Nitrogen fixation and acetylene reduction in decaying conifer boles: effects of incubation time, aeration, and moisture content

WARWICK B. SILVESTER,[1] PHILLIP SOLLINS, THOMAS VERHOEVEN, AND STEVEN P. CLINE
Department of Forest Science, Oregon State University, Corvallis, OR, U.S.A. 97331

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Free-living microaerophiles fixed $^{15}N_2$ and reduced acetylene in fallen tree boles at two old-growth *Pseudotsuga menziesii* stands in western Oregon. Acetylene reduction was most rapid under an atmosphere of 2–10% O_2, whereas under prolonged anaerobic conditions it was at or below detection limits. Acetylene reduction rates increased up to fourfold during long-term incubations in acetylene (> 12 h). Ratios of acetylene reduction to N_2 fixation frequently exceeded 6.0 during such long-term incubations but averaged 3.5 when samples were incubated < 7 h; consequently, long-term incubation of low-activity material in acetylene should be avoided. A preliminary survey indicated that N_2 fixation by free-living organisms in fallen boles was less than other potential N inputs to fallen boles and to the forest ecosystem.

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Des organismes micro-aérophiles autonomes ont fixé $^{15}N_2$ et ont réduit l'acétylène dans des troncs d'arbres renversés chez deux peuplements âgés de *Pseudotsuga menziesii* en Orégon occidental. Ayant atteint sa rapidité maximale dans un milieu contenant de 2 à 10% d'O_2, la réduction de l'acétylène a diminué, dans des conditions anaérobiques prolongées, à un niveau égal ou inférieur aux limites de détection. La capacité de réduction de l'acétylène se quadrupla durant des incubations prolongées (> 12 h) dans de l'acétylène. Les rapports de la réduction d'acétylène à la fixation de N_2 ont dépassé fréquemment la valeur 6,0 durant ces incubations prolongées mais se sont situées autour de 3,5 quand l'incubation des échantillons n'a pas dépassé 7 h; on devrait donc éviter une incubation prolongée dans l'acétylène d'organismes à activité restreinte. Un inventaire sommaire a montré que la fixation de N_2 par des organismes autonomes dans des arbres tombés s'avère inférieure à d'autres moyens potentiels d'enrichissements en N dans les arbres tombés et dans l'écosystème forestier.

[Traduit par le journal]

Introduction

Decaying wood is a conspicuous feature of many forests, especially the temperate coniferous forests of the Pacific Northwest, where amounts of coarse woody debris (> 15 cm diameter) can exceed 500 Mg/ha and can account for 10–25% of the total biomass (Franklin and Waring 1980). Coarse woody debris is a major input to the forest floor in both mature and old-growth stands of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), often exceeding input from leaf fall (Sollins 1982).

Decomposition of this woody material is slow, in part because nutrients such as N and P are present at very low concentrations (Cowling and Merrill 1966). Some nutrients presumably enter the wood in litter fall and crown wash, which strikes fallen boles and penetrates the wood through cracks and insect galleries (Grier 1978; Sollins *et al.* 1980). Wood-invading fungi lack N_2-fixing ability and obtain needed N by decomposition of a large wood volume; however, N_2 fixation by free-living bacteria could provide an important N input to decaying logs.

Nitrogenase activity has been demonstrated in decaying fallen wood at a number of sites (Cornaby and Waide 1973; Sharp and Millbank 1973; Todd *et al.* 1975; Larsen *et al.* 1978; Roskoski 1980); however, its significance remains unclear. For example, $^{15}N_2$ fixation was demonstrated in only two studies involving woody material. In one (Sharp 1975), rates of N_2 fixation and acetylene reduction were not compared. In the other (Roskoski 1980, 1981), a series of 31 samples was incubated first with $^{15}N_2$, then with acetylene; however, molar ratios of N_2 fixed to acetylene reduced were highly variable. The range for the 26 samples that reduced acetylene was 0–20 (mean = 5.0, SE = 1.2); four additional samples fixed N_2 but did not reduce acetylene (ratio = ∞). Thus, the relation between measured nitrogenase activity and actual N_2 fixation remains tentative.

Furthermore, the proper conditions for nitrogenase assay in fallen wood have not been thoroughly investigated. Most workers have incubated samples anaerobically for about 24 h (e.g., Cornaby and Waide 1973; Todd *et al.* 1975; Larsen *et al.* 1978), although

[1]Permanent address: Biology Department, University of Waikato, Hamilton, New Zealand.

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Roskoski (1980, 1981) used a 24-h aerobic incubation. To our knowledge, however, no one has investigated the effect of incubation atmosphere on nitrogenase activity in decaying wood. Effect of incubation time is also unstudied, despite widespread acknowledgement that long-term incubations in acetylene can cause derepression of nitrogenase synthesis, thus artificially stimulating acetylene reduction and causing the ratio of acetylene reduction to N_2 fixation to increase with time (David and Fay 1977). It thus seemed desirable to investigate the conditions affecting N_2 fixation and nitrogenase activity in decaying wood to develop an accurate assay. Results of that investigation are reported here.

Materials and laboratory techniques

Study sites

Fallen boles were sampled at two stands of old-growth Douglas-fir. One site was located at 500 m elevation along Lookout Creek at the H. J. Andrews Experimental Ecological Reserve (HJA) in the Oregon Cascade Range. (A similar old-growth stand about 1 km north (WS-10) was the focus of intensive studies by the Coniferous Forest Biome, US/IBP (Grier and Logan 1977; Sollins *et al.* 1980).) A second site was located at 1200 m elevation along the Woods Creek Rd. on Marys Peak in the Oregon Coast Range about 15 km southwest of Corvallis. These old-growth stands consist of large Douglas-fir 300–500 years old with varying amounts of western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and several other coniferous and broad-leaved species in the overstory and understory (Franklin *et al.* 1981).

Decay classification

Decay classes of fallen boles are based on a system developed by R. Fogel, M. Ogawa, and J. M. Trappe (unpublished data): class I, bark and all wood intact, fine twigs attached; class II, bark and heartwood intact but sapwood partly soft, twigs absent; class III, bark loosened, sapwood decayed but still present, heartwood structurally sound; class IV, sapwood partly to totally deteriorated and often sloughed off, heartwood not structurally sound; and class V, wood largely fragmented, forming an ill-defined, elongate mound on the forest floor. Details of the classification system are provided by Triska and Cromack (1980), Franklin *et al.* (1981), and Sollins (1982).

Acetylene reduction assays

Wood was removed from fallen logs in circular cores or as bulk pieces and transported the same day to the laboratory, where it was stored in an incubator at field temperature. Acetylene reduction was assayed by placing the material in glass jars (50–500 cm^3) fitted with septum-capped lids. A known volume of acetylene, generated by action of water on calcium carbide, was added to each jar to bring it to 10% C_2H_2. All jars were incubated at 22°C in the laboratory unless otherwise noted. Ethylene production was measured with a Hewlett Packard 5830A gas chromatograph; acetylene was used as the internal standard. Both endogeneous C_2H_4 production and background C_2H_4 levels were checked regularly; rates reported here are net C_2H_2 reduction rates after subtraction of background levels and endogenous rates. After acetylene reduction was measured, samples were oven-dried to determine moisture content and dry weight.

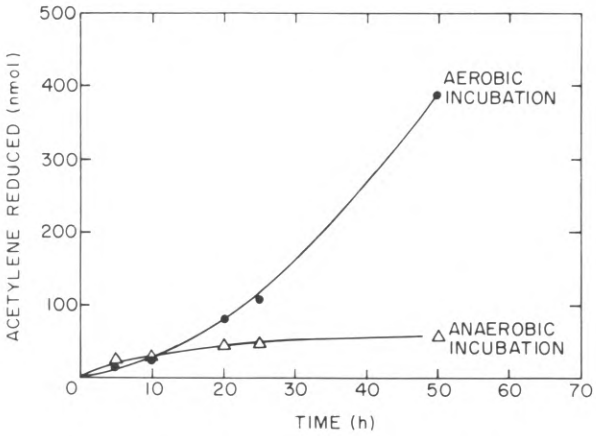

FIG. 1. Cumulative amount of acetylene reduced in decayed Douglas-fir heartwood. Each point is mean of six samples. Material collected 27 March 1980 at HJA from class III bole that fell ca. 1932.

For anaerobic assays, jars were flushed for 10 min with a stream of N_2 via an 18-gauge syringe needle inserted through the septum seal. At least 20 volumes of N_2 were flushed through each jar.

$^{15}N_2$ uptake

Gas containing 95 atom % excess ^{15}N was obtained from Isomet Co., mixed with oxygen to give an O_2 concentration of 20%, and stored over water. Wood samples were placed in 13- or 130-cm^3 jars, which were evacuated for 30 s, and then a slight positive pressure of gas mixture was added by syringe to each bottle. Because the above process can result in contamination with air, each completed jar was tested for ^{15}N and O_2 concentrations by removing 0.2 cm^3 of gas and analyzing it on a mass spectrometer (Associated Electrical Industries MS10). Oxygen content was adjusted to the desired value, and a gas sample was removed for isotopic analysis.

After incubation in $^{15}N_2$ for 42–48 h, each wood sample was oven-dried and finely ground. Duplicate 200-mg aliquots were placed in micro-Kjeldahl flasks along with 3 mL concentrated H_2SO_4, 1 g K_2SO_4, 30 mg $CuSO_4$, and 20 mg SeO_2, and digested at 360°C until the solution had been clear for 1 h. Concentrated NaOH was added; then the NH_3 was distilled from the digestate and trapped. The resulting solution was titrated immediately with H_2SO_4, further acidified, concentrated by evaporation, and placed in small vials for ^{15}N analysis.

N isotope composition was determined at Los Alamos Scientific Laboratories with an automatic isotope-ratio mass spectrometer (McInteer and Montoya 1981). ^{15}N enrichment was calculated in relation to a measured reference value of 0.366% obtained for three samples of untreated wood.

Experiments and results

Our first experiment was designed to investigate the

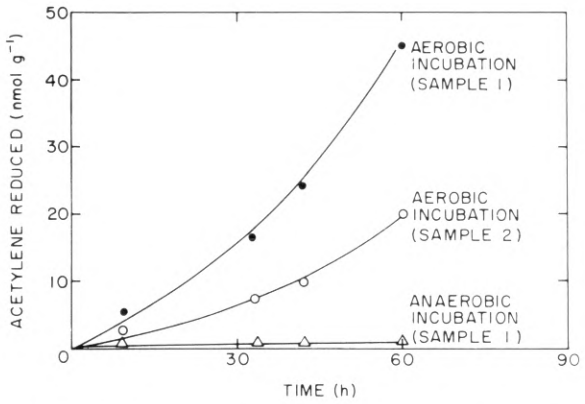

FIG. 2. Cumulative amount of acetylene reduced in decayed Douglas-fir heartwood crushed into fine fragments (<5 mm). Each point is mean of four replicate samples. Material was the same as in Fig. 1. Sample 2 contained denser heartwood than sample 1.

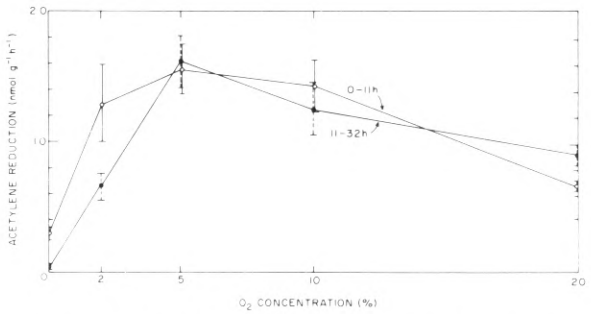

FIG. 3. Effect of oxygen concentration on acetylene reduction rate in decayed Douglas-fir sapwood. Each point is mean of five replicates (SE shown as error bars). Material obtained 20 May 1980 from class II bole at Marys Peak.

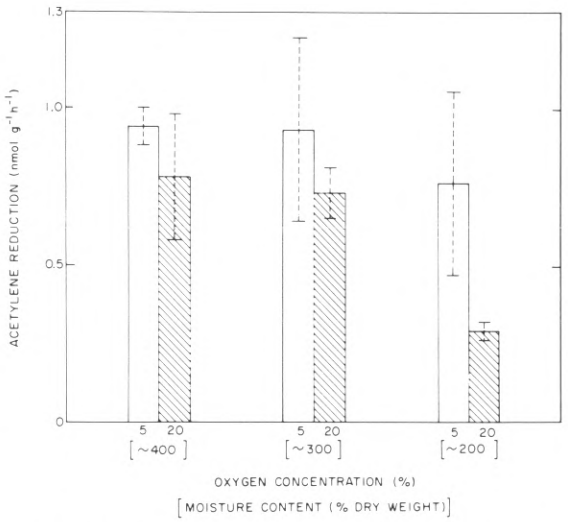

FIG. 4. Effects of moisture content and oxygen concentration on acetylene reduction rate in decayed Douglas-fir sapwood. Values are means of three replicates with SE shown as error bars. Material collected 3 June 1980 from class II fallen bole at Marys Peak.

course of acetylene reduction during aerobic and anaerobic incubation in 10% acetylene. In this experiment, the acetylene reduction rate, initially 0.14 nmol g^{-1} h^{-1}, had increased fourfold after 50 h (Fig. 1). This initial experiment also showed much higher nitrogenase activity during aerobic than during anaerobic incubations.

Because large pieces of wood were used in the first experiment, we were concerned that anaerobic microsites might have been present within the samples. If so, then facultative anaerobes, such as had been isolated previously from decay zones in live trees, might have been responsible for the measured nitrogenase activity. Accordingly, some of the material not used in the first experiment was fragmented into pieces less than 5 mm thick to facilitate gas diffusion; the wood was then incubated at 22°C in 10% acetylene with and without oxygen. Under these conditions, the acetylene reduction rate was well maintained in air but ceased almost immediately under pure N_2 (Fig. 2).

Effects of O_2 concentration were investigated further by incubating wood in 10% acetylene at four subambient O_2 levels. During the initial 11 h of incubation, the acetylene reduction rate was maximum at 5% O_2 (Fig. 3), an effect that became more pronounced during an additional 21 h of incubation. At 0% O_2 the rate was low during the first 11 h, decreasing to nearly zero during hours 11–32.

Two other lines of evidence indicated an aerobic diazotroph. First, anaerobic odors were never detected in any of the freshly collected or aerobically incubated wood samples but were readily detectable after 48-h anaerobic assays. Second, we were unable to stimulate nitrogenase activity by adding glucose, sucrose, or mannitol under anaerobic conditions. This last result helped rule out the possibility that the diazotroph was an anaerobe dependent on wood-decaying fungi for soluble carbohydrate and that the latter were inactive under anaerobic conditions.

Moisture content typically affects gas diffusion through porous media. Wood collected in the field was moist but not saturated, and we anticipated that water content might strongly influence O_2 diffusion and, therefore, response to the incubation atmosphere. This possibility was tested by incubating wood in acetylene at ambient and subambient O_2 levels for 17 h at three different moisture contents: saturation (ca. 400% dry weight), field capacity (ca. 300%), and moist (ca. 200%). At the two wetter levels, acetylene reduction

TABLE 1. Rates of $^{15}N_2$ uptake and N_2 fixation in decaying Douglas-fir boles

Sample[a]	Subsample	Dry weight (g)	N (%)	^{15}N excess (atom %)	^{15}N uptake (μg)	N_2 fixation[b] (ng $N\ g^{-1}\ h^{-1}$)
1	Light sapwood	12.4	0.09	0.013	1.46	2.80
	Dark sapwood	1.8	0.14	0.013	0.33	4.37
	Fungal mycelium	0.26	1.11	0.0	0.0	0.0
	Frass	2.39	0.15	0.019	0.69	6.87
						$\bar{X}$ = 3.50
2	Sound heartwood	8.24	0.09	0.013	0.98	2.83
	Dark heartwood	2.10	0.22	0.009	0.40	4.54
	Dark sapwood	2.70	0.13	0.006	0.22	1.94
	Fibrous sapwood	0.09	0.03	0.049	0.12	31.75
	Frass	1.20	0.19	0.014	0.32	6.35
						$\bar{X}$ = 3.39

[a]Sample 1 was sapwood collected from a fallen class II bole at Marys Peak on 5 May 1980. Sample 2 was collected on 27 March 1980 from a class III bole at HJA that had been on the ground ca. 48 years.
[b]$\bar{X}$ gives weighted mean for entire sample.

TABLE 2. Molar ratio of acetylene reduction to dinitrogen fixation in decaying Douglas-fir boles

Acetylene reduction assay period (h)	Ratio (acetylene reduced : N_2 fixed)[a]	
	Sample pair 1	Sample pair 2
0–6	4.16	3.40
6–18	5.07	4.70
18–26	4.55	6.01
26–44	0.51	7.01
Total period	3.03	5.89

[a]Ratio calculated by dividing acetylene reduction rate for each period by mean rate of N_2 fixation over the entire 42-h period (Table 1).

TABLE 3. N_2 fixation and C_2H_2/N_2 ratio in decaying Douglas-fir boles at two oxygen concentrations

O_2 concentration (%)	C_2H_2 incubation period (h)	N_2 fixation (ng $N\ g^{-1}\ h^{-1}$)	C_2H_2 reduced / N_2 fixed
7.5	0–2.5	14.0(5.2)	3.16(0.51)
	2.5–7		3.24(0.37)
20	0–2.5	5.07(1.2)	4.36(0.54)
	2.5–7		3.33(0.62)

NOTE: Values are means of five replicates with SE shown in parentheses. All material was collected from one location on a class II bole at Marys Peak on 16 June 1980.

was only slightly inhibited at atmospheric O_2 levels (Fig. 4), whereas in the driest samples a 20% O_2 level clearly inhibited acetylene reduction.

The nonlinear accumulation of ethylene over time strongly suggested the need for an isotope experiment to confirm the actual rates of N_2 fixation. Two cores from two decayed boles were incubated in $^{15}N_2$ for 42 h. Samples were incubated at approximately constant temperature and in sealed containers so that moisture content was presumably constant; consequently, we have no reason to expect that N_2 fixation varied during the incubation period. At the end of the experiment each core was dissected and the components were analyzed separately for total N and ^{15}N. All of the components took up significant amounts of $^{15}N_2$ except for fungal mycelia collected from the cambial region (Table 1).

Two similar, undissected cores were incubated in 10% acetylene and 20% O_2 under identical conditions and assayed for acetylene reduction sequentially over a 44-h period. (The extra 2-h incubation under acetylene was unintentional but should not have affected results.) The ratio of acetylene reduction rate to mean rate of N_2 uptake increased markedly during the 44-h incubation period (Table 2), suggesting that acetylene reduction rates had increased with time. The ratio doubled in wood sample 2, while in sample 1 the ratio climbed, flattened, and then decreased abruptly as the acetylene reduction rate decreased. Of particular interest is the average ratio of 3.03 for sample 1 over the total 44-h time course. This value is remarkably close to the theoretically expected ratio of 3.0, but as is evident from the preceding data, it is an artifact and masks a complex and rapidly changing pattern.

In the preceding experiment, we assumed that the rate of N_2 fixation remained constant during the 42-h incubation and that the pairs of samples were comparable. The first assumption seemed reasonable given our objective of testing for a constant rate of acetylene reduction. Additional data seemed desirable, however, to establish the ratio of acetylene reduction to N_2 fixation

TABLE 4. Acetylene reduction rates typical of various components of Douglas-fir ecosystem

Species	Decay class	Residence time on forest floor (year)	No. of logs sampled	Material	No. samples analyzed	Acetylene reduction (nmol g^{-1} h^{-1})	
						Mean	SE
Douglas-fir	II	24	3	Sapwood	10	0.68	0.21
				Heartwood	19	0.03	0.01
Douglas-fir	III	46	2	Sapwood	3	0.18	0.11
				Heartwood	16	0.11	0.03
Douglas-fir	IV	119	1	Heartwood[a]	8	0.17	0.06
Douglas-fir	V	144	2	Heartwood[a]	9	<0.02	0
Western hemlock	II	12	2	Sapwood and heartwood	5	0.13	0.10
Western hemlock	III	18	1	Sapwood and heartwood	16	0.20	0.03
Douglas-fir snag	—	ca. 15	1	Sapwood and heartwood	16	<0.02	0
Douglas-fir snag	—	ca. 100	1	Heartwood[a]	16	0.04	0.01
Douglas-fir	—	<1	—	Fallen needles	20	7.5	2.5
Western hemlock	—	<1	—	Fallen needles	12	<0.02	0

[a]No sapwood present.

during the first 6–12 h. Consequently we designed a second N isotope experiment in which the same sample could be incubated first in $^{15}N_2$, then in acetylene. Simultaneously, we checked for an effect of O_2 concentration of $^{15}N_2$ uptake. In this second isotope experiment, wood samples were incubated at 22°C at 7.5 and 20% oxygen in the presence of $^{15}N_2$. After 47.5 h incubation in $^{15}N_2$, 10% acetylene was added to stop the biological uptake of $^{15}N_2$. Gas samples were removed after an additional 2.5 and 7 h and assayed for acetylene reduction. The results (Table 3) showed that when the incubation period in acetylene was kept short, the mean of the C_2H_2/N_2 ratios was 3.52 (SE = 0.28), close to the theoretically expected ratio of 3.0. N_2 fixation was 2.8 times greater at 7.5% O_2 concentration than at 20%, again suggesting that the diazotrophs are microaerophilic, but the response to O_2 concentration could have been due to chance ($p < 0.10$).

On the basis of the results presented above, we undertook a preliminary survey of acetylene reduction rates in coarse woody debris and fallen needles. Samples were incubated 6–8 h at field temperature under an atmosphere of 10% acetylene and 20% oxygen. (We used an atmosphere of 20% O_2 rather than 5% because we reasoned that logs were exposed to the former in the field. Clearly, data are needed on O_2 concentrations at the site of N_2 fixation.) Consistently high rates were found only in sapwood from decay class II boles (Table 4). Rates were variable; however, the standard error was about 30% of the mean with a sample size of 10. Lower rates were found in sapwood from class III Douglas-fir boles, in heartwood from class IV Douglas-fir boles, and in class II hemlock boles in which sapwood and heartwood could no longer be distinguished. Highest rates were found in fallen Douglas-fir needles, not in woody litter. Fallen hemlock needles showed essentially no nitrogenase activity.

Discussion

Methodology

Despite the heterogeneous nature of fallen wood and the uneven spatial and temporal distribution of microbial activity therein, several aspects of the methodology of assessing nitrogen fixation in such wood have been resolved in these experiments. First, because nitrogenase activity is strongly affected by O_2 concentration, anaerobic incubation is not recommended unless it is established in an initial survey that the diazotrophs are in fact anaerobes, not microaerophiles.

Second, rates of oxygen diffusion are strongly affected by moisture content of the material. It is thus important to take samples of reasonably large volume to reduce the surface area : volume ratio and thus prevent drying. For example, activity of very wet material will be largely unaffected by sample collection and incubation in air, whereas activity of drier material may be significantly reduced by such treatment.

Third, the time over which samples are incubated is critical, both because of the potential for oxygen depletion and because of the increase in nitrogenase activity during long-term incubation with acetylene. This increase was noted by David and Fay (1977), who suggested that, because acetylene inhibits N_2 fixation, amino N levels in the bacteria will be depleted, in turn stimulating activity and (or) synthesis of the nitrogenase enzyme. They warned that long-term incubations could produce gross overestimates, a warning that has received little attention. Our results show that long incubations can introduce an error of at least 100% into estimates of N_2 fixation. Our results also show that,

over a long incubation, a close approximation to the theoretically expected ratio of 3.0 can be fortuitous and need not indicate a satisfactory method. We recommend that all workers carefully investigate the effect of acetylene on nitrogenase activity and incubate low-activity material a maximum of 12 h.

Microbiology

Earlier work of Seidler *et al.* (1972) and Aho *et al.* (1974) focused attention on facultative anaerobes as agents of nitrogenase activity in decaying wood. Consequently, they and later workers (e.g., Cornaby and Waide 1973; Sharp 1975; Larsen *et al.* 1978) used anaerobic assays. While we do not doubt that facultative anaerobes capable of nitrogen fixation can be isolated from a wide variety of woody substrates, we found no evidence that such organisms were responsible for the activity we detected. We conclude from our physiological evidence that the diazotrophs we studied are obligate aerobes whose ability to reduce nitrogen is greatest at O_2 concentrations between 2 and 10%. We conclude that initial nitrogenase activity under anaerobic incubations was due to incomplete flushing of O_2 from the wood samples. Initially, the internal O_2 concentration was lowered close to the optimum and nitrogenase activity was stimulated. Later, the internal O_2 supply was exhausted and activity ceased.

Nitrogen cycling

Although logs were not selected systematically and the number sampled was small, we can calculate a probable maximum for N_2 fixation in decaying boles in an average stand of old-growth Douglas-fir. If we assume an acetylene reduction rate of 0.2 nmol g^{-1} h^{-1} (Table 4), a substrate mass of 100 Mg/ha, and a C_2H_2 reduction / N_2 fixation ratio of 3.5, then the N input amounts to 1.4 kg N ha^{-1} $year^{-1}$.

Other potential N inputs to logs are larger. Since fallen wood occupies 15–30% of the land area in these old-growth Douglas-fir stands, similar percentages of the needle fall and crown wash strike the fallen boles. Once the bark begins to fragment, the N in intercepted crown wash and that released in decomposition of the fallen litter can enter the sapwood. One might argue that N is not released immediately from the intercepted litter. However, Douglas-fir needles become totally unrecognizable in about 10 years (K. Cromack and R. Fogel, unpublished data), a short time in relation to the time fallen boles persist. Moreover, much of the N in fallen leaves is released within the first few weeks of decomposition (Berg and Staaf 1981; K. Cromack and R. Fogel, unpublished data). Thus, N from intercepted litter fall could constitute an important input to fallen boles. At a nearby stand, N in crown wash and non-woody litter fall amounted to 19.6 kg ha^{-1} $year^{-1}$ (Sollins *et al.* 1980). Fifteen to 30% of this would amount to between 3 and 6 kg N ha^{-1} $year^{-1}$, several times larger than the probable input to fallen boles from N_2 fixation.

It should be noted, however, that N_2 fixation could still be important in initiating wood decay. A careful study of the microfloral and faunal succession in relation to changes in physical and chemical structure of the wood is needed to determine the significance of N_2 fixation in decay of fallen boles.

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