

Lessons from the Sandbox: Is Unexplained Nitrogen Real?

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ABSTRACT

In their review of 24 studies of forest nitrogen (N) budgets, Binkley and others (2000) found that only one of them supported the conclusion that an N accumulation of more than 25 kg N ha⁻¹ y⁻¹ is possible without known symbiotic N₂-fixing plants. They contended that, given how well the N cycle is known, new N accumulation pathways are unlikely. They also concluded that the Hubbard Brook sandbox study (Bormann and others 1993) was insufficiently replicated and had low precision in vegetation and soil estimates. Here we reevaluate and extend the sandbox analysis and place the findings in a broader context. Using multiple methods of estimating vegetation N accumulation in pine sandboxes, we arrived at results that differed from the reported rates but still strongly supported large biomass N accumulation. The original study's conclusions about soil N changes were strengthened when new evidence showed that N accumulated in

lower horizons and that the sandboxes were successfully homogenized at the beginning of the experiment. Unexplained ecosystem N accumulation ranged from about 40 to 150 kg ha⁻¹ y⁻¹, with 95% confidence intervals that did not include zero. No evidence was found that could balance the sandbox ecosystem N budgets without adding unexplained N. Unreplicated experiments, such as the sandboxes, can explore the possibility that N can accumulate in ways not explainable by mass balance analysis, but they cannot quantify the frequency and extent of the phenomenon. New studies should combine substantive microbiological, mass balance, and process research using multiple direct measures of N₂ fixation.

Key words: unexplained nitrogen accumulation; lysimeters; mesocosms; ecosystem budgets; mass balance; nitrogen fixation.

INTRODUCTION

A vigorous debate has emerged about whether nitrogen (N) can accumulate at rapid rates (exceeding 25 kg ha⁻¹ y⁻¹) in ecosystems without known symbiotic N₂ fixers. The evidence for such rapid accumulation comes from studies of grass ecosystems lacking known symbiotic N₂ fixers, including those

by Whitt (1941), Chapman and others (1949), Karriker and others (1950), Smith and others (1954), App and others (1980), Lima and others (1987), and Cavalcante and Dobereiner (1988). The rapid accumulation of N has been reported in forest ecosystems as well (Dickson and Crocker 1953; Fisher and Eastburn 1974; Day and others 1975; Jenny 1980; Turvey and Smethurst 1988; Son and Gower 1992; Bormann and others 1993; Eriksson and Rosen 1994; Johnson and Todd 1998).

In a recent review of many of the forest studies,

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Binkley and others (2000) concluded that there is “no widespread evidence of high rates of occult [unexplained] N input in forests.” This conclusion appears to be based on their assessment of the experimental designs, the size of the experimental errors, the measures of inputs or outputs, and a belief that “the N cycle of forest ecosystems is understood relatively well.” We agree that skepticism is appropriate; however, skepticism should not lead to dismissal based on conventional wisdom.

Small-scale experimental mesocosm studies have a number of advantages over field experiments because they can control treatment effects, limit errors, and allow the measures needed for more complete mass balance analyses. The Hubbard Brook sandbox experiment was designed as a mesocosm study to examine whether pioneer plants that appear to grow well in N-poor environments but are not known to be symbiotic N₂ fixers, are somehow associated with unexplained N accumulations on an ecosystem basis. The researchers concluded that the evidence for an unexplained net N accumulation—of 50 kg ha⁻¹ y⁻¹ or more—was strong in two pine ecosystems (Bormann and others 1993).

How, then, did the Binkley review conclude that high error and poor design did not yield high confidence for rapid N accumulation in the sandbox study? In this paper, we analyze these conflicting conclusions by reevaluating the original data, examining sources of uncertainty in greater detail, and presenting previously unreported data. Alternative methods of estimating unexplained N are compared, and new results are placed in the context of our understanding of the N cycle.

BACKGROUND AND METHODS

The sandbox study was initiated in 1982 as an adaptation of the small-watershed approach to nutrient cycling, as applied to the cycling of N in experimental ecosystems (Bormann and others 1987). The study is described in detail in Bormann and others (1993) and summarized briefly by Binkley and others (2000).

Sandbox ecosystems were built in two sizes, 2.5 × 2.5 m wide and 7.5 × 7.5 m wide by 1.5 m deep, lined with Hypalon polymer landfill liner, and filled with screened, homogenized, low-N fluvial outwash sand from a glacial deposit in central New Hampshire. Various species were planted, each in its own box, including two pines—red (*Pinus resinosa* Ait.) and pitch (*Pinus rigida* Mill.)—European black alder (*Alnus glutinosa* (L.) Gaertn.), and black locust (*Robinia pseudoacacia* L.). In addition, a control “no veg” box was built in which vascular plants

were not allowed to grow. Outside buffers (three or six rows of seedlings) were planted to create uniform stand conditions by reducing edge effects inside the boxes. The sandboxes were allowed to settle for a year, and initial (t₀) soil samples were taken 1 year after planting, before the seedlings began to grow rapidly.

In an ecosystem without significant N₂ fixation, dry deposition, or denitrification, N accumulation in vegetation (ΔN_{veg}) and soil (ΔN_{soil}) is limited to net meteoric input—that is, input in bulk precipitation (N_{bp}) less loss in drainage (N_{dr}):

$$N_{bp} - N_{dr} = \Delta N_{veg} + \Delta N_{soil}$$

If the accumulation cannot be explained by net meteoric input, a term (N_{unexplained}) must be added to the lefthand side of the equation:

$$N_{bp} - N_{dr} + N_{unexplained} = \Delta N_{veg} + \Delta N_{soil}$$

which can be rearranged to give:

$$N_{unexplained} = \Delta N_{veg} + \Delta N_{soil} - N_{bp} + N_{dr} \quad (1)$$

The terms on the right side of Eq. (1) were evaluated by using standard analytical techniques (Bormann and others 1993) for samples representing conditions at t₀ and for samples collected 4 to 5 years later, representing final “5-year” conditions. Initial soil samples were collected using a cylindrical 5.3-cm-diameter soil corer. Because of the corer’s poor performance when roots were present, a steel box sampler was used for the final sampling of all soil under the growing space (the 0.5 × 0.5 m area under a sampled tree). Change in vegetation N was estimated as the sum of final root, litter, and aboveground biomass N—based on the sampling and processing of entire trees—less seedling N. An alternative method was also used to estimate N accumulation in vegetation aboveground: regressing N mass (kg ha⁻¹) on tree heights of sampled trees and extending the predictions to a sandbox with heights measured on all trees. Standard methods (Cochran 1977) were used to estimate a mean and confidence interval based on the regression. Rarely, a few small-diameter or missing trees resulted in a negative estimate of N mass; these were set to zero. We did not measure denitrification or dust inputs and assume these fluxes to be very small. The three- to six-tree outside buffers reduced dust inputs, and we saw little indication of extraneous litter.

We used an approximate formula for estimating confidence intervals for the sums of components added to calculate ecosystem unexplained N values,

Table 1. Original Estimates (Bormann and others 1993) and 95% Confidence Intervals for Components^a of Unexplained Nitrogen (N) Accumulation or Loss Estimated by Mass Balance

Sandbox	ΔN_{veg} (kg N ha ⁻¹ y ⁻¹)	$\Delta N_{soil(0-20)}$ (kg N ha ⁻¹ y ⁻¹)	N_{bp} (kg N ha ⁻¹ y ⁻¹)	N_{dr} (kg N ha ⁻¹ y ⁻¹)	$N_{unexplained}$ (kg N ha ⁻¹ y ⁻¹)
Alder	175 ± 12	86 ± 52	5	1 ^b	255 ± 56
Locust	52 ± 18	43 ± 60	5	1 ^b	90 ± 70
Red pine	83 ± 8	-17 ± 23	5	1	62 ± 34
Pitch pine	70 ± 40	-19 ± 25	5	1 ^b	49 ± 47
No veg	0	-96 ± 77	5	7	-94 ± 75

^aComponents: ΔN_{veg} and $\Delta N_{soil(0-20)}$ are changes in storage in vegetation and upper soil, N_{bp} is input in bulk precipitation, N_{dr} is loss in drainage, and $N_{unexplained}$ is the net unexplained N needed to balance the equation ($N_{unexplained} = \Delta N_{veg} + \Delta N_{soil(0-20)} - N_{bp} + N_{dr}$).

^bDrainage for alder, locust, and pitch pine was based on the estimate from a red pine sandbox.

following Bormann and others (1993). This formula takes the square root of the sum of the squared 95% half-intervals for vegetation and soil N values (Meyer 1975). Interval estimates for sums have two possible complications: They assume no covariance (which could increase or decrease the intervals), and they do not account for unequal sample sizes (L. Ganio personal communication). The data suggest that there is little correlation between aboveground vegetation and soil N estimates, likely because the roots extend far beyond the 0.5 × 0.5 m space sampled beneath each tree.

The results of the mass balances, as reported in 1993, are summarized in Table 1. For reasons discussed below, accumulation in soil was based only on analyses of the upper 20 cm. Our reassessment of the sandbox data centered on checking the original calculations, reevaluating sources of uncertainty, devising ways to lower estimates of accumulations, and reevaluating the scope of our inferences. By these means, we tried to find, in the same spirit of skeptical assessment expressed by Binkley and others (2000), any reasonable grounds to overturn our original conclusions.

RESULTS

Binkley and others (2000) focused on vegetation and soil N pools in the red pine and pitch pine ecosystems. Our reexamination has a parallel focus.

N in Vegetation

Binkley and others (2000) noted that there were large, within-plot confidence intervals associated with the 1993 estimates of aboveground pine biomass. On rechecking, we found an error in the 1993 confidence intervals for aboveground biomass and litter, which were shown with 99% intervals, not

95% intervals as stated in the text. The actual means and 95% intervals for 5-year aboveground N accumulation are 63 ± 34 kg ha⁻¹ y⁻¹ in red pine and 45 ± 21 kg ha⁻¹ y⁻¹ in pitch pine (Table 2). This error was not found in any of the other mass balance components reported, and it was not carried to the 1993 calculation of intervals for ecosystem N accumulation.

Binkley and others (2000) also expressed concern about the sample size, especially for the small red pine and alder sandboxes containing 16 trees planted at a 0.5-m spacing inside of three outside-buffer rows. We evaluated the effect of tree position on height in the small red pine and alder sandboxes. The 12 inside-box buffer trees in the red pine sandbox were 14% shorter than the central four trees but were not significantly different ($P = 0.31$); the opposite pattern was observed in the alder sandbox, where the central four trees were 10% shorter than the 12 inside-box buffer trees but not significantly so ($P = 0.24$).

We sought an alternative estimate of aboveground red pine biomass N based on a regression of aboveground N mass (kg ha⁻¹) on height (cm) of sampled red pine trees:

$$\text{Aboveground red pine N} = -383.1 + 4.448 \cdot \text{height} \quad (2)$$

We used this method to calculate N in all interior trees. The regression produces an estimate and 95% confidence interval for aboveground N accumulation of 55 ± 22 kg N ha⁻¹ y⁻¹, which differs from the original estimate of 63 ± 34 kg N ha⁻¹ y⁻¹ (Table 2). A single dead tree in the buffer contributed strongly to the lower estimate of the regression-based mean. The confidence interval is smaller, even though the slope coefficient is not

Table 2. Original and Revised Estimates of Annual N Change and 95% Confidence Intervals for Aboveground Vegetation and Soil in Pine Sandboxes^a

Mass Balance Components	Red Pine		Pitch Pine	
	1993 Study ^b (kg N ha ⁻¹ y ⁻¹)	This Study (kg N ha ⁻¹ y ⁻¹)	1993 Study ^b (kg N ha ⁻¹ y ⁻¹)	This Study (kg N ha ⁻¹ y ⁻¹)
ΔN_{veg} aboveground ^c	63 ± 34		45 ± 21	
Regression mean		55 ± 22		36 ± 7
Weighted mean				44 ± 22
Unweighted mean				45 ± 21
NonADJ trees mean ^d				42 ± 22
ΔN_{soil} ^e				
Upper (0–20 cm)	-17 ± 23	-28 ± 18	-19 ± 25	-9 ± 13
Lower (20–135 cm)	0	98 ± 11	0	73 ± 9
Total soil	-17 ± 23	70 ± 21	-19 ± 25	64 ± 16

^aAnnual rates can be multiplied by 5.2 years for red pine and 5.3 years for pitch pine vegetation and 3.9 years for soils to obtain N changes between the beginning and end of the experiment.

^bFrom Bormann and others (1993).

^cThese values do not include belowground biomass and litter as in Table 1.

^dNonADJ trees are trees not adjacent to hybrid or missing-tree cells.

^eUpper- and lower-soil changes are shown and compared to other sandboxes in Figures 1 and 2.

significant ($r^2 = 0.33$, s.e. $\beta_0 = 386$, s.e. $\beta_1 = 2.39$, $n = 6$, $P = 0.14$).

For the large pitch pine sandbox, alternative estimates for aboveground N accumulation are motivated by an accidentally introduced source of uncertainty. Seedling mortality in the 1st year, and accidental replanting with pitch loblolly hybrids, led Bormann and others (1993) to divide the population into three groups with various numbers of individuals (n): cells with younger trees, planted where hybrids were removed (two); cells with trees adjacent to hybrid cells, which may have benefited from more growing space (nine); and cells with trees not adjacent to hybrid cells (37). Initially reported aboveground N mass (Table 2) was based on weighted averages for trees randomly selected from the adjacent and nonadjacent populations.

We sought an alternative estimate of aboveground pitch pine biomass N based on a regression of aboveground N mass (kg ha⁻¹) on height (cm) of sampled pitch pine trees:

$$\text{Aboveground pitch pine N} = -624.5 + \text{height} \cdot 5.195 \quad (3)$$

We then extended it to all trees in the population of 84 cells ($r^2 = 0.91$, s.e. $\beta_0 = 88$; s.e. $\beta_1 = 0.50$; $n = 12$, $P < 0.001$). The regression approach reduces the estimate of average N accumulation in pitch pine aboveground from 45 ± 21 to 36 ± 7 kg ha⁻¹ y⁻¹ (Table 2).

Further, we investigated alternative approaches to the pitch pine, hybrid neighbor problem. One approach allocated the growing space of each missing or hybrid cell to the four adjacent trees and weight samples on areas of nonadjacent and adjacent plus hybrid cells before calculating N accumulation. With this method, adjacent and nonadjacent tree means are more similar than they were in the original estimates (differences are less significant at $P = 0.57$), producing a mean and 95% confidence interval of 44 ± 22 kg N ha⁻¹. When all values from adjacent trees are disregarded, we obtained 42 ± 22 kg N ha⁻¹; when means from all samples are not weighted at all, we obtained 45 ± 21 kg N ha⁻¹ (Table 2). All of these estimates support the conclusion that pitch pine has a large, significant aboveground N accumulation of a magnitude similar to that of red pine.

N in Soil

Binkley and others (2000) questioned the assumption that the initial soil conditions were similar among the sandboxes and further asked whether this and some additional uncertainty in the final soil N estimates might mask a large depletion of soil N translocated into aboveground biomass. Here we evaluate these two sources of uncertainty.

The first uncertainty concerns the 1993 assumption of initial interbox similarity of the N masses in the deep (20–135 cm) soil. Lower soil was sampled at t_0 only in the nonvegetated box. Because the boxes

contained intensively homogenized soils, repeating the difficult deep sampling on other boxes was deemed to be unnecessary. Additional analysis shows that differences in the percentage of N in lower-soil samples from the nonvegetated box and composited grab samples—taken from piles after soils were mixed but before they were placed in the other boxes—were small (2%) and nonsignificant (two-tailed *t*-test, $P = 0.83$). Upper soil, which was measured in all boxes at t_0 , was also similar and not significantly different (ANOVA, $P = 0.61$). Average tree heights in the three replicate red pine boxes were similar in 1987: 132, 155, and 155 cm, for an average of 147 ± 42 cm, which suggests similar initial conditions.

The second uncertainty concerns a possible measurement error associated with changing bulk density measurement techniques. When the bulk densities of 5-year upper (0–20 cm) soils were compared with soils at t_0 , there were small but significant differences in both red pine and pitch pine—but these differences could have been due to changes in sampling technique, rather than being caused by soil expansion or collapse, as originally assumed (Bormann and others 1993). Here we report an alternative simplified method, where the upper and lower soil masses at 5 years are assumed to also represent the soil masses at t_0 and are then multiplied by both the initial and the 5-year N concentrations to obtain the initial and 5-year soil N masses. The results for upper soils (Figure 1 and Table 2) showed significant (one-tailed *t*-test), large changes in N mass for the alder ($P < 0.001$) and nonvegetated ($P = 0.04$) treatments but small, nonsignificant N mass changes for both the red pine ($P = 0.14$) and pitch pine ($P = 0.50$). The large 95% confidence interval for the nonvegetated box at t_0 is influenced by one value. For the lower (20–135 cm) soils, highly significant (two-tailed *t*-test, $P < 0.001$) changes in N concentration and mass were found in the pitch pine, red pine, alder, and nonvegetated boxes (Figure 2 and Table 2). These lower-soil increases for the trees were taken as zero by Bormann and others (1993) because of their limited confidence in initial values; our reanalysis showing small, nonsignificant differences in the initial conditions increases that confidence. In the nonvegetated treatment, the loss of upper-soil N appears to be nearly balanced by the gain of lower-soil N (Figures 1 and 2). The two pines showed highly significant total soil gains of 70 ± 21 and 64 ± 16 kg ha^{-1} (Table 2).

Recalculating N Budgets in the Pine Sandbox Ecosystems

Methods based on N–height regressions reduced the estimates of the accumulation of total vegetation N by 7% to 11% and decreased uncertainty.

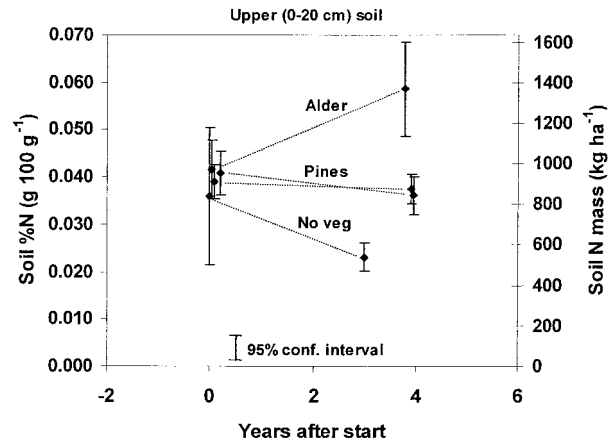


Figure 1. Changes in N concentration and mass in upper soil layers (0–20 cm), assuming a fixed soil mass in four sandboxes. Changes were significant with a two-tailed *t*-test in alder ($P = 0.01$) and nonvegetated ($P = 0.03$) boxes and nonsignificant in red pine ($P = 0.27$) and pitch pine ($P = 0.57$) boxes.

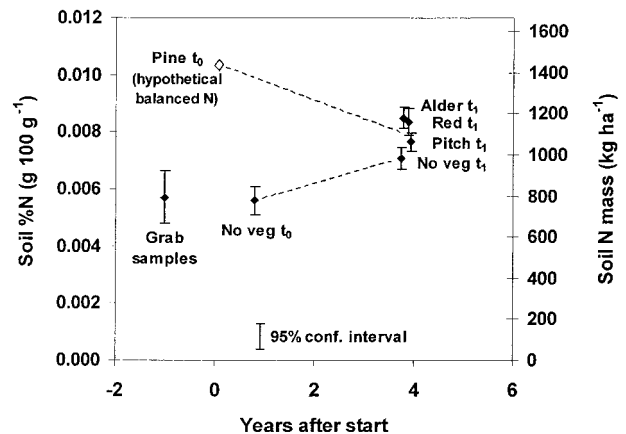


Figure 2. Changes in N concentration and mass in lower soil layers (20–135 cm), assuming a fixed soil mass in four sandboxes. Initial nonvegetated lower-soil samples did not differ from composited grab samples taken before boxes were filled ($P = 0.83$). The hypothetical value needed to balance pine vegetation N gains (\diamond) is nearly double the actual t_0 values.

Estimated losses of N from the upper soil by the new fixed-mass method were increased for red pine and decreased for pitch pine. The fixed-mass method, based on strengthened assumptions about the initial conditions, revealed large N gains for lower soil with small confidence intervals. New estimates for accumulation of N in the entire sandbox soil (0–135 cm), based on the fixed-mass method, were also large with small confidence intervals: 70 ± 21 $\text{kg ha}^{-1} \text{y}^{-1}$ for red pine and 63 ± 16 $\text{kg ha}^{-1} \text{y}^{-1}$ for pitch pine.

Table 3. Revised Calculation^a of Unexplained Nitrogen (N)-using Alternative Approaches

Ecosystem N	Approach ^b	Red Pine	Pitch Pine
Components and sums			
Aboveground veg	N–height regression	54 ± 22	36 ± 7
Roots and litter	1993 method	20 ± 3	29 ± 5
ΔN _{veg} total	With regression estimate	74 ± 22	65 ± 9
ΔN _{veg} total	1993 method	83 ± 8	70 ± 40
Upper soil (0–20 cm)	Fixed-mass (Figure 1)	–28 ± 18	–10 ± 13
	1993 method	–17 ± 33 ^c	–19 ± 25
Lower soil (20–135 cm)	Fixed-mass (Figure 2)	98 ± 11	73 ± 9
	1993 method ^d	0 ± na	0 ± na
ΔN _{soil} total	Fixed-mass	70 ± 21	63 ± 16
ΔN _{soil} total	1993 method	–17 ± 33	–19 ± 25
N _{dr} –N _{bp} (output–input)	1993 methods ^d	–4 ± na	–4 ± na
Alternative estimates of ecosystem unexplained N ^e			
Minimum rate	N–height regression, most negative upper- and no lower-soil N changes	42 ± 29	42 ± 26
Reported rate	1993 methods	62 ± 34	49 ± 47
Maximum rate	1993 ΔN _{veg} , fixed-mass upper and lower soil	149 ± 23	130 ± 43

^aAll units are kg ha^{–1} y^{–1} ± 95% confidence intervals.

^bAlternatives include estimates from Bormann and others (1993), with minor corrections, regression-based vegetation, and fixed-mass soil N estimates.

^cThe upper-soil interval was incorrectly reported as 23, but reported ecosystem intervals were unaffected by this typographical error in Bormann and others (1993).

^dConfidence intervals were not attempted on inputs and outputs because of their small effect on the ecosystem mass balance.

^eEcosystem unexplained N is the sum of annual vegetation, roots and litter N changes (ΔN_{veg} total), upper- and lower-soil N changes (ΔN_{soil} total), and output–input. For example, the minimum rate in red pine is 74 – 28 – 4 = 42.

Estimates of unexplained ecosystem N change (Table 3) ranged from 42 kg ha^{–1} y^{–1} (based on the aboveground N estimate from the regression method and assuming no N change in lower soil) to 130–149 kg ha^{–1} y^{–1} (based on including aboveground vegetation N means and lower-soil N change estimates). Viewed slightly differently, the aboveground accumulation of N in pine vegetation could be explained by the loss of N in lower soil (as suggested by Binkley and others 2000) only if such N losses were considered so large as to be inconsistent with what we know about initial conditions (Figure 2). We believe that the weight of evidence cannot support such interpretive choices. We cannot avoid the conclusion that unexplained N has accumulated at modest to large rates in these sandboxes.

DISCUSSION

A major advance in the understanding of the N cycle may well be imminent, but confidence in the conclusion that N accumulates rapidly without symbiotic N₂-fixing plants at ecologically important

scales of time and space will not be achieved easily. Mesocosm studies will likely play a critical role in this discovery because they can speed the detection of changes in soils and account for other important processes. However, to acknowledge a point raised implicitly by Binkley and others (2000): Mesocosm experiments are a challenging undertaking that requires large commitments of resources to achieve the experimental control and measurement accuracy needed to detect changes over a few years. Our finding of significant and large changes in lower-soil horizons in the sandboxes should also raise concerns about studies that fail to quantify deep soil changes, as difficult as that is.

In addition to controlling errors and bias better, future studies aimed at quantifying the potential for high rates of unexplained N accumulations more accurately are likely to be more successful if they focus on plants, soils, and climates that have already been implicated, such as red and pitch pine on unweathered glacial soils. Given the evidence supporting unexplained N in a few well-documented cases, the time is ripe to invest in studies that can help us to understand how this unex-

plained N is accumulating. Future studies should attempt to directly quantify ecosystem N₂ fixation rates with multiple approaches, including more field ¹⁵N studies. Microbiological studies, such as those already underway in tropical grasses (James and Olivares 1997; James 2000), are also needed to look for N₂-fixing bacteria in soils, rhizospheres, and inside the roots of temperate zone pines and other species adapted to low-N environments.

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