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Presence and ecological role of nitrogen-fixing bacteria associated with wood decay in streams

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With 2 figures and 3 tables in the text

Introduction

In the Cascade Mountains of the Pacific Northwest, virgin forests of Douglas-fir *(Pseudotsuga menziesii)* provide large inputs to both terrestrial and aquatic environments as giant trees are felled by wind, age and disease. In small streams where these wood inputs are not readily exported, wood storage can amount to 0.74 metric tons/m of channel (FROEHLICH 1973). Although large boles dominate the standing crop of organic debris, finer branches, twigs and bark can constitute a larger annual input. This finer wood input is also processed more rapidly by the decomposer community. Recent findings of nitrogen fixation associated with wood in the terrestrial environment (CORNABY & WAIDE 1973; SHARP & MILLEBANK 1973; AHO et al. 1974; SHARP 1975) raised questions concerning a similar role for N fixation in the mineralization of wood from lotic environments. Even low fixation rates could exert a controlling influence in wood decomposition since the C/N ratio of wood is high (235 for twigs; 324 for bark; 701—1343 for Douglas-fir heartwood) and because small Cascade streams often have low dissolved N concentrations (FREDRIKSEN 1972). To evaluate the potential role of N fixation on wood in old-growth forest streams, a study was conducted at a second order channel (Watershed 2) in the H. J. Andrews Experimental Forest. The study had three objectives:

1) to determine if N fixation was occurring on fine wood debris;
2) to isolate N-fixing organisms, conduct preliminary identification, and document their capacity to fix N in pure culture;
3) if fixation occurred at detectable levels, to determine its potential role as a N input to a small watershed stream.

Study site

The study was conducted in the H. J. Andrews Experimental Forest, a 6000 ha watershed in the western Cascades of Oregon, U. S. A. (Fig. 1). Watershed 2 (WS 2) in this drainage covers 60 ha and rises from 525 m elevation at the gauging station to 1060 m at its highest point (ROTHACHER et al. 1967). The stream channel at WS 2 consists of an 1100 m network which rises 460 m from the gauging station. Overall slope of the watershed is 60 %. Recorded stream discharge has varied from a low of 3.68×10^{-4} m³/sec during October 1972 to a high of 0.99 m³/sec during December 1964. The channel is controlled by bedrock with some pools and gravel areas. Both pools and gravel areas are formed primarily behind accumulations of wood debris, except at an alluvial fan downstream from the gauging station. Mean annual precipitation is 225 cm. Temperature range is between 1 and 18 °C. Monthly mean temperature is typically above 10 °C from mid May through September.

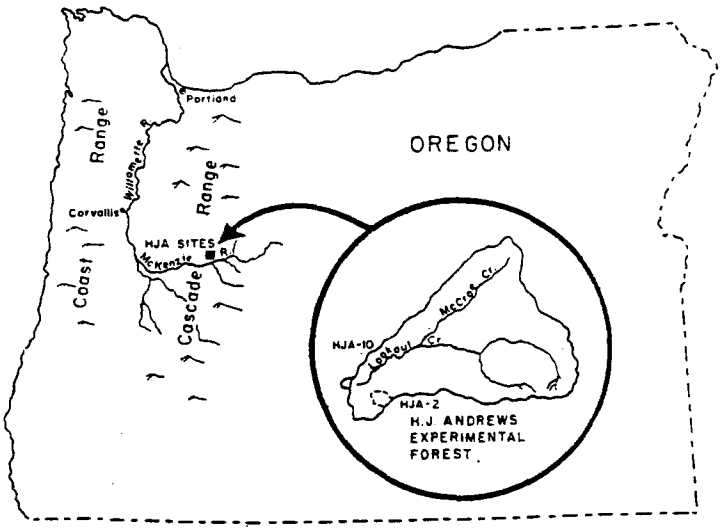

Fig. 1. Map of the H. J. Andrews Experimental Forest indicating the study area of Watershed 2.

Materials and methods

N fixation rates were estimated by the acetylene reduction method (STEWART et al. 1967; POSTGATE 1972). Four Douglas-fir wood substrate types were used in the study: heartwood blocks, twigs, bark and wood chips. Wood substrates were dried (50 °C), weighed and placed into litter bags (800 μm mesh nylon). Bags were incubated in the stream at WS 2 and removed seasonally. Upon collection, the sample was divided in half and placed in jars fitted with serum stoppers. Excess water was excluded from these samples to minimize aqueous and vapor phase problems (FLETT et al. 1976). One-tenth atmosphere of the treatment jar was replaced with acetylene generated in the field from calcium carbide. The second jar served as a control. Acetylene gas samples were also collected as a control for ethylene contamination. Samples were either incubated in the stream for 24 hours or returned to the laboratory in a styrofoam cooler filled with streamwater and incubated for 24 hours at the temperature of the stream. Following incubation, a gas sample was removed and stored in a non-sterile vacutainer (SCHELL & ALEXANDER 1970) for later analysis by gas chromatography. Ethylene was measured on a F and M Scientific gas chromatograph, at ambient temperature using a Porpak R six-foot column. The theoretical ratio of three moles acetylene reduced to 1 mole N fixed was used as a conversion factor (BERGERSEN 1970). Tissue samples were dried at 50 °C and weighed for conversion of results to a fixation rate/gram basis.

In addition to field estimates of N fixation, isolations for N-fixing bacteria were also conducted. Samples collected at Watershed 2 were returned to the laboratory, placed in Tris buffer and macerated for 15 seconds on a Waring blender. The resultant mixture was inoculated in dilution series to 10^{-6} into H-tubes (Fig. 2) containing a N-free media (HINO & WILSON 1958). The media was modified slightly as recommended by AHO et al. (1974). Following inoculation, the H-tubes were purged free of oxygen by bubbling ultrapure N gas through the system for 15 minutes. Alkaline pyrogallol on the adjacent arm of the H-tube scrubbed out residual oxygen and served as a check for atmospheric oxygen contamination by a color change from amber to black. The overall procedure selected for facultative anaerobes. H-tubes were incubated at room temperature for 10 days. Cultures were then injected with a 10 % acetylene

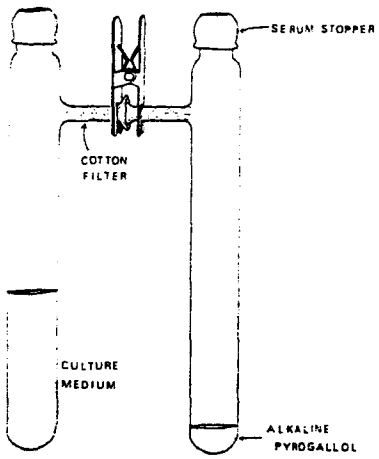

Fig. 2. Modified H-tube used for isolation of nitrogen-fixing bacteria (after CAMPBELL & EVANS 1969).

atmosphere and gas samples were removed for analysis after 24 hours. Analysis procedures were identical to field samples except that direct injection into the gas chromatograph was employed wherever possible. Positive cultures in the H-tubes were further purified for preliminary identification using standard taxonomic sources (BUCHANAN & GIBBONS 1974; LENNETTE et al. 1974) and then rechecked for acetylene reducing ability.

To estimate the role of N fixation in a small old-growth forest stream, results from this study were extrapolated to Watershed 10, a nearby stream where the standing crop of wood had previously been determined (FROEHLICH et al. 1972). Their determination conducted by line transect methodology resulted in an estimate of 1.73 kg/m² of fine wood debris (1—10 cm) when corrected for the immediate stream channel. N contribution by fixation was based on the acetylene reduction estimates of wood blocks from this study. Since the method of FROEHLICH concentrated primarily on branch material, an additional wood component of 0.86 kg/m² must be added to consider the finest twigs, bark pieces and wood within the sediments (TRISKA unpubl. data). This latter determination was made by coring the stream bottom. Samples were sorted by size and substrate type, equivalent to those employed in this study. Rates were extrapolated by calculating a summer mean (March—September) and a winter mean (October—February). Estimates of fixation were not applied to wood in excess of 10 cm in diameter, or for small particulates less than 4 mm in diameter.

Results and discussion

Some nitrogenase activity was observed from all four substrate types incubated in Watershed 2 (Table 1). Highest rates of acetylene reduction were observed in twigs and chips, probably due to higher surface to volume ratio. Denser blocks and bark exhibited the lowest rates of acetylene reduction. Rates of acetylene reduction were generally highest April—September, the warmest months of the year, although rates were also high during March, 1976 on chips and twigs. Major seasonal differences are attributed to temperature since the nitrogenase enzyme system is inhibited at colder temperatures, particularly below 5 °C (PAUL 1975). H-tube isolations in dilution series largely confirmed the field data (Table 2). For example, at 187 days when acetylene reduction rates were high in field samples, they were also high in H-tubes. At 247 days, when no activity or low activity was observed on field samples, enrichment for N-fixing bacteria

Table 1. Acetylene reduction rates (n moles/g/day) for wood substrates incubated in Watershed 2, H. J. Andrews Experimental Forest.

Date	4/1/75	6/1/75	9/3/75	11/3/75	3/17/76	5/19/76
Days in Place	31	94	187	248	383	446
Twigs	713	1104	2827	31	1820	108
Bark	274	112	405	—	152	80
Chips	172	227	286	—	1151	4066
Blocks	—	451	105	—	—	—

also failed to produce positive results. Acetylene reduction rates from the stream at Watershed 2 are somewhat higher than the few available rates from decomposing wood in terrestrial systems. CORNABY & WAIDE (1973) estimated acetylene reduction rates of 30.5 n moles ethylene/gram dry wt/day on decomposing chestnut logs. SHARP & MILLIBANK (1973) report 73.6 n moles ethylene/day on beech cubes (1 cm^3) after 20 days of soil contact with 0.5 % glucose amendment. Oak and beech veneers which approximate the chip samples in this study had rates of only 0.86 and 4.97 n mole/day, respectively, after 20 days of soil contact with no glucose amendment. Weight of wood samples were not provided in the latter study. More recently, SHARP (1975) reported ethylene production rates of 28—720 n moles/day from 1 cm^3 of Douglas-fir. Various faces were sealed and cubes placed in soil contact. Assuming a density of 0.54 g/cm^3 (MACMILAN pers. comm.), the latter data are more in the range of our results.

Preliminary culture work from chips and twigs has thus far produced two isolates of *Enterobacter agglomerans* (EWING & FIFE 1972) and six isolates of related *Enterobacter* sp. (Table 3). The finding of *E. agglomerans* is especially significant since this N-fixing species has previously been isolated from decaying wood of white fir (AHO et al. 1974). In addition, PETRIKUS & BREZNAK (1977) have isolated *E. agglomerans* from the gut of termites and found it an important

Table 2. Acetylene reduction rates (n moles/10 ml culture/day) in anaerobic HINO-WILSON media from incubated wood substrates.

Substrate	Dilution Days in Place	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Twigs	187	47,789	41,242	63,168	67,929	144,469	137,541
	248	1,144	—	—	—	—	—
	383	4,977	15,233	8,955	5,543	—	—
Bark	187	6,741	411	—	515	589	—
	248	219	—	—	—	—	—
	383	10,685	14,392	12,204	—	—	—
Chips	187	162,752	123,424	73,667	105,069	67,715	34,695
	248	—	—	—	—	—	—
	383	9,669	11,804	6,579	—	5,688	—
Blocks	187	50,360	59,525	68,977	134	1,775	—
	248	—	—	—	—	—	—
	383	—	7,298	—	—	—	—

factor in the termite
established in wood
In the cultures of
at the rate of 28,281
and 17,000 n moles
Enterobacter sp. in

Table 3. Identification s
wood substrates.

Classification	Acetylene reduction Rate n moles/culture/day
Enterobacter agglomerans	28,281
Enterobacter sp.	17,000
Enterobacter sp.	4,300

of AHO et al. (1974) w
day for other wood d
To assess the role
field rates attained in
10, as described previ
N-fixing activity on
0.70 grams N/m^2 strea
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Acetylene reduction
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When results of field d
standing crop are know
N may play an importa
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factor in the termite N economy. Thus, the role of this N-fixing species is now established in wood processing from both terrestrial and aquatic environments.

In the cultures of *E. agglomerans* isolated in this study, acetylene was reduced at the rate of 28,281 n moles/culture/day in HINO-WILSON media. Rates of 4,300 and 17,000 n moles/10 ml culture/day have been recorded from the other *Enterobacter* sp. in the same media. The latter rates are comparable to those

Table 3. Identification scheme for fermentative, gram-negative bacteria from incubated wood substrates.

Classification	Acetylene reduction Rate n moles/culture/day	No. of isolates	Triple sugar iron agar	Indole	Methyl red	Voges-proskauer	Citrate	Motility	Lysine decarboxylase	Ornithine decarboxylase	Arginine dihydrolase	Phenylalanine deaminase	Urease	Gelatin	Gram stain	Type of substrate
Enterobacter agglomerans	28,281	2	A/A+G	—	—	+	+	+	—	—	—	+	—	—	G—	Chips Twigs
Enterobacter sp.	17,000	5	A/A+G	—	—	+	+	+	—	—	+	+	—	—	G—	Twigs Chips
Enterobacter sp.	4,300	1	A/A+G	—	±	+	+	+	+	+	+	—	—	—	G—	Twigs

of AHO et al. (1974) who found rates between 497—9507 n moles/10 ml culture/day for other wood decaying species of the same genus in HINO-WILSON media.

To assess the role of N fixation as an input to a small wood choked stream, field rates attained in our study were extrapolated to a nearby stream, Watershed 10, as described previously. Based upon field estimates of acetylene reduction, N-fixing activity on the total standing crop of wood debris is estimated at 0.70 grams N/m² stream channel. This is a significant input when compared to other major litter inputs. For example, annual needle litter input contributes 0.90 g N/m² and deciduous leaf litter contributes 0.45 g N/m² (TRISKA unpubl. data).

Summary and conclusion

Acetylene reduction was observed on all four substrate types from Douglas-fir incubated on WS 2. Rates of ethylene formation were highest on substrates with high surface to volume ratio (chips and twigs) and lower on denser substrates (wood blocks and bark). Rates were generally highest during warmest months of the year, April—September, and very low during cold, winter months. Isolates from field samples in selective media confirm the presence of N-fixing organisms. *Enterobacter agglomerans*, a N-fixing bacteria on wood in terrestrial environments, was also isolated from WS 2. When results of field data were extrapolated to WS 10, a stream whose N inputs and standing crop are known, fixation was a potentially significant input. This fixation of N may play an important role both in the decomposition of wood in lotic systems and in the N economy of old-growth forest streams of the Pacific Northwest.

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