentified soluble compound[f]
22.590–22.619	0.339	0.316	0.363	Methylene, CH_2 (ω_2)
24.699–24.781	0.464	0.368	0.445	Methylene, CH_2 (C3α and C3β)
25.409–25.458	—	0.123	0.280	Allyl, CH_2–CH=CH– (L11)
27.057–27.143	0.734	0.551	0.593	Allyl, CH_2–CH=CH– (O8,11 and L8,14)
29.267–29.717	1	1	1	Methylene, CH_2 (C4–C8)
31.443–31.465	—	0.096	0.214	Methylene, CH_2 (ω_3)
31.860–31.912	0.385	0.276	0.290	Methylene, CH_2 (ω_3)
33.534–33.905	0.343	0.247	0.288	Methylene, CH_2 (C2α and C2β)
39.299–39.355	—	—	0.105[d]	Unidentified
40.471–40.490	—	—	0.097	CH_2N^+, PE
60.741–60.756	—	—	0.137	CH_2O, PC
61.712–61.976	0.299	0.157	0.134	(Tri-) glycerol, CH_2 (G11, G13)
62.493–62.519	—	0.064	0.320	Unidentified soluble compound[f]
66.291–66.499	—	—	0.078	CH_2N^+, PE
68.850–68.947	0.140	0.082	0.166	(Tri-) glycerol, CH (G12)
70–76	—	—	0.131[e]	Mono-, diglycerides
~77	0.036[b]	NA	NA	Deuterated chloroform, (CDC13)
127.795–127.862	—	0.144	0.272	Olefin, C=C (L12)
129.491–129.586	0.406[c]	0.269	0.347	Olefin, C=C; (O9,10 and L9,10)
171.596–172.861	0.071	0.101	0.113	Acyl, C=O (C1 β)
172.902	0.130	—	—	Acyl, C=O (C1 α)

L, linoleic; O, oleic; G, glycerol; PE, phosphotidylethanolamines; PC, phosphotidylcholines.
All peaks were normalized by the methylene group intensity at ~29.5 ppm.
[a]See Refs. Everts and Davis (2000), Gunstone (1991b), Gunstone (1993), Gunstone (1994), Lie Ken Jie and Mustafa (1997), Mannina et al. (1999), Vlahov (1996).
[b]Three nearly equal peaks produced by CDC13.
[c]Average of two peaks (cf. Fig. 2, plot C).
[d]Only two samples contained this peak.
[e]Average of the greatest peak of each spectrum within 70–76 ppm (cf. Fig. 2, plot B).
[f]See the Spectral Analysis Section.

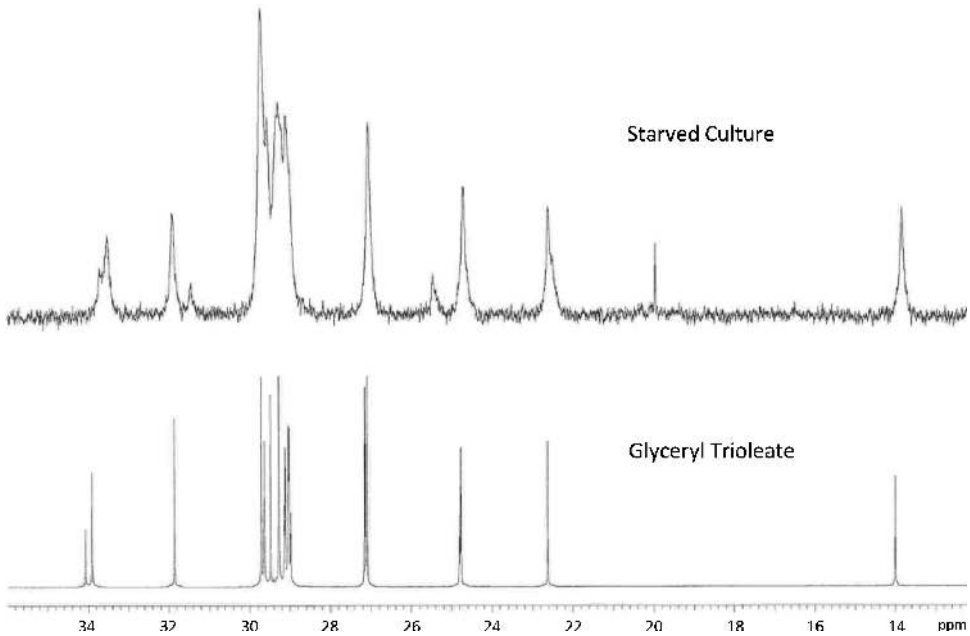

Figure 3. The peak widths of the ^{13}C NMR algal spectra (a nitrogen-starved culture's spectrum is shown) (**top**) are larger than those in the glyceryl trioleate spectrum (**bottom**). These spectra were processed with 1 Hz line broadening and demonstrate the effect of inhomogeneous field line broadening on the algal spectra (note: vertical scales vary).

minor fluctuations in environmental conditions and lack of direct internal reference among all samples.

Table I lists carbon-containing functional groups that are proposed to be responsible for each of the prominent peaks in the spectra (e.g., olefin groups, acyl groups, etc.). These assignments were made based on data in the literature and can be supported by computer programs that generate NMR spectra, such as *ChemDraw*. The peaks located at ~19.97 and ~62.50 ppm in all of the algal spectra are significantly narrower than the other peaks listed in Table I. This difference is illustrated in Figure 3 for the peak at ~19.97 ppm. All of the peaks in each spectrum were inspected using both 1 and 10 Hz line broadening to evaluate potential peak width distortion, and of the distinct peaks listed in Table I, only those located at ~19.97 and ~62.50 ppm were noticeably distorted (cf. Figs. 2 and 3). The specific compounds responsible for these two peaks are not known.

Significant peaks of the glyceryl trioleate spectrum are present in both the healthy and nitrogen-starved algal spectra (cf. Table I), which indicate the presence of triglyceride in both algal samples. The only possible differences may be the acyl peaks located at 172.515 and 172.902 ppm, which are not distinct in the algal spectra when processed with 10 Hz line broadening. In the glyceryl trioleate spectrum and other studies, the two acyl peaks produced by the C1 carbon–oxygen bonds are separated by about 0.41 ppm (Gunstone, 1993; Lie Ken Jie and Mustafa, 1997), and this separation is obscured in the algal spectra due to line broadening. The presence of the two acyl peaks is more evident for the starved samples.

The chemical shifts produced by the carbon atoms in glycerol of triglyceride (i.e., G11–13) have been reported to be ~62.1, ~68.9, and ~62.1 ppm (Gunstone, 1991b, 1993; Lie Ken Jie and Mustafa, 1997; Vlahov, 1996), and the algal samples contained peaks at ~61.8, ~68.9, and ~61.8 ppm. The discrepancy between the reported glycerol carbon atom chemical shifts and those measured in this study likely results from different environmental conditions and line broadening. Furthermore, an approximate 2:1 ratio of the relative, normalized peaks at ~61.8 and ~68.9 ppm was recorded for the starved culture and the glyceryl trioleate sample. This ratio is produced by the symmetry of the two end glycerol carbon atoms (~61.8 ppm) about the middle glycerol carbon atom (~68.9 ppm) of triglyceride (cf. plot C of Fig. 2 and Table I) (Gunstone, 1991b, 1994). The peaks in the healthy culture are less distinct in the glycerol region (i.e., ~60–70 ppm) and do not coincide with the peaks of the glyceryl trioleate spectrum as closely as those of the nitrogen-starved sample. This result suggests that the healthy sample contains fewer lipids and a more diverse assortment of neutral lipids (likely including monoglyceride and diglyceride, which produce "asymmetric" glycerol signals in the NMR spectra (Gunstone, 1991b, 1994; Vlahov, 1996)). This result is expected because, as Hu et al. (2008) explain, under nutrient deprivation, many algae alter their biosynthetic pathways to mainly produce triglyceride, which is supported by data in the literature for *N. oleoabundans* (Pruvost et al., 2009; Tornabene et al., 1983) and other algal species (Yamaberi et al., 1998). Independent HPLC analysis supported these results, also indicating that most of the nitrogen-starved culture's neutral lipids were triglyceride, while a significant percentage of the healthy culture's neutral lipids were of non-triglyceride types (Poenie, 2009).

Methylene Peak Indicator of Lipid Content

Figure 4 presents one spectrum from the healthy culture and one from the nitrogen-starved culture, which were acquired with identical instrument settings and are plotted on the same scale. These spectra are representative of the other spectra obtained for each culture. Nitrogen starvation promotes lipid accumulation and lipids produce strong methylene group signals in ^{13}C NMR (specifically at

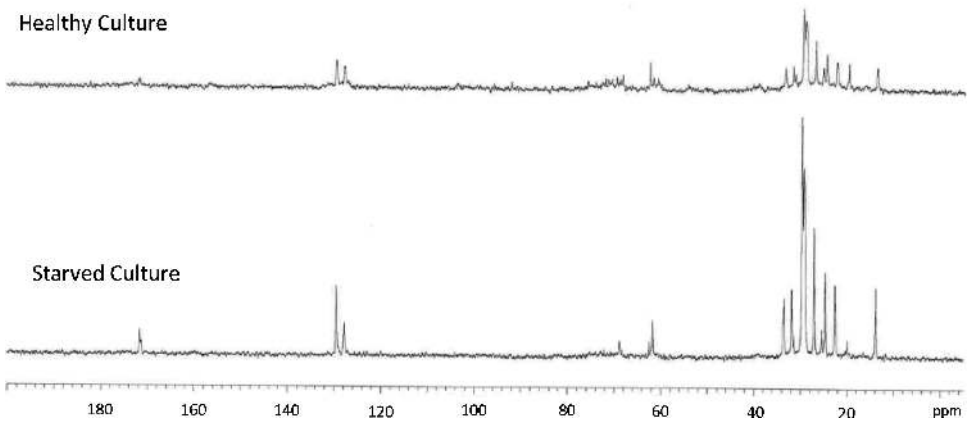

Figure 4. The healthy culture (**top**) produced an average methylene group signal-to-noise ratio of 49 and the nitrogen-starved culture (**bottom**) produced an average methylene group signal-to-noise ratio of 120. These ^{13}C NMR spectra are plotted on the same vertical and horizontal scales and 10 Hz line broadening was used.

~29.5 ppm) (Falch et al., 2006; Gunstone, 1994; Lie Ken Jie and Mustafa, 1997; Mannina et al., 1999; Sacchi et al., 1997). Therefore, the methylene peak signal intensity can be used as an indicator of lipid content. The healthy culture and the nitrogen-starved culture produced average methylene peak (~29.5 ppm) signal-to-noise ratios of 49 and 120, respectively. Although the ~29.5 ppm methylene peak is not necessarily specific to neutral lipids (since many compounds contain methylene groups, such as phospholipids), the interference analysis described below indicates that the algal spectra are primarily comprised of the contributions from neutral lipids (but not exclusively triglyceride). Therefore, the increased signal-to-noise ratio of the ~29.5 ppm methylene peak of the starved culture, as compared to that of the healthy culture, provides further evidence that the NMR spectra effectively indicate the presence of neutral lipids in *N. oleoabundans* grown in these conditions.

Interference Analysis

Although triglyceride produces characteristic signals in ^{13}C NMR spectra, other compounds can contribute to, and thus interfere with, these characteristic peaks. As with all spectroscopy methods, interferences must be accounted for. Due to the dominant methylene envelope signals produced by the algal samples, the list of potential compounds that would cause significant interferences can be limited to compounds with long hydrocarbon chains (i.e., lipids). Several studies have presented the lipid composition of algal species (Behrens and Kyle, 1996; Guschina and Harwood, 2006; Hu et al., 2008; Pruvost et al., 2009; Tornabene et al., 1983), and the common lipids can be categorized as neutral lipids (including mono-, di-, and triglyceride) and polar lipids (including phospholipids). There are a variety of other compounds present in the algal samples, as in all organisms, but most of these compounds were not present in sufficient concentrations to produce distinct peaks in the algal spectra. For instance, characteristic chemical shifts for biological extracts of chlorophyll (~11 ppm), carotenoids (~13–15, ~125–145 ppm), sulfoquinovosyldiacylglycerol (SQDG, ~99, ~75, ~73, ~70, ~66, ~64, and ~55 ppm), and monogalactosyldiacylglycerol (MGDG, ~103, ~74, ~73, ~71, ~70, ~68, and ~62 ppm) have been reported (de Souza et al., 2006; Pollesello et al., 1993), and these compounds did not contribute prominent, discernable signals (cf. Figs. 1 and 2). Furthermore, except for the unidentified peak in two of the healthy culture's spectra at ~39.3 ppm and the narrow peaks located at ~19.97 and ~62.50 ppm (as discussed above), all of the major peaks in the algal spectra can be attributed to lipids (cf. Table I). While unforeseen interferences may exist, the most likely compounds that could cause significant interference with the triglyceride signals are monoglyceride, diglyceride, and phospholipids. If other compounds produced significant interference, characteristic peaks for those compounds would be prominent in the algal spectra.

^{13}C NMR signals produced by mono- and diglyceride have been shown to be slightly different than those produced by triglyceride (Gunstone, 1991b, 1994; Vlahov, 1996). The spectral analysis above indicated that most of the starved culture's neutral lipids were triglyceride, whereas the healthy culture's neutral lipids contained a larger percentage of mono- and diglyceride. As a result, the methylene peak at ~29.5 ppm for the healthy culture's spectra likely contains a contribution (and thus interference) from monoglyceride and/or diglyceride. Although mono- and diglyceride are considered interferences with the triglyceride signal, they are useful for biodiesel production. Therefore, the overlap of signal contribution to the methylene peak from these species does not necessarily detract from this approach to lipid analysis for biodiesel applications.

Phospholipids (and other complex lipids) are less well suited for biodiesel production than neutral lipids and therefore, it is important to discriminate between the contributions of phospholipids and neutral lipids to the NMR spectra. Based on the results presented by Tornabene et al. (1983), which indicated phospholipids accounted for <10% of total lipids in nitrogen-starved *N. oleoabundans*, it is expected that neutral lipids are more prevalent than polar lipids (such as phospholipids). This expectation is consistent with data presented in other studies (Hu et al., 2008; Sriharan et al., 1991). Generally, phospholipids can be distinguished in NMR spectra by the peaks associated with the polar head group (e.g., CH_2N+, CH_2O) or the acyl carbon atoms (i.e., C1). For instance, phosphotidylethanolamines (PE) and phosphotidylcholines (PC) have been shown to produce characteristic peaks in ppm at ~173, ~71, ~64, ~63, and either ~62 and ~41 for PE or ~66, ~59, and ~54 for PC (Everts and Davis, 2000; Falch et al., 2006; Gunstone, 1994; Pollesello et al., 1993). The starved culture's spectra lack significant, distinct peaks at almost all of these locations. The healthy culture's spectra, on the other hand, contain several minor peaks that are similar to those of PE and PC listed above, specifically between ~60 and 72 ppm and at ~41 ppm. However, many of the minor peaks between ~60 and 72 ppm may be produced by monoglyceride and diglyceride glycerol carbon atoms (cf. Table I; Gunstone, 1994; Vlahov, 1996). Furthermore, the characteristic PE peaks at ~174 and ~64 ppm and the characteristic PC peaks at ~174, ~64, and ~54.3 ppm are not prominent in any of the algal spectra. For the prominent peaks that are possibly produced by phospholipids in the healthy culture spectra (~40.5, ~60.7, and ~66.4 ppm), all of the averaged, normalized peak intensities are <0.14. Based on these results, it is reasonable to expect that if phospholipids contribute interfering signals to the algal spectra, they are small in comparison to the neutral lipid signals.

Since phospholipids are known to be present in the cell membrane, the lack of distinct phospholipid signals is unexpected. To further investigate this result, liquid state ^{31}P NMR was conducted on a different batch of healthy and nitrogen-starved *N. oleoabundans*. Phospholipids should

produce strong signals in ^{31}P NMR, since ^{31}P NMR is nearly 400 times more sensitive than ^{13}C NMR (Schiller et al., 2001). The resulting spectra are externally referenced to phosphoric acid and displayed in Figure 5 along with lists of the associated peak locations.

The spectra contain prominent, sharp peaks between ~1 and ~−2 ppm and a dominant, broad peak at ~−23.4 ppm. The peak locations around 0 ppm are similar to those published for phospholipid extracts in other studies (Branca et al., 1995; Culeddu et al., 1998; Meneses and Glonek, 1988; Meneses et al., 1993; Schiller et al., 2001, 2007; Sotirhos et al., 1986) but could be produced by other soluble phosphate-containing compounds in algae (e.g., soluble orthophosphates, polynucleotides, and soluble nucleotides). The shape of the dominant peak at ~−23.4 ppm (a broad, shouldered peak) resembles that produced by intact membrane phospholipids reported in other studies (Cullis and De Kruijff, 1979; McLaughlin et al., 1975; Murphy et al., 1989; Schiller et al., 2007). Thus, it is believed that the broad peak at ~−23.4 ppm is produced by membrane phospholipids (e.g., PC and PE) and the sharp peaks located around 0 ppm are produced by soluble phosphate-containing compounds.

The lack of clear phospholipid signal in the ^{13}C NMR algal spectra can be attributed to reduced signal clarity resulting from inhomogeneous field line broadening, which may be more severe for the "liquid-crystalline" phospholipid bilayer membrane. However, it is believed that the phospholipids contributed to the liquid state NMR signals in this study (for both ^{13}C NMR and ^{31}P NMR), based on peaks in the ^{31}P spectra and results from other studies in literature (Cullis and De Kruijff, 1979; McLaughlin et al., 1975; Murphy et al., 1989; Schiller et al., 2007). The phospholipid signal in the ^{13}C spectra could also be reduced due to relatively low phospholipid concentration (as compared to neutral lipids). This effect may be overcome in ^{31}P NMR because ^{31}P NMR is so much more sensitive than ^{13}C NMR that the phospholipid signals can be resolved (Schiller et al., 2001). Since phospholipids contain fatty acid methylene tails and contribute liquid state NMR signals, it is possible that the ^{13}C NMR methylene peaks contain contributions from phospholipids. It is not clear how great this contribution is, if it exists at all. In order to use the ^{13}C signal to quantify neutral lipids in algae in future work, the specific contributions from phospholipids, mono-, di-, and triglycerides need to be deciphered.

In addition to considering interferences, it is important to understand which compounds produce the peaks that are not present in the glyceryl trioleate spectrum but are present in the algal spectra. Linoleic acid (whether in mono-, di-, or

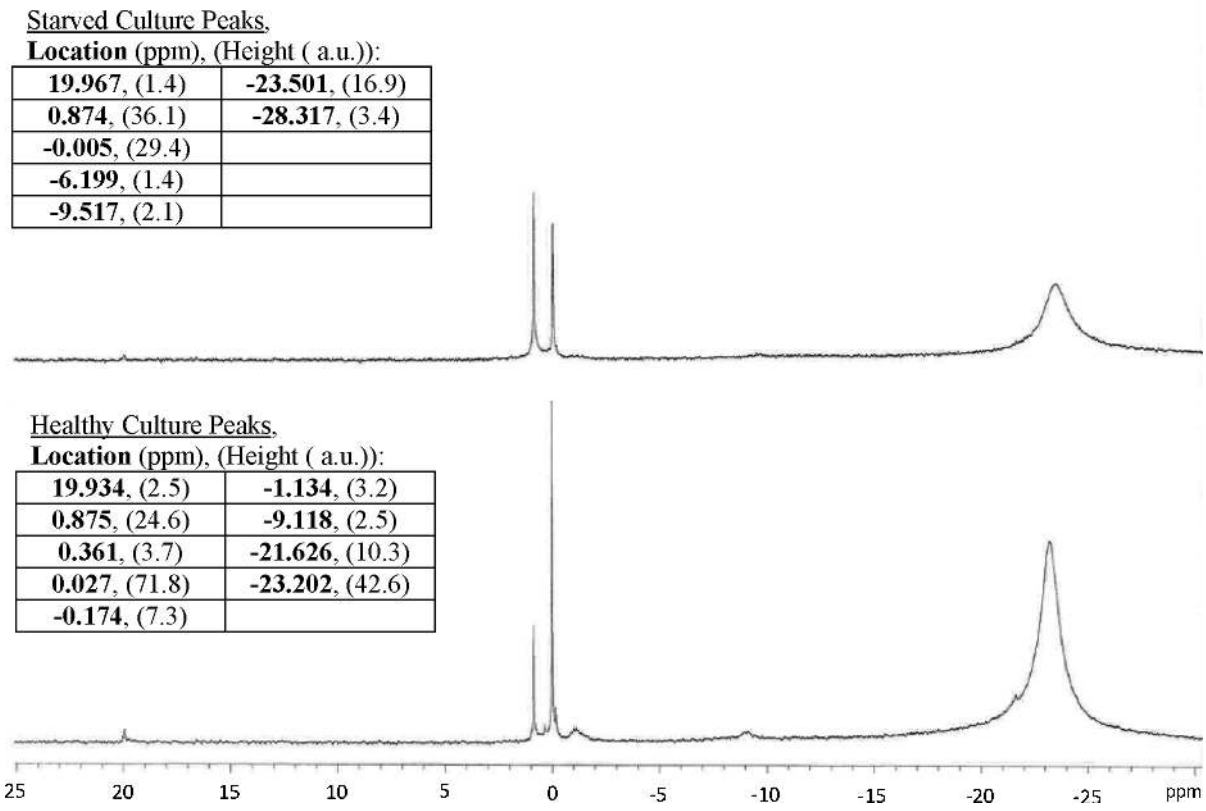

Figure 5. The nitrogen-starved culture (**top**) and healthy culture (**bottom**) ^{31}P NMR spectra and peak locations are shown. These spectra are plotted on the same vertical and horizontal scales and 4 Hz line broadening was used.

triglyceride) is likely present in the algal samples, given the signals at ~127.83 and ~25.43 ppm, which are of similar normalized magnitude (Gunstone, 1991a, 1994). The other peak present in the algal spectra that is not present in glyceryl trioleate spectrum is located at ~31.45 ppm, which can be produced by an ω_3 methylene group (note the peaks at ~19.97 and ~62.50 ppm are discussed above). Consequently, we conclude that liquid ^{13}C NMR is reasonably robust against the expected types of interferences that might be present for analyzing neutral lipids in algae.

Conclusions

NMR provides information that is not available from other analysis tools, such as specific chemical structure, and can be used to analyze bulk samples of intact algae. Thus, ordinary liquid state NMR can be a useful complementary analysis tool to supplement data provided by other analysis methods (e.g., solvent extraction and chromatography).

This study demonstrates proof-of-principle for the use of liquid state NMR to identify the presence of triglyceride in algal samples. Triglyceride was determined to be present in the algal cultures by comparing the ^{13}C NMR algal spectra to that of a pure triglyceride standard (glyceryl trioleate). Every peak in the standard triglyceride spectrum was also present in the algal spectra (healthy and nitrogen-starved). In addition, the nitrogen-starved culture produced methylene signals (~29.5 ppm) that were, on average, ~2.5 times greater than those of the healthy culture. This result was expected because nitrogen-starved algae contain greater lipid content and further supports the ability of NMR to evaluate algal lipid content.

Interference analysis indicated that mono- and diglycerides were also likely present in the algae, and that these compounds were more prevalent in the healthy culture than the starved culture. Characteristic phospholipid signals were not distinct in the ^{13}C NMR algal spectra (likely due to inhomogeneous field line broadening) but were present in ^{31}P NMR spectra. Thus, it is probable that the methylene peaks in the algal spectra contain contributions from mono-, di-, and triglycerides and may possibly contain contributions from phospholipids. The extent of each contribution is not known. Therefore, the methylene peaks in the ^{13}C NMR spectra indicate the content of a broad range of lipids, while compound-specific peaks are needed to identify particular compounds, such as the characteristic triglyceride peaks at ~62.1 and ~68.9 ppm.

While this exploratory study verifies the potential viability of NMR as a diagnostic tool, the sensitivity or quantitative capability of ordinary NMR for algal lipids was not investigated. Because this proof-of-principle study demonstrates the application of ^{13}C NMR analysis for one alga that was grown in the laboratory, further research is recommended to determine the ability for NMR to characterize lipids in a range of algal species and cultures grown under different conditions. Furthermore, development of post-processing methods that improve the specificity of NMR to individual compounds of interest would also be valuable. In conclusion, ^{13}C NMR is a tool with potential for analyzing intermediate products in the algal biodiesel production pathway, including algal cultures.

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