Physicochemical and biochemical characterization of non-biodegradable cellulose in Miocene gymnosperm wood from the Entre-Sambre-et-Meuse, Southern Belgium

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Abstract

Specimens of Miocene fossil wood from the Entre-Sambre-et-Meuse karsts (southern Belgium) were examined using physicochemical and biochemical techniques in order to understand the reasons for the exceptional preservation of these fossilized remains after 15 million years. Structural and chemical changes were assessed by comparing the structural features of the fossil samples with those of their modern counterpart, *Metasequoia*. Solid state 13Cnuclear magnetic resonance (NMR) and microscopic analysis showed good preservation of the cellulose structure in the fossil wood from the Florennes peat deposit. Despite the substantial cellulose fraction available in the fossil tissue, an enzymatic degradation test and a biochemical methane potential assay showed that the fossil cellulose could not be degraded by cellulases and anaerobic microorganisms usually involved in the biodegradation of organic matter. Moreover, the cellulose structure (crystallinity and surface area) seemed to have no effect on cellulose biodegradability in these Miocene fossil wood samples. On the basis of our observations, we suggest that the presence of a modified lignin structure could greatly influence cellulose preservation/biodegradability.

1. Introduction

In terrestrial environments wood is progressively degraded by biochemical and chemical processes (fungal and/or bacterial activity, hydrolysis andoxidation) so that its basic components can return to the natural cycle (Staccioli et al., 1997; Opsahl and Benner, 1999). Depending on geological events, wood tissue may also be fossilized in a variety of ways, including mineralization, permineralization, petrifaction, charcoalification and incorporation into peat and lignite (Creber and Chaloner, 1984). The relative speed and severity of degradation, compression and mineralization determine whether the potential wood fossil will be more or less preserved (Horwood, 1991).

Previous work on biodegraded and coalified wood has shown that the gradual transformation from recent woody cell walls to lignitic material is dominated by similar processes (Hatcher et al., 1982; Hedges et al.,

1985; Stout et al., 1988). These processes encompass the relatively rapid removal of hemicelluloses, biochemical depolymerisation of the cellulose early in peatification and the apparent gradual removal of all carbohydrate material at the lignite stage (presumably by anaerobic microbial and/or geochemical processes). These early diagenetic changes also result in a gradual biotransformation of the lignin macromolecule, leading to an aromatic hydrocarbon network (Venkatesan et al., 1993). Indeed, solid state 13C NMR and analytical pyrolysis studies (Bates and Hatcher, 1989; Hatcher et al., 1989a) have demonstrated that, during peatification, the lignin of decayed wood is altered by depolymerisation, demethylation, demethoxylation and further defunctionalization.

In a few instances, the various components forming the wood structure resist degradation in different ways and this resistance in turn affects the process of ageing and fossilization (Fengel, 1991). Structural biopolymers can indeed survive microbial degradation but their preservation in gymnosperm and angiosperm wood has been rarely reported (Fengel, 1991; Moers et al., 1994; Yang et al., 2005). Although these studies have provided insight into wood preservation in different environments, the biochemical explanation of this exceptional conservation remains unclear. The existence of well preserved fossil wood in the Entre-Sambre-et-Meuse (ESEM) area in Belgium is known (Fairon-Demaret, 1992; De Putter et al., 1996) but no chemical characterization of this material has been carried out. In this study, we have investigated the nature of the biomolecules in fossil wood buried in two peats located in the ESEM area. By combining chemical and biochemical approaches we hoped to relate the extraordinary preservation state to the non-biodegradability of the lignocellulose content.

2. Samples and methods

Wood samples were collected from two peat deposits in the ESEM area at Florennes (50°15'27N, 4°38'888E) and Onhaye (sand pit – 50°14'986N,4°50'014E). Two traditional approaches were used for their identification. First, thin sections were obtained, following the transversal, radial and tangential planes of the wood tissue. After clearing and mounting, the preparations were examined under a light microscope. Second, orientated fractured surfaces were prepared, gold-coated using a SPI-MODULE^{TM} Sputter Coater and observed with a JEOL JSM-5800 scanning electron microscope. Identification of the wood at the genus level was obtained by comparing the characters of the fossil material with those of modern wood, using the usual keys (Greguss, 1955), followed by an in depth analysis of the fossil species described in the literature (e.g., Kraüsel, 1949; Fairon-Demaret, 1992; Van Der Burgh and Meijer, 1996; Figueiral et al., 1999). Investigation of the palynological content of the embedding peat led us to consider that the wood fragments were deposited in a continental environment under a warm temperate climate. They have been dated to the base of the Middle Miocene (Russo Ermolli, 1991).

Carbohydrates were analysed after acid hydrolysis carried out on the wood powder according to the method reported by Rodriguez et al. (2005). Briefly, samples were incubated in a 72% w/v H_2SO_4 solution. The solubilised individual sugars were separated and analysed using high pressure liquid chromatography (HPLC) with an Agilent 1100 series apparatus (Agilent Technologies, Massy, France) equipped with a C-610-H ion exchange column (300 mm x 7.8mm, Supelco, Bellefonte, PA) and a refractometer detector.

Lignin was determined gravimetrically following extraction with triethyleneglycol (Edwards, 1973) and clean up of the waste material with a modified neutral detergent fibre (NDF) pre-treatment (Rowland and

Roberts, 1999). Milled wood (600 mg) was extracted in a boiling flask, connected to a water cooled condenser, with 100ml NDF for 1h, followed by digestion for 1h at 121 °C with 30 ml triethyleneglycol/0.2% HCl (v/v). The suspension was filtered, washed with hot distilled water and methanol, and dried at 105 °C to constant weight. Lignin content was calculated as loss in weight from this treatment.

X-ray diffractograms were recorded at room temperature with a SIEMENS D5000 powder diffractometer, using Ni-filtered Cu Kα radiation. The operating voltage and current were 40 kV and 50 mA, respectively. Samples were scanned for a range of 2θ from8° to 40° at 0.02°/min. The relative degree of crystallinity (CrI) was calculated according to the empirical equation of Segal et al. (1959).

For NMR, 13C cross polarization (CP) magic angle spinning (MAS) spectra were recorded with 4mm zirconia rotors spinning at 7 kHz, using a Bruker Avance DSX 400WB spectrometer (B_0= 9.04 T) working at the Larmor frequency of 100.6 MHz. Spectra were obtained under high power proton decoupling (83 kHz) with a delay time of 5 s and a contact time of 2 ms. The experiments were run with 5000 scans. The chemical shifts were determined using the Carbon-1 signal as internal standard (Kono et al., 2002). The relative proportions of the different carbon types were determined by integrating peak areas in various chemical shift ranges using an electronic integrator. The quantitative reliability of NMR data for fresh and degraded wood samples was established using a variable contact time (VCT) experiment. The different signal intensities were plotted vs. contact time (t_{cp}) and fitted into the classical and simplified equation $M = M_0$ (1 - exp (-t_{cp}/T_{CH})) exp (-t_{cp}/$T_{1\rho H}$), where M is the measured magnetization intensity, M_0 the initial intensity, T_{CH} and $T_{1\rho H}$ are two characteristic relaxation times of the system. The usual assumptions for applying this equation (Kolodziejski and Klinowski, 2002) were fulfilled. M_0 values were computed by fitting the experimental intensity M recorded at different contact times t_{cp} into the above equation. Therefore, for a given contact time, the correction factors to be applied at the different signal intensities and their corresponding integrals were easily calculated. It appeared that a spin lock time of 2 ms was optimal for reaching the maximum polarization of all the wood carbons.

Enzymatic hydrolysis was performed with a mixture of cellulases and hemicellulases as described by Rodriguez et al. (2005). Each sample (50 mg) was incubated at 40 °C with 4ml of an enzymatic solution that had 332.3 m International Unit (IU) per ml of cellulolytic activity and 906.6 mIU/ml of hemicellulolytic activity. Biodegradability was assessed as the mass of monosaccharides released, determined using HPLC, per mass of sample hydrolysed.

Sulfitization of the wood fibres, leading to a partial delignification, was carried out according to the method reported by Desmons (1987). The neutral sulfite ‘‘cooking liquor’’ consisted of 10% w/v sodium sulfite, 4% w/v sodium carbonate and 4% w/v sodium hydrogencarbonate. A 2.5g wood sample (dry weight) was suspended in 15 ml of the sulfite liquor and incubated for 2h at 121 °C. Following sulfitization, the fibres were washed extensively with distilled water until the pH of the filtrate reached neutrality and dried at 105 °C.

Oxygen delignification was performed by treatment with 5% H_2O_2 as described by Gould (1984) ; 1g wood (dry weight) was suspended in 50 ml of a 5% H_2O_2 solution. The suspension was adjusted to pH 11.5 with

0.1N NaOH and stirred for 24h at 28 °C. The insoluble residue was collected by filtration, washed with distilled water until the pH of the filtrate reached neutrality and dried at 105 °C.

The biochemical methane potential assay (BMP), based on measurement of CH_4 produced during anaerobic degradation of organic matter, was per-formed following the procedure described by Wang et al. (1994).CH_4 in the biogas was analysed using a gas chromatograph equipped with a thermal conductivity detector (TCD) and a GasPro GSC column (30 m x 0.32 mm) coupled to a CP-Carboplot P7 column (27.5 m x 0.53 mm). He N45 was used as carrier and reference gas. Calibration was per-formed using gas mixture standards (Air liquide, Liege, Belgium).

3. Results and discussion

3.1. Microscopy and botanical attribution

On the basis of their anatomical characteristics, the wood fragments from Florennes belong to the Taxodioideae subfamily of the *Cupressaceae. Taxodioid* pits in the cross-fields (Fig.1b) and abundant wood parenchyma with smooth or slightly pitted transversal walls enabled us to assign them particularly to the fossil species *Taxodioxylon gypsaceum* Göppert. The fragments also exhibit some characters attributed to Juniperoxylon pachyderma (Göppert) Kraüsel, i.e., cupressoid pits in the cross-fields and juniperoid thickenings in the vertical walls of the ray parenchyma cells (Fig. 1c). However, in the absence of other macrofossil remains, taxodiaceous secondary xylem is particularly difficult to assess because of overlapping characters (Basinger, 1981; Van Der Burgh and Meijer, 1996). Nevertheless, both fossil species mentioned above show the nearest anatomical convergence to that of the single living species of *Metasequoia*, M. *glyptostroboides* Hu and Cheng (Fig.1d–f).

In the Florennes samples, the three-dimensional shape of the peatified wood has been maintained (intact intracellular spaces). Cell walls and protoplasm has not been drastically affected by mineralization or coalification, as delicate structures such as bordered pits and the composite nature of the tracheid walls appear physically intact (Fig.1a and b). On the basis of classification of preservation states (Harris, 1958; Schopf, 1975; Horwood, 1991; Gerards and Gerienne, 2003) and examination using light and electron microscopy, the transformation of the samples has reached the level of peatification. Even though the gross morphology of the Onhaye fossil wood remained intact, its fine anatomical details have been destroyed in the Onhaye samples and their identification was therefore not possible. Nevertheless, the samples were used for comparative studies, as their age and environmental conditions of deposition were quite close to those from Florennes (Russo Ermolli, 1991).

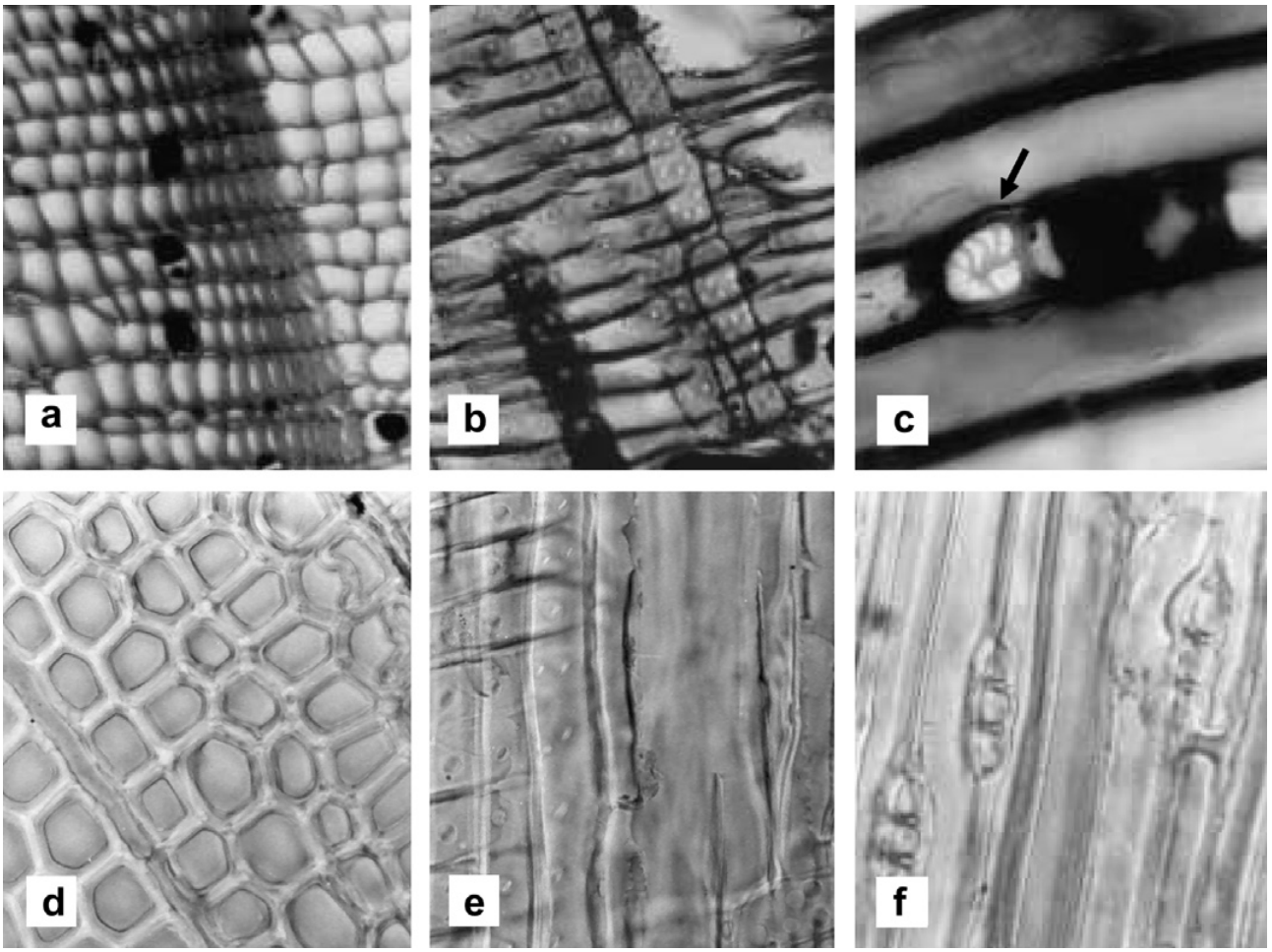

Fig. 1. Microscopic features of Florennes fossil wood: (a) transverse section, showing filling of tracheids with resin or tannin (x250) ; (b) radial sections, showing taxodioid pits in cross-fields (x250) and (c) tangential section, showing juniperoid thickening (indicated by arrow) in vertical walls of ray parenchyma cells (x660) ; (d–f) microscopic features of modern *Metasequoia*: (d) transverse section (x250), (e) radial section, cross-field pits (x250) and (f) tangential section, uniseriate rays (x250).

3.2. (Hemi)cellulose and lignin content

Chemical analysis of the Florennes wood revealed the same cellulose content as that found in the living species *Metasequoia* (Table 1). In contrast, the cellulose content of samples from Onhaye is two times lower than that of the Florennes fossil wood. This probably reflects a greater state of deterioration that can be explained by all the processes (transport and deposition) preceding burial of the plant remains in the sediment. Hemicelluloses are generally more susceptible to hydrolysis because of their branched structure and lower molecular weight (Goldstein, 1991). Our analysis of Florennes and Onhaye woods also showed that hemicelluloses were preferentially degraded compared to cellulose. According to several authors, the initial biochemical stage of coalification is characterized by a complete loss of hemicelluloses and a significant reduction in cellulose, which is completely degraded after a short span of geological time (Rollins et al., 1991; Orem et al., 1996; Hatcher and Clifford, 1997; Palanti et al., 2004). However, nearly all the cellulose, the major component of wood, is well preserved in our samples, suggesting that the ESEM fossil wood is at a very early stage of peatification. To allow such cellulose preservation over 15 million years,

these wood fragments should have benefitted from the presence of protective physicochemical conditions before and during sedimentation (surrounding clay layers, anaerobic conditions, etc.) and should have been buried quickly.

As shown in Table1, the lignin content, revealed by the single extraction with triethyleneglycol, was significantly reduced by 40% approximately in the Florennes and Onhaye fragments compared to the modern wood. This suggests that lignin deteriorated during its fossilization or was modified to a form unreactive to the triethyleneglycol treatment. Indeed, large amounts of lignin were released following subsequent acid hydrolysis of the cellulose part of the wood tested (Table1). This suggests that a fraction of the fossil wood equivalent to the lignin material is still present but is chemically different from the lignin of *Metasequoia*.

Table 1 Lignocellulosic composition of *Metasequoia*, and Florennes and Onhaye fossil wood (values in parentheses = standard deviation of mean)

Sample	Cellulose[a] (wt%)	Hemicellulose[a] (wt%)	Lignin[b] (wt%)	Residual lignin[c] (wt%)
Metasequoia, fresh	27.9 (2.1)	12.0 (0.6)	44.6 (1.5)	7.0 (2.8)
Florennes wood	26.0 (2.7)	0	3.6 (0.6)	54.7 (1.5)
Onhaye wood	13.4 (0.9)	0	4.4 (2.2)	55.5 (4.3)

[a] Determined by acid hydrolysis (Rodriguez et al., 2005).
[b] Determined by extraction with triethyleneglycol (Edwards, 1973).
[c] Determined by extraction with triethyleneglycol (Edwards, 1973) followed by acid hydrolysis (Rodriguez et al., 2005).

3.3. Susceptibility to enzymatic and microbial degradation

As a complementary approach, we wanted to simulate the biodegradation of cellulose compounds by way of enzymatic or microbial activity. An enzymatic cellulose degradation (ECD) test was carried out using an optimised mixture of cellulases/hemi-cellulases in order to analyse the cellulose fraction still available for biological degradation. The ECD test was performed first on wood fragments in their native state in order to preserve their physicochemical characteristics. Whatman no.1 filter paper samples were also included in the test as cellulose standards. The results showed that the cellulose present in the Florennes and Onhaye fossil woods appeared almost unreactive, while a low but significant extent of hydrolysis occurred for the modern *Metasequoia* (7% after 42 h; Table 2). This suggests that the susceptibility of fossil cellulose to enzymatic hydrolysis is affected by the ‘‘bioavailability’’ of these complex lignocellulose materials. Bioavailability is defined by several parameters such as crystallinity (Weimer et al., 1990), degree of polymerisation (Cao and Tan, 2004), particle size (Hu et al., 2005), surface area (Mooney et al., 1999) and fibre size or pore volume (Gama et al., 1994). The lignin barrier (Mooney et al., 1998) and other phenolic compounds in plant cell walls might also result in a reduction of the rate and extent of polysaccharide degradation.

In order to determine the impact of surface area of cellulose fibres on their susceptibility to enzymatic degradation, wood samples were reduced to a fine powder (60 mesh) to favour direct physical contact between the enzymes and the cellulose surface. Surprisingly, results from the ECD test applied to these

ground samples showed an enhanced hydrolysis rate for the modern *Metasequoia* wood but the increase was limited for the fossil substrates (Table 2).

Lignin is intimately associated with cellulose in wood tissue and its resistance is thought to delay biodegradation of the cellulose material (Stinson and Ham, 1995; Rivard et al., 1994; Sewalt et al., 1997). If lignin inhibits cellulose decomposition in the fossil wood, partial removal or deterioration of this polymer should increase the number of sites available for enzyme action and should favour the hydrolysis of lignocellulose substrates. In an attempt to evaluate whether lignin influences the enzymatic hydrolysis of lignocellulose substrates, chemical delignification with sulfite was carried out before the ECD test. As a result, the *Metasequoia* wood sample was rendered more susceptible to enzyme attack (Table2).

Therefore, partial delignification resulted in a significant increase in the amount of cellulose available for decomposition. In contrast, no benefit from the sulfite treatment was observed for the Florennes wood. This lack of efficiency of the sulfite treatment on cellulose availability suggests the hypothesis of a chemical modification of the original Florennes wood lignin, which seems to be more resistant than the original lignin in the modern wood. However, enzymatic hydrolysis extent appears to be greater (12%) after oxidative treatment with H_2O_2. In this case, the oxidation mechanism could have opened up the cell wall structure to allow greater access to the cellulose (Morrison, 1988).

Cellulose decomposition was also investigated through BMP experiments. This test measures the CH_4 generated during the decomposition of organic matter (i.e., mainly cellulose and hemicelluloses) by microorganisms under anaerobic conditions similar to those taking place in a machuria or peat. As light but effective production (8.9 ml/g) was observed for *Metasequoia* wood after 60 days incubation but no gas production could be measured with the fossil woods. These samples were thus not susceptible to microbial degradation despite the relatively high cellulose content of the Florennes wood (Table1).

To confirm the biochemical observations, fossil and modern woods resulting from the BMP test were submitted to microscopic investigation using SEM. It appears that *Metasequoia* samples used as control, have undergone a slight extent of degradation characterized by shrinkage and homogenization of the cell walls (Fig. 2a and b). Onhaye material exhibits the poorest quality of tissue preservation, showing only few identifiable anatomical details on transverse and longitudinal sections (Fig.2c). The Florennes fossil wood fragments have a similar external appearance to modern wood. When viewed under SEM, these fragments, buried in Tertiary clay, show homogenized as well as slightly cracked cell walls (Fig. 2d). There is also very little infilling material within and between the cells. After 60 days of anaerobic incubation, the fine structure of the cell wall did not change so, compared to modern *Metasequoia*, these fossil wood samples are seemingly not affected by microbial activity. The SEM observations are consistent with the ECD results indicating that the fossil woods are not susceptible to degradation.

Table 2 Enzymatic hydrolysis of lignocellulose (*Metasequoia*, and Florennes and Onhaye fossil woods) and cellulose (Whatman no.1 filter paper) substrates

Sample	Cellulose degradation (%)			
	Native state (42 h)	Grinding (48 h)	Grinding + sulfitization (48 h)	Grinding + oxygen delignification (48 h)
Metasequoia, fresh	7.5	29.3	48.2	17.1
Florennes wood	0	5.4	5.4	11.7
Onhaye wood	0	6.2	ND[a]	52.1
Whatman	92.7	96.3	89.8	87.1

[a] Not determined.

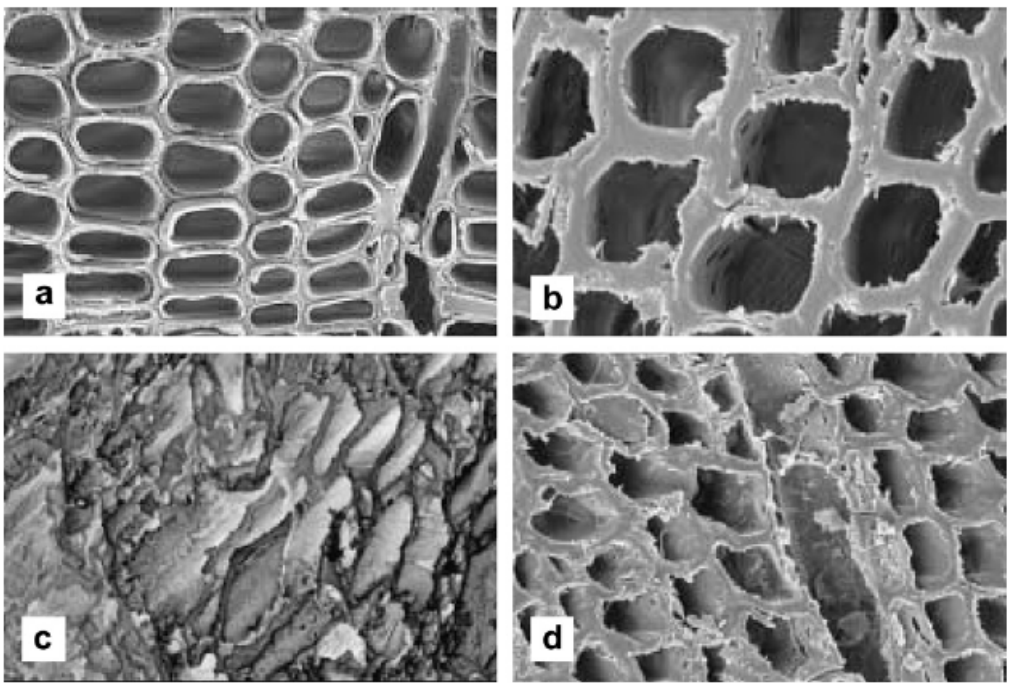

Fig. 2. SEM micrographs of cell walls of *Metasequoia* (a) Onhaye (c) and Florennes (d) wood in native state (respectively, x500, x1000, x500) and (b) tissues of *Metasequoia* after 60 days anaerobic degradation (x1000).

3.4. Physicochemical characterization of lignocellulose material

3.4.1. X-ray diffraction

X-ray diffraction was used to investigate the crystalline structure of cellulose in the fossil wood and to determine whether the degree of crystallinity affected its biodegradability or not. The diffraction patterns indicate that cellulose in the modern as well as in the two fossil woods is indeed cellulose I (Fig.3). This exhibitsa characteristic curve, displaying peaks at 15°, 16°and 23° 2θ due to 101, $10\bar{1}$ and 202 reflections, respectively (Stewart and Foster, 1976). The diffractogram obtained for pure cellulose is well resolved and the degree of crystallinity calculated with the Segal method (1959) is greater (99.4%) than for the wood samples. The Florennes fossil wood displays a greater degree of crystallinity (52%) than its modern counterpart *Metasequoia* (42%). This increase in crystallinity could result from a slight degradation of the amorphous fraction of cellulose with aging. On the other hand, a 15% degree of crystallinity was measured

for the Onhaye fossil wood. Its low cellulose content (Table 1), together with its lower crystallinity proportion, suggests that this wood was exposed to more unfavourable degradation conditions at early stages of fossilization than the Florennes wood. Our results point out that the degree of crystallinity should not affect the bioavailability of cellulose in the fossil woods. This hypothesis is reinforced by the results obtained on various fossil woods from different countries (data not shown), which display no cellulose degradation, despite a crystallinity index ranging from 15% to 56%. This implies that other structural parameters must also be taken into account to explain the low hydrolysis extent.

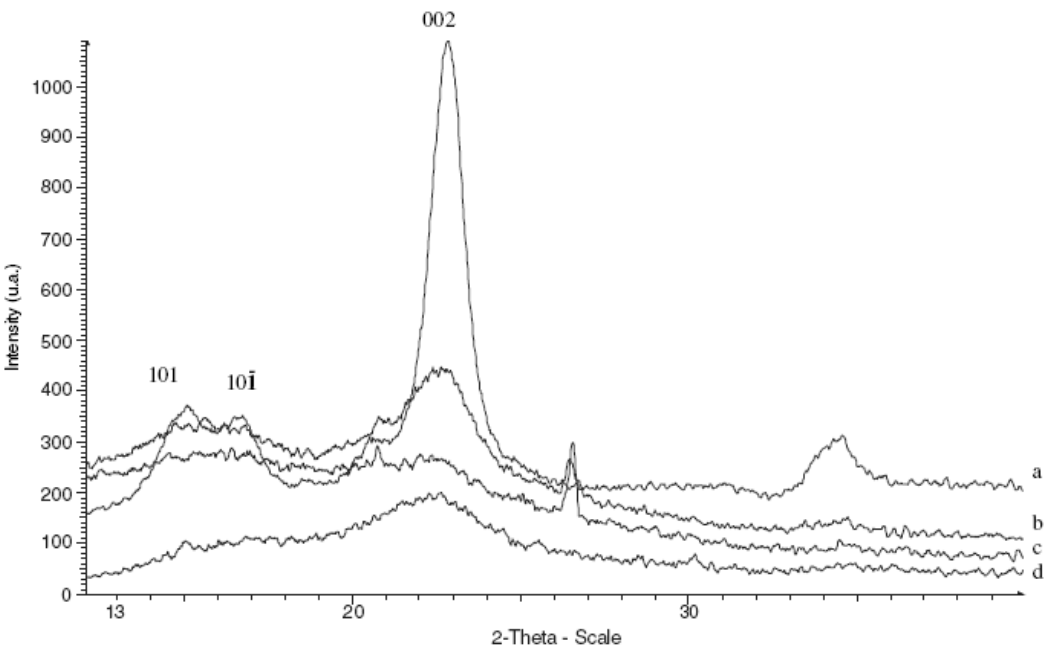

Fig. 3. X-ray diffractograms of: (a) pure cellulose; (b) Florennes cellulose; (c) Onhaye cellulose and (d) *Metasequoia* cellulose. These celluloses exhibit a characteristic curve displaying peaks at 15° ,16° and 23° 2θ due to 101, 10$\bar{1}$ and 202 reflections, respectively (Stewart and Foster, 1976).

3.4.2. CPMAS ^{13}C NMR

Solid state CPMAS ^{13}C NMR has been used to obtain qualitative and quantitative data about the chemical structure of fossil and modern woods. This non-destructive technique is well adapted to samples with restricted solubility, e.g., residual lignin, or when a physical structure such as cellulose morphology is being studied (Gil and Neto, 1999; Maunu, 2002). The CPMAS ^{13}C NMR spectra of the modern and peatified woods are shown in Fig. 4. Major peaks include signals corresponding (Orem et al., 1996) to aliphatic (0–50 ppm), methoxyl (57 ppm), carbohydrate (64–106ppm), aromatic (110–153 ppm), phenolic (148–160 ppm) and carboxyl/amide carbons (174 ppm). Peak areas for carbon atoms in these different environments are shown in Table 3 as a percentage of the area representing total organic carbon. The fossil wood samples show many of the NMR resonance characteristics of lignin and cellulose, which are the major constituents of the modern wood.

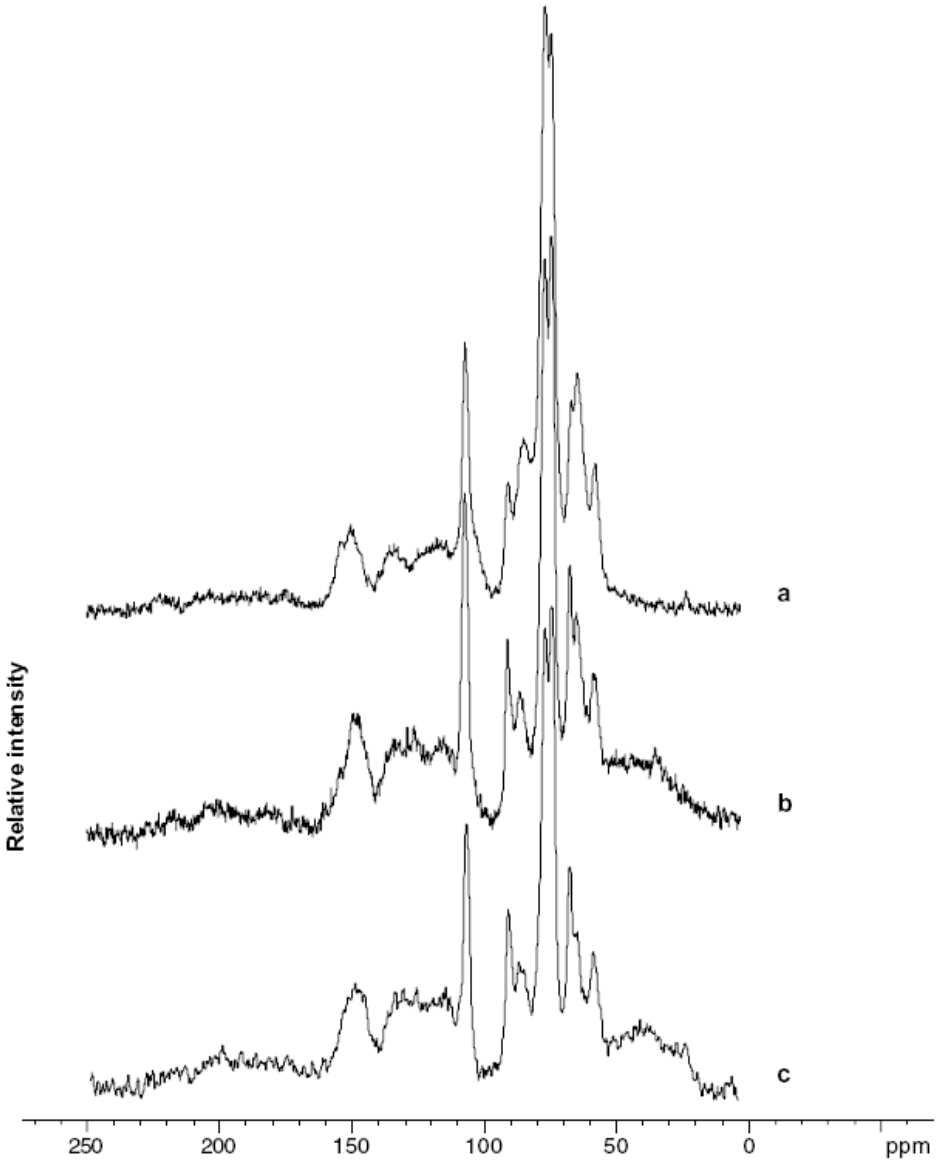

Fig. 4. CPMAS ^{13}C NMR spectra of: (a) fresh xylem from *Metasequoia*; (b) Florennes fossil wood and (c) Onhaye fossil wood. Spectra were obtained with a contact time of 2 ms.

3.4.2.1. Cellulose structure. The resonances of the carbohydrate components in both modern and fossil woods are at 64, 67, 72, 85, 90 and 106 ppm (Gilardi et al., 1995; Maunu, 2002). The Florennes and Onhaye fossil samples have ^{13}C NMR spectra dominated by a peak related to carbohydrates at 72 and 106 ppm, indicating that the samples are well preserved and retain much of the cellulose present in the lignocellulose structure. However, the samples have undergone some degradation during the peatification process, resulting in a slight loss of carbohydrate compared to the modern *Metasequoia* (Table 3). The shoulder at 102 ppm on the signal for cellulose C-1 and the signal for the methyl carbons of acetyl groups (20 ppm), assigned to hemicelluloses, are absent from the spectra of the fossil woods (Fig.4). This lack of signal is consistent with a loss of hemicelluloses from the wood structure.

Table 3 Carbon distributions for modern *Metasequoia* and fossil (Florennes/Onhaye) wood from peak integration of CPMAS ^{13}C NMR spectra

	Sample		
	Metasequoia, fresh	Florennes fossil wood	Onhaye fossil wood
% Carbohydrate carbon[a]	76.0	53.9	50.6
% Aliphatic carbon[a]	–	17.2	13.3
% Aromatic carbon[a]	19.5	24.0	31.2
$-OCH_3$/ring[b]	1.4	1.2	1.1
Aryl-O/ring[b]	1.0	0.9	0.7

[a] Cellulose, aliphatic and aromatic carbon peak areas expressed as % total carbon resonances.

[b] Average number of methoxyl groups and oxygen substituents (aryl-O) per ring calculated by integrating peaks at 56 and 148 ppm (Fig. 4), respectively, normalizing to total area for aromatic carbons (110–153 ppm) and multiplying by 6 (Bates and Hatcher, 1989).

3.4.2.2. Lignin structure. Lignin in fossil gymnosperm wood shows NMR signals at 56ppm for methoxyl carbons and in the 110–160ppm region for aromatic structures, though in different proportions compared to the modern wood.

Compared to the lignin in modern *Metasequoia*, the number of methoxyl groups per ring (OCH_3/ R) slightly decreases in the fossil wood, indicating a progressive change in the nature of lignin (Table 3). According to Hatcher et al. (1989b), during the early coalification of gymnosperm woods, there is indeed a progressive demethylation or demethoxylation of lignin, resulting in increasing proportions of catechol-based products. However, demethoxylation seems not to play an important role in lignin alteration since the number of oxygen substituents per ring (Aryl-O/R) does not vary significantly between modern and Florennes fossil woods (Table 3).

According to Nimz et al. (1981), the resonances at 148 and 153 ppm in the aromatic region are related to oxygen-substituted aromatic carbons. Specifically, the resonance at 153 ppm is due to the C-3 and C-4 carbons of guaiacyl units with all β-O-4 linkages still intact. This resonance is shifted to 146 ppm when β-O-4 linkages are hydrolysed to yield free phenols. It should also be mentioned that the resonance at 135 ppm results from C-1 carbon resonances. Moreover, peaks at 115–120 and 125 ppm are related to C-2, C-5 or C-6 aromatic carbons (Bates et al., 1991).

The decrease in relative intensity of the resonances at 153 and 148 ppm in Florennes fossil wood (Fig. 5) suggests that its lignin is relatively depleted in arylether linkages compared to the modern wood. On the other hand, the increase in the relative intensity of the signal at 146 ppm indicates the formation of free phenolic hydroxyl groups. Both transformations result in the formation of catechol-like structures lacking in the modern gymnosperm, so this could explain the non-reactivity of the residual lignin to the triethyleneglycol treatment. Even though the structural integrity of the lignin seems to be damaged, the decrease in the intensity of the resonance at 115–120 ppm and the emergence of a new resonance at 125 ppm, resulting from a shift in the 120 ppm resonance (Bates and Hatcher, 1989), indicate that condensed

guaiacylstructures were already present in the modern lignin and the percentage of alkyl bonds (C-5 carbons in guaiacyl units) was preserved during fossilization. The resonance at 135 ppm remains unchanged in the Florennes wood spectra relative to modern *Metasequoia*, suggesting that the C-1 alkyl linkage has been retained.

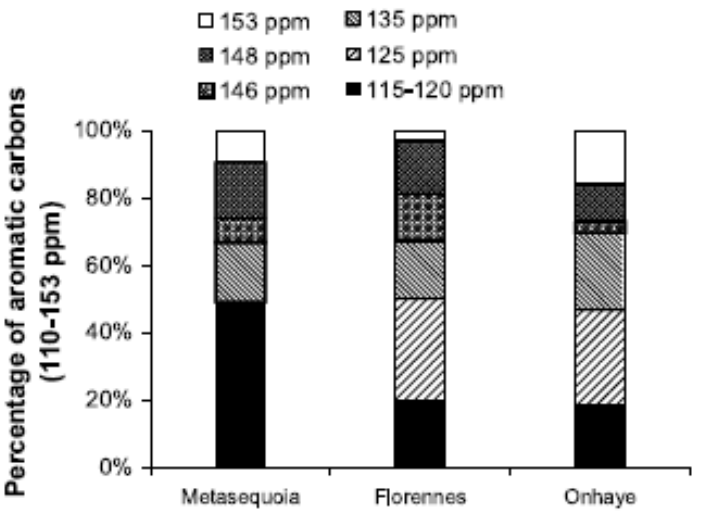

Fig. 5. Distribution of aromatic carbons characteristic of gymnosperm lignin for modern *Metasequoia* and fossil (Florennes/ Onhaye) wood. Percentages were calculated by integrating peak areas at 115–120, 125, 135, 146, 148 and 153 ppm and normalizing to total area for aromatic carbons (110–153 ppm).

4. Conclusions

The objective of the investigation was to try to explain the reasons for the exceptional preservation of Miocene fossil wood from the Entre-Sambre-et-Meuse Belgian karsts. Indeed, fragments up to large tree stumps or trunks seem to have weathered the centuries without any extensive damage. This fossil wood has not undergone significant coalification as evidenced by the fact that it still contains relatively high amounts of cellulose. In contrast, most of the studies on peatified and early coalified wood have demonstrated that the first stage in the transformation of wood to bituminous coal involves the hydrolysis and loss of cellulose (Hatcher et al., 1981, 1982, 1989a,b). Our biochemical experiments have shown that the Florennes and Onhaye fossil woods are not biodegraded by cellulases and hemicellulases or by microorganisms usually involved in organic matter biodegradation under aerobic and anaerobic conditions. These results suggest that the biodegradation of these complex lignocellulose fossil substrates is influenced by their bioavailability. We show that the chemical structure of cellulose from ESEM fossil wood is quite similar to that of *Metasequoia*, the nearest modern wood species. Therefore, cellulose structure in itself could be discarded as a factor in explaining the difference in the extent of cellulose hydrolysis. Although there is some evidence that surface area negatively influences the hydrolysis rate of modern wood, it seems not to be a major limiting factor in fossil cellulose hydrolysis. In contrast, lignin appears to be a major determinant of cellulose digestion in fossil wood. Moreover, we have demonstrated that the lignin structure had only undergone slight chemical alteration such as demethylation, cleavage of some β-O-4 linkages and alkylation of the resulting catechol-like structures. These transformations could have

maintained the structural integrity of the Florennes fossil wood as observed with microscopy. Therefore, the results imply that the bioavailability of the cellulose substrate slows as lignin modification proceeds. Another possible route for investigating the non-biodegradability of cellulose in peatified wood would be to correlate substrate accessibility with the presence of polyphenolic compounds, such as tannins, which may also inhibit extensive bacterial degradation.

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