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Ann. For. Sci. 60 (2003) 503–508
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DOI: 10.1051/forest:2003043

Original article

Inter-annual mobility of nitrogen between beech rings: a labelling experiment

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(Received 23 October 2002; accepted 25 March 2003)

Abstract – Nitrogen concentration [N] and isotopic composition ($\delta^{15}N$) in annual growth rings of 16-year-old beech trees (*Fagus sylvatica* L.) were measured before and after treatment of wood using organic solvents. The trees, grown under field conditions in Northeastern France, were labelled with ^{15}N-enriched urea solution at leaf level for three successive years (1993, 1994 and 1995), and were sampled in 2001. $\delta^{15}N$ values in this experiment ranged from –4 to –7‰ for non-labelled tree. Labelling resulted in markedly increased $\delta^{15}N$ of whole wood for years 1994, 1995 and 1996. Significant labelling-induced increases, compared to non-labelled rings, in $\delta^{15}N$ were also found in the rings formed before and after the labelling period, pointing to an important mobility of N across the rings. The extraction removed 36% of total nitrogen and 14% of total carbon. The treatment improved the inter-annual resolution of [N] and $\delta^{15}N$, but did not reduce the ^{15}N signal in rings prior to labelling to the same level as in control tree as was expected. After extraction, significant correlation between August precipitation and [N] variations at the inter-annual level was found for the 1990–2000 period.

nitrogen 15 / isotopic labelling / internal transfer / tree ring / *Fagus sylvatica*

Résumé – Mobilité de l'azote dans les cernes annuels de hêtre : une expérience de marquage. La concentration [N] et la composition isotopique ($\delta^{15}N$) de l'azote ont été mesurées dans les cernes annuels d'arbres d'un jeune peuplement de hêtre (*Fagus sylvatica* L.) du Nord-Est de la France, âgés de 16 ans. Les analyses ont été effectuées sur des échantillons de bois avant et après l'extraction des composés labiles par des solvants organiques. Les arbres ont été soumis à un enrichissement en ^{15}N par une solution d'urée pulvérisée sur les feuilles. L'enrichissement a été effectué durant trois années successives : 1993, 1994 et 1995. Les arbres ont été échantillonnés en 2001. Le $\delta^{15}N$ du bois de l'arbre non marqué variait entre –4 et –7 ‰. Le marquage a conduit à une augmentation significative de $\delta^{15}N$ dans le bois brut pour les années 1994, 1995 et 1996. On a également trouvé des valeurs élevées de $\delta^{15}N$ dans les cernes formés avant et après la période de marquage. Cela traduit une forte mobilité de N entre les cernes. L'extraction a éliminé 36 % de l'azote total et 14 % du carbone. Le traitement appliqué a amélioré la résolution inter-annuelle de [N] et $\delta^{15}N$, mais n'a pas ramené le niveau du signal ^{15}N dans les cernes formés avant l'expérience de marquage au même niveau que ceux de l'arbre témoin. Après extraction, on a observé une corrélation significative entre les précipitations du mois d'août et les variations inter-annuelles de [N] pour la période 1990–2000.

azote 15 / marquage isotopique / transfert interne / cerne de croissance / *Fagus sylvatica*

1. INTRODUCTION

Increasing atmospheric deposition of nitrogen, as a consequence of fossil fuel and biomass combustion, fertilizer consumption and manure management is suspected to be a major factor driving current forest dynamics and increasing productivity, thereby enhancing the terrestrial carbon sink [16]. Several dendroecological studies showed an increasing growth trend of forest stands as a result of environmental changes in Western Europe [2, 3, 5, 22]. This increase in productivity may be attributed to a combination of several factors: increasing CO_2 concentration, nitrogen deposition, silvicultural management or land-use history. We cannot yet clearly separate the exact relative roles of these different factors. Retrospective studies of plant chemical and isotopic composition could help in assessing the role of N in this increasing growth trend. There is a widespread view that ^{15}N cannot be used as a tracer in soil-plant natural systems due to the fractionations that occur during metabolic processes leading to loss of the original ^{15}N signal [12, 13]. On the other hand, other authors suggest that ^{15}N is a useful tracer in some particular species and circumstances [1, 14, 23].

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Long-term changes in tree N status have already been observed. Resampling permanent plots, Duquesnay et al. [8] and Flückiger et al. [9] observed an increase of beech leaf N content during the last decade. Using herbarium leaf material and tree-growth rings, Peñuelas and Estiarte [18] observed a decreasing trend in $\delta^{15}N$ values for *Quercus pubescens* during the last century. Stable isotope measurements in tree rings might provide an annually resolved record of such changes in N supply. However, Poulson et al. [19] concluded from an experiment on *Tsuga canadensis* that nitrogen concentration in tree rings cannot provide such information on the availability of nitrogen during ring formation due to the recycling of nitrogen by the tree during maturation. This view was confirmed by the pioneering work of Nõmmik [15] who studied N translocation between rings after a labelling experiment.

To date there have been no successful retrospective tree ring studies based on [N] or $\delta^{15}N$ to get insights on tree N supply, due to two major difficulties: one is linked to the biochemistry of wood and the other to the interpretation of $\delta^{15}N$ variations.

The components of wood can be classified into two major groups: structural and non-structural compounds. The first group is mainly composed of polysaccharides (cellulose and hemicelluloses), lignin and other structural material such as cell wall proteins. The second group comprises inter alia: tannins, polyphenols, fats, waxes, resins, alkaloids and other extractive material. Nitrogen is partly bound to structural forms such as lignin polymers [4, 7]. It would be of particular interest to access this N pool which could be a better indicator of inter-annual variations of nitrogen metabolism than the total N pool. The use of whole wood for elemental and/or isotopic analyses leads to confounding effects. Sheppard and Thompson [21] suggested an extraction pre-treatment of wood by organic solvents, before interpreting N in tree rings, to remove sap and soluble forms of nitrogen which may obscure the environmental signal of nitrogen concentration and/or isotopic composition.

It is difficult to test whether such a pre-treatment improves the accuracy of the annual resolution of N analysis in tree rings without any additional information on the ^{15}N content of yearly tree N uptake. Thus, we used a ^{15}N labelling experiment in which young trees had been subjected temporarily to high ^{15}N levels for three successive years. Our objective here was to test the effect of the proposed extraction pre-treatment on the inter-annual resolution of elemental and isotopic compositions of nitrogen in the annual growth rings of beech.

2. MATERIALS AND METHODS

Three dominant beech trees (*Fagus sylvatica* L.), aged 16 years and between 4 and 5 meters high, were sampled on a calcareous soil in a large natural regeneration area at Puvenelle forest in north-eastern France (long. 6° 3´ 17" E, lat. 48° 54´ 19" N). The trees were cut in autumn 2001. Two trees (tree A and B) were sampled from a ^{15}N labelled plot, and one tree, considered as a control, was sampled from a non-labelled plot, ca. 50 meters away from the labelled plot. The labelling was done with urea solution (99.5% ^{15}N, pH 6.2) at leaf level for three successive years. The following amounts of ^{15}N were applied per tree: 56.6 mg, 26.9 mg and 58.2 mg on September 1993, August 1994 and August 1995 respectively [26]. These amounts of nitrogen induced a significant isotopic signal without affecting the overall N supply to the trees.

A 5 cm thick cross-section of basal stem was cut into 10–12 thin discs (2–3 mm thickness) which were lyophilised. The annual rings from 1987 to 2001 were then separated manually under an optical microscope using a razor blade. Tree rings were measured along 4 radii in each disc. Ring measurements were crossdated using five different methods: matching among radii, among discs, among trees and comparing radial growth variation with height growth and with regional climatic data. For the control tree, the inner rings corresponding to the years 1987 and 1988 were mixed due to the small material yield after rings separation. The samples were oven-dried at 60 °C to constant weight, and milled in a planetary mill (MM 200, Retsch, Haan-Germany) before analysis.

The C, N and $\delta^{15}N$ analyses were performed on samples before and after extraction.

Soluble N forms were extracted in a Soxhlet apparatus for 4 h in a mixture of toluene and ethanol 1:1, then in 100% ethanol for the same time, and then 1 h in distilled water, according to the protocol named "short duration" described by Sheppard and Thompson [21]. The samples subjected to this extraction are referred to as treated wood (TW), and the samples which had not been extracted are referred to as non-treated wood (NTW) in the following.

In addition, we extracted lignin in two samples: one from the control tree, mixing all rings from the pith to the outmost ring into a composite sample, and the other from a mix of treated trees A and B, including all rings formed before the labelling date (1987 to 1992). Each 15 g wood sample was reduced to powder by ultramilling and lignin extracted following the protocol of Tollier et al. [24].

Between 5 and 7 mg of each sample were processed for determination of nitrogen concentration with a continuous flow elemental analyser (Carlo Erba Analyser-NA 1500, CE instruments, Rodano, Italy), following Dumas combustion of organic material to CO_2 and N_2, interfaced with an isotope ratio mass spectrometer (Finnigan Mat, Delta S, Bremen, Germany) for isotopic determination.

The $\delta^{15}N$ was expressed as:

$$\delta^{15}N‰ = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000,$$

where R_{sample} is the isotopic ratio ($^{15}N/^{14}N$) of the sample, and $R_{standard}$ is the isotopic ratio of the standard which is the N_2 of air.

3. RESULTS AND DISCUSSION

3.1. Nitrogen concentration and C/N ratio

On average, N concentration decreased from 0.22% before extraction to 0.15% after extraction, while C concentration went from 46.4% to 44.1%. Taking into account the loss of material during extraction, 36% of initial nitrogen and 14% of carbon were removed by the extraction pre-treatment. As a consequence, the C/N ratio increased in almost all annual samples (Fig. 1), in a similar manner for the three trees under study. The average increase amounted to 16, 39 and 24% in trees A, B and control, respectively. Enough N remained for isotopic and elemental analyses. Sheppard and Thompson [21] report that mainly soluble forms of nitrogen are likely to have been removed by the treatment applied, considering the structural forms are mostly incorporated and crosslinked to lignin and are insoluble in organic solvents and water. However it is possible that an exchange of N from the storage pool into the

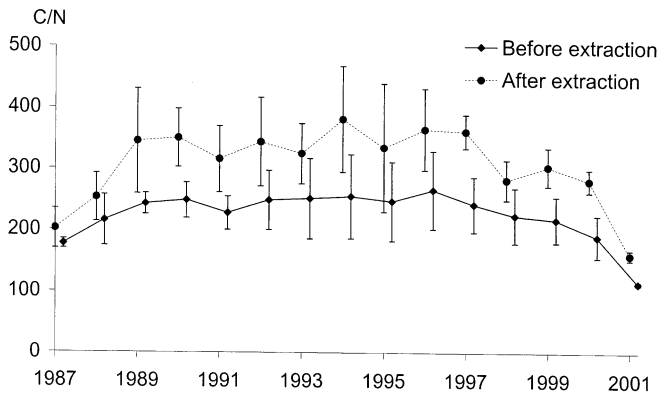

Figure 1. Average annual variations of carbon to nitrogen ratio in tree rings of ^{15}N-labelled trees A, B and a control tree of *Fagus sylvatica* L. before and after extraction by organic solvent. Vertical bars indicate SE ($n = 3$).

structural protein takes place as was suggested by Sauter et al. [20] in a detailed study of protein storage of young poplar (*Populus* x *canadensis*) wood.

[N] values in rings for these young trees were high and ranged from 0.14 to 0.41% in the non-treated samples (Fig. 2a). In the southern part of the Belgian Ardennes, Penninckx et al. [17] reported ranges of 0.07–0.15% and 0.12–0.25% in rings of 130–160 year old *Fagus sylvatica* L. and *Quercus robur* L., respectively. Before and after extraction, the most recent ring (2001) showed much higher values than previous rings. If we consider that the nitrogenous compounds are mostly in soluble forms, the pre-treatment applied should have reduced the [N] in this external ring to the same level as the previous annual rings. This lack of difference in the external ring before and after extraction suggests either a possible "contamination" by structural forms of nitrogen coming from tissues external to cambium (phloem and bark), which are difficult to separate during ring cutting, or an insufficient pre-treatment extraction of mobile N.

The results shown in Figure 2a confirm that [N] varies across the rings from the pith to the external ring. Many factors can lead to such variations: age effect, environmental conditions or availability of nitrogen. Interestingly, extraction pre-treatment made the [N] signal in tree rings more consistent and similar between trees in the treated plot (trees A and B) than before extraction. Excluding the most external ring, the inter-annual correlation coefficient ranges from $r = -0.13$ ($P > 0.10$) before extraction to $r = 0.85$ ($P < 0.001$) after extraction. The control tree sampled at ca. 50 meters from the labelled trees did not display this pattern. The similar pattern observed in trees A and B (Fig. 2b), after elimination of soluble compounds, might be indicative of a common environmental influence.

We searched for correlations between this common annual [N] signal and main climatic parameters (monthly precipitations and temperatures). August precipitation was negatively correlated with [N] at the inter-annual level during the 1990–2000 period [$r = -0.86$ and $r = -0.94$ ($P = 0.01$), for tree A and B, respectively (Fig. 3)]. This relationship could be the indirect consequence of the role of precipitation during late summer on lignification. However, this preliminary result needs further confirmation before suggesting any causal relationships between tree-ring [N] and climate variations.

3.2. Nitrogen isotopes

In ^{15}N-enriched A and B trees, the labelling period (1993–1995) was strongly marked by an increase of the δ^{15}N (Fig. 4), lagged by one year. Patterns of δ^{15}N variation in tree rings are not the same before and after the labelling period: whereas there is a gradual decrease of δ^{15}N after the end of labelling, δ^{15}N values are constant in rings formed before labelling. This pattern is very similar between trees A and B and between treated and non-treated wood.

The one year lag between labelling and δ^{15}N peak is probably due to the late date in the year (September) of ^{15}N-enriched urea spraying. This suggests that wood-N metabolism is too slow at this time of the year to allow a strong impact of foliar uptake on ring N composition. The slow decrease of δ^{15}N after the end of labelling, during at least 4 years, shows that a large part of N in each ring was derived from previous years.

Dendrochemical studies are based on the fact that the concentration of elements in tree rings reflects the conditions of environment at the time of ring formation. Concerning nitrogen, there is a limitation to this approach due to the inter-annual mobility of nitrogen between rings. The constant value of δ^{15}N observed in trees A and B before labelling is much higher than the values observed for the same years in the control tree (Fig. 4). This demonstrates that nitrogen is highly mobile across rings, consistently with other works [6]. This mobility of nitrogen can be attributed to the living cells of the abundant ligneous rays which are the vector of translocation towards the inner part of the tree and explain the homogenous redistribution of N especially for young trees. Nõmmik [15] also observed a large inward shift of labelled ^{15}N in young *Pinus sylvestris* and *Picea abies* trees. Beech is known for its special anatomical pattern of living cells. There are numerous living cell patches even far inside the trunk suggesting a weak transformation of sapwood into heartwood. This is in agreement with previous observations of a lack of defined boundary between heartwood and sapwood in *Fagus sylvatica* [11]. The higher concentrations of nitrogen in the outer rings are mostly explained by the presence of high proportion of livings cells [17].

Several studies on nitrogen isotopes have been conducted in soil-plant systems [10, 23, 25]. However there are few published data on natural δ^{15}N values in tree rings. The control tree showed negative δ^{15}N (–4 to –7‰) (Fig. 4), decreasing from the pith to the ring formed in 1992, and then a linear increase in 1992–2001.

The extraction slightly decreased the δ^{15}N for almost all annual rings by –0.4‰ on the average, showing that, in natural conditions, labile forms of nitrogen contain heavier N isotope. Thus, removing extractives from wood results in a decreasing δ^{15}N.

In the period before labelling, the δ^{15}N for TW was lower than NTW by 14 and by 9% for tree A and B, respectively. Rings formed during this period presented high δ^{15}N compared

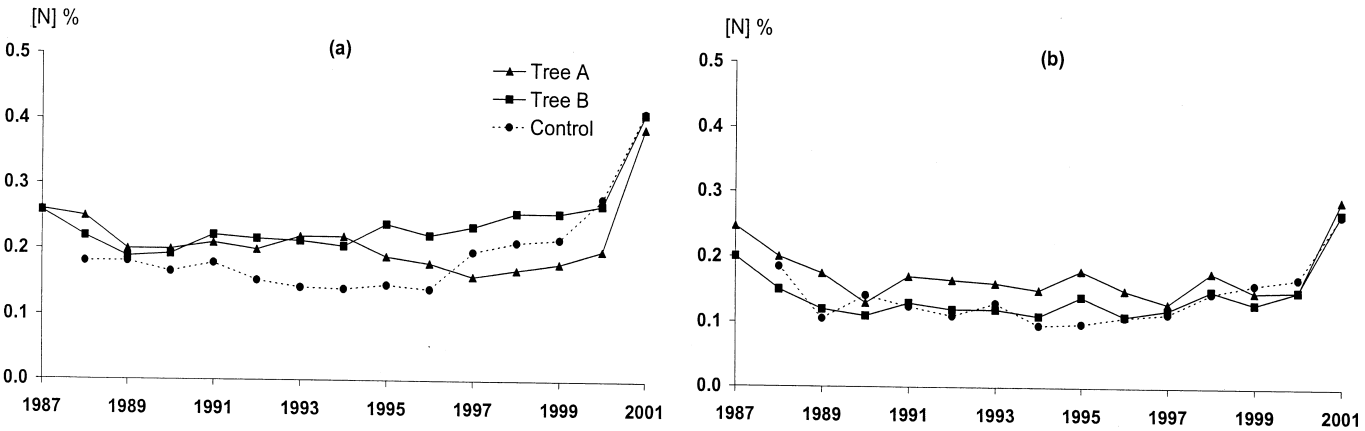

Figure 2. Annual variations of nitrogen concentration in tree rings of trees A, B and control of *Fagus sylvatica* L. before (a) and after (b) extraction by organic solvent, following ^{15}N-enrichment during the period 1993–1995 for trees A and B.

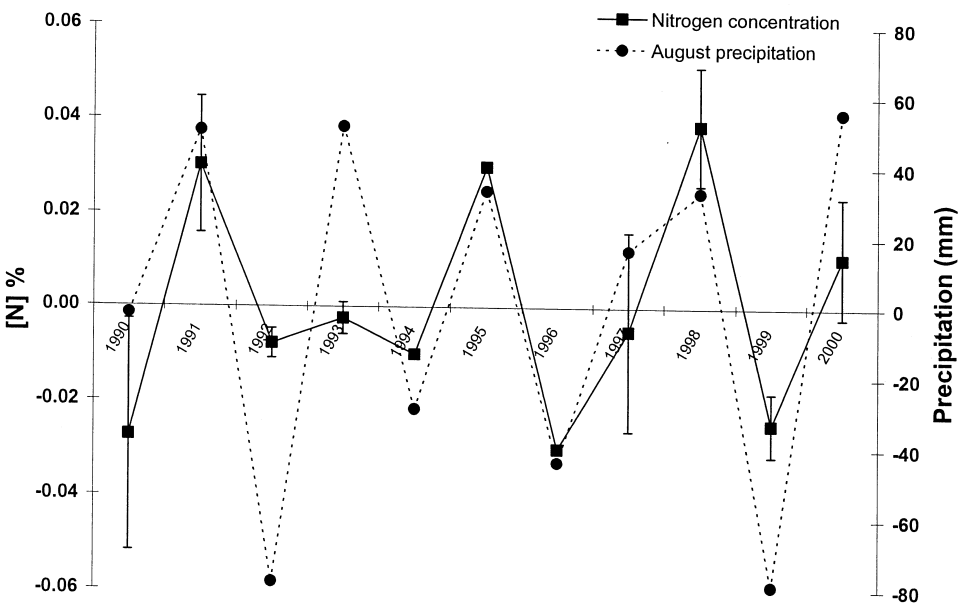

Figure 3. Inter-annual variations of August precipitation and average N concentration in trees A and B. Precipitation and [N] were detrended before analysis by taking first differences. The y-axis for precipitation (on the right) is reversed. Vertical bars indicate SE (n = 2).

with control, demonstrating that N is highly mobile across rings, because the rings formed before labelling period in labelled trees (A and B) should have similar values as in the control tree. We believe that the method applied could not remove all the soluble forms of exchangeable N between rings, and/or an exchange of labile N pool with structural one took place. Lignin extraction did not improve the efficiency of labelled ^{15}N removal: the $\delta^{15}N$ value in lignin extracts was the same as after application of the previous Sheppard and Thompson's method, although 2 to 5% only of initial N was retained in these lignin extracts.

During the labelling period, we found higher values for TW than NTW. The extraction caused an amplification of $\delta^{15}N$. It increased by 35% in tree A and by 26% in tree B [values calculated for the ring with maximum enrichment (1994)]. This difference in treatment effects before and during labelling supports the hypothesis of a large incorporation of N in structural components during the year when N was taken up by the trees. In contrast, in the rings formed prior to the labelling period, $\delta^{15}N$ decreased after extraction. This probably reflects the elimination of labile ^{15}N compounds which accumulated in these rings (N reserves compounds), thus decreasing the ^{15}N signal.

We do not know the $\delta^{15}N$ value of the source of N found in rings formed before labelling (1987–1992). Taking as a reference the maximum observed $\delta^{15}N$ value (ring 1994), we can

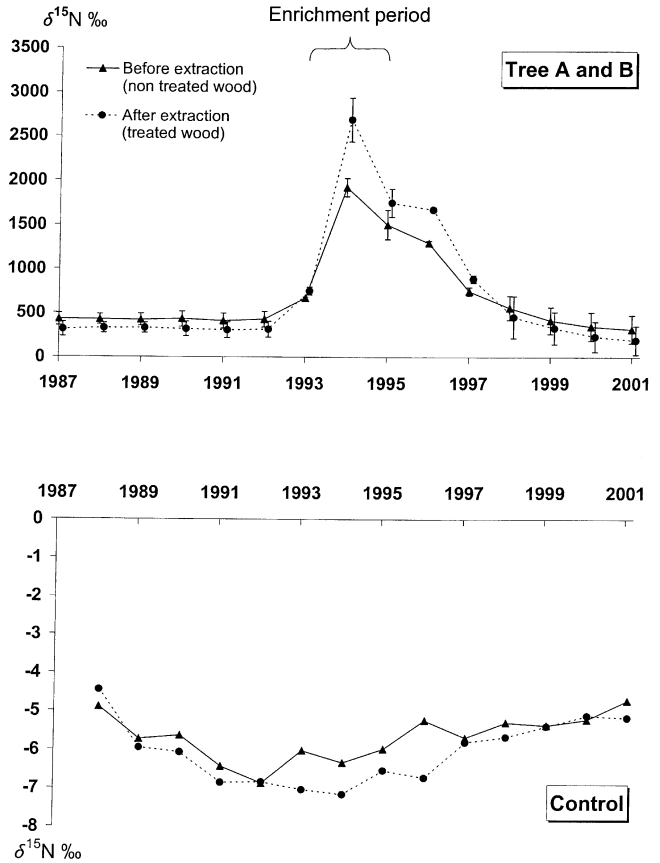

Figure 4. Annual variations of $\delta^{15}N$ in tree rings of trees A, B (average) and control of *Fagus sylvatica* L. before and after extraction by organic solvent and following ^{15}N-enrichment during the period 1993–1995 for trees A and B. Vertical bars indicate SE ($n = 2$).

estimate the percentage of N in 1987–1992 rings coming from this N source. We calculated that 19 and 26% for trees A and B, respectively, came from the arbitrary source before wood pre-treatment. After the extraction, ^{15}N-enriched was still present in the rings formed before labelling and represented 12 and 18% for trees A and B, respectively.

4. CONCLUSIONS

In spite of the relative dilution of the $\delta^{15}N$ signal around the rings corresponding to the years of enrichment, indicating the mobility of labile nitrogen forms, we found a high and pronounced peak corresponding to the enrichment period. This temporal resolution could be interesting if the $\delta^{15}N$ found after treatment in rings prior to the labelling period was reduced to a similar value as in the control tree. The extraction left enough N for subsequent isotopic analyses and improved the annual resolution of concentration patterns. We need a higher resolution if we are to gain a significant environmental signal. Further experiments are needed to test the efficiency of other methods in isolating structural compounds, such as some cell wall protein, which should be stable and not subjected to fractionation, similar to cellulose in the $\delta^{13}C$ dendroecological approach.

Acknowledgements: We thank Dr. Etienne Dambrine who initiated the labelling experiment. We also thank Dr. Brigitte Chabbert for the lignin extraction. This work was supported by the European Community's Human Potential Programme under contract HPRN-CT-1999-00059, and by the French Ministry of Environment GICC Programme.

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