Preservation of proteinaceous material during the degradation of the green alga Botryococcus braunii: A solid-state 2D $^{15}$N $^{13}$C NMR spectroscopy study

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Abstract—Using solid-state cross-polarization-magic-angle-spinning (CPMAS) $^{13}$C and $^{15}$N nuclear magnetic resonance (NMR) and 2-D double cross polarization (DCP) MAS $^{15}$N $^{13}$C NMR techniques, microbially degraded Botryococcus braunii was analyzed to study the chemical nature of organic nitrogen in the algal residue. The amide linkage, as found in protein, was observed as the major nitrogen component in 201-day-old degraded algae. No significant amount of heterocyclic nitrogen, or evidence for melanoidin products, was found. The results strongly suggest that proteinaceous material can survive early diagenesis and be preserved via its encapsulation by refractory, macromolecular, organic matter. Copyright © 2001 Elsevier Science Ltd

1. INTRODUCTION

Proteins, the most abundant nitrogen-containing substances in many organisms, traditionally have been considered relatively labile in the environment. The recent observation of proteinaceous material preserved in terrestrial and aquatic systems, therefore, has drawn considerable attention (Yamamoto and Ishiwatari, 1992; Mitterer, 1993; Keil and Kirchman, 1994; Knicker et al., 1996; Nguyen and Harvey, 1997, 1998, 2001; Schulten and Schnitzer, 1997; Bada, 1998; Stankiewicz et al., 1998; Walton, 1998; Pantoja and Lee, 1999; Poinar and Stankiewicz, 1999; Zang et al., 2000). Several solid-state $^{15}$N cross-polarization-magic-angle-spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopic studies have suggested that a significant part of refractory organic nitrogen (RON) in peat (Zang et al., 2000), algal-derived sapropel (Knicker and Hatcher, 1997), humin (Knicker and Hatcher, 1997), and algaenan (Derenne et al., 1993), is bound in amide and amino forms. Such findings challenge the traditional depolymerization-recondensation hypothesis, which argues that heterocyclic nitrogen comprises a significant amount of the RON (Schulten and Schnitzer, 1997). However, it has been argued that the CPMAS $^{15}$N NMR spectroscopy technique underestimates the amount of certain types of heterocyclic-nitrogen that have no directly bound $^1$H (e.g., pyridine-type-nitrogen) (Knicker, 2000). The significant overlap of the amide-nitrogen signal with some types of heterocyclic-nitrogen (e.g., pyrrole-nitrogen) signals also makes it difficult to distinguish different nitrogen functionalities (Knicker, 2000). In a recent study (Knicker, 2000), a solid-state double cross polarization (DCP) MAS NMR technique was applied to a degraded algal sample, which only exhibited signals of $^{13}$C or $^{15}$N that interact intra and intermolecularly. DCP MAS NMR has demonstrated great promise as a technique with which we can better understand the chemical nature of RON in the environment.

While the preservation of organic matter could be attributed to sorptive protection by minerals (Ensminger and Gieseking, 1942; Marshmann and Marshall, 1981; Mayer, 1994; Keil et al., 1994; Hedges and Keil, 1995), evidence does exist that proteinaceous material can survive in systems where minerals are absent, or only present in low amounts (Knicker and Hatcher, 1997; Nguyen and Harvey, 1997, 1998, 2001; Derenne et al. 1998). One hypothesis proposed for the protection of “labile” protein is encapsulation (Knicker and Hatcher, 1997), which states that proteins incorporated within the macromolecular matrix forming sedimentary organic matter are sterically protected from bacterial hydrolysis. A recent hypothesis suggests that proteins may also undergo hydrophobic and other non-covalent aggregations that enhance preservation (Nguyen and Harvey, 2001).

In this study, the green microalga Botryococcus braunii was labeled with $^{13}$C and $^{15}$N isotopes during growth and subsequently degraded under oxic conditions in a flow-through system. B. braunii occurs in freshwater, brackish, and saline lakes around the world, and is known to contain insoluble, nonhydroyzable, and highly refractory cell wall material termed algaenan (Berkaloff et al.1983; Largeau, 1995). Algaenan is thought to originate from polymerization of high molecular mass lipids, and appears as a trilaminar sheath surrounding the classical, polysaccharidic cell wall (Gelin et al., 1999). The aim of this research was to investigate the potential for preservation of proteinaceous material during early diagenesis in an aquatic system devoid of minerals, and thus to evaluate the role of encapsulation by refractory, macromolecular, organic matter (e.g., algaenan), or for abiotic reactions. The fate of organic nitrogen was monitored by the solid-state 2-D DCP MAS $^{15}$N $^{13}$C-NMR technique, as well as by traditional CPMAS $^{13}$C and $^{15}$N NMR techniques.

2. MATERIAL AND METHODS

2.1. Algal Cultures and Degradation Experiment

The experimental design and sample-processing scheme is illustrated in Figure 1. Two cultures of B. braunii race A were grown in modified CHU medium (Largeau et al., 1980) in 20-L carboys. The culture medium was supplemented with NaHCO$_3$ at a final concentration of 1 lean. 

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mM, representing a typical concentration of bicarbonate in river water. One culture was grown in medium containing NaH13CO3 (98 atom%; from Isotec, Inc., Miamisburg, OH, USA) which was added to obtain a 13C isotopic enrichment of 20 atom%; the other was grown in medium containing K15NO3 for a 15N enrichment of 99 atom% with no carbon isotopic enrichment. To the former culture, NaH13CO3 was added aseptically (by syringe through a septum vial inserted into the carboy stopper) every 3 days during growth, with the total addition estimated to yield a 10 to 20% 13C label. Total bicarbonate addition was based on the following: (1) the culture would reach a maximum dry biomass of 0.8 g L\(^{-1}\) and would contain 60% organic carbon by weight, based on pilot growth experiments; (2) one mole of reduced algal carbon (CH\(_2\)O) is produced per mole of CO\(_2\) utilized; and (3) one mole of HCO3\(^{-}\) is formed per mole of CO\(_2\) dissolved. The cultures were grown (110 –170 \(^{\circ}\)C; 12:12 L:D cycle; 25\(^{\circ}\)C; 6 L min\(^{-1}\) aeration with 0.2 \(\mu\)m filtered air) to late-stationary phase of growth, with dry weights used to assess growth (Casadevall et al., 1985). The pH of both cultures was initially 7.8 and increased to 9.5 during growth; therefore, loss of H\(^{13}\)CO\(_3\) as 13CO\(_2\) was not expected to be significant. The two cultures, one containing 13C- and the other 15N-labeled algae, were subsequently mixed together in a 40-L carboy and allowed to degrade as previously described using a flow-through system (Harvey et al., 1995; Nguyen and Harvey, 1997). This mixing resulted in ca. 50% and 5 to 10% of the algae being 15N- and 13C-labeled, respectively.

2.2. Extraction of Organic Components from Algal Cells and Detritus

Stationary-phase B. braunii and 201-d old detritus were treated with multiple solvents to remove lipids, pigments, and extractable proteins. Lyophilized material (500 mg) was weighed into 50-mL Teflon centrifuge tubes. All extractions were performed with 35-mL solvent volumes, unless noted otherwise, using gas chromatography grade reagents, and with the assistance of ultra-sonication (30 W). Centrifugation following each extraction was at 20000 \(g\) for 30 min. Initial extraction was with ice-cold 10% (w/v) trichloroacetic acid in acetone (containing 0.1% \(\beta\)-mercaptoethanol) as described by Nguyen and Harvey (1998). Extraction was continued with acetone (containing 0.1% \(\beta\)-mercaptoethanol), and this was performed until the supernatant was clear (three times). Since pigments could not be removed completely with the previous treatments, extraction with 1:1 CH\(_2\)Cl\(_2\):methanol was performed (six times). The organic solvent treated residue was then dried under nitrogen. Removal of “easily-extractable” protein was achieved with extraction (1 h; 25\(^{\circ}\)C) in 15 mL of 0.1 N NaOH, with tubes placed on a rotary shaker. A short, room temperature extraction was performed to avoid protein hydrolysis as well as artifactual melanoidin formation, which likely would have occurred at higher temperatures and with longer extraction times (Hedges, 1978). The insoluble residues remaining after NaOH extraction were washed thoroughly with deionized water until the pH of the supernatant was neutral. A final extraction with 1:1 CH\(_2\)Cl\(_2\): methanol was performed (two times) on the residues. The postsonolvent residues were finally dried at 50\(^{\circ}\)C under a stream of nitrogen gas before analysis by NMR spectroscopy.

2.3. Solid-State CP/MAS 13C NMR

Solid-state 13C NMR spectroscopy utilizing cross polarization magic angle spinning (CP-MAS), a ramp-CP procedure, and TSPM (two pulse phase modulation) was performed using a Bruker DMX-300...
solid-State CPMAS $^{15}$N NMR

Solid-state $^{15}$N NMR spectroscopy utilizing CP-MAS and TPPM was performed using a Bruker DMX-400 MHz NMR spectrometer with a $^1$H frequency of 400 MHz, or a $^{13}$C resonance frequency of 40.5 MHz. Spectra were obtained with a pulse delay of 250 ms, a contact time of 2 ms, and a magic angle spinning speed of 5 kHz. Chemical shifts were calibrated using the carboxyl signal of glycine as a reference ($-347.6$ ppm).

2.5. Solid-State 2-D DCP MAS $^{15}$N $^{13}$C NMR

For the solid-state 2-D DCP MAS $^{15}$N $^{13}$C NMR spectra, a Bruker DMX 400 instrument with a triple resonance 7 mm probe was used. The $^{15}$N resonance frequency was 40.5 MHz; that of the $^{13}$C was 100.61 MHz. A consecutive matched spin-lock transfer was used, first from $^1$H to $^{15}$N (contact time $t_1 = 0.7$ ms) and then from $^{15}$N to $^{13}$C (contact time $t_2 = 3$ ms). During the latter, protons were decoupled using a frequency-shifted Lee-Goldburg sequence. During $t_1$ a ramped $^1$H-pulse was used, and during $t_2$ a ramped $^{15}$N-pulse was used. Both pulses were shaped from 100% down to 99% (Peersen et al., 1993). For postsolvent residues of stationary-phase $B$. braunii and 201-d detritus, spectra were obtained with mixing times increasing at an increment of 0.062 ms and at a spinning speed of 5.5 kHz. For the stationary-phase algal residue, 9216 scans with a pulse delay of 1.5 s were accumulated for each spectrum. For the degraded algal residue, 61440 scans with a pulse delay of 1.5 s were accumulated for each spectrum. The $^{15}$N chemical shifts are referenced to the nitromethane scale ($= 0$ ppm) and were calibrated with glycine ($= -347.6$ ppm). The $^{13}$C chemical shifts are referenced to the tetramethylsilane ($ = 0$ ppm) and were also calibrated with glycine (carboxylic carbon $= 176.04$ ppm).

3. RESULTS AND DISCUSSION

The 2-D DCP MAS NMR technique used here has previously been applied to a mixed algal culture that has been degraded under aerobic conditions for two months (Knicker, 2000). The $B$. braunii samples are different from the previous mixed algal culture in several ways. Firstly, the mixed algal culture was comprised of strains of $^{13}$C and $^{15}$N dually labeled Chlamydomonas, Chlorella, Closterium, and Scenedesmus. $B$. braunii for our degradation experiment was generated by mixing two cultures that were labeled separately with $^{13}$C and $^{15}$N. Such an approach allows testing of the depolymerization-recondensation hypothesis (Tissot and Welte, 1984; Bada, 1998).

The identification of enriched levels of $^{13}$C's bonded to enriched $^{15}$N's would confirm condensation reactions, whereas lack of such bonding would indicate that the biopolymers in each algal pool have maintained their original structural integrity. The power of the isotopic label approach for NMR spectroscopy is that the sensitivity of detection of any potential melanoidin reaction can be improved by one to greater than two orders of magnitude over natural abundance studies. A second difference between our experimental design and that of Knicker (2000) is that our algae were degraded for 201 d in a flow-through system, with active microbial consortia present to mediate the decay. The decay time used is three times longer than that of the previous study of Knicker (2000). Importantly, the $B$. braunii degradation was carried out in a mineral-free medium; consequently, we can rule out any observed protein preservation as due to sorptive protection by minerals. Finally, we performed multiple solvent extractions on the fresh algae and the detritus to remove soluble lipids and unshielded proteins to evaluate the potential role of encapsulation by the refractory cell wall.

Solid-state $^{13}$C NMR spectra of the stationary-phase- and 201-d-degraded $B$. braunii residues are shown in Figure 2. The dominant signals in the $^{13}$C NMR spectrum of the stationary-phase algal residue (Fig. 2 a) are those for carbohydrates (74 and 105 ppm), paraffinic carbons (0 – 45 ppm), olefinic carbons (129 ppm), and carboxyl and amide carbons (173 ppm), which corresponds to the primary composition of algae, including proteins, carbohydrates, and lipids. Our spectrum for the stationary-phase alga is not directly comparable to other spectra obtained for various green algal species (e.g., Zelibor et al., 1988), since we performed solvent extractions. Varied growth conditions, species composition, and NMR acquisition parameters would also affect comparisons.

Degradation of $B$. braunii under oxic conditions in a flow-through system significantly alters the carbon component (Fig. 2 b). The signals from aliphatic carbon components (30 ppm and 129 ppm) that are inherently chemically resistant to bio-degradation become more intense relative to other components at 74, 105, and 173 ppm. These changes suggest that biologically "labile" substances, such as proteins and carbohydrates, are preferentially lost during oxic degradation. The peak at 129 ppm can mainly be assigned to an isolated unsaturated carbon in a long paraffinic chain, e.g., those in algaenan, as this signal has been observed for chemically isolated algaenan (Derenne et al., 1997). The ultimate product of the biodegradation of $B$. braunii can be speculated to be the algaenan residue that is selectively enriched, as other more labile carbon components are lost.

$^{15}$N CPMAS NMR spectra of the postsolvent residues of stationary-phase algal and algal detritus are illustrated in Figure 3. Although the algae have been degraded under oxic conditions for 201 d, both of the spectra show almost identical 2-D DCP MAS NMR spectra of the post solvent residues of $B$. braunii for our degradation experiment was generated by mixing two cultures that were labeled separately with $^{13}$C and $^{15}$N. Such an approach allows testing of the depolymerization-recondensation hypothesis (Tissot and Welte, 1984; Bada, 1998). The identification of enriched levels of $^{13}$C's bonded to enriched $^{15}$N's would confirm condensation reactions, whereas lack of such bonding would indicate that the biopolymers in each algal pool have maintained their original structural integrity. The power of the isotopic label approach for NMR spectroscopy is that the sensitivity of detection of any potential melanoidin reaction can be improved by one to greater than two orders of magnitude over natural abundance studies. A second difference between our experimental design and that of Knicker (2000) is that our algae were degraded for 201 d in a flow-through system, with active microbial consortia present to mediate the decay. The decay time used is three times longer than that of the previous study of Knicker (2000). Importantly, the $B$. braunii degradation was carried out in a mineral-free medium; consequently, we can rule out any observed protein preservation as due to sorptive protection by minerals. Finally, we performed multiple solvent extractions on the fresh algae and the detritus to remove soluble lipids and unshielded proteins to evaluate the potential role of encapsulation by the refractory cell wall.
region of some of these heterocyclic compounds can expand to 
−270 ppm (Knicker, 2000). Consequently, the ability to deter-
mine their contribution may be diminished by the broad amide 
signal. To distinguish the contribution of amide-nitrogen and 
heterocyclic-nitrogen to the total signal intensity of the peak at 
−257 ppm, we have applied a solid-state two-dimensional 
double-cross-polarization magic angle spinning (2-D DCP 
MAS) NMR technique to the solvent extracted, stationary-
phase- and 201-d-degraded algal residues. During the 2-D DCP 
MAS NMR experiment, magnetization is transferred first from 
1H to 15N and then to 13C (Knicker, 2000). The resulting 
spectrum shows cross peaks correlating 15N signal intensity to 
13C chemical shifts of carbons that have strong dipolar inter-
actions with neighboring 15N. Solid-state 2-D DCP MAS 15N 
13C NMR spectra of the stationary-phase algae and the de-
graded algae are shown in Figures 4 and 5. In both cases, cross 
peaks are observed only for carboxyl/amide-C (185 – 160 ppm) 
and N-substituted alkyl-C (60 – 45 ppm). The cross peaks at 
−230 ppm and 120 ppm of the 13C dimension are spinning side 
bands of the signal at 173 ppm. No cross peaks are detected for 
C in the sp2 13C chemical shift region that could give direct 
evidence for pyrrole-type nitrogen (−145 ppm). Thus, the peak 
at −257 ppm in the CPMAS 15N NMR spectrum of the 
degraded algal residue (Fig. 3 b) can be assigned solely to 
amide-nitrogen. A 1-D spectrum version of the 2-D DCP MAS 
15N 13C NMR experiment is also shown in Figures 4 and 5. 
Two major signals at 185 – 160 ppm and 60 – 45 ppm with 
comparable intensities are observed, indicating almost all of the 
nitrogen in amide bonds is additionally bound to alkyl carbon, 
as would be expected for peptides.

A further examination of the 1-D spectrum of the degraded 
algal residue revealed a poor signal to noise ratio, demonstrat-
ing a lack of extensive resonance signal from direct bonding 
between 13C–15N (Fig. 5). In the case of the stationary-phase 
sample (Fig. 4), the 13C NMR spectrum shows poor signal-to-
noise because the 13C enriched structure are bound to 15N 
present only at natural abundance levels (0.36 atom%); this is 
due to the fact that the isotopic labels were applied to separate 
B. braunii cultures which were then mixed before initiating the 
oxic decomposition. The enriched 15N-containing stationary-
phase algae are not 13C enriched, and the enriched 13C-con-
taining stationary-phase algae are not 15N enriched. If cross-
coupling reactions were occurring during diagenesis, we would 
expect the 15N-enriched proteins in one subculture to react with 
the 13C-enriched components of the other subculture, thereby 
showing a significant gain in signal-to-noise ratio. The fact that 
a higher signal-to-noise ratio was not observed in the case of 
the degraded sample indicates that 13C-enriched components 
do not cross couple to 15N-enriched components; thus, the 
signals observed are from residual proteinaceous materials 
rather than newly synthesized material produced during the 
decomposition process. Since the number of scans accumulated 
for the spectrum of the degraded algal residue is 6.7 times more 
than that accumulated for the spectrum of the stationary-phase 
agal residue, we should observe a decrease in the signal to 
noise ratio if the number of scans for both samples were
Fig. 3. Solid-state $^{15}$N CPMAS NMR spectra of $^{15}$N- and $^{13}$C- enriched, (A) stationary-phase- and (B) 201-$d$-degraded *B. braunii* residues. Asterisks indicate spinning side bands of the signal at $-257$ ppm.

Fig. 4. Solid-state 2-D DCP MAS $^{15}$N $^{13}$C-NMR spectrum of $^{15}$N- and $^{13}$C- enriched, stationary-phase *B. braunii* residues.
A decrease in the signal to noise ratio would be mainly due to the loss of proteinaceous material during diagenesis. Since bacteria are reliant on the algal derived C and N to synthesize compounds necessary for their survival, we would expect the $^{15}$N- and $^{13}$C-labeled organic matter to be hydrolyzed and recombined. Thus, in the 2-D experiment we might expect to observe an increased resonance signal from direct bonding between $^{13}$C-$^{15}$N. Several reasons could explain why recombination (due to bacterial metabolism) was not observed. Firstly, past studies of oxic algal decay have indicated that bacteria generally contribute less than 10% of total particulate organic carbon by the end of the incubations (Harvey and Macko, 1997). With the current B. braunii degradation experiment, bacteria appear to contribute only 1 to 2% of the total particulate organic carbon content during the entire degradation experiment (Nguyen et al., manuscript in prep.). We hypothesize that some $^{15}$N- and $^{13}$C-labeled amino acids from algae were incorporated by bacteria (for de novo synthesis of proteins containing both $^{15}$N and $^{13}$C labels), but the bacterial contribution was not significant enough to observe an increased resonance signal from direct bonding between $^{13}$C-$^{15}$N. Secondly, although the decomposer organisms may have used much of the biologically available ("un-encapsulated") algal protein, most of the newly synthesized protein was likely degraded near the end of the degradation experiment. Finally, another explanation for the observed lack of increased bonding between $^{13}$C-$^{15}$N lies in the fact that we are looking at the residue remaining after organic solvent and NaOH extraction. It is possible that only minor amounts of bacterial proteins are associated with the solvent-treated residue, due to the bacteria’s lack of refractory, algaenan-like material. We hypothesize that most of the solvent-treated residues (stationary phase algae and detritus) are comprised of algal proteins intimately associated with algaenan.

The results strongly suggests that biopolymers in the $^{15}$N and $^{13}$C labeled algal pools have maintained their original structural integrity and that depolymerization-recondensation reactions among biopolymers were very unlikely during the microbially-mediated degradation of B. braunii. The NMR data support other evidence indicating the minor importance of the melanoidin reaction during early diagenesis, for example, in recent work applying a cleaving agent (N-phenacylthiazolium bromide) for glucose-derived protein-protein cross-links (Nguyen and Harvey, 2001).

4. CONCLUSIONS

(1) Proteinaceous material remains the major organic nitrogen component in B. braunii residual organic matter after over 200 d of oxic degradation by natural microbial assemblages.

(2) Results of 2-D DCP MAS $^{15}$N $^{13}$C NMR do not support the formation of heterocyclic-nitrogen compounds resulting from depolymerization-recondensation reactions during the microbial degradation of B. braunii. The finding of both proteinaceous and highly aliphatic materials in the degraded algae supports the "encapsulation" hypothesis of Knicker and...
Hatcher (1997), whereby the highly aliphatic macromolecular network (e.g., algaenan) physically protects intrinsically labile proteins from bacterial hydrolysis.

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