Characterization of Lignocellulose Decomposition in Stream Wood Samples Using ^{14}C and ^{15}N Techniques

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Nicholas G. Aumen

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Abstract approved: Peter J. Bottomley

A study was conducted on the distribution and lignocellulolytic activity of the microbial community on a Douglas fir log (Pseudotsuga menziesii) in a Pacific Northwest stream. Scanning electron microscopy, plate counts, and [^{14}C]lignocellulose degradation experiments show that microbial colonization and lignocellulolytic is mainly a surface phenomenon. Incubations of [^{14}C]-lignocelluloses and wood samples in a nitrogenless mineral salts medium showed no increase in $^{14}CO_2$ evolution as compared to incubations in distilled water. $(NH_4)_2SO_4$ or organic nitrogen stimulated lignocellulose decay, with the greatest effect observed from $(NH_4)_2SO_4$ addition. Subsequent incubations revealed that KNO_3 stimulated lignin decomposition more than $(NH_4)_2SO_4$ or NH_4NO_3, whereas all three were equally favorable to cellulose decomposition. Glucose repressed both lignin and cellulose decay. Decomposition of [^{14}C]lignocelluloses was greatest when incubated in stream water collected from four different sources than in distilled water. Phosphate and nitrate additions alone and in

combination to a stream water medium yielded increases in [^{14}C]-cellulose decay, whereas [^{14}C] lignin decomposition responded only to both in combination. Decomposition of [^{14}C] lignocelluloses was greatest when KNO_3 was added in concentrations $\geq$10 mg N·l^{-1} to a mineral salts medium. Decomposition rates increased three- to seven-fold over a range of temperatures from 5 to 22^{o}C. Accumulation of filtrate NH_4^+ N (2-4 mg N·l^{-1}) was always observed in incubations with KNO_3 addition, and was independent of NO_3^- concentrations $\geq$10 mg N·l^{-1}. An incubation was conducted of stream wood samples with [^{14}C] lignocelluloses in a mineral salts medium with 10 mg N·l^{-1} K$^{15}NO_3$ (50% ^{15}N). Evolution of $^{14}CO_2$, distribution of ^{15}N, filtrate nitrogen, denitrification, nitrogen fixation, and respiration were measured at 6, 12, and 18 days. The organic nitrogen in the lignocellulose/wood sample mixture increased two-fold over the first 6 days. [^{14}C] lignocellulose decomposition rates were greatest during the first 6 days, then diminished over the remaining 12 days, as did the rate of overall respiration. Filtrate NH_4^+ N increased from background levels to a final value of 57 ug N per treatment. Filtrate NO_3^- disappeared by day 6, and organic nitrogen showed a slight decline between 12 and 18 days. The majority of the ^{15}N that was accounted for (53%) was in the particulate organic fraction. By 18 days, the ^{15}N enrichments of the filtrate NH_4^+, the organic fraction, and the inorganic nitrogen associated with the organic fraction had all increased to 20% ^{15}N. Nitrogen fixation and denitrification measurements indicated insignificant gain/or loss of nitrogen from the incubations by

these processes. The role of wood in streams is discussed from a geomorphic and biological standpoint, along with the effect of nitrogen on decomposition of lignocelluloses.

APPROVED:

Associate Professor of Microbiology and Soil Science in charge of major

Chairman of the Department of Microbiology

Dean of Graduate School

Date thesis is presented July 3, 1984

Typed by Shirley Clark for Nicholas G. Aumen

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CHARACTERIZATION OF LIGNOCELLULOSE DECOMPOSITION IN STREAM WOOD SAMPLES USING ^{14}C AND ^{15}N TECHNIQUES

CHAPTER I

INTRODUCTION

Coniferous forests of the Pacific Northwest are characterized by extremely large biomass accumulations of evergreen coniferous trees. The combination of mild, wet winters and warm, relatively dry summers lead to an old-growth forest biomass that is unequalled by any other coniferous forest ecosystem in the world (Franklin and Waring, 1980). Natural mortality caused by wind throw, disease, or catastrophic events such as fire lead to huge accumulations of downed wood on the forest floor and stream channels (Froehlich et al., 1972; Grier and Logan, 1977; Franklin and Waring 1980; Triska and Cromack, 1980). In fact, the greatest concentrations of large woody debris (LWD) are found in or near the sharply incised stream channels with steep adjacent hillslopes.

Traditionally, LWD has been considered an impediment to reforestation and stream quality, and management practices still call for complete removal of LWD from stream beds following logging (Triska and Cromack, 1980). Recent research, however, has demonstrated the important role that LWD plays in stream ecosystems of the Pacific Northwest, from both a geomorphic and biological standpoint (Triska and Cromack, 1980). LWD exerts its greatest geomorphic influence in high gradient headwater streams, where

accumulations of downed wood can dominate the channel structure (Keller and Swanson, 1979; Triska and Cromack, 1980; Swanson et al., 1982). Bole wood, alone and in debris jams, can control the direction of the stream flow, creating large pools that lead to sediment and organic matter accumulations (Bilby and Likens, 1980). The resulting stair-step profile of the channel dissipates stream hydraulic energy, and prevents scouring of the stream bed.

The presence of wood debris accumulations increase retention of organic matter that normally would be transported downstream and allows greater time for decomposition and recycling of this material (Bilby and Likens, 1980). LWD also creates habitat for stream organisms, particularly in the pools formed upstream of the jams. Gravel accumulations in these regions can create spawning areas for fish, and the debris itself provides cover and protection from predators (Hall and Baker, 1975; Baker, 1979). The debris accumulations also increase habitat diversity for the wood-associated insect populations found in streams (Anderson et al., 1978; Dudley and Anderson, 1982).

The biological role of LWD in streams is less well known, but has been hypothesized to be a slow release source of nutrients through the process of decomposition (Triska and Cromack, 1980). Wood decomposition occurs through a combination of factors, including microbial activity, invertebrate tunneling and feeding activity, and physical processes such as abrasion by stream flow (Anderson et al., 1978; Triska and Cromack, 1980). Of these

processes, microbial activity has been the least studied to date, and is the focus of this research project.

Wood consists primarily of lignin and cellulose, which are structural components comparatively resistant to microbial degradation. Cellulose is an unbranched polymer of glucose residues joined by β-1,4 linkages, while lignin is a complex, amorphous polymer consisting of oxyphenylpropane units (R. Crawford, 1981). Lignin and cellulose exist in close association with one another, and can compromise more than 50% of the organic matter in decomposing wood (Adler, 1977). Traditionally, methods for assessing the decomposition dynamics of organic matter usually involve the measurement of substrate weight loss (Findlay, 1934; Schmitz and Kaufert, 1936; Hungate, 1940; Savory, 1954; Merrill and Cowling, 1966a; 1966b; Abbot and Crossley, 1982; Melillo et al., 1983). Although these studies have provided valuable information, the experiments require many months of incubation before significant results can be obtained. Even then, the decomposition dynamics of individual chemical components in woody tissue are difficult to assess. It is for similar reasons that traditional methods of measuring microbial activity are less useful. Although wood is colonized and decomposed by non-filamentous bacteria, fungi, and actinomycetes, their activities are relatively low when compared to activities on more labile substrates such as proteins and non-structural carbohydrates (R. Crawford, 1981).

A major breakthrough in methodology occurred a decade ago with the advent of radiotracer techniques to label lignin and cellulose compounds. Kirk and others developed techniques for synthesizing lignin model compounds in the laboratory that could be used as substrates in microbial decomposition experiments (Haider and Trojanowski, 1975; Kirk et al., 1975). A major advantage of this procedure is that the carbon atoms of the model compounds can be radiolabelled with ^{14}C in specific positions, such as in the ring structures, aliphatic side chains, or ring substitution groups. This proved extremely valuable in investigating the ability of individual groups of microorganisms to attack specific components of the lignin structure. A drawback of this technique, however, is that rates and patterns of microbial degradation of these model compounds may not be the same as for the degradation of natural lignin, and their manufacture requires considerable experience and knowledge of synthetic organic chemistry (R. Crawford, 1981).

The need for a radiolabelled substrate in a more natural form was addressed by the Crawford brothers, who developed a technique for preferentially labelling the lignin and cellulose components of lignocellulose with ^{14}C (Crawford and Crawford, 1976; Crawford and Crawford, 1978; Crawford et al., 1980). The method requires incubation of a cut branch of the desired plant with a ^{14}C-labelled lignin precursor, such as [^{14}C]phenylalanine, and [^{14}C]glucose as a cellulose precursor. The ^{14}C-labelled precursors are taken up by the stem, and are incorporated into newly synthesized lignin,

cellulose, and other plant components. An extensive extraction procedure follows whereby the undesired ^{14}C-labelled extractable plant components are largely removed, leaving a non-extractable lignocellulose enriched product preferentially labelled with ^{14}C in the lignin or cellulose fraction (R. Crawford, 1981). The major advantage of this procedure is that lignin and cellulose are in close association in natural substrates (Adler, 1977), and this procedure yields a product that closely resembles that composition. A disadvantage is that undesired ^{14}C-labelled components, such as proteins, not removed by the extraction procedure lead to an overestimate of lignocellulose breakdown. This is of the most concern for [^{14}C]lignin-lignocelluloses labelled with [^{14}C]phenylalanine, where protein synthesis could lead to incorporation of some of the precursor. Plant species, such as grasses, which have a higher protein content to start with, are more of a problem than coniferous species, for example, and may require the use of a more specific lignin precursor, such as [^{14}C]cinnamic acid (Crawford, 1978; Crawford et al., 1980; Benner et al., 1984a; 1984b).

Once a suitable labelled material has been prepared, it must be thoroughly characterized for the distribution of ^{14}C among its various components. The necessary procedures include a Klason digestion with 72% (wt/wt) H_2SO_4 (Effland, 1977) to determine the distribution of ^{14}C between the lignin and cellulose fractions, followed by paper chromatographic separation of wood sugars in

Klason acid hydrolysates for determination of their radioactivity (Crawford, 1981; Aumen et al., 1983). The extent of protein contamination in the [^{14}C]lignocelluloses is estimated by Kjeldahl nitrogen analysis and determination of the specific radioactivity of the substrate before and after protease digestion.

Once the [^{14}C]lignocelluloses have been prepared, they can be used in decomposition experiments which involve incubation with homogenized stream wood samples in a suitable medium, and measurement of the $^{14}CO_2$ evolution resulting from lignocellulose decomposition (R. Crawford, 1981). The release of water soluble ^{14}C-labelled lignin breakdown products into the culture medium observed by other researchers in pure culture studies was shown not to occur here (Crawford and Sutherland, 1979; Reid et al., 1982; Reid, 1983).

Two major directions of research have developed since the inception of these techniques in the middle 1970's. One has been to utilize [^{14}C]lignin model compounds and natural [^{14}C]lignocelluloses in pure culture studies to elucidate the biochemistry and microbial physiology of lignin decomposition (R. Crawford, 1981). [^{14}C]lignin model compounds have proven to be particularly useful in this regard, as the decomposition of specific fractions of the lignin structure can be studied. Natural [^{14}C]lignocelluloses have also been important in the continuing search for microorganisms that are capable of delignifying waste lignocellulosics with the eventual conversion of the lignins to

products of economic value (D. Crawford, 1981; R. Crawford, 1981). In the course of this applied research, much valuable information has been gained on lignin decomposition (D. Crawford, 1981; R Crawford, 1981).

The second major research direction using the radiotracer techniques has centered on ecological studies conducted on samples from various ecosystems. [^{14}C]lignocelluloses have been utilized to study lignin and cellulose decomposition in samples from soils, aquatic sediments, and surfaces of organic detritus (Hackett et al., 1977; Crawford et al., 1977a, 1977b; Martin and Haider, 1979; Federle and Vestal, 1980; Maccubbin and Hodson, 1980; Benner et al., 1984a; 1984b). The present study utilized natural [^{14}C]lignocelluloses prepared from Douglas fir (Pseudotsuga menziesii) to examine the microbial community capable of degrading lignocelluloses on a downed log of Douglas fir in a Pacific Northwest stream. Previous investigations on the role of LWD in streams of the Northwest have alluded to the importance of the microbial community, but few actual studies have been undertaken. Statements are frequently made in the literature that stream LWD decomposition is slow, but important, to the ecosystems, given the large accumulations observed (Triska and Cromack, 1980). Preliminary studies on decomposition of woody substrates incubated in the stream and in the surrounding terrestrial environment demonstrated that after two years of incubation, only the stream incubated wood had begun to show any significant weight loss

(Triska and Cromack, 1980). Another interesting observation was that the woody substrates in the streams had increased in nitrogen content over time. Nitrogen fixation was measured, but was determined not to be responsible for the majority of the nitrogen increase (Buckley and Triska, 1979; Triska and Cromack, 1980). Any mechanism leading to substrate nitrogen increase would be of importance given the large concentrations of LWD in streams and the paucity of nitrogen that exists (Triska and Cromack, 1980; Triska et al., 1984).

In order to further the knowledge of the microbiology of lignocellulose decay on wood substrates in Mack Creek, a third-order stream in the Cascade Mountains of Oregon, a series of studies were initiated utilizing [^{14}C]lignocellulose methods in conjunction with standard microbiological techniques. Mack Creek was chosen as the study site because of the abundance of data that had already been collected under the auspices of the Coniferous Forest Biome Project of the U.S. International Biological Program (IBP) and research supported by the National Science Foundation's Long Term Ecological Reserve program. Mack Creek was also the site of preliminary investigations on wood decomposition in streams, and had ongoing research that would produce much relevant data.

Initially, the approach was to investigate the microbiology on a chronosequence of downed logs in Mack Creek. Techniques by which residence times of downed logs could be estimated had already been developed, and included dating of Western Hemlock (Tsuga

heterophylla) seedlings established on Douglas fir logs, dating of scars on trees adjacent to the felled log, and ^{14}C dating of the outer growth rings. Initial results of the study indicated that much variability existed in samples from logs of the same approximate age, and that the most recently felled logs supported the highest rates of respiration and [^{14}C]lignocellulose decomposition.

A single Douglas fir log from the initial sample series was selected for intensive study based on its relatively high microbial activity, amount of surface area wetted by stream water that was available for sampling, and ease of access during the winter. The log was wind-felled into Mack Creek during a storm event in the fall of 1977. Sampling was limited to cores taken with an increment borer, and surface samples obtained with a carpenter's plane from bark-free, stream wetted areas.

Chapter II of this thesis describes in detail the development and use of the [^{14}C]lignocellulose technique in assessing the distribution and lignocellulolytic activity of the microflora sampled from the downed log. The effect of various organic and inorganic nitrogen supplements to a mineral salts medium was investigated. Additional techniques were also utilized and included standard plate counts, respiration measurements by gas chromatography, and scanning electron microscopy. Chapter III describes the use of the [^{14}C]lignocellulose technique to study the effects of nitrogen and phosphorus on lignocellulose decay, along

with the effects of varying nutrient concentration and temperature. Chapter IV describes a study which utilized ^{15}N-labelled nitrate to follow the fate of nitrate after addition to incubations with [^{14}C]lignocelluloses and stream wood samples.

CHAPTER II

Microbial Decomposition of Wood in Streams: Distribution of the Microflora and Factors Affecting [^{14}C]lignocellulose Mineralization

Running Title: Microbial Decomposition of Wood in Streams

Nicholas G. Aumen[1], Peter J. Bottomley[2*], G. Milton Ward[3], and Stan V. Gregory[4]

Department of Microbiology[1], Departments of Microbiology and Soil Science[2], Department of Fisheries and Wildlife[4], Oregon State University, Corvallis, Oregon 97331-3804, and Department of Biology[3], University of Alabama, University, Alabama 35486.

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*Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804
Telephone No. (503) 754-4441

ABSTRACT

The distribution and lignocellulolytic activity of the microbial community was determined on a large log of Douglas fir (Pseudotsuga menziesii) in a Pacific Northwest stream. Scanning electron microscopy, plate counts, and degradation of ^{14}C-labeled lignocelluloses prepared from Douglas fir and incubated with samples of wood taken from the surface and within the log revealed that most of the microbial colonization and lignocellulose degrading activity occurred on the surface. Labeled lignocellulose and surface wood samples were incubated in vitro with nutrient supplements to determine potential limiting factors of [^{14}C]-lignocellulose degradation. Incubations carried out in a nitrogen-less mineral salts and trace elements solution were no more favorable to degradation than those carried out in distilled water alone. Incubations supplemented with either $(NH_4)_2SO_4$ or organic nitrogen sources showed large increases in the rates of mineralization over mineral salts and trace elements alone, with the greatest effect being observed from an addition of $(NH_4)_2SO_4$. Subsequent incubations with $(NH_4)_2SO_4$, KNO_3 and NH_4NO_3 revealed that KNO_3 was the most favorable for lignin degradation, whereas all three supplements were equally favorable to cellulose degradation. Supplementation with glucose repressed both lignin and cellulose mineralization. The results reported in this study indicate that nitrogen limitation of wood decomposition may exist in streams of the Pacific Northwest. The radiotracer technique was

shown to be a sensitive and useful tool for assessing relative patterns of lignocellulose decay and microbial activity in wood, along with the importance of thoroughly characterizing the experimental system prior to its general acceptance.

INTRODUCTION

The large biomass of old-growth coniferous forests of the Pacific Northwest can result in substantial accumulations of logs on the forest floor and in the streams which drain the surrounding watershed (Franklin and Waring, 1980). Standing crops of wood in Cascade Mountain stream channels can exceed 40 kg m^{-2} (Keller and Swanson, 1979). The determination of the role large woody debris plays in ecosystems is essential, especially in view of current management strategies for logging operations which may significantly decrease the amounts of debris available for habitat formation and biological processing (Triska and Cromack, 1980; J. D. Hall and C. O. Baker, A Workshop on Logging Debris in Streams, Oregon State University, Corvallis, OR, 1975, and F. J. Swanson and G. W. Lienkaemper, USDA Forest Service Gen. Tech. Report PNW-69, 1978).

A major component of this woody debris is lignocellulose, which represents more than one-half of the total carbon present in wood. Recently developed radiotracer methodologies that specifically label the lignocellulose fraction of plant tissue have led to ever increasing amounts of information on the microorganisms, biochemical processes, and environmental factors which affect the degradation of lignocellulose (R. Crawford, 1981).

In recent years the literature shows that much emphasis has been placed upon studies involving specific microorganisms in pure culture (Haider and Trojanowsi, 1975; Kirk et al., 1975; Crawford

and Crawford, 1976; Crawford, 1978; Keyser et al, 1978; Crawford et al., 1980; Barder and Crawford, 1981; Reid, 1983). While this is of undeniable importance, such information cannot be extrapolated easily to natural systems. Research that uses radiotracer methods with environmental samples has been limited to soil and sediment systems (Crawford and Crawford, 1976; Crawford et al., 1977a; Crawford et al., 1977b; Hackett et al., 1977; Federle and Vestal, 1980; Maccubbin and Hodson, 1980). Large woody debris differs from soils and sediments in that the substrate has a very small surface area to volume ratio, is structurally recalcitrant, and sometimes occurs in flowing water environments which are frequently low in nutrients.

This paper describes the development and use of a radiotracer assay to determine the characteristics of lignocellulose degradation in wood. The assay was used to determine patterns of microbial decomposition in large logs of Douglas fir (Pseudotsuga menziesii) which have been lying for known periods of time in a stream channel of an old-growth forest.

MATERIALS AND METHODS

Preparation and characterization of ^{14}C-labeled lignocelluloses

Lignocelluloses of Douglas fir labeled specifically either in the lignin or in the cellulose fraction were prepared according to the method of Crawford (R. Crawford, 1981). Freshly-cut ends of Douglas fir branches, each approximately 1 m in length, were immersed in aqueous solutions of either 50 µCi of L-[U-^{14}C]phenylalanine (10 mCi mmol^{-1}) or 50 µCi of D-[U-^{14}C]glucose (3 mCi mmol^{-1}) to initiate the labeling of lignin and cellulose respectively. Radiochemicals were obtained from Amersham/Searle Corp., Arlington Heights, IL. Just before uptake of the radioactive solution was completed, additional water was added as needed to keep the cut ends immersed and the stems were allowed to metabolize the radiolabels under constant illumination until wilting began to occur (approximately 1 week). The cambial tissue was stripped from the main stem and dried at 50°C for 2 weeks. The labeled tissue was ground to pass a (#40) mesh screen, and subjected to a sequence of hot water, ethanol/benzene, and ethanol extractions to remove undesired labeled plant constituents, as described in detail by R. Crawford (1981). The extractive-free tissue was dried at 50°C and stored in a desiccator at room temperature.

The specific activities of the extractive-free [^{14}C]lignin and [^{14}C]cellulose lignocelluloses were determined by combustion of 10 mg samples in a Model 306 Packard Tri-carb oxidizer and the

resulting $^{14}CO_2$ trapped and counted by standard liquid scintillation techniques.

Distribution of ^{14}C within the labeled material was determined by the modified Klason procedure of Effland (1977). Triplicate 200 mg portions of each labeled substrate were digested for 1 h with 72% (wt/wt) H_2SO_4. The digest was diluted (1:28), heated at 120°C and at a pressure of 103 kPa for 1 h, and then filtered through tared, fritted glass crucibles. The residue was dried overnight at 105°C and weighed to determine the initial lignin content of the woody tissue. The specific radioactivity was determined using the Packard oxidizer as described previously. The Klason filtrate was adjusted to pH 4.5 with $CaCO_3$ and stored at 4°C for subsequent carbohydrate determinations. A portion of the filtrate was utilized for determination of its radioactivity by pipetting 0.1 ml aliquots onto 5.5 cm diameter Whatman #1 filter papers, which were combusted and the $^{14}CO_2$ quantified as described above.

The distribution of radiolabel in wood sugars was determined by TAPPI method T250 pm-75 (The Technical Association of the Pulp and Paper Industry, Atlanta, GA), with the following modifications. Portions (100 ml) of the Klason filtrates were lyophilized to concentrate the filtrate and then rehydrated with 5 ml of distilled water. Incorporation of ^{14}C into wood sugars was determined by descending paper chromatography using a butanol:pyridine:water (10:3:3 v/v) solvent system. Solutions of glucose, mannose, and xylose were used as standard markers. The sections of

the chromatograms corresponding to glucose, mannose, and xylose were removed and analyzed for ^{14}C activity by oxidation.

The total nitrogen remaining in the radiolabeled lignocelluloses was estimated by micro-Kjeldahl analyses. Radioactivity associated with the protein fraction was estimated by incubating 10 mg samples of the labeled substrates with 6 units of Type XIV bacterial protease (Sigma Chemical Co., St. Louis, MO) in 0.05 M phosphate buffer, pH 7.5, at 37°C for 2 h. Samples were analyzed in quintuplicate and changes in specific radioactivity of the residue determined by sample oxidation before and after protease incubation.

Study site and sample collection

Wood samples used as inocula for [^{14}C]lignocellulose degradation experiments were obtained from Mack Creek, a third-order stream in an old-growth section of the H. J. Andrews Experimental Ecological Reserve, located at an elevation of 830 m in the Cascade Mountain Range, Oregon. Surface scrapings were obtained with a carpentry plane and cores were taken with a 12 mm diameter increment borer (Forestry Suppliers, Inc., Jackson, MS) from bark-free, stream-wetted portions of an old-growth Douglas fir log that fell into the stream in 1977. Samples were placed in sterile Whirlpak bags, stored on ice, and transported to the laboratory and processed within 48 h. Wood cores were split in the laboratory with a sterile knife and subsampled to avoid contamination that may have been carried down from the surface by use of the increment borer.

Sample preparation

Wood samples were homogenized in sterile distilled water for 8 minutes at a setting of 30 on a Virtis Model 45 homogenizer. The duration of homogenization had been determined previously to yield the highest numbers of microorganisms from wood samples by use of standard microbiological plate counts on dilute tryptic soy agar (data not shown). Experimental treatments were prepared by placing 1 ml portions of homogenate into 60 ml glass serum bottles containing 20 ml of an incubation medium and fitted with glass capillary (2 mm dia.) bubbler tubes and sleeve-type rubber stoppers. The treatments were either bubbled continuously or once per day for 15 minutes with filtered, humidified, CO_2-free air. Incubations were carried out at room temperature (21 ± 2°C). Outflow gas from the incubation bottles was passed through 8% (w/v) NaOH to absorb $^{14}CO_2$, and radioactivity was determined by acidification of the alkali with concentrated H_2SO_4 and the $^{14}CO_2$ was trapped on filter paper soaked with β-phenylethylamine (free base, Sigma Chemical Co., St. Louis, MO). Filter papers were then placed in 15 ml of liquid scintillation cocktail and counted on a Beckman Model LS8000 liquid scintillation counter. Counting efficiency was determined using a quench series and the external standard technique with data corrected for efficiency and background. The liquid scintillation cocktail consisted of Spectrafluor (Amersham/Searle Corp., Arlington Heights, Ill.), methanol and toluene (16:100:125 v/v). The removal of $^{14}CO_2$ from

the alkali traps was shown to be complete by using known quantities of $Na_2{}^{14}CO_3$.

Spatial distribution and [^{14}C]lignocellulose decomposition experiments

The spatial distribution of the microbial community in the study log was determined by obtaining surface scrapings and core samples from a depth of 25 cm in July and September, 1981. Plate counts were performed on samples of homogenate by spreading 0.1 ml of serial dilutions (3×10^{-2} to 3×10^{-4} in distilled water) of wood homogenate on the surface of one tenth strength tryptic soy agar (TSA) plates. Incubation was carried out at 30°C for 5-7 days, both under aerobic and anaerobic conditions. Anaerobic incubation conditions were achieved by incubating plates inside BBL GasPak anaerobic jars (BBL Microbiology Systems, Cockeysville, MD) placed in a 30°C incubator. Preliminary studies had revealed that diluted TSA yielded a higher recovery of microorganisms from the homogenate than did full strength media.

Wood homogenate from the surface scrapings and core samples were also incubated in the presence of either 10 kdpm of [^{14}C]lignin lignocellulose or 10 kdpm of [^{14}C]cellulose lignocellulose for 27 days in distilled water, with the evolved $^{14}CO_2$ trapped and quantified at approximately seven day intervals. Portions of the sample obtained before incubation were prepared for scanning electron microscopy by fixation with 0.33% (v/v) glutaraldehyde solution, dehydrated in a graded ethanol

series and dried in a Model DCP-1 critical-point dryer (Denton Vacuum, Inc., Cherry Hill, N.J.). Samples were then mounted on stubs, coated with 10 nm of gold-palladium, and observed on a ETEC Autoscan scanning electron microscope.

The effect of varying amounts of added [^{14}C]lignin lignocellulose on $^{14}CO_2$ evolution was determined by incubating 0.026 g (dry weight) portions of wood inoculum (collected in October, 1982) with 10, 20, 30, and 40 kdpm of labeled substrate in distilled water.

The effects of various media supplements on [^{14}C]lignocellulose mineralization were examined by incubating 0.013 and 0.025 g (dry weight) of wood inoculum (collected in February and April, 1983, respectively) in media supplemented with organic and inorganic components. All incubation treatments contained either 20 kdpm of [^{14}C]lignin lignocellulose or 10 kdpm of [^{14}C]cellulose lignocellulose as the labeled substrates. The various incubation media (20 ml) used in the experiments were as follows: distilled water alone, mineral salts solution alone and in combination with one of the following amendments at a final concentration given in grams per liter; $(NH_4)_2SO_4$, 3.0; yeast extract, 9.0; glucose, 1.8; KNO_3, 2.3; or NH_4NO_3, 0.9. The mineral salts solution was composed of the following components in grams per liter of distilled water: $CaCl_2$, 0.6; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; Fe-citrate, 0.1; $K_2HPO_4 \cdot 3H_2O$, 0.5; KH_2PO_4, 0.188; and 10 ml of a trace elements solution containing (per liter) H_3BO_3, 143 mg; $MnSO_4 \cdot 4H_2O$, 102 mg;

$ZnSO_4 \cdot 7H_2O$, 22 mg; $CuSO_4 \cdot 5H_2O$, 8 mg; $CoCl_2 \cdot 4H_2O$, 10 mg; and $Na_2MoO_4 \cdot 2H_2O$, 5 mg. Controls for all experiments consisted of distilled water and the appropriate labeled lignocellulose. Incubations were conducted in quintuplicate for 3 to 4 weeks at room temperature and the $^{14}CO_2$ evolved was determined at weekly intervals. Results were plotted as accumulated kdpm per gram dry weight of wood inoculum, and the standard error of the mean was determined for values obtained at each sampling time.

RESULTS

[^{14}C]lignocellulose characterization

The specific activities and distribution of ^{14}C within the labeled lignocellulose are summarized in Table 11-1 and correspond favorably to values reported in the literature for Douglas fir [^{14}C]lignocelluloses, as do the results of the Klason analysis (Crawford et al., 1980). Chromatographic analysis of the Klason filtrate of the [^{14}C]cellulose lignocellulose demonstrated that all of the radioactivity in the hydrolysate was found in the three sugars analyzed (glucose, mannose, and xylose), with 82% residing in the glucose fraction. In contrast, only 42% of the total radioactivity of the [^{14}C]lignin Klason filtrate could be accounted for in the three sugars, with 22% found in the glucose fraction (presumably from the acid hydrolysis of contaminating labeled cellulose). Similar patterns of label distribution within the Klason filtrate are reported by Maccubbin and Hodson (1980) for pine lignocelluloses and Crawford and Crawford (1978) for lignocelluloses from several different sources. The 58% of the radioactivity in the [^{14}C]lignin Klason filtrate that is unaccounted for by the carbohydrate analysis is presumably in the form of either acid-soluble lignin or amino acids liberated by acid hydrolysis. In fact, it has been noted that a serious drawback of the Klason analysis is that considerable 72% H_2SO_4-soluble lignin is present in many lignocelluloses (Crawford et al., 1980; R. Crawford, 1981). Protease digestion of the labeled lignocelluloses

used in this study resulted in a 5% loss of radioactivity when compared to undigested substrate. A total protein content of 3.5%, assuming protein = N X 6.25, was obtained from micro-Kjeldahl analysis.

Spatial distribution and [^{14}C]lignocellulose decomposition experiments

A series of samples taken to investigate the spatial distribution of the microbial community on decaying wood showed that most of the colonization and activity occurred on the outer surface of the log. Plate counts revealed 1.44 x 10^7 CFUs per gram of wood from the aerobically incubated surface samples, and 2.22 x 10^6 CFUs per gram of wood from the anaerobically incubated surface samples. Wood samples obtained from a depth of 25 cm in the log yielded no CFUs either under aerobic or anaerobic conditions.

Evolution of $^{14}CO_2$ from the ^{14}C incubation experiments demonstrated that the greatest lignocellulose mineralization activity was in the surface sample incubated with [^{14}C]cellulose (Fig. 11-1). Decomposition of the [^{14}C]cellulose in the surface samples was more than four times that of the interior sample, with a similar relationship observed for the [^{14}C]lignin treatments. The SEM study also revealed a colonization pattern consistent with the results reported above (Fig. 11-2, A-C). One can see substantial evidence of microbial colonization on the exposed side of the surface scraping, including individual bacterial cells, actinomycete-like filaments, and possibly fungal hyphae (Fig. 11-

2A). There is very little microbial colonization apparent on the underside of surface scrapings or on the sample obtained from a depth of 25 cm (Fig. 11-2,B-C).

Preliminary experimentation indicated that in the case of the [^{14}C]lignin lignocellulose, caution must be exercised in the selection of the appropriate amounts of radiolabel added to incubation treatments. Generally accepted amounts (10-30 kdpm per treatment) may result in suboptimal mineralization rates, depending on the type and amount of inoculum added. Results of an experiment illustrating the effect of varying amounts of labeled substrate with a fixed quantity of inoculum are presented in Fig. 11-3. Increasing rates of [^{14}C]lignin lignocellulose mineralization were evident with increasing ^{14}C substrate additions. Significant differences were observed between the 10 kdpm addition and the other three treatments.

Supplementation of the incubation treatments with either inorganic or organic nitrogen sources resulted in [^{14}C]lignocelluloses labeled in the cellulose fraction being mineralized to $^{14}CO_2$ at rates three- to nine-fold faster than that of the [^{14}C]lignin fraction (Fig. 11-4). Organic and inorganic nitrogen supplements affected the rates of mineralization of the [^{14}C]lignin more so than [^{14}C]cellulose mineralization rates when compared to samples incubated either in distilled water or in nitrogen-less mineral salts solution. Glucose had a substantial inhibitory effect on [^{14}C]cellulose mineralization and less of an effect on

TABLE II-1. Distribution of ^{14}C within [^{14}C]lignocellulose

Substrate	Sp act (dpm/mg)	% Original radioactivity			
		In Klason residue	In Klason filtrate	Recovered	After protease digestion
[^{14}C-lignin]lignocellulose	1211	76.5	27.6	104.1	94.5
[^{14}C-cellulose]lignocellulose	560	28.8	72.1	100.9	95.0

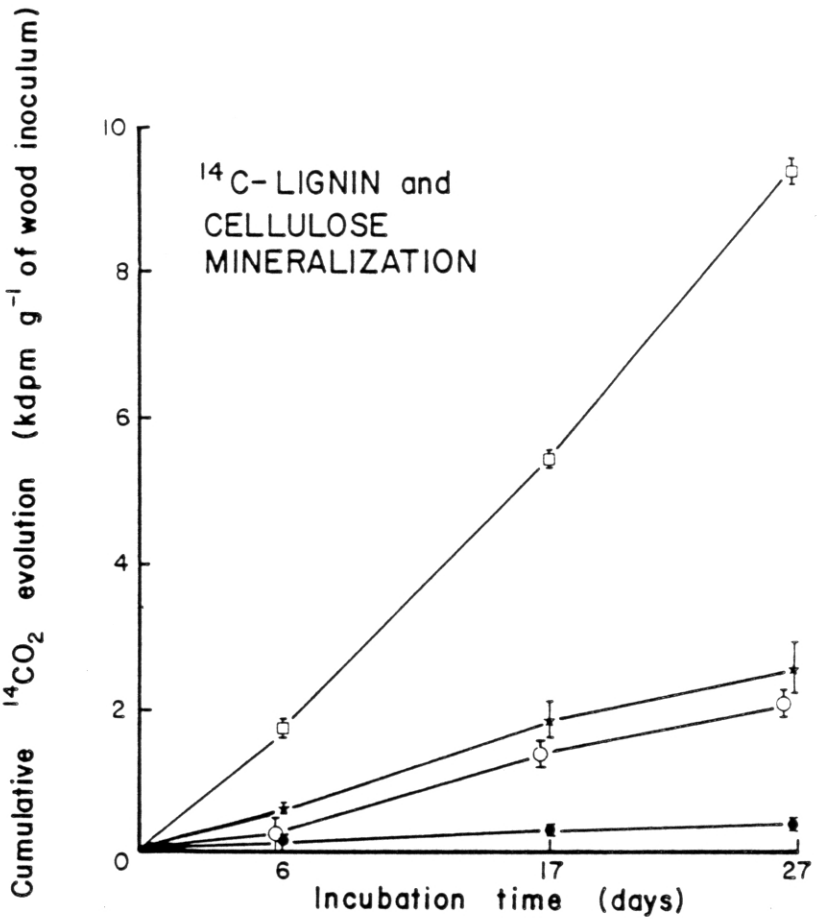

Figure II-I. [^{14}C]lignocellulose degradation in surface scrapings and core samples (25-cm depth) from a wetted log. Symbols: □, surface [^{14}C]cellulose; ○, core [^{14}C]cellulose; ★, surface [^{14}C]lignin; ●, core [^{14}C]lignin. Each point is the mean of two replicates. Bars, Standard errors of the means.

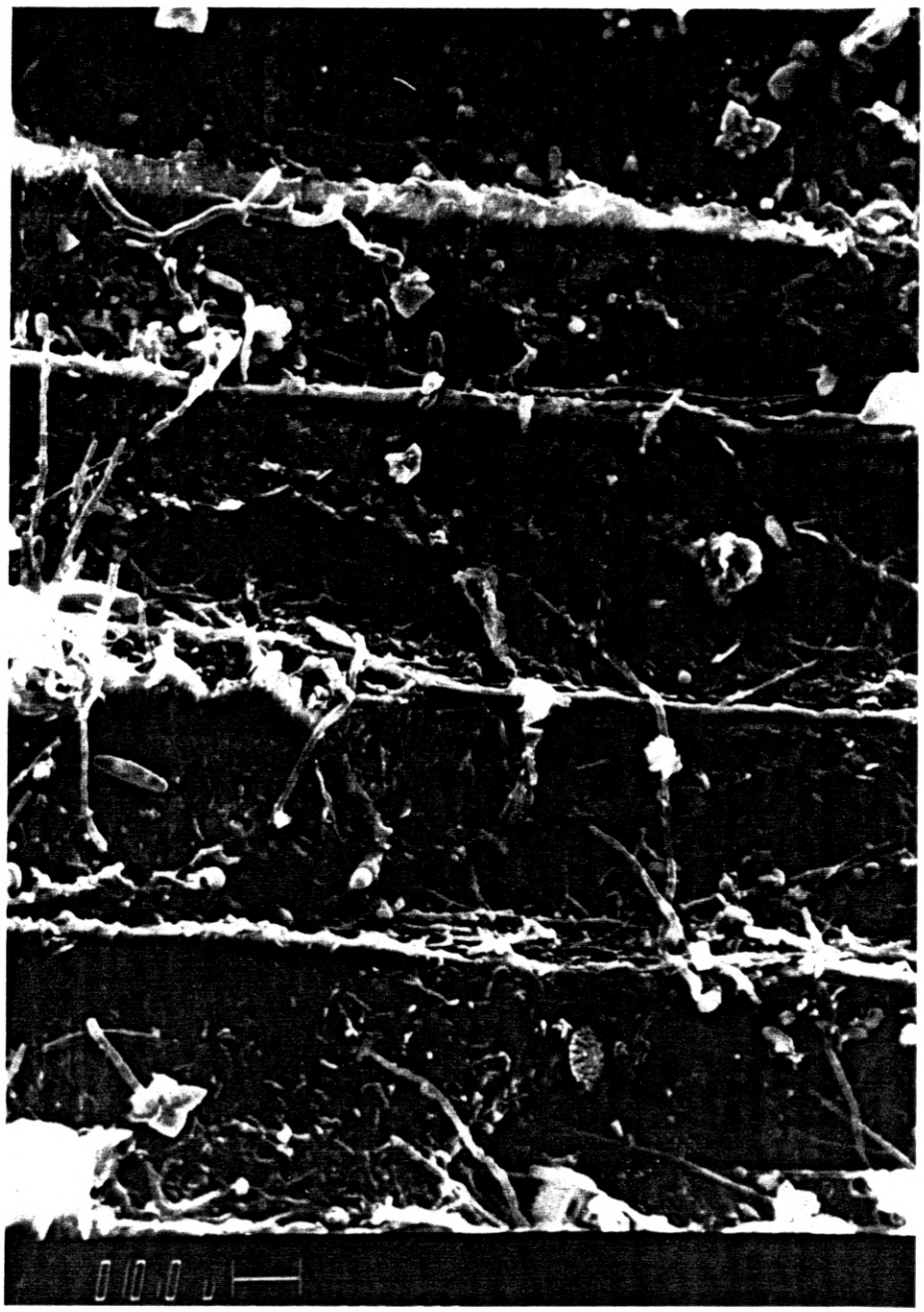

Figure II-2. SEM of wood samples from the study log. (A) Exposed side of a surface scraping. Bar, 10 μ.

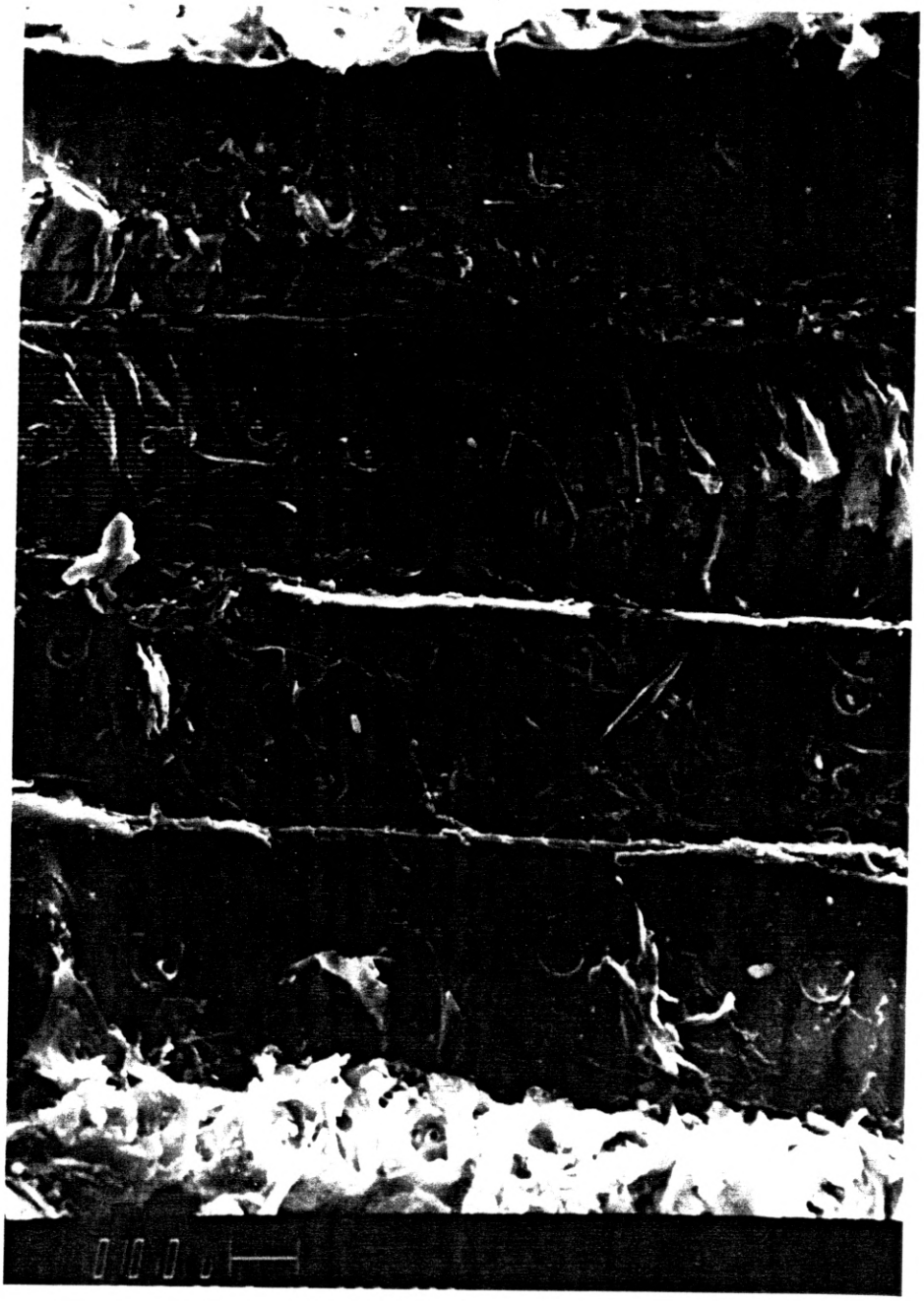

Figure II-2. SEM of wood samples from the study log. (B) Underside of a surface scraping. Bar, 10 μ.

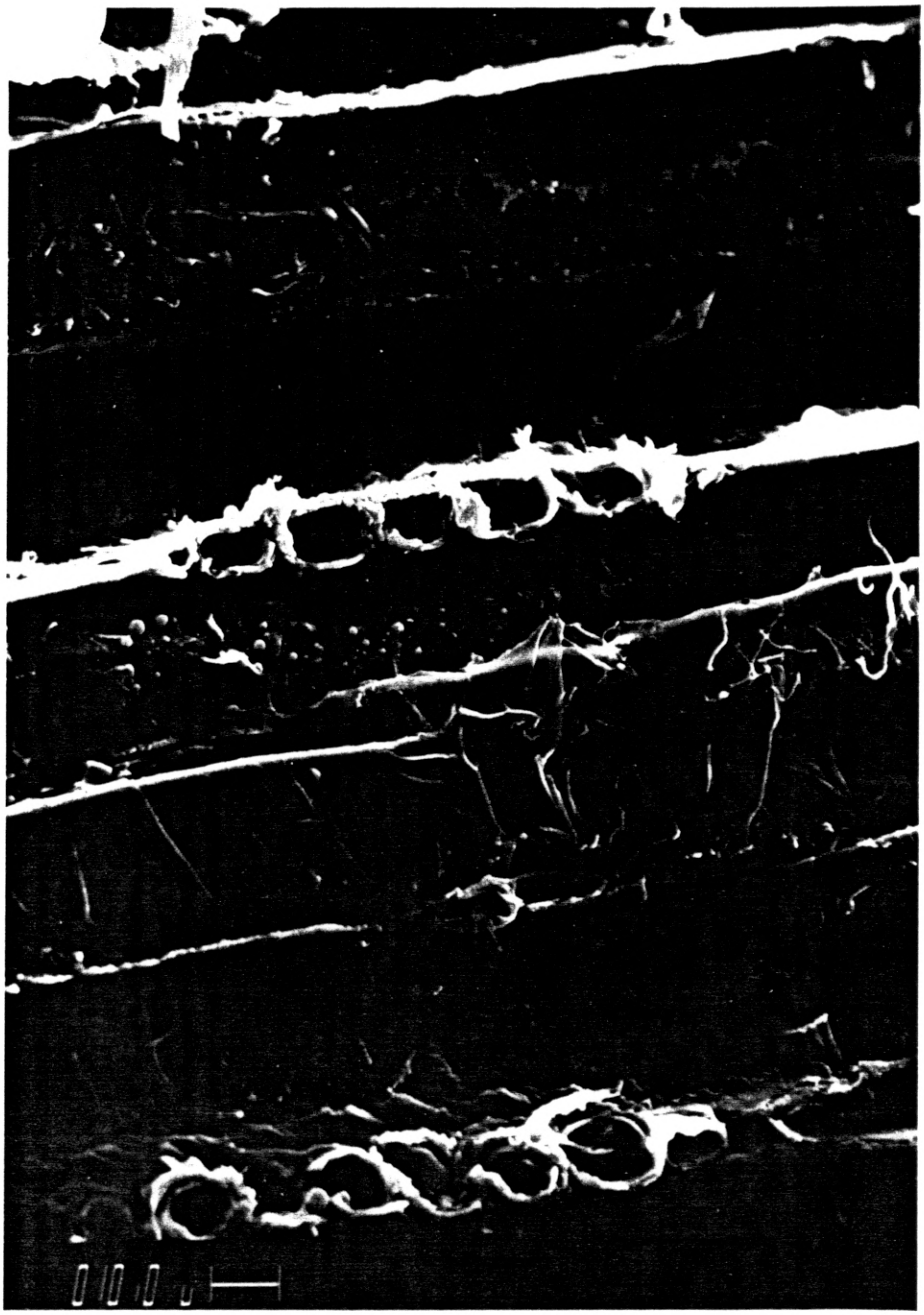

Figure II-2. SEM of wood samples from the study log. (C) Core sample from a 25-cm depth. Bar, 10 μ.

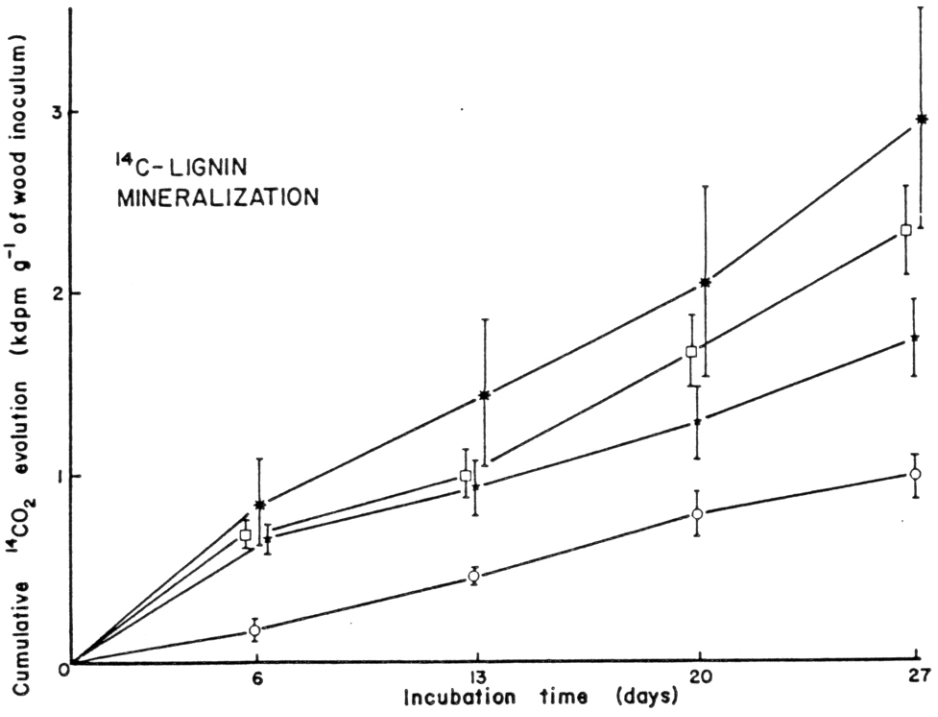

Figure II-3. Effects of various amounts [^{14}C]lignin-lignocellulose on mineralization rates with a fixed quantity of wood inoculum. Symbols: ○, 10-kdpm addition; ★, 20-kdpm addition; □, 30-kdpm addition; ✱, 40-kdpm addition. Each point is the mean of five replicates. Bars, Standard errors of the means.

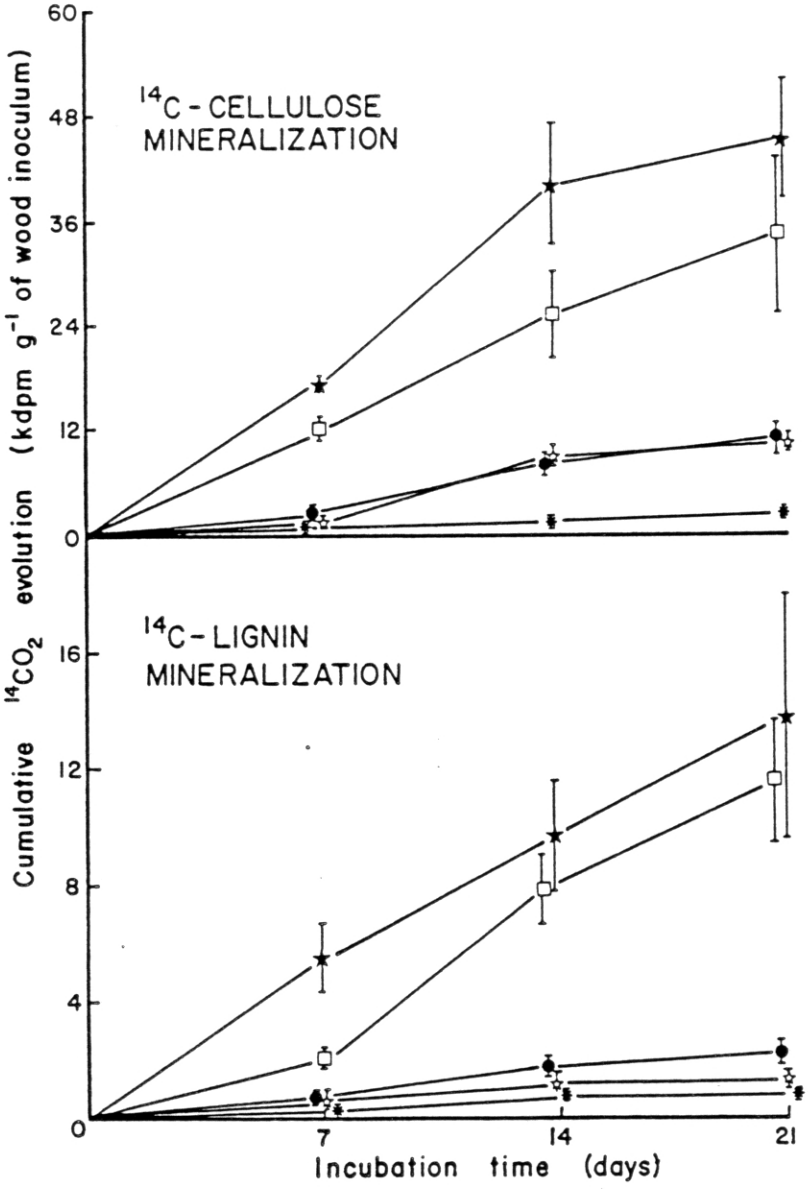

Figure II-4. Effects of medium supplementation on [^{14}C]lignocellulose mineralization. Symbols: ☆, distilled water alone; ●, mineral salts solution; ✱, salts plus glucose; □, salts plus yeast extract; ★, salts plus $(NH_4)_2SO_4$. Each point is the mean of five replicates. Bars, Standard errors of the means.

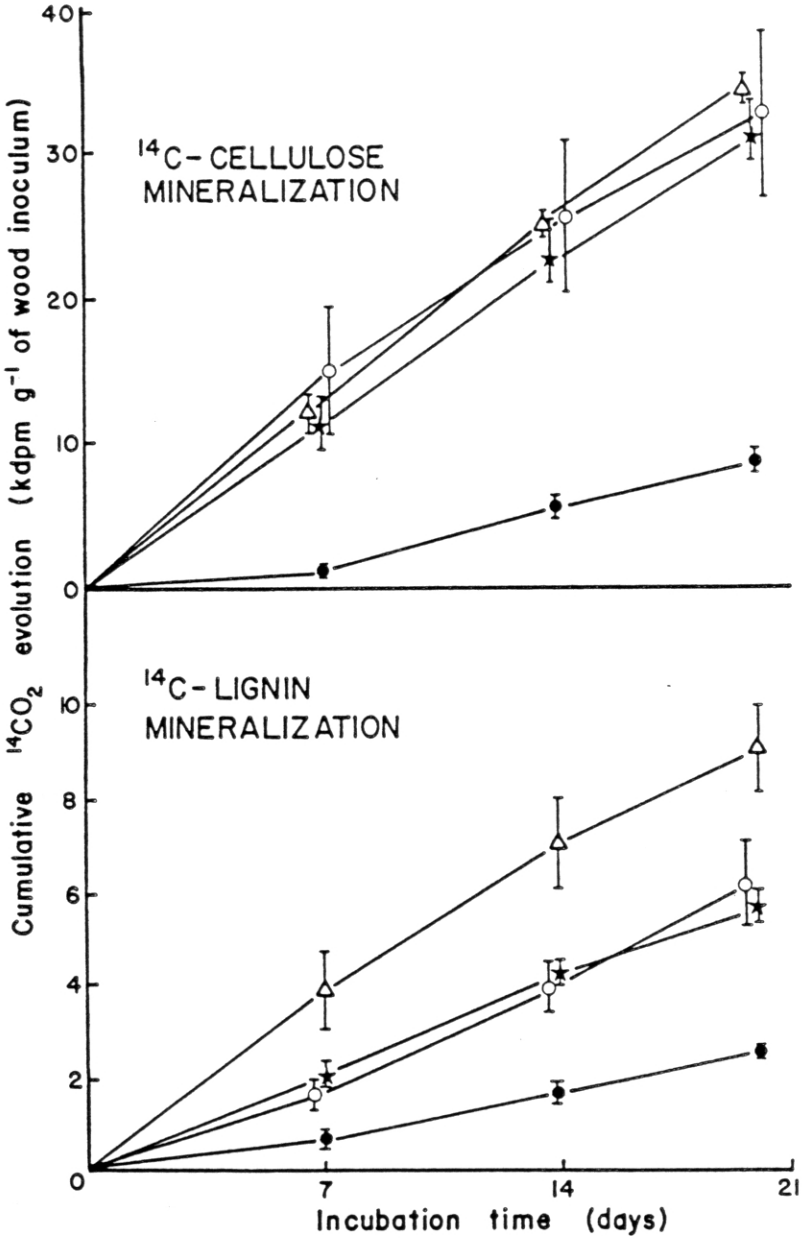

Figure II-5. Effects of various inorganic nitrogen species on [^{14}C]lignocellulose mineralization. Symbols: ●, mineral salts solution; ★, salts plus $(NH_4)_2SO_4$; ○, salts plus NH_4NO_3; △, salts plus KNO_3. Each point is the mean of five replicates. Bars, Standard errors of the means.

DISCUSSION

Degradation of [^{14}C]lignocelluloses has been shown by this study to be a sensitive and useful tool for assessing relative patterns of lignocellulose decay and microbial activity in wood. Application of this technique should prove particularly attractive to those interested in studying wood decay when compared to conventional weight loss studies. Measurements of wood decomposition by substrate weight loss may require many months of substrate incubation, and provide no information on decay rates of specific chemical components.

Before the radiotracer technique is adopted for use in a particular experimental system, however, it is essential that a thorough chemical characterization of the [^{14}C]lignocellulose be obtained. For example, degradation of small amounts of contaminating [^{14}C]protein residing in the lignin label could result in non-lignin derived ^{14}C being evolved, leading to an overestimate of lignin degrading capability. The quantities of ^{14}C-labeled protein contained in the lignin label used here were low (5%) and were well within ranges reported by other investigators using Douglas fir [^{14}C]lignocelluloses (Crawford, 1978; Crawford et al., 1980). The results of Klason and carbohydrate analyses of the labeled material and the ratios between lignin and cellulose mineralization rates were also similar to other values reported in the literature and indicate that evolution of $^{14}CO_2$ from the treatments represents lignocellulose

mineralization (Crawford et al., 1977a; Crawford, 1978; Crawford and Crawford, 1978; Crawford et al., 1980; Maccubbin and Hodson, 1980).

Rates of in vivo [^{14}C]lignin decomposition were maximized so that the effects of physical and chemical manipulations could be more readily measured. This was first accomplished by varying the amount of ^{14}C label with a fixed inoculum size (Fig. 11-3). Even though the 30 and 40 kdpm additions were slightly more favorable than the 20 kdpm addition by the fourth week of incubation, the latter amount was selected to conserve labeled substrate and to restrict incubations to a maximum of three weeks. The appropriate level of ^{14}C substrate addition should be determined for all applications of the described technique and, surprisingly, has only recently been considered in the literature (Baker, 1983).

Degradation of [^{14}C]lignocelluloses, plate counts, and SEM all demonstrated that microbial colonization on the log was mainly a surface phenomenon. This supports suggestions in the literature that microbial activity is generally restricted to the surface of decomposing wood in aquatic environments (Savory, 1954; Anderson et al., 1978; Triska and Cromack, 1980; Dudley and Anderson, 1982). The observed pattern of surface-related microbial activity probably reflects the lack of gallery-forming insect activity in aquatic wood; a factor considered a potentially important microbial distribution mechanism in logs decomposing in terrestrial environments (Anderson et al., 1978; Dudley and Anderson, 1982).

Waterlogging and the absence of tunneling insects may limit the access of oxygen into the log, which is a requirement for significant microbial breakdown of natural lignin (Zeikus, 1980; R. Crawford, 1981). Oxygen limitation would also restrict the growth of the hyphae of aerobic lignin degrading fungi into the inner parts of the wood (Kirk et al., 1978). Restriction of microbial activity to the surface of aquatic wood results in extremely slow rates of decomposition given the small surface area to volume ratio of large logs and the recalcitrant nature of lignocellulose.

Though significant [^{14}C]lignocellulose decay occurred during incubation in distilled water, rates of breakdown were greatly enhanced by supplementation of media with inorganic nitrogen (Fig. 11-4). The observation that NO_3^- N enrichment enhanced [^{14}C]lignin degradation more so than $(NH_4)_2SO_4$ or NH_4NO_3 is particularly worthy of note. Preliminary experimental evidence obtained by us suggests that nitrate ammonification may be occurring in the KNO_3 enriched incubation treatment (data not published). This observation warrants further study to determine if it is related to the favorable effect of NO_3^- N addition on lignin degradation. When NO_3^- N is supplied in combination with NH_4^+ N, however, [^{14}C]lignin mineralization rates are similar to the $(NH_4)_2SO_4$ treatment alone, suggesting that a classical repression of NO_3^- metabolism by NH_4^+ may be in effect (Stouthamer, 1976).

In contrast to the stimulatory effects of nitrogen supplementation, the repression resulting from glucose addition

suggests that the microbial community present in decaying wood will utilize more favorable carbon and energy sources when they are available. Whether or not this repressive effect is directly on the ligninolytic community *per se* or indirectly on other members of the community is in need of further study. Repression of lignocellulose mineralization by simple sugars has been noted before, both in environmental samples and in pure culture work (Ander and Eriksson, 1975; Skowronska, 1977).

There are few published results of studies concerning the effect of nutrient amendments on [^{14}C]lignocellulose decomposition in natural samples, and none exist for wood substrates to the best of our knowledge. Positive correlations have been observed between synthetic [^{14}C]lignin degradation rates and NO_3^- N concentrations in sediments, and additions of nitrogen to arctic lake sediments enhanced [^{14}C]cellulose mineralization and had no effect on [^{14}C]lignin decay (Hackett *et al.*, 1977; Federle and Vestal, 1980). Pure culture work on the physiology of white-rot fungi and lignin degrading actinomycetes also show contrasting effects of nitrogen amendments (Keyser *et al.*, 1978; Kirk *et al.*, 1978; Barder and Crawford, 1981; Reid, 1983). In non-radioactively labeled decomposition studies, however, several investigators report that added N can stimulate wood decomposition by fungi (Findlay, 1934; Schmitz and Kaufert, 1936; Hungate, 1940).

The enhancement of lignocellulose degradation by mineral nitrogen supplementation reported here suggests that nitrogen

limitation may exist in decomposing wood in Pacific Northwest streams. Nitrogen concentrations in wood are usually low, with faster decay rates correlating with higher nitrogen content of the woody tissue (Merrill and Cowling, 1966a). Even in the later stages of decomposition when nitrogen content has been shown to increase, it may not be in a form available for use by microorganisms (Merrill and Cowling, 1966b; Rice, 1982). These observations indicate that the microbiota on decaying wood should respond favorably to an external source of N. Streamwater sources of nitrogen are low in lotic ecosystems of the Pacific Northwest where nitrogen limitation has been observed (Triska et al., 1982), and could be responsible for the extremely slow biological processing rates of wood in these natural environments. Obviously, further studies in situ will need to be carried out.

Other nutritionally important mineral elements do not appear to be limiting to the degradation process, as evidenced by no substantial increase in ^{14}C mineralization rates in the presence of a nitrogen-less complete mineral salts and trace elements source (Fig. 11-4). Correlative studies in other stream ecosystems, however, have suggested that decay rates of wood may respond to increased phosphorus concentrations in streamwater (Melillo et al., 1983) and to the initial quality of the substrate (Fogel and Cromack, 1977; Melillo et al., 1982).

Results from the study have demonstrated the applicability of the [^{14}C]lignocellulose technique in assessing the activity of

lignocellulolytic microbial communities in decaying wood. Now that optimal conditions have been established for laboratory mineralization studies, this technique can be utilized in conjunction with other methodology to further characterize the microbial communities involved in wood decay in the stream environment and the physico-chemical and nutritional factors that affect these rates of activity.

ACKNOWLEDGEMENTS

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CHAPTER III

Microbial Decomposition of Wood in Streams: Impact of Mineral Nutrients and Incubation Conditions on [^{14}C]lignocellulose Decomposition

Nicholas G. Aumen[1], Peter J. Bottomley[2], and Stan V. Gregory[3]

Department of Microbiology[1], Departments of Microbiology and Soil Science[2], and Department of Fisheries and Wildlife[3], Oregon State University, Corvallis, Oregon 97331-3804.

ABSTRACT

Nutritional and physical factors affecting the decomposition of [^{14}C]lignocelluloses prepared from Douglas fir (Pseudotsuga menziesii) were examined by incubating the labelled substrate with homogenized surface wood scrapings obtained from a Douglas fir log in a Pacific Northwest stream. Incubations were conducted in either distilled water, stream water collected from four sources, or a defined mineral salts solution alone or supplemented with KNO_3. Decomposition rates of [^{14}C]lignocelluloses, as measured by $^{14}CO_2$ evolution, were greater in stream water than in distilled water alone. Decomposition experiments conducted in stream water media with the addition of defined mineral salts demonstrated that [^{14}C]cellulose decomposition was stimulated by additions of either nitrate or phosphate alone and enhanced significantly more by a combination of both. In contrast, [^{14}C]lignin decomposition responded only to a combination of added nitrogen and phosphorus. Decomposition of [^{14}C]lignocelluloses was greatest when supplemental KNO_3 was supplied in concentrations of at least 10.0 mg $N \cdot l^{-1}$ to a mineral salts background medium. [^{14}C]lignocellulose decomposition increased as the incubation temperature was raised and NO_3^- N supplementation further increased these rates between three- and seven-fold over the range of temperatures examined (5 to 22°C). Accumulation of NH_4^+ (2-4 mg $N \cdot l^{-1}$) was always observed in culture filtrates of incubations which had been supplemented with KNO_3; the quantity being independent of NO_3^- concentrations >10 mg

$N \cdot l^{-1}$. The role of supplemental NO_3^- in the decomposition of [^{14}C]lignocelluloses is discussed in relation to wood decomposition and the low concentrations of nitrogen found in stream ecosystems of the Pacific Northwest.

INTRODUCTION

Large woody debris has been shown to be an important component of streams in the Pacific Northwest, both from a geomorphic and a biological standpoint. Wood debris serves to stabilize stream channels and to reduce stream bed scouring by dissipating the effects of gradient on stream flow (Keller and Swanson, 1979). Large logs in the channel create habitat for stream organisms, retain organic matter, and are potential slow-release sources of large amount of nutrients to the lower trophic levels through the process of decomposition (Bilby and Likens, 1980; Triska and Cromack, 1980).

Methods traditionally used to assess the decomposition dynamics of woody substrates involve weight loss studies requiring many months of incubation before significant results can be obtained. Recently developed methods for labelling natural lignocelluluoses with ^{14}C have proven to be valuable tools for the microbial ecologist in that the dynamics of lignocellulose decay can be studied over relatively shorter periods of time (R. Crawford, 1981; Aumen et al., 1983). Lignocellulose is the major structural component of woody tissue and contains the majority of carbon associated with decomposing wood. Lignin in itself is a recalcitrant substrate, and often proves to be the ultimate rate-limiting constituent to decomposition of natural detritus (Fogel and Cromack, 1979; Melillo et al., 1982).

Previous work in our laboratory has shown that microorganisms capable of degrading lignin and cellulose are restricted to the surface of downed logs in streams and respond to the addition of supplemental nitrogen with increased rates of decomposition (Aumen et al., 1983). The results have shown that inorganic nitrogen in the form of KNO_3 stimulates lignin degradation to the greatest extent.

Our previous studies were restricted to high concentrations of nitrogen added to a background mineral medium of defined but complex composition, and the incubations were conducted at room temperature. The present study was undertaken to characterize in more detail the influence of supplemental inorganic nitrogen on the decomposition of [^{14}C]lignocelluloses, and to investigate the effects of the concentration of nitrogen, lower incubation temperatures, and to ascertain if any specific components of the mineral salts medium were critical for the stimulation by nitrate. Based on preliminary evidence of NH_4^+ N accumulation in culture filtrates (Aumen et al., 1983), the fate of nitrogen additions to laboratory cultures of stream wood samples incubated with [^{14}C]lignocelluloses was investigated.

MATERIALS AND METHODS

Study sites and sample collection

Wood samples used as the source of lignocellulose degrading microorganisms were obtained from Mack Creek, a third-order stream situated at an elevation of 830 m in the H. J. Andrews Experimental Ecological Reserve, in the Cascade Mountain Range, Oregon. A carpentry plane was used to collect surface wood scrapings of 1 mm thickness from an old-growth Douglas fir log (*Pseudotsuga menziesii*) that was wind-felled into Mack Creek in 1977. The scrapings were taken from bark-free portions of the log that were either immersed in, or constantly wetted by, the stream flow. Wood scrapings were placed in sterile whirl-pak bags, stored on ice, and returned to the laboratory for processing.

The stream water used in the incubation media for [^{14}C]lignocellulose decomposition experiments was collected from Mack Creek and the following three locations. Quartz Creek, located near the H. J. Andrews Reserve, represents a lower elevation site (550 m) that flows through a forty-year-old second-growth alder dominated reach. Grasshopper Creek also is in the vicinity of the Andrews Reserve at a slightly higher elvation (900 m) and flows through a five-year-old clear-cut site now dominated by herbaceous vegetation. Sulfur Creek is located in the Coast Mountain Range of Oregon (10 m elevation), and drains an alder dominated watershed. The stream water was collected in sterile Nalgene containers, placed on ice, returned to the laboratory and filtered through a

Nuclepore membrane (0.2 μ pore size, Nuclepore Corp., Pleasanton, Calif.).

Sample preparation

Surface wood scrapings were homogenized in sterile distilled water for 8 min at a setting of 30 on a VirTis Model 45 homogenizer (VirTis Co., Inc., Gardiner, N.Y.). Aliquots of this homogenate were used as inocula for [^{14}C]lignocellulose decomposition experiments as described in detail elsewhere (Aumen et al., 1983). Briefly, 1.0 ml portions of homogenate (approximately 0.015 g dry weight of wood inoculum) were added to 20 ml of an incubation medium in a glass serum bottle (60 ml capacity) containing either [^{14}C]lignin- or [^{14}C]cellulose-lignocellulose whch was prepared from Douglas fir and thoroughly characterized as described previously (Aumen et al., 1983). The bottles were capped with sleeve-type serum stoppers fitted with glass capillary bubbling tubes (2 mm dia.), and the treatments were bubbled for 15 min each day with filtered, CO_2-free air. Outflow gas was passed through 8% (wt/vol) NaOH to trap $^{14}CO_2$ which was quantified as described elsewhere (Aumen et al., 1983).

Characterization of [^{14}C]lignocellulose decomposition

The incubation media used in the decomposition experiments varied, and consisted either of sterile distilled water, filter-sterilized stream water, or a defined mineral salts medium whose components are described in Aumen et al. (1983). Nitrogen additions were in the form of KNO_3, and were added to the various background media described above.

(i) Effect of stream water and defined mineral salts media

The effect of stream water collected from various sources on the decomposition of lignocelluloses was examined by incubating radiolabelled lignocelluloses and surface wood homogenate with filter-sterilized stream water collected from Quartz, Grasshopper, Mack, and Sulfur Creeks. The NO_3^- N concentrations of the aforementioned stream waters were 0.012, 0.043, 0.063, and 0.123 mg $N \cdot l^{-1}$ respectively. NH_4^+ N and NO_2^- N were at the lower limits of detection (<0.005 mg $N \cdot l^{-1}$).

(ii) Effects of nitrogen and phosphorus

The effects of specific components of the defined mineral salts medium on the decomposition of lignocelluloses was examined by incubating [^{14}C]lignocelluloses and wood inoculum in the presence of individual components of the medium. The various components were added to a background of stream water (SW) collected from Mack Creek containing a NO_3^- N concentration of 0.537 mg $N \cdot l^{-1}$. The experimental treatments were prepared in triplicate, and included SW alone, SW plus 22.7 mM KNO_3 (318.0 mg $N \cdot l^{-1}$), SW plus 1.4 mM KH_2PO_4 and 2.2 mM $K_2HPO_4 \cdot 3H_2O$ (110 mg $P \cdot l^{-1}$), SW plus KNO_3 and KH_2PO_4/K_2HPO_4, and SW plus the complete mineral salts medium plus KNO_3 (Aumen et al., 1983). An additional treatment of SW plus 10 mM MOPS buffer (morpholinopropane sulfonic acid, Sigma Chemical Co., St. Louis, Mo.) adjusted to pH 7.0 was included to discriminate between the effects of phosphate as a source of P and as a buffering agent. In this experiment and those that follow,

accumulation of $^{14}CO_2$ was determined at weekly intervals over the course of a 21 day incubation period at room temperature.

(iii) Effect of nitrate concentration

A series of incubations were conducted to ascertain the response of lignocellulose mineralization to varying concentrations of NO_3^- N. Wood homogenate was incubated with [^{14}C]lignocelluloses in the presence of the complete mineral salts medium supplemented with KNO_3 to give final concentrations of 0, 0.01, 0.1, 1.0, 10.0, 100.0, and 318.0 mg N$\cdot 1^{-1}$. Incubations were conducted in quintuplicate, with additional replicates for the 318.0 mg N$\cdot 1^{-1}$ treatment. Release of $^{14}CO_2$ was followed as described above. The temporal pattern of NH_4^+ N accumulation in the treatments amended with 318.0 mg N$\cdot 1^{-1}$ was examined by sacrificing four replicates at each weekly sampling point for autoanalysis of the culture filtrate.

Total bacterial numbers in the samples were determined by epifluorescence microscopy using 4',6-diamidino-2-phenylindole (DAPI, Sigma) (Porter and Feig, 1980). Four replicates of the 0, 1.0, and 318.0 mg N$\cdot 1^{-1}$ treatments, and a three-week time series on the 318.0 mg N$\cdot 1^{-1}$ treatment were examined. The particulate organic matter remaining after filtration of the autoanalyzer samples was utilized in the counts. The Nuclepore filter (0.2 μ pore size) with the residue was suspended in 25 ml of a solution of 0.1% (wt/vol) partially hydrolyzed gelatin in a 0.1 M $(NH_4)_2HPO_4$ solution and shaken for 5 min on a wrist action shaker (Burrell

Corp., Pittsburgh, Penn.) to help disassociate bacterial cells from the particulate organic fraction. A flocculant (0.125 g of 2:5 mixture of $Ca(OH)_2$ and $MgCO_3$) was then added and the sample was shaken for an additional 5 min (Kingsley and Bohlool, 1981). The contents were transferred to 30 ml test tubes and allowed to settle for approximately 6 hrs. A 1.0 ml aliquot was removed and stained for 5 min with 0.11 ml of DAPI (1.0 $\mu g \cdot l^{-1}$), filtered through a 0.2 μ Nuclepore filter, and counted under a Zeiss IV Fl epifluorescence condenser microscope fitted with a mercury illuminator and filters BP 365, FT 395, and LP 397. Twenty fields were counted per filter, with four replicates per experimental treatment.

(iv) Analysis of culture filtrates

The form and concentrations of inorganic nitrogen species in the culture filtrates were determined upon termination of the incubations. The contents of duplicate serum bottles were centrifuged at 20,000 X G for 15 min in a Beckman Model J2-21 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.), and the supernatants passed through a 0.45 μ Millipore membrane (Millipore Corp., Bedford, Mass.). The filtrates were stored at 4°C for <24 hrs, and the concentrations of NO_3^- N, NH_4^+ N, and NO_2^- N were determined on a Technicon Autoanalyzer II (Technicon Instrument Corp., Cherrytown, N.Y.).

(v) Effects of temperature

The role of temperature in [^{14}C]lignocellulose decomposition was studied by conducting incubations at 5, 10, and 22°C. Labelled

substrate and inoculum were incubated either in Mack Creek stream water (0.073 mg$\cdot 1^{-1}$ NO_3^- N), defined mineral salts medium, or mineral salts medium amended with KNO_3 (10.0 mg N$\cdot 1^{-1}$). The sub-ambient temperature experiments were conducted inside walk-in incubators, and the 22°C (±2°C) incubation was the ambient laboratory temperature.

RESULTS

The incubation of labelled lignocelluloses with distilled water, mineral salts solution, and stream waters from various sources demonstrated that the decomposition of lignocelluloses is greater in stream water than in distilled water alone (Fig. III-1). This pattern was evident for both [^{14}C]lignin- and [^{14}C]cellulose-lignocelluloses, with stream water supporting decomposition rates up to two times that of distilled water alone.

Decomposition of [^{14}C]lignocellulose was greatest when the substrate was incubated in the presence of a complete mineral salts medium with NO_3^- N supplementation (Table III-1). A NO_3^- N amendment on a background of stream water yielded no increase in decay of [^{14}C]lignin, but did enhance [^{14}C]cellulose decomposition. A similar pattern was observed with the addition of phosphorus to stream water, with phosphorus stimulating cellulose mineralization as much as nitrogen did. The addition of phosphorus and nitrogen together enhanced decomposition almost as much as the complete complement of mineral salts and nitrogen together. The addition of MOPS buffer to stream water showed no enhancement over stream water alone (data not shown).

An experiment with various concentrations of NO_3^- N supplementation demonstrated that a minimum concentration of 10.0 mg N$\cdot 1^{-1}$ yields the maximum lignocellulose mineralization (Fig. III-2). Additions of NO_3^- N at concentrations of 0.01 and 0.1 mg N$\cdot 1^{-1}$ show no differences in lignocellulose decay from the

nitrogenless treatment. Supplementation with 1.0 mg $N \cdot l^{-1}$ yields some enhancement of decomposition, but the largest effect is associated with 10.0, 100.0, and 318.0 mg $N \cdot l^{-1}$. Both [^{14}C]lignin- and [^{14}C]cellulose-lignocellulose exhibit the same response to NO_3^- N stimulation. The accumulation of NH_4^+ N in the culture filtrate first appears in the 10.0 mg $N \cdot l^{-1}$ treatment, and is of the same magnitude in the two higher supplementations (Fig. III-3A). NO_3^- N is no longer detectable in the filtrates of the 0 through 10.0 mg $N \cdot l^{-1}$ treatments, but is still present in high concentrations in the 100.0 and 318.0 mg $N \cdot l^{-1}$ treatments (data not shown). A time course of NH_4^+ N appearance in culture filtrates show the greatest increase occurring after 7 days of incubation (Fig. III-3B).

Epifluorescence cell counts after three weeks of incubation show no major differences between the different nitrogen concentration treatments, with cell counts ranging from 3.5×10^7 to 7.34×10^7 cells per treatment. A time series conducted on replicates from the 318.0 mg $N \cdot l^{-1}$ treatment show a moderate increase in cell numbers from seven to twenty-one days incubation (5.50×10^6 to 7.34×10^7 cells per treatment). The cells observed under epifluorescence illumination were often associated with the particulate organic material and were extremely small (0.2 μ in length).

Rates of [^{14}C]lignocellulose mineralization responded dramatically to temperature and nutrient variations (Fig. III-4). NO_3^- N supplementation (10.0 mg $N \cdot l^{-1}$) on a background of mineral

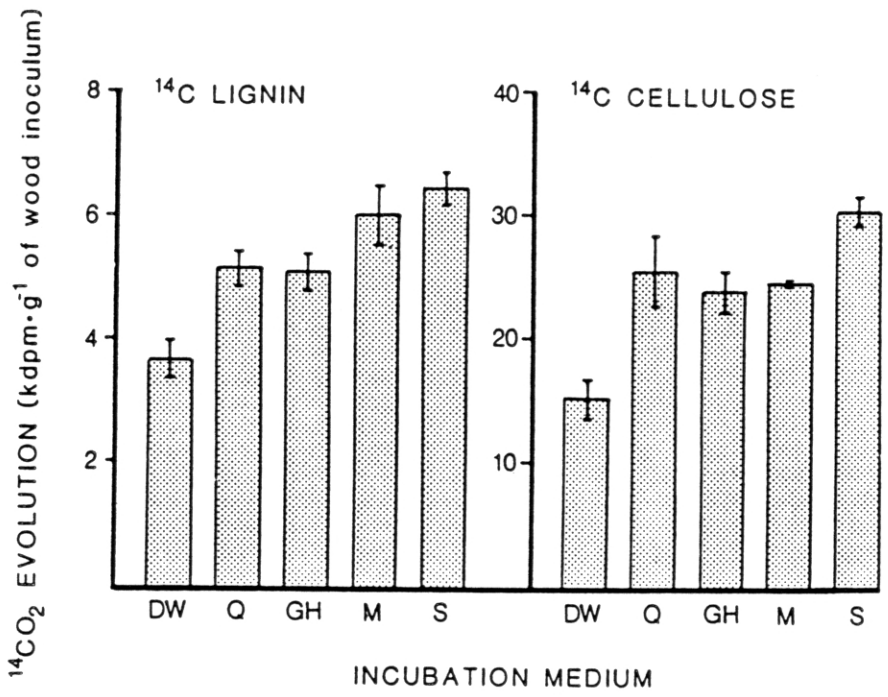

Figure III-1. Effects of stream water and distilled water media on [^{14}C]lignocellulose decomposition. Incubation medium: DW, distilled water; Q, Quartz Creek; GH, Grasshopper Creek; M, Mack Creek; S, Sulfur Creek. Each value is the mean of three replicates after 21 days incubation. Bars, Standard errors of the means.

TABLE III-1. Effect of medium composition on [^{14}C]lignocellulose decomposition. Each value is the mean of three replicates. Standard errors of the means are in parentheses.

Medium composition[1]	$^{14}CO_2$ Evolution (dpm·g dry wt wood sample) over 21 days incubation	
	[^{14}C]lignin	[^{14}C]cellulose
SW	7717 (647)	23439 (1373)
SW + NO_3^-	6937 (387)	35723 (3753)
SW + $PO_4^{\equiv}$	7111 (1911)	35160 (234)
SW + NO_3^- + $PO_4^{\equiv}$	12769 (561)	62665 (5868)
SW + mineral salts + NO_3^-	14847 (1201)	64602 (6751)

[1]SW, stream water; NO_3^-, KNO_3 (318 mg N·l^{-1}); $PO_4^{\equiv}$, KH_2PO_4/K_2HPO_4 (110 mg P·l^{-1}); mineral salts, complete mineral salts mixture with $PO_4^{\equiv}$.

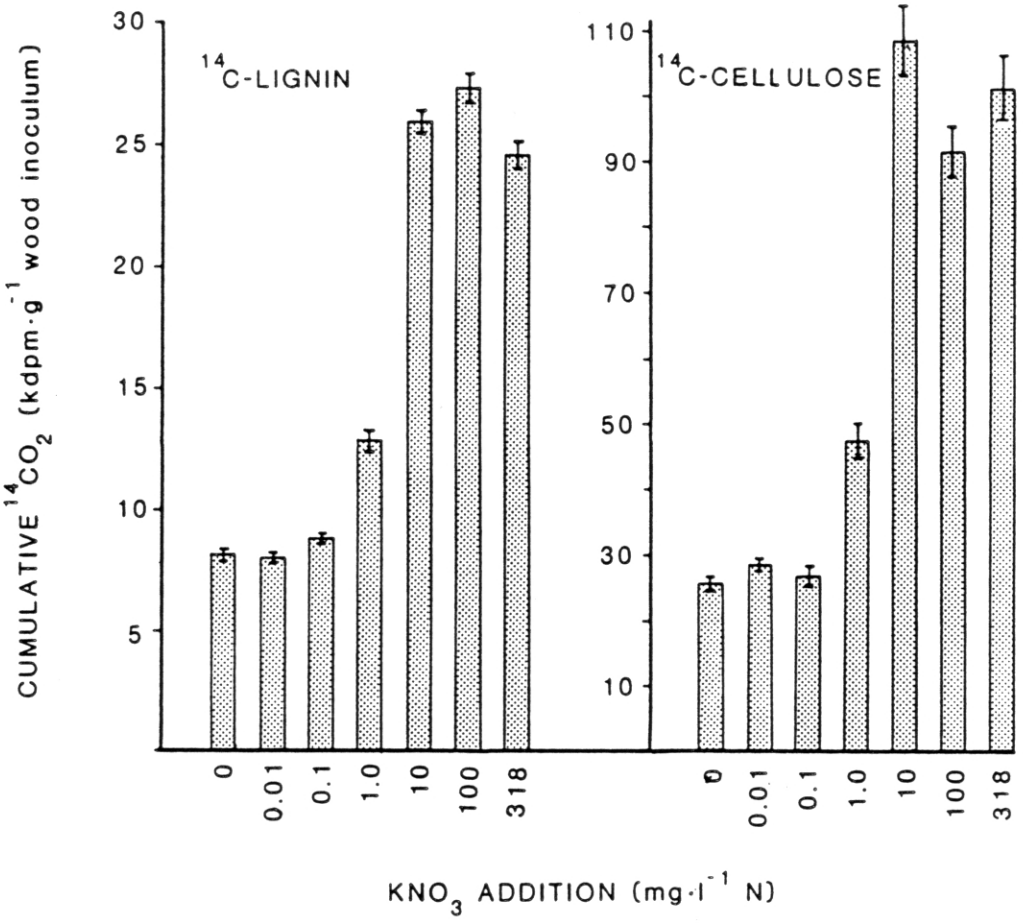

Figure III-2. Effects of varying concentrations of supplemental KNO_3 on [^{14}C]lignocellulose decomposition. Each value is the mean of five replicates after 21 days of incubation. Bars, Standard errors of the means.

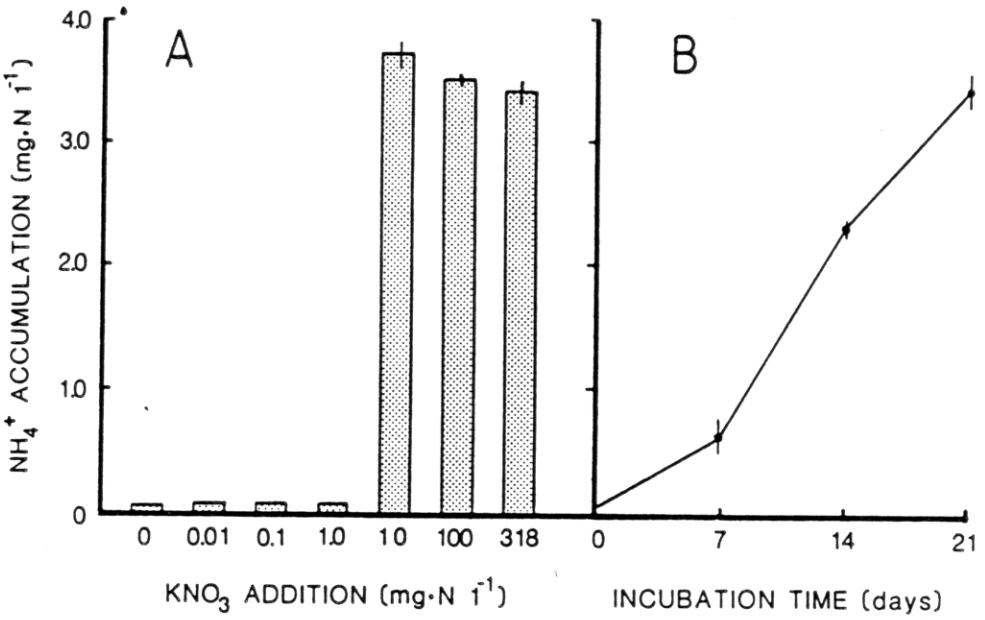

Figure III-3. (A) Effects of varying concentrations of supplemental KNO_3 on the accumulation of filtrate NH_4^+ N. (B) Accumulation of filtrate NH_4^+ N over time in incubations supplemented with KNO_3 (318 mg N·l^{-1}). Each value is the mean of four replicate samples. Bars, Standard errors of the means.

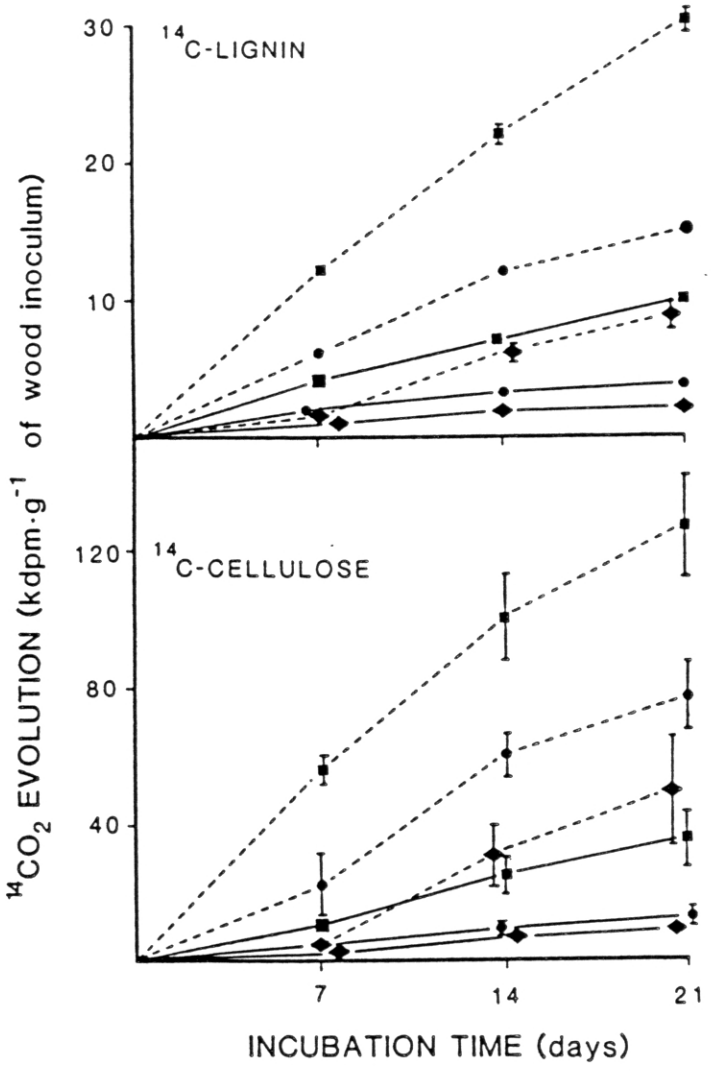

Figure III-4. Effects of incubation temperature on [^{14}C]lignocellulose decomposition. Incubation medium: ——, mineral salts solution; ---, mineral salts solution with KNO_3 (10 mg N·l^{-1}). Symbols: ■, 22°C; ●, 10°C; ◆, 5°C. Each point is the mean of four replicates. Bars, Standard errors of the means.

DISCUSSION

It is evident from data presented here that while decomposition of [^{14}C]lignocelluloses by stream wood microflora can occur even in a distilled water environment, the process is enhanced by incubation in natural stream water (Fig. III-1). Since these rates of decomposition were much lower than those observed previously in a nitrate supplemented complete mineral salts medium, it was considered of interest to determine what mineral nutrients might limit the rate of lignocellulose decomposition in stream water. The results show that lignin decomposition responds only to the combined presence of phosphorus and nitrogen, whereas the rate of cellulose decomposition can be enhanced by either nutrient alone (Table III-1). This suggests that the microbial community capable of cellulolytic activity on wood substrates is either a diverse one, or metabolically flexible and can readily respond to increases in external supply of either nitrogen or phosphorus.

Studies to date on nutrient limitation of decomposition have centered on stream detritus in general, not individual chemical components. The role of nitrogen in lignin degradation, a major component of organic matter in streams, is variable even at the pure culture level (Keyser et al., 1978; Kirk et al., 1978; Barder and Crawford, 1981; Reid, 1983). Extrapolation of these findings to the natural stream environment where a diverse microbial community exists under a very low nutrient regime is not feasible. However, our findings that low concentrations of NO_3^-

added to closed incubation systems were just as stimulatory as high concentrations to both lignocellulose decomposition and NH_4^+ accumulation (discussed below) suggest that the effects of nitrogen observed in our studies at high concentrations are relevant to lower concentrations. Further study is desired using flow-through systems where experiments can be conducted at actual stream concentrations of nutrients.

The constant level of filtrate NH_4^+ accumulation observed in treatments with at least 10.0 $mg \cdot l^{-1}$ added nitrogen is in accord with the results of [^{14}C]lignocellulose processing, which showed no further increase in $^{14}CO_2$ evolution beyond the threshold concentration of 10.0 mg $N \cdot l^{-1}$ (Fig. III-2). The demonstration of greater accumulation toward the second and third weeks of incubation suggest mineralization processes as its source (Fig. III-3B). Lower temperatures retard the nitrogen flux in the system, with a small amount of NO_3^- N still being present in the filtrate at 5°C after three weeks of incubation, and less NH_4^+ N accumulation evident.

Even in the presence of an abundance of all major nutrients, the rate of decomposition of lignocellulose cannot be enhanced beyond a certain point. This implies that carbon quality and/or availability becomes the controlling factor, even in the presence of sufficient nitrogen and phosphorus (Melillo et al., 1984). There is evidence that decay of lignin-rich organic detritus proceeds at the fastest rate initially, then becomes progressively

more resistant to microbial attack (Suberkropp et al., 1976; Rice, 1982). A suggested mechanism for this pattern is the condensation of microbially-altered lignin subunits with microbial extracellular products to form chemical complexes that are degraded at a much slower rate than observed initially (Martin and Haider, 1980; Stevensen, 1982).

An environmental factor which must be considered along with nutrient status is temperature, which was shown to exert an effect on decomposition rates of lignocelluloses, whether incubated in stream water, mineral salts, or mineral salts with NO_3^- N supplementation (Fig. III-4). Stream water temperatures in Mack Creek average 3°C during the winter months, and rarely rise above 16°C in the warmest part of the year. Melillo et al., (1984), suggest that temperature affects the rate of detrital processing, but not the long-term pattern. In other words, the initial chemical composition of the substrate determines the extent of humification processes and nitrogen immobilization, and that temperature only affects how fast it reaches that predetermined point.

The fate of external nitrogen additions to decomposing lignocelluloses is one of considerable interest considering reports of nitrogen limitation in streams of the Pacific Northwest (Triska et al., 1982; Aumen et al., 1983; Triska et al., 1984). Concentrations of dissolved inorganic nitrogen in the stream water are low, and are mainly in the form of NO_3^- N. Large woody debris

may serve as an important nitrogen sink in these streams, particularly if its microbial flora can mediate efficiently the conversion of NO_3^- N to NH_4^+ N and organic N and serve as a slow release source of this readily utilizable form of nitrogen to other micro and macroorganisms. Preliminary data (not shown) indicate that the organic nitrogen content of the lignocelluloses and wood inoculum used in our experimental systems increase two-fold over a short incubation period when supplied with relatively low levels of NO_3^- N. Absolute accretion of nitrogen has been reported in long term studies on decomposition of detritus, and includes woody substrates decomposing in streams (Staaf, 1980; Berg and Staff, 1981; Marinucci, 1982; Melillo et al., 1982; Rice, 1982; Melillo et al., 1983; Triska et al., 1984). The mechanisms hypothesized for this nitrogen increase have included nitrogen fixation, although rates measured in the environment fail to account for a major portion of nitrogen increase (Triska et al., 1984). Another possible explanation is an increase in microbial standing crop, although actual measurements of microbial population densities by a variety of methods suggest they are not responsible for the increase in the amount of nitrogen observed (Iverson, 1973; Rublee et al., 1978; Lee et al., 1980; Marinucci et al., 1983). Our measurements of direct counts using the DAPI reagent support this latter observation. Assuming a bacterial nitrogen content of 10% and dry weight per cell of 1.0×10^{-13} g, the greatest microbial standing crop could only account for ~2% of the total nitrogen

increase. An attractive explanation which has gained recent attention is the microbial release of reactive extracellular substances containing nitrogen. It is suggested that these compounds condense with microbial degradation products of organic matter, becoming resistant to further degradation (Rice, 1982; Melillo et al., 1984). This hypothesis could explain nitrogen increases with time on detritus colonized by low population densities of microorganisms, but remains to be tested.

The nitrogen dynamics of our experimental system have considerable implications from an ecosystem perspective, particularly if it can be established that the nitrogen accretion of decomposing wood results from external nitrogen addition. Triska et al. (1984) notes that the biological role of the large nitrogen pool in wood is largely unknown, but may involve 25% of the total nitrogen budget. Although the mineralization postulated here from this large pool may be slow, the total contribution could be extremely large.

ACKNOWLEDGEMENTS

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Chapter IV

Microbial Decomposition of Wood in Streams: Use of ^{15}N-labelled Nitrate to Study the Dynamics of Nitrogen Addition to [^{14}C]lignocellulose Decomposition Experiments

Nicholas G. Aumen[1], Peter J. Bottomley[2], and Stan V. Gregory[3]

Department of Microbiology[1], Departments of Microbiology and Soil Science[2], and Department of Fisheries and Wildlife[3], Oregon State University, Corvallis, Oregon 97331-3804

ABSTRACT

Surface wood samples obtained from a Douglas fir log (*Pseudotsuga menziesii*) in a Pacific Northwest stream were incubated *in vitro* with [^{14}C]lignocelluloses prepared from Douglas fir in a defined, mineral salts medium supplemented with 10 mg N•l^{-1} ^{15}N-labelled nitrate (50% ^{15}N). Evolution of $^{14}CO_2$, distribution and isotopic dilution of ^{15}N, filtrate nutrient concentrations, denitrification, nitrogen fixation, and respiration were measured at 6, 12, and 18 days of incubation. The results show that the organic nitrogen in the lignocellulose/wood sample mixture increased two-fold over the first 6 days of incubation, from 132 µg N to a maximum of 231 µg N per treatment. [^{14}C]lignocellulose decomposition rates were greatest during the first 6 days, then began to diminish over the remaining 12 days. Respiration rates measured by CO_2 evolution were also highest at day 6, and declined steadily over the duration of the incubation. Filtrate NH_4^+ N increased from background levels to a final value of 57 µg N per treatment. Filtrate NO_3^- N completely disappeared by day 6, and organic nitrogen showed a slight decline between day 12 and day 18. The results of the ^{15}N analyses show that the majority of the ^{15}N that can be accounted for appeared in the particulate organic fraction by day 6 of the incubation period (41 µg N), and that the filtrate NH_4^+ N fraction contained 11 µg ^{15}N. The ^{15}N enrichment values of the filtrate NH_4^+ and the inorganic nitrogen associated with the particulate fraction had increased to

approximately 20% ^{15}N by 18 days incubation, whereas the particulate organic fraction reached its highest enrichment by day 6. Nitrogen fixation and denitrification measurements over the time course of the study indicated insignificant gain/or loss of nitrogen from the experimental system by these processes. The data show that wood debris in stream ecosystems might function as a rapid and efficient sink for exogenous nitrogen, resulting in stimulation of wood decomposition and subsequent activation of other nitrogen cycling processes.

INTRODUCTION

Stream ecosystems in the Cascade Mountains of the Pacific Northwest are known to possess large accumulations of woody debris in the channels, particularly in old-growth forests dominated by Douglas-fir (Pseudotsuga menziesii) (Triska and Cromack, 1980). Although earlier studies have shown that wood decomposition in streams is a slow process, it may provide significant amounts of mineralized carbon and nitrogen to the biological community given the large accumulations present (Triska and Cromack, 1980; Triska et al., 1984). Virtually nothing is known of the physico-chemical and nutritional factors which affect the decomposition processes.

We previously demonstrated that decomposition studies conducted with ^{14}C-labelled natural lignocelluloses prepared from Douglas fir are a sensitive and useful tool for investigating the dynamics of lignocellulose decomposition by the stream wood microflora (Aumen et al., 1983). The results of our studies show that the microbial community on downed logs is restricted to the surface, and responds to mineral nutrient supplements of N and P with increased rates of [^{14}C]lignocellulose mineralization. The concentrations of supplemental mineral nutrients and variations in incubation temperature also affect decomposition rates (see Chapter III). Previously it was shown that additions of nitrate in concentrations as low as 10.0 mg $N \cdot l^{-1}$ can result in increased rates of [^{14}C]lignocellulose decay and concurrent accumulation of NH_4^+ in the culture filtrate. Preliminary data also demonstrated

that the nitrate additions lead to a two-fold increase in the organic N fraction of the [^{14}C]lignocellulose/wood inoculum mixtures (see Chapter III). The kinetics of nitrate utilization and the subsequent fate of the nitrogen in decomposing wood are of considerable interest given the low initial nitrogen content of the substrate and the low stream water nitrogen concentrations (Triska et al., 1982; 1984).

In order to determine more clearly the source of the filtrate NH_4^+ and the organic nitrogen increase, along with its relation to [^{14}C]lignocellulose decay, a study was conducted using a combination of stable and radioisotopes. [^{14}C]lignocelluloses and stream wood samples were incubated in a medium of mineral salts with the addition of $K^{15}NO_3$. Measurements of $^{14}CO_2$ evolution, isotope ratio determinations, chemical analysis of culture filtrates, and gas chromatographic studies were performed on replicate samples to follow the nitrogen dynamics and lignocellulose decomposition in laboratory incubations over an 18 day period.

MATERIALS AND METHODS

Sample collection and preparation

Surface wood scrapings were obtained in April, 1984, from a stream-wetted section of an old-growth Douglas fir log wind-felled seven years previously into Mack Creek, a third-order stream located in the H. J. Andrews Experimental Ecological Reserve, at an elevation of 830 m in the Cascade Mountain Range, Ore. (Aumen et al., 1983). Wood samples were placed in sterile whirl-pak bags, stored on ice, and returned to the laboratory for processing. The scrapings were homogenized in distilled water for 8 minutes at a setting of 30 in a VirTis Model 45 homogenizer (VirTis Co., Inc., Gardiner, N.Y.). Aliquots (1.0 ml) of the homogenate containing approximately 0.008 g (dry weight) of homogenized wood were used as microbial inocula for the series of decomposition studies described below.

[^{14}C]lignocellulose decomposition experiments

[^{14}C]lignocelluloses were prepared by incubating Douglas fir branches with [^{14}C]phenylalanine or [^{14}C]glucose to preferentially label the lignin and cellulose fractions, respectively. The methods used to prepare and thoroughly characterize the radiolabelled lignocelluloses are those of R. Crawford (1981) and are described in detail elsewhere (Aumen et al., 1983). Wood homogenate and [^{14}C]lignocelluloses (20 kdpm per treatment of lignin, 10 kdpm per treatment of cellulose) were incubated in 60 ml glass serum bottles with a mineral salts solution supplemented with

KNO_3 to a final concentration of 10.0 mg $N \cdot l^{-1}$. Bottles were bubbled once per day with filtered, CO_2-free air, and outflow gases were trapped and quantified for the presence of $^{14}CO_2$ (Aumen et al., 1983). The incubations were conducted over 18 days with $^{14}CO_2$ accumulation measured at 6-day intervals.

^{15}N studies

A series of experimental incubations were prepared as described above using a medium of mineral salts solution in which the supplemental nitrogen (10.0 mg $N \cdot l^{-1}$) contained $K^{15}NO_3$ (50% abundance, Amersham Corp., Arlington Heights, Ill.). The culture filtrate and particulate organic matter of ten replicate bottles were analyzed for the distribution of ^{15}N (Hauck, 1982) after 6, 12, and 18 days of incubation. The entire contents of each serum bottle were filtered through 0.45 μ Millipore filters (Millipore Corp., Bedford, Mass.) and the particulates remaining on the filter rinsed with 5 ml of distilled water. A KCL extraction step was not included as preliminary experiments had shown that this treatment did not increase recovery of NH_4^+ N in the filtrate. A portion of the filtrate was reserved for NO_3^-, NO_2^-, and NH_4^+ analysis on a Technicon Autoanalyzer II (Technicon Inst. Corp., Cherrytown, N.Y.). The remainder was stored at 4°C for no longer than 24 hrs before distillation. NH_4^+ N in 5.0 ml of filtrate was recovered by steam distillation for 7 min in the presence of NaOH in an all-glass microdistillation apparatus with Teflon stopcocks. Distillate was collected in 20 ml distilled water (pH 5.5) from

which a subsample (7 ml) was removed for autoanalyzer determination of NH_4^+ N, and the remainder acidified with 2 drops of 0.05 M H_2SO_4. Acidified samples were spiked with 50 µg N as unenriched $(NH_4)_2SO_4$ (0.3683% ^{15}N) and evaporated to dryness. An unenriched sample of $(NH_4)_2SO_4$ was distilled after each ^{15}N sample to eliminate the chance of cross-contamination of ^{15}N between distillates.

The particulate organic matter remaining on the Millipore filter was dried at 50°C and digested for 10 hrs in 2 ml of Kjeldahl catalyst digestion solution. The digest was diluted with 11 ml of distilled water, and 5 ml used for distillation and recovery of NH_4^+ N as described above. NO_3^- + NO_2^- N in the digest was recovered as NH_4^+ N by the addition of 0.2 g of Devarda's alloy and distillation for an additional 7 min.

N_2 was produced from the reconstituted samples by the addition of sodium hypobromite, and the isotopic ratio was determined in a Nuclide RMS 3-60 mass spectrometer by Isotope Services, Inc., Los Alamos, N.M. Two to four aliquots of each sample were analyzed, and the mean determined.

Gas chromatography

Additional replicates of the nitrogen enriched incubation treatments were sacrificed at 6, 12, and 18 days for determination of the rates of respiration, denitrification, and nitrogen fixation. Respiration measurements on six replicate samples were made by injecting 0.2 ml samples of head space gas into a Hewlett-

Packard 5700A gas chromatograph fitted with a 1.5 m Porapak Q column and a thermal conductivity detector. Gas samples were removed from each bottle at 0, 2, 4, and 6 hours. Results were standardized against injections of a 100 ppm CO_2 in N_2 standard gas mixture. Six replicate samples for denitrification determinations by the acetylene block technique (Payne, 1981) were prepared by injecting calcium carbide-generated acetylene to a final concentration of 10% (vol/vol) in air. An additional six replicates contained no acetylene to assay for acetylene-independent N_2O production. Samples of head space gas (0.2 ml) were injected at 0, 2, 4, and 6 hr intervals into a Hewlett-Packard 5840A gas chromatograph fitted with a 4 m Porapak Q column and a ^{63}Ni electron capture detector. The sample bottles containing 10% acetylene were also assayed for acetylene reduction over the same time intervals by injecting 0.2 ml gas samples into a Hewlett-Packard 5830A gas chromatograph containing a 1.5 m Porapak R column and a flame-ionization detector. The denitrification and nitrogen fixation results were standardized by injections of a 3.7 ppm mixture of N_2O in N_2 and a 2.0 ppm mixture of ethylene in N_2, respectively. Results were corrected for background levels of N_2O and ethylene contained in the acetylene, and the CO_2 in air.

RESULTS

Previous data from our laboratory have shown that high concentrations of nitrate (318.0 mg $N \cdot l^{-1}$) stimulate the decomposition of both the lignin and cellulose fractions of [^{14}C]lignocellulose. Results presented in Fig. IV-1 show that a lower concentration of nitrate (10.0 mg $N \cdot l^{-1}$) also supports relatively high rates of $^{14}CO_2$ evolution from both fractions, with the greatest rates observed between day 0 and day 6 of the incubation. The subsequent rates of lignocellulose decomposition began to diminish over the remaining 12 days of the incubation period. This pattern coincided with a decrease in the overall rate of respiration measured by gas chromatography on samples which had been incubated for 6, 12, and 18 days, respectively (Fig. IV-1).

Data obtained on the nitrogen budget of these nitrate supplemented systems show a large increase (112 µg N per treatment) in the Kjeldahl nitrogen fraction over the first 6 days of incubation (Fig. IV-2). The initial nitrate addition (200 µg N per treatment) decreased below the limits of detection by the sixth day of incubation (Fig. IV-2). Since filtrate NH_4^+ and NO_2^- N concentrations were very low (~3 µg N per treatment) over the same time period, the data suggest that nitrate is efficiently assimilated into organic fractions and not simply reduced to NH_4^+ and immediately excreted into the medium. Filtrate NH_4^+ N was seen to increase to 17 and 57 µg N per treatment by days 12 and 18, respectively. In fact, this latter value represents 20% of the

total combined nitrogen found in the 18 day sample, and coincides with a small, but significant, decrease in the organic nitrogen fraction of the particulates. The increases in filtrate NH_4^+ N occur during the time when nitrate was undetectable in the medium. A point of some significance is the fact that addition of Devarda's alloy to the Kjeldahl samples following distillation of the NH_4^+ resulted in the detection of additional NH_4^+ (10 - 15 µg N per treatment) which we infer originated from nitrate sequestered by the woody material and not reduced by microbiological activity.

Results of the ^{15}N analyses of the samples show that the majority of ^{15}N that can be accounted for was associated with the particulate fraction (Fig. IV-3). The ^{15}N incorporated into the organic fraction between day 0 and day 6 amounted to 44 µg N per treatment. When this value is corrected for the 50% of ^{15}N in the nitrate amendment, 81% of the nitrogen increase in the particulate organic fraction originated from the nitrate supplement. The amounts of ^{15}N found in the filtrate NH_4^+ N fraction were small, but significantly above the natural abundance of ^{15}N, over the first 12 days of incubation. There was a large increase in the filtrate ^{15}N content between days 12 and 18, with 11 µg of ^{15}N eventually recovered in the filtrate NH_4^+ N fraction at day 18. It can be seen that 53 µg of the original 100 µg of ^{15}N per treatment could be accounted for in the filtrate and particulates at the end of the incubation period.

The data presented in Fig. IV-4 provide some insight into the complexity of the nitrate processing within the system. The initial large increase in ^{15}N enrichment of the organic nitrogen fraction and the inorganic nitrogen fraction associated with the particulate organic matter coincides with increases in absolute amounts of ^{15}N shown in Fig. IV-3. An observation of interest is that the filtrate NH_4^+ N fraction is enriched to 4.5% ^{15}N within 1 h of the addition of the $K^{15}NO_3$ to the [^{14}C]lignocellulose/wood inoculum mixture. The % ^{15}N values decline between that time and day 6 of the incubation, and then increase to a final value of 20% ^{15}N. By 18 days of incubation, the enrichment of all three nitrogen fractions were similar. The data suggest that although the nitrate was rapidly sequestered and metabolized into organic N, further turnover of this fraction, involving mineralization and nitrification became significant during the later stages of the incubation and coincided with the decrease in respiration (see Fig. IV-1).

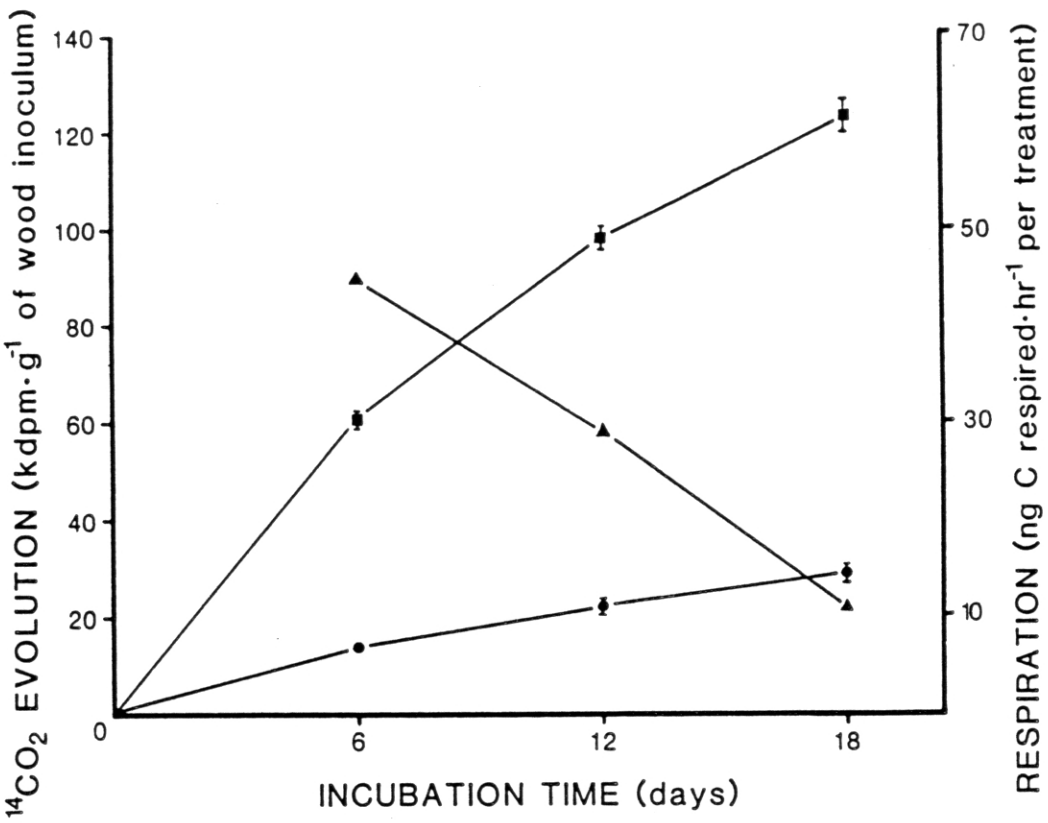

Figure IV-1. [^{14}C]lignocellulose decomposition and respiration rates over time in samples incubated in mineral salts solution with supplemental KNO_3 (10 mg N·l^{-1}). Symbols: ■, [^{14}C]cellulose; ●, [^{14}C]lignin; ▲, respiration rate. Each point is the mean of five or six replicates. Bars, Standard errors of the means.

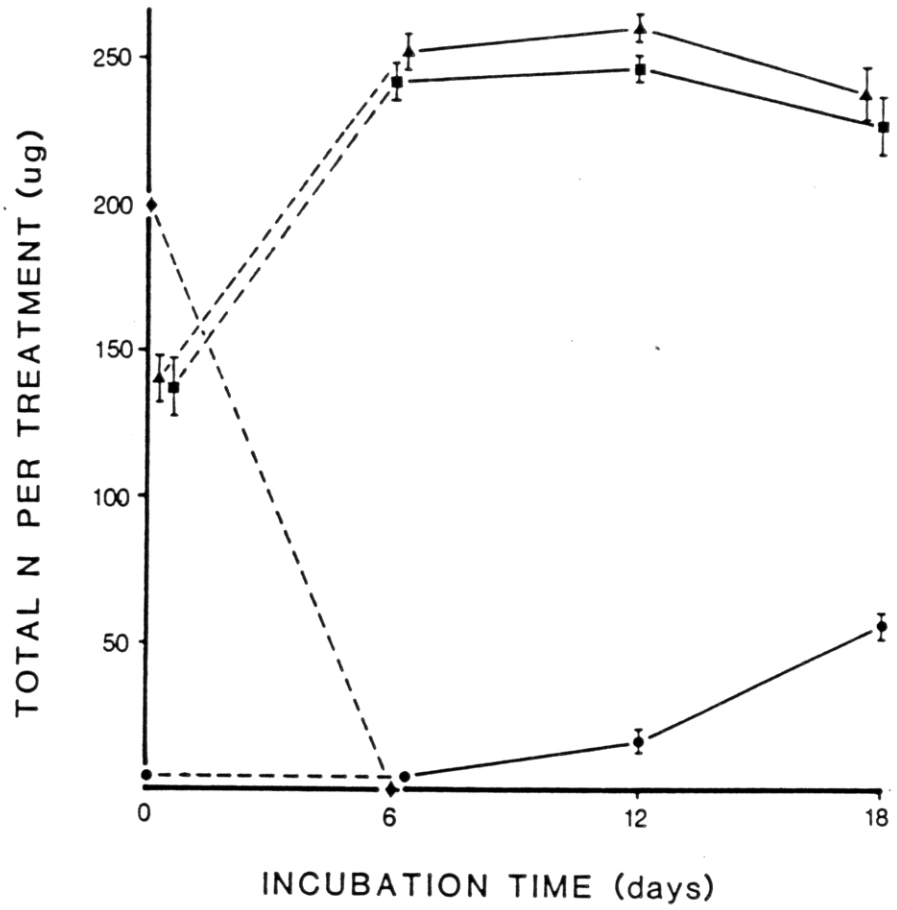

Figure IV-2. Distribution of nitrogen in [^{14}C]lignocellulose/wood sample incubations over time. Symbols: ■, organic nitrogen; ▲, organic nitrogen plus inorganic nitrogen associated with particulate organics; ●, filtrate NH_4^+ N; ◆, filtrate NO_3^- N. Each point is the mean of ten replicates. Bars, Standard errors of the means.

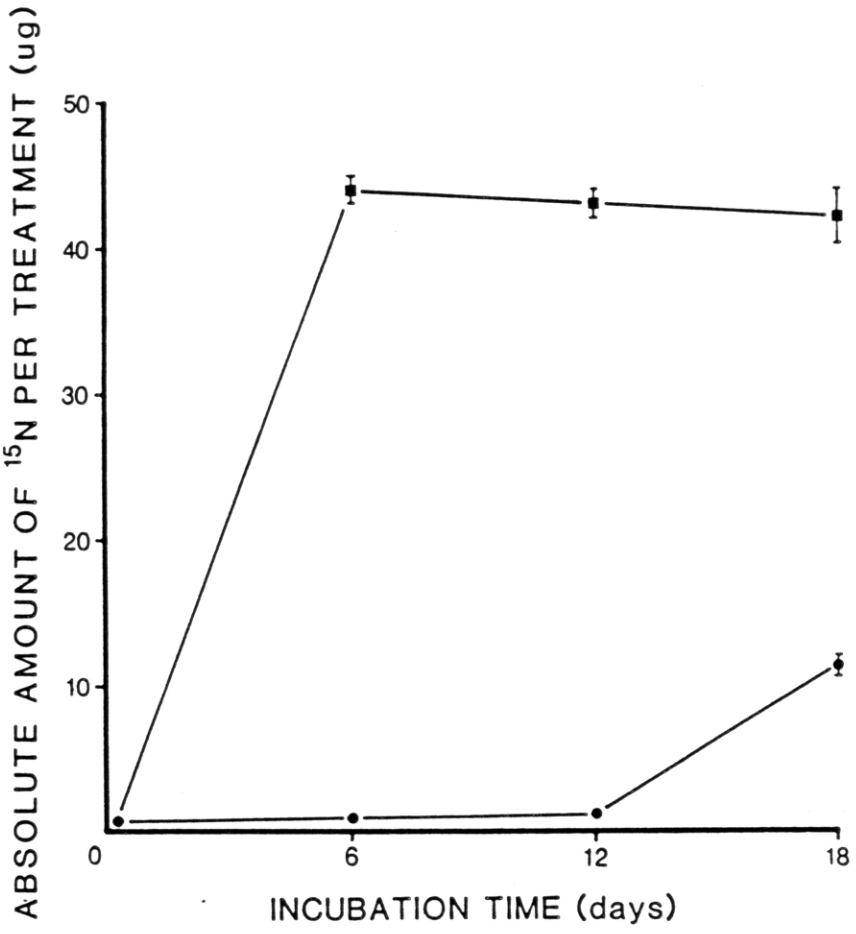

Figure IV-3. Distribution of ^{15}N in [^{14}C]lignocellulose/wood sample incubations over time. Symbols: ■, organic nitrogen; ●, filtrate NH_4^+ N. Each point is the mean of ten replicates. Bars, Standard errors of the means.

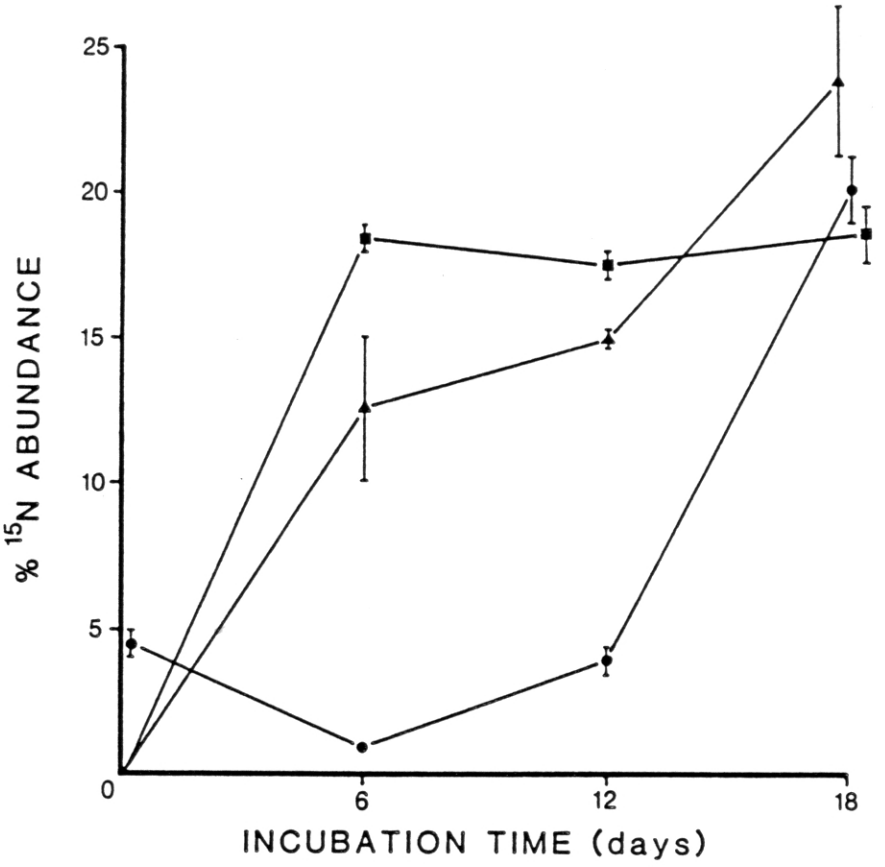

Figure IV-4. % ^{15}N abundance in [^{14}C]lignocellulose/wood sample incubations over time. Symbols: ■, organic nitrogen; ▲, inorganic nitrogen associated with particulate organics; ●, filtrate NH_4^+ N. Each point is the mean of ten replicates. Bars, Standard errors of the means.

DISCUSSION

The experimental results presented here represent the first use of a combination of stable and radioisotopes to examine the interaction of supplemental combined nitrogen with lignocellulose decomposition in laboratory incubations of stream wood samples. The data from the ^{15}N analyses demonstrate that the addition of NO_3^- N to laboratory incubations of stream wood samples and [^{14}C]lignocelluloses can lead to nitrogen accretion in the organic fraction (Figs. IV-2 and IV-3). Even though the organic substrate in this experiment consists mainly of lignocelluloses which are decomposed relatively slowly, the potential exists for rapid immobilization of nitrate from the surrounding medium. Absolute increases in nitrogen content of organic detritus have been reported, both from laboratory decomposition experiments and from weight-loss studies conducted *in situ* (Staaf, 1980; Berg and Staaf, 1981; Marinucci, 1982; Melillo *et al.*, 1982; Rice, 1982; Melillo *et al.*, 1983; Triska *et al.*, 1984). The mechanisms for this nitrogen increase are largely unknown, but have been suggested to include nitrogen fixation (Triska *et al.*, 1984), and microbial uptake of combined nitrogen from the surrounding environment (Melillo *et al.*, 1984). A third possible mechanism not often considered is abiotic surface charge phenomena such as anion and cation exchange. While the latter two pathways can only be speculated upon at this point, the acetylene reduction assays performed on replicates of our experimental treatments suggest that nitrogen fixation is certainly not a substantial source of combined nitrogen in this instance.

The most intense biological activity in the experimental treatments appears to occur during the first 6 days of incubation. The greatest rates of [^{14}C]lignocellulose degradation and respiration (as measured by CO_2 production), and all of the nitrogen uptake occur during this period (Figs. IV-1 and IV-2). Although surprising from the standpoint of lignocellulose recalcitrance to microbial decomposition, the rapid response to mineral nutrient supplementation agree with our previous observations of nitrogen and phosphorous enhancement of lignocellulose decay (Aumen et al., 1983; see Chapter III). Given the low concentrations of nitrogen in lignocelluloses and in the stream waters of the Pacific Northwest, external nitrogen supplementation could be expected to stimulate an initial pulse of microbial activity. The subsequent decline in activity observed in our studies and those of other investigators suggest that carbon limitation eventually becomes the controlling factor (Martin and Haider, 1980; Rice, 1982; Stevenson, 1982; Melillo et al., 1984). Further study using flow-through systems with continual additions of nitrogen are needed, however, to eliminate the nutrient depletion in our closed systems.

The increase in filtrate NH_4^+ enriched in ^{15}N toward the latter stages of incubation suggest that mineralization of the immobilized nitrogen is taking place (Fig. IV-2). If, in fact, the utilizable carbon sources become depleted as indicated by the decline in [^{14}C]lignocellulose decomposition and overall respiration, the

subsequent use of microbial carbon and release of immobilized nitrogen could increase. This possibility is supported by the observation of a decrease in the organic nitrogen fraction which coincided with the increase in filtrate NH_4^+ N between days 12 and 18. The potential for nitrification developing as filtrate NH_4^+ N concentrations increase is supported by the steady increase in ^{15}N abundance in the NO_3^- N associated with the particulate organic fraction.

Even though several different pathways of nitrogen processing can be inferred by the results of this experiment, a discrepancy exists in that 15% of the total nitrogen that was present at the beginning of the incubation period is unaccounted for at day 18. An apparent nitrogen loss such as this is typically explained by denitrification activity, yet our measurements of denitrification in replicate samples at days 6, 12, and 18 by the acetylene block technique suggest that an insignificant amount of nitrogen is lost as N_2. Although it is conceivable that the bulk of denitrification could have occurred during the initial several days of incubation before the filtrate NO_3^- N was depleted, several earlier denitrification studies in our laboratory on [^{14}C]lignocellulose/wood inoculum incubations under optimal denitrification conditions (high NO_3^- N concentrations, anaerobic incubations) also show insignificant losses of nitrogen (unpublished data). The percentage of ^{15}N unaccounted for at the end of this study (47%) was greater than the percentage of total nitrogen missing (15%).

This observation implies that the missing nitrogen might be sequestered in its original form of NO_3^- by the lignocellulose and wood substrate, and not recovered quantitatively by the procedures utilized in this study.

The appearance of ^{15}N enrichment in the filtrate NH_4^+ N fraction after only 1 h of incubation could result from dissimilatory reduction of NO_3^- to NH_4^+. This process has been reported in soils, aquatic sediments, and water, although usually under oxygen limited conditions (Buresh and Patrick, 1978; Koike and Hattori, 1978; Sørensen, 1978; Caskey and Tiedje, 1979; Samuelsson and Rönner, 1982). Even then, the process is usually responsible for only a small percentage of the total nitrogen flux, as was the case here (Tiedje et al., 1981; Smith et al., 1982; Kaspar, 1983). The results do indicate, however, that a microbial population capable of this pathway exists on decomposing wood substrates in stream environments.

The rapidity and efficiency of nitrate uptake exhibited by our laboratory systems is extremely interesting when considered from an ecosystem perspective. Stream water concentrations of nitrogen are low in Northwest streams, and are mainly in the form of NO_3^- (Triska et al., 1982). Large increases in NO_3^- N concentrations do occur during the high discharges in the fall, and might be related to the wetting and leaching of organic matter stored along the stream banks. Instead of these occasional pulses of NO_3^- being lost to the stream community, the presence of extremely large accumulations of

woody debris, along with their resident surface microflora, could serve as a rapid sink for this nitrogen. Whether the mechanism of nitrogen uptake is biotic or abiotic, the increase could provide for carbon release from lignocelluloses and the eventual slow release of NH_4^+ N through mineralization for use by other micro and macroorganisms. While these possibilities are interesting, they point to the need for further experimentation, both in the laboratory and in the field, particularly with measurements of nitrogen cycling and being made over shorter time intervals.

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