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RAPID N_2 FIXATION IN PINES, ALDER, AND LOCUST: EVIDENCE FROM THE SANDBOX ECOSYSTEM STUDY[1]

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Abstract. Not all nitrogen (N) inputs have been accounted for in forested ecosystems. We sought to account for N_2 fixation and dry deposition using a lysimeter mass–balance approach. Large sand-filled, field lysimeters were used to construct 5-yr nitrogen budgets for two N_2-fixing trees, two pines, and a nonvegetated control soil. This approach is a promising and straightforward technique for quantifying otherwise difficult-to-measure fluxes. Accurate assessment of changes in N storage combined with direct measurement of N inputs in precipitation and losses from leaching allowed us to estimate fluxes. Gains of N in pine systems were greatest in vegetation and litter, overshadowing combined losses from mineral soil and leaching by about threefold. Rapid acetylene reduction in pine rhizospheres and in cultures from washed roots suggests that unexplained gains are due to associative N_2 fixation. These results provide strong evidence for N_2 fixation in pine systems of ≈ 50 kg·ha^{-1}·yr^{-1} N. The symbiotic N_2-fixing trees black locust and black alder fixed 2 and 5 times more N_2, respectively, than did pines. In all systems, input in precipitation and dry deposition were relatively unimportant to the N budget. Unexplained losses of N from the nonvegetated control suggest that denitrification is an important flux. Mineral soil organic matter declined sharply and significantly in pines (20%) and even more so in the nonvegetated control (40%). Symbiotic N_2-fixing trees caused a small, nonsignificant increase in mineral soil organic matter and large, significant increases in litter layer organic matter. Bulk density (0–20 cm) declined by 5% under symbiotic N_2-fixing trees and increased by 5% in one pine sandbox. Correction for soil expansion or collapse did not greatly alter estimates of unexplained N or N_2 fixation. Pines with rhizospheres that fix N_2 at the rates we observed might be used to restore degraded land and to create silvicultural systems that are N self sufficient. We first need to better understand the microbiology, tree genetics, and soil conditions that lead to rapid N_2 fixation in pine ecosystems.

Key words: acetylene reduction; associative nitrogen fixation; dry deposition; Hubbard Brook; land restoration; mass–balance; nitrogen volatilization; organic matter losses; rhizosphere; soil expansion; sustainable forestry; unexplained nitrogen.

INTRODUCTION

A wide range of scientific approaches has helped to shape a consensus view on the importance of symbiotic N_2 fixation at the ecosystem level. The process of biological N_2 fixation was discovered only after the studies of N mass–balance in field plots by Boussingault in the 19th century (Russell 1973). These were the first experiments to demonstrate large unexplained N accumulations with leguminous crops. Modern ecosys-

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tem experiments and comparisons have repeatedly noted a rapid accumulation of N in a variety of ecosystems containing symbiotic N_2 fixers (e.g., Chapman et al. 1949, Tarrant and Miller 1963, Van Cleve et al. 1971, Cromack et al. 1979, Bormann and DeBell 1981). At the same time, rapid N_2 fixation in symbiotic N_2 fixers is suggested by direct measures of nitrogenase activity extrapolated to an area and yearly basis (e.g., Binkley et al. 1982, Bormann and Gordon 1984).

There is a fundamental inconsistency, however, in the nonsymbiotic N_2 fixation literature. While rapid accumulation of N is also repeatedly observed in ecosystems without symbiotic N_2 fixers, direct measurements of nitrogenase activity of associative and free-living N_2 fixers very rarely can explain these accumulations. For example, abandoned agricultural fields without known N_2-fixing plants can gain N at 22–65 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Greenland and Nye 1959, Jenkinson 1971). Rapid rates of N accumulation (in kilograms per hectare per year) occur in cultivated bluegrass (112, Whitt 1941; 31–51, Karraker et al. 1950), Sudan grass (45–54, Chapman et al. 1949), rangelands (22–34, Smith et al. 1954), and rice (App et al. 1980). Some hardwood forest ecosystems without known symbiotic N_2 fixers may accumulate 14–37 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Bormann et al. 1977, Bowden 1991). Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) stands can accumulate N at 33 $kg \cdot ha^{-1} \cdot yr^{-1}$ in vegetation and litter without losing N from the upper 20 cm of soil (Bormann 1977). Ponderosa pine ecosystems developing on mudflows can accumulate 21–63 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Dickson and Crocker 1953). Richards and Bevege (1967) report gains of N in vegetation and soil of 22, 30, and 50 $kg \cdot ha^{-1} \cdot yr^{-1}$ for *Pinus caribaea* var. *hondurensis, P. elliottii* var. *elliottii,* and *P. taeda,* respectively.

By themselves, these N accumulation studies are not fully convincing because of: (1) uncertainty about site history; (2) inappropriate controls; (3) high soil variability; (4) lack of measures of some pools; and (5) failure to include various other N inputs and outputs. For example, many accumulation studies do not measure increased or decreased storage of N in soils; others do not measure precipitation, dry deposition inputs, or losses from leaching or volatilization. Comparison of N budgets on treated and adjacent areas are questioned because of inherent variability and the potential for large unmeasured differences in the rates of volatilization and leaching (Rennie and Rennie 1983, Boddey and Dobereiner 1988).

Direct measures of nitrogenase activity of associative and free-living N_2 fixers usually suggest only slow rates, especially when extrapolated to estimate N_2 fixation in the ecosystem over yearlong periods. For example, Postgate's (1974) review points to an N accumulation rate of 0.3 $kg \cdot ha^{-1} \cdot yr^{-1}$ for *Azotobacter* in agricultural soil. Kaputska and Rice (1978) estimate 0.71 $kg \cdot ha^{-1} \cdot yr^{-1}$ for tall grass prairie. Assorted North American forest ecosystems were estimated to have: 0.002 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Grant and Binkley 1987); 0.1 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Vance et al. 1983); 0.3 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Cushon and Feller 1989); <1 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Hendrickson 1990); and 1 $kg \cdot ha^{-1} \cdot yr^{-1}$ (Heath et al. 1988).

Rapid nitrogenase activity has been observed in some grasses and conifers with endo- and ectomycorrhizae, suggesting the possibility of rapid associative N_2 fixation at the ecosystem level. For example, Dobereiner et al. (1972) measured nitrogenase activity (by acetylene reduction) on the roots of sugar cane and other tropical grasses that extrapolated to an annual N accumulation rate of 67 $kg \cdot ha^{-1} \cdot yr^{-1}$. Isotope dilution techniques, when extrapolated to the field, suggest rates as high as 200 $kg \cdot ha^{-1} \cdot yr^{-1}$ for sugar cane (Lima et al. 1987). Plants, including pines, not known to be symbiotic N_2 fixers have been shown to accumulate ^{15}N when exposed to $^{15}N_2$ gas (Stevenson 1959, Richards and Voigt 1964, Richards 1973, Bevege et al. 1978).

Extrapolation of nitrogenase activity measurements on seedlings, cores, or excised roots to annual per-unit-area rates under field conditions has been criticized (Shearer and Kohl 1986). Acetylene-reduction measurements are often thought to overestimate N_2 fixation because of an uncertain ratio of C_2H_4 fixed to N_2 fixed (Silvester 1983). Acetylene reduction may underestimate free-living or associative N_2 fixation when roots and soil are exposed to high O_2 concentrations or when gas diffusion into and out of intact soil cores is limited (van Berkum and Bohlool 1980). Natural abundance ($^{15}N/^{14}N$) and isotope dilution techniques (Binkley et al. 1985, Shearer and Kohl 1986) depend on a comparison with a presumed physiologically similar but non-N_2-fixing species.

We designed an experiment to search for and explain N accumulations. We sought to overcome the limitations of previous N accumulation studies and to use the latest methods to directly measure nitrogenase activity. Our method was to compare measured changes in N storage to measured N inputs minus outputs and to determine, to the degree possible, probable sources of unexplained N including biological N_2 fixation, dry deposition, and biological and chemical volatilization. We also evaluated the activity of N_2-fixing bacteria in the rhizosphere with measures of acetylene reduction under low O_2 conditions.

Methods

General approach

The Hubbard Brook "sandbox" experiment (Bormann et al. 1987) was designed to minimize problems associated with previous mass–balance studies. The method is based on lined pits filled with homogenized sandy soil of known N content, hereafter referred to as sandboxes. Sandboxes were planted with seeds or seedlings of known N content, allowed to revegetate naturally, or maintained vegetation free. We report here N budgets for five individual sandboxes contain-

ing monospecific stands of European black alder (*Alnus glutinosa* (L.) Gaertn.), an actinorrhizal N_2 fixer; black locust (*Robinia pseudoacacia* L.), a leguminous N_2 fixer; pitch pine (*Pinus rigida* Mill.); red pine (*P. resinosa* Ait.); and a sandbox kept free of vegetation. After 5–6 growing seasons, N budgets were constructed by measuring: (1) changes in storage; (2) precipitation inputs; and (3) output in drainage water.

Changes in storage were separated into soil (ΔN_{soil}) and vegetation (ΔN_{veg}). N_{veg} here includes litter, herb, and tree biomass, including roots, to the bottom of the sandbox. Additionally, we used the Hubbard Brook bulk precipitation record to estimate N input in bulk precipitation (N_{bp}) and directly measured output of N in drainage (N_{dr}) on several sandboxes.

We calculated changes in N storage ($\Delta N_{storage}$) with the equation:

$$\Delta N_{storage} = \Delta N_{soil} + \Delta N_{veg}. \quad (1)$$

To see if changes in N storage were balanced by measured N inputs minus outputs, we used the equation:

$$\Delta N_{storage} = N_{bp} - N_{dr}. \quad (2)$$

By rewriting Eq. 2 to estimate what fraction of $\Delta N_{storage}$ could not be explained by the mass balance equation ($N_{unexplained}$), we obtained:

$$N_{unexplained} = \Delta N_{storage} - N_{bp} + N_{dr}. \quad (3)$$

Probable sources of $N_{unexplained}$ included biological N_2 fixation (N_{bnf}), dry deposition (N_{dd}), and biological and chemical volatilization (N_{vol}), as follows:

$$N_{unexplained} = N_{bnf} + N_{dd} - N_{vol}. \quad (4)$$

Sandbox construction

The research design was implemented in a 0.3-ha mowed pasture adjacent to the U.S. Department of Agriculture Forest Service headquarters at the Hubbard Brook Experimental Forest in central New Hampshire. The surface soil (0–0.5 m) was removed by a bulldozer in October 1982, exposing a sandy gravelly substrate of ice-contact stratified drift. The topsoil (0–10 cm) was stockpiled for later use. Location of each sandbox was determined by surveyors' transit and staked. For the entire study, not entirely reported here, 12 small (2.5 × 2.5 m) pits and 6 large (7.5 × 7.5 m) pits were dug to a depth of 1.5 m with a backhoe. Frames were built at the surface and pits were lined on the sides with a 0.09-mm reinforced membrane (Hypalon, a DuPont Polymer) used to eliminate seepage from lagoons and reservoirs. Before installation, the Hypalon was exposed to sun and leached with rain for 6–8 wk to remove possible contaminants. Three of the large pits were fully lined and had a network of bottom drains leading to an underground chamber where drainage and water chemistry could be monitored. A 15-cm layer of stones, 1.9–3.8 cm in diameter, was placed in the bottom of each sandbox. Then, 1.3 m of 0.95-cm screened "sand" obtained from a sand and gravel company was added. This "sand" was glacial outwash of granitic and metamorphic origin and contained some finely ground particles. It was carefully mixed using a front-end loader before being loaded into dumptrucks. Soil previously excavated from the pits was spread outside of the sandboxes. Construction was completed in November 1982.

To enhance the survival of seedlings, 5 cm of carefully mixed topsoil removed from the field was added to the sandboxes. Topsoil was incorporated into the upper 20 cm of the sand layer with a rototiller. Topsoil was also added and rototilled in a buffer zone 0–3 m around each sandbox. This was completed by 16 May 1983.

In this paper, we report results from two large sandboxes with drains (pitch pine and nonvegetated), one large sandbox without a drain (locust), and two small sandboxes (alder and red pine). These sandboxes were sampled first because these species had grown the fastest and were beginning to approach a size that would bring into question edge effects. Also it seemed likely that the fastest growing species would have the greatest N accumulations.

Calculation of unexplained N accumulation or loss

We quantify $N_{unexplained}$ on an annual basis (in kilograms per hectare per year) by dividing each component of the right side of Eq. 1 by the final minus initial sampling time (Eqs. 5–6):

$$\Delta N_{veg} = \frac{N_{veg(final)} - N_{veg(initial)} + N_{litter(final)}}{t_{final} - t_{initial}}, \quad (5)$$

$$\Delta N_{soil} = \frac{N_{soil(final)} - N_{soil(initial)}}{t_{final} - t_{initial}}. \quad (6)$$

$N_{unexplained}$ is calculated by substituting the above values in Eq. 3 and adding average annual values for N_{bp} and N_{dr}:

$$N_{bp} = \frac{\sum_{t_{initial}}^{t_{final}} (N_{bp})}{t_{final} - t_{initial}}, \quad (7)$$

$$N_{dr} = \frac{\sum_{t_{initial}}^{t_{final}} (N_{dr})}{t_{final} - t_{initial}}. \quad (8)$$

We believe the three principal components to $N_{unexplained}$ are: addition of N from dry deposition (N_{dd}), by dust, aerosols, direct NH_3, HNO_3, or NO_x uptake; addition of N from N_2 fixation (N_{bnf}) by symbiotic, free-living, or rhizosphere organisms; and losses of N from biological and chemical volatilization (N_{vol}). The relations between $N_{unexplained}$ and these unmeasured inputs and outputs are represented mathematically in Eq. 4.

Initial soil conditions

Planting, including replacements for failed seedlings, was completed for most sandboxes by autumn of 1984. We had hoped to establish initial values for soil N by coring the soils in the summer of 1983 but found that standard soil tubes would not remove intact cores of the sandy soil. A piston-type soil corer, 5.3 cm in diameter, was designed in the spring of 1983–1984; it was tested during the early summer of 1984 (Ingersoll et al. 1987). During autumn 1984, 0–10 and 10–20 cm cores were removed from all sandboxes with this corer. Sixteen cores were collected from the small sandboxes and 18 cores from the large sandboxes. Cores were taken at points farthest from seedlings. To minimize soil compaction during coring, walking in the sandboxes was limited to boards placed on the soil surface. The exact location of each core was recorded and holes created by coring were back-filled with sand. Cores were expelled directly from the corer into previously weighed and labeled 177-mL flint-glass jars. Jars with soil were oven-dried to a constant mass at 65°C and then sealed and stored for future chemical analyses.

We removed five deep cores to depths of 50–75 cm from the nonvegetated sandbox in October 1985. Core sections could not be retrieved from deeper depths, and no initial cores were taken below 20 cm in vegetated sandboxes. N content of deep cores was indistinguishable from grab samples of bulk sand before it was added to the sandboxes; thus, we assumed that initial deep cores in the nonvegetated sandbox were representative of the deep soil layers in the other sandboxes. Use of the deep-soil N content in the nonvegetated sandbox for initial conditions in the other sandboxes is discussed in the *Results*. Our idealized experimental design was slightly disrupted because initial samples of vegetation and soil were not obtained at the same time.

Final soil conditions

Mineral soil was sampled below the four central alder and red pines in the small sandboxes to avoid possible edge effects of the sandbox walls, and below 8 of the 16 sampled locusts and 6 of the 12 sampled pitch pines. The upper 30 cm of mineral soil was collected with a 0.50 × 0.50 × 0.32 m open-ended frame made from four 6.3 mm thick steel plates welded together and sharpened on the bottom. This box-shaped sampler was needed, instead of the 5.3-cm corer, to cut through the large roots that had developed and to encompass new variation associated with tree position. The box sampler, corresponding to the growing space allotted to each tree, was centered around a stump and driven 30 cm into the soil. Four soil layers were collected: 0–5, 5–10, 10–20, and 20–30 cm. Depth gauges were used to delineate soil layers. Entire-layer samples were first sieved through 6.35-mm mesh hardware cloth to remove larger roots and coarse fragments. A subsample (8–25% of total wet mass) of the remaining soil was obtained with a riffle subsampler. The subsample was further divided into fine earth (<2 mm) and smaller coarse fragments (2–6.35 mm). Small root fragments missed in the first sieving were meticulously separated from these subsamples. A 5.3 cm diameter corer was used to collect 10-cm core sections from 30 cm to the bottom of the sandbox (1.35 m) at one point under each tree for soil analyses, except root biomass. After coring, a 0.25-m^2 monolith was excavated to a depth of 1 m to collect remaining deep roots.

Soil samples were air-dried for several weeks and then oven-dried (65°C). Additional subsamples were oven-dried at 105°C. Nutrient analyses of soil samples were carried out only on material dried to 65°C, but results are based on a 105°C dry mass.

Initial vegetation conditions

Seedlings were planted in 1983: alder on 10 July; locust on 17 August; red pine on 6 June; and pitch pine on 18 May. Before planting, random subsamples of seedlings were collected, dried (65°C), and stored for later chemical analysis. Some replanting of failed seedlings was required in the locust, red pine, and pitch pine sandboxes. Weeds were pulled and dropped in place several times during the first few years in some sandboxes to ensure seedling survival. No dead seedlings or weeds, or the N they contained, were removed from any of the sandboxes.

All seedlings were planted at 0.5-m spacing, leaving them with an aboveground growing space of 0.25 m^2. A buffer of three and six rows of the same species and spacing was planted outside of the sandboxes in the small and large sandboxes, respectively. All seedlings had a soil growing space of 0.25 m^2, except for those planted adjacent to the sandbox walls. Because the position of the walls did not match the spacing of the seedlings, the row just inside the wall had a soil growing space of 0.38 m^2, and the row just outside the wall had a soil growing space of 0.13 m^2.

Final vegetation conditions

Aboveground portions of individual trees in the 0.25-m^2 growing spaces were harvested during August and September 1988 (alder and pines) and July 1989 (locust). In the small alder and red pine sandboxes with 16 trees, we collected the tops of all of the central four trees and randomly selected two trees along the border row remaining inside of the sandbox. Height growth of border trees was not statistically different ($P > .05$, t test) from nonborder trees.

In the large locust and pitch pine sandboxes, one-third of the sandbox running along one of the four sides was randomly designated for sampling. The other two-thirds was reserved for future sampling. We did not sample trees in the border row inside the frame of the sandbox.

About 1% of the pitch pine seedlings died during the

1st yr and were inadvertently replanted with pitch × loblolly hybrid seedlings. Early in 1987, we decided to cut these hybrids down leaving a few vacant growing spaces. A preliminary analysis of tree diameters indicated that trees growing directly adjacent to these vacant growing spaces had a significantly ($P < .05$, t test) larger diameter than nonadjacent trees. As a result, trees were stratified on the basis of whether they were directly adjacent to empty growing spaces. We randomly selected 4 of the 9 adjacent trees and 8 of the 37 nonadjacent trees.

Tree height varied considerably in the locust sandbox, prompting us to stratify by tree size. Four trees were randomly drawn from each of four size strata for a total of 16 sample trees.

Tree tops were cut 5 cm above the soil surface and separated into foliage, branch, and stems. Roots, obtained while processing soil samples, were divided into <2 mm, ≥2 mm, and nodules (if present). Stumps included 5 cm of stem above the soil surface and a 5 cm length of each root attached to the root stock. Herbs, grasses, and mosses were collected from the aboveground growing space (0.25 m^2) associated with each sampled tree and processed like the other biomass samples.

Litter was treated as part of the vegetative component (ΔN_{veg}) because it was not present initially and because it was sampled at the same intensity as vegetation. Litter was collected from the 0.25-m^2 space under all sample trees (6 in red pine and alder, 12 in pitch pine, and 16 in locust), dried at 65°C, and ground in a large blender. Minor amounts of litter were blown into and out of the sandboxes. We assume that these were roughly equal or otherwise insignificant to the N budget.

After oven-drying (65°C), entire samples, except stem and stump samples, were finely ground in a large blender or Wiley mill. Stem segments and stumps were randomly subsampled before being ground. A single composite sample, combining all ages, was constructed for foliage, branches, stems, and reproductive parts. The amount of sample representing each age in the composite was proportional to the total dry mass of the corresponding plant part of that age.

Time used in calculations

To determine annual rates of N change, we divided differences (Eqs. 5 and 6) and sums (Eqs. 7 and 8) by appropriate time intervals. For vegetation and litter, we used the decimal years between planting and harvesting: 5.0 yr for alder, 5.2 yr for red pine, 5.3 yr for pitch pine, and 5.9 yr for locust. For soils, we used the time since initial soil sampling, September–October 1984, to the final sampling: 3.8 yr for alder, 3.9 yr for red pine, 3.9 yr for pitch pine, and 4.8 yr for locust. For the nonvegetated sandbox, we used 3.0 yr. Average annual sums of N_{dr} and N_{bp} were derived for the soil measurement period.

Input and output to the sandboxes

Input of N in bulk precipitation was measured weekly in a nearby Hubbard Brook long-term biogeochemistry monitoring station. Seepage discharge rates were estimated from two of the completely lined, large sandboxes, one that contained red pine and one that was nonvegetated. Water samples were collected approximately weekly during the frost-free season, from late 1982 until late 1986. Initially, discharge rates were continuously monitored with a "V"-notch weir. This system, however, proved to be insufficiently sensitive to the range of flow rates we observed (0 to ≈6000 mL/min, typically 50–200 mL/min). Consequently, instantaneous discharge estimates were measured weekly or more often during storm events and snowmelt. In 1989, we devised a method to measure outflow continuously with a tipping-bucket recorder. These later measurements show the same trends as the 1984–1988 instantaneous measurements.

We estimated N export (N_{dr}) from the sandboxes based on 3-mo averages. We were concerned that a few unusually large instantaneous discharge values, which might have lasted for only minutes to hours, when multiplied by their corresponding concentration values and averaged over a week between sampling, would bias the export calculations in favor of large losses. Consequently, we interpolated discharge rates between known days and summed the discharge over 3-mo seasons, roughly corresponding to hydrologic seasons in the sandboxes. Next, we averaged the NO_3^- concentrations over the season. Export was estimated from the product of the interpolated, seasonal, accumulated water yield and average seasonal concentration in the discharge.

Acetylene reduction measurements

Lastly, we sought to determine whether unexplained N gains could be due to associative N_2 fixation. We obtained measurements of acetylene reduction in the fall of 1990 and the spring of 1991. Methods followed Li et al. (1992). Detached roots with soil attached were placed in incubation tubes and flushed for 15 min to remove O_2. Acetylene (10%) and O_2 (1%) were added and ethylene production was followed for 24 h. Gas samples withdrawn from the tubes were analyzed for acetylene and ethylene with a Hewlett Packard 5830A gas chromatograph fitted with a flame ionization detector and a 2 m × 2.1 mm, 80–100 mesh Poropak R, column at 70°C; the injection port and detector temperature were adjusted to 100°C. Both endogenous acetylene production and background ethylene levels were checked regularly. Nitrogenase activity was calculated as net acetylene reduced after subtracting background levels and endogenous rates. After incubations were completed, roots were separated from soil, oven-dried (70°C), and weighed.

Small root sections were also washed with distilled

water, cut into pieces, and placed on selected media, in an attempt to identify organisms present in pure cultures. Three N-free media described by Burk (1930), Rennie (1981), and Dobereiner and Day (1976) were used in 33-mL vials fitted with septa. Acetylene reduction of successful cultures was also evaluated in these vials.

Chemical analysis

Our primary interest was to compare initial and final conditions. To minimize systematic analytical biases, we followed the convention of analyzing initial and final samples at the same time and on the same equipment. Locust samples were analyzed 1 yr later than other samples with a different block digestor. Because of this, we limited the comparison of initial and final conditions in the locust plot to the 0–20 cm layer.

Samples of vegetation and soil were digested using sulfuric acid and hydrogen peroxide as oxidizers, selenium as a catalyst, and lithium sulfate to elevate the boiling point (Parkinson and Allen 1975). Sample mass was 0.2 g for soils and 0.1 g for vegetation. Digestions were performed in 75-mL reflux tubes in a Technicon BD-40 block digestor. Soils were predigested overnight at room temperature and then for 4–5 h, beginning at 200°C with 50°C increases every hour to a maximum of 350°C. Vegetation samples were also predigested overnight at room temperature and then for 20 min at 250°C and 1.5–2 h at 450°C. The digest was diluted to 75 mL and a portion was analyzed for total Kjeldahl N colorimetrically with a Technicon Autoanalyzer II (D'Elia et al. 1977). National Bureau of Standards citrus leaves (SRM N. 1572) were included as a primary reference in all digestions and analyses. Other subsamples of soil were analyzed for organic matter content by loss on ignition (600°C).

Water samples were analyzed for nitrate (NO_3^-), ammonium (NH_4^+), and dissolved organic nitrogen (DON). Nitrate was analyzed colorimetrically on an autoanalyzer using an alkaline hydrazine reduction method (Technicon 1969). Ammonium was analyzed similarly by the alkaline phenate method (Technicon 1971). DON was measured as NO_3^- after digestion in alkaline persulfate (Smart et al. 1981). Nitrate and NH_4^+ were subtracted from the postdigestion NO_3^- to estimate DON by difference.

Statistical design and analysis

Our primary goal was to estimate sources of N accumulation over time. The null hypothesis that we sought to evaluate is Eq. 2, that change in storage equals measured inputs and outputs. The "target populations" were initial and final N content of trees and their associated growing spaces in each sandbox. The individual tree comprised the sample unit. We assumed a uniform distribution of N in the soil at the beginning of the experiment because all sandboxes were filled with the same homogenized sand and topsoil. We concluded that a significant N accumulation or loss occurred if the 95% confidence interval of the difference between initial and final condition did not include zero. The variances of components of $N_{\text{unexplained}}$ (Eq. 3) were summed with standard sampling procedures (Cochran 1963) and variance rules (Freese 1978).

Results

We have calculated rates of unexplained N accumulation and loss that exceed most published values. In the following sections, we establish a foundation to support these high rates. First, we review development of vegetation and soil in the sandboxes; we present values for the components of Eq. 3; and then calculate rates of unexplained N accumulation (loss). Lastly, we discuss dry deposition, volatilization, and provide acetylene reduction and microbiological data that support our estimates of biological N_2 fixation.

Development of sandbox ecosystems

Vegetation.—Mortality of planted seedlings was low in alder (0%), locust (8%), and pitch pine (9%). Red pine, however, had 41% mortality in the 1st yr. Nearly complete survival was achieved after replanting in all species.

The herb layer that developed under the sampled sandboxes varied widely. The composition was similar in the first few years, dominated by *Rumex acetosella* L., grasses including *Panicum* sp., and *Mollugo verticillata* L. Herb cover changed by the time of sampling, dropping to near zero in pines and alder. Locust had 30% herb cover (*Panicum* sp., *Polygonella* sp., *Potentilla* sp., *Rumex* sp., and others). A tiny annual sedge, *Bulbostylis capillaris* (L.) Clarke, invaded the nonvegetated sandbox and repeatedly reseeded each spring after being meticulously uprooted in late summer.

Relative growth among the sandbox species was not as we expected. Black alder clearly grew the fastest, even though it is not an indigenous species (Fig. 1). Pines also grew more rapidly than we expected. Accumulation and increment of biomass far exceeded our expectations for three of the four vegetated sandboxes we sampled (Fig. 2). Pitch pine biomass reached 80 Mg/ha in just six growing seasons (Table 1). Red pine (77 Mg/ha) and black alder (66 Mg/ha) were close to pitch pine. Black locust accumulated $\approx$¼ the biomass of the other species (18 Mg/ha).

Changes in soil properties.—Dramatic changes were observed in organic matter content of the mineral soil. The nonvegetated sandbox lost on average $\approx$4 $Mg \cdot ha^{-1} \cdot yr^{-1}$ of organic matter from the mineral soil (Fig. 2). Cumulatively, 12 Mg/ha of organic matter ($P < .05$) or $\approx$40% of the original 29 Mg/ha was lost.

Pine systems lost 8 Mg/ha of organic matter in the mineral soil ($P < .05$) or $\approx$20% of their initial values. The net amount of organic matter in mineral soil in alder and locust systems did not change significantly, although organic matter in alder increased 3% ($P =$

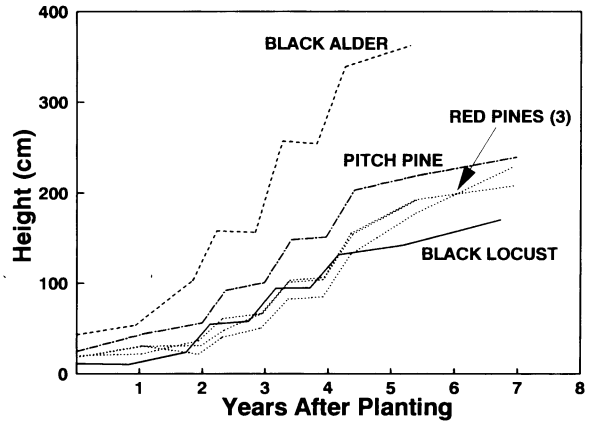

FIG. 1. Height of trees in alder, pitch pine, locust, and three red pine sandboxes.

.33). Possible reasons for losses of organic matter from the mineral soil of pines relative to alder and locust include: (1) pine foliage is retained 2–3 yr longer; (2) pine needle litter may decompose more slowly; (3) pines may gain control over the site more slowly; and (4) microbes associated with pines may be more efficient at consuming soil humus.

Other important changes in sandbox soils were observed. In the alder and locust systems, mass of fine soil (<2 mm) in the top 20 cm volume of soil actually declined by 5% ($P < .05$) and 2.5% ($P = .14$), respectively. The most likely explanation is that the soil expanded upward because of root activity or by addition of soil organic matter, but other mechanisms such as filtering of fine soil downward or changes in sampling techniques cannot be ruled out. Fine soil (0–20 cm) also declined under pitch pine, probably because of a large root biomass in that layer, 11.2 Mg/ha, compared to only 5.2 Mg/ha of roots (0–20 cm) in red pine (Fig. 3). Low root biomass and decreased organic matter content are probably responsible for a 4% increase in fine soil (0–20 cm) in red pine ($P < .05$). These changes affect calculation of changes in mineral soil N content and are discussed later.

Changes in bulk density mirrored changes in soil organic matter, as expected. Bulk density declined significantly ($P < .05$) under alder (1.38–1.32 g/cm^3; −4%) and pitch pine (1.37–1.30 g/cm^3; −5%), but increased under red pine (1.36–1.43 g/cm^3; +5%). The mass of soil (<2 mm) or bulk density did not change significantly ($P > .05$) in the nonvegetated sandbox, even though large amounts of organic matter were decomposed.

We also measured a 14% increase ($P < .05$) in dry mass of coarse fragments (soil ≥2 mm) in the upper 20 cm of the alder and nonvegetated sandboxes. We are uncertain of the underlying causes. Aggregation, cementation, settling of fines, or frost-heaving may have occurred. The amount of coarse fragments influenced the mass of fine earth and hence the calculation of changes in mineral soil N content. These influences are discussed later, in the soil expansion and collapse section. Pine and locust sandboxes did not have a significant ($P < .05$) change in coarse fragment mass.

Rate of accumulation of N in vegetation and litter: ΔN_{veg}

Concentrations of N were generally 2–4 times greater in alder and locust biomass components than pines (Table 1). The two pines had similar N concentrations. Net accumulation of N (in kilograms per hectare) in tree biomass above and below the ground was 549 in alder, 290 in locust, 403 in red pine, and 348 in pitch pine. Little N accumulated in herbs in all systems, reaching only 10 kg/ha under locust. Litter N accumulations were large under alder (340 kg/ha) compared to locust and red and pitch pine (72, 28, and 44 kg/ha, respectively). Initially, planted seedlings had only small amounts of N (1–11 kg/ha).

The average rate of net N accumulation (ΔN_{veg}; in kilograms per hectare per year) was very high 5–6 yr after planting. Alder accumulated 175 kg·ha^{-1}·yr^{-1} in biomass and litter. This rate was more than double that observed in locust (52 kg·ha^{-1}·yr^{-1}), red pine (83 kg·ha^{-1}·yr^{-1}), and pitch pine (70 kg·ha^{-1}·yr^{-1}). Alder accumulated large amounts of biomass with a high N concentration. Locust had N concentrations similar to alder but had low biomass production. Pines accumulated the same or greater biomass than alder, but the biomass had a low N concentration. Thus, for different reasons, pines and locust had a similar ΔN_{veg}.

Changes in mineral soil N: $\Delta N_{soil(0-20)}$ *and* $\Delta N_{soil(0-135)}$

We measured change in mineral soil N in two ways: from the mineral soil surface to a depth of 20 cm, $\Delta N_{soil(0-20)}$, and that found in soil from the surface to the bottom of the sandbox, $\Delta N_{soil(0-135)}$. The reliability of these measurements differs. We consider the

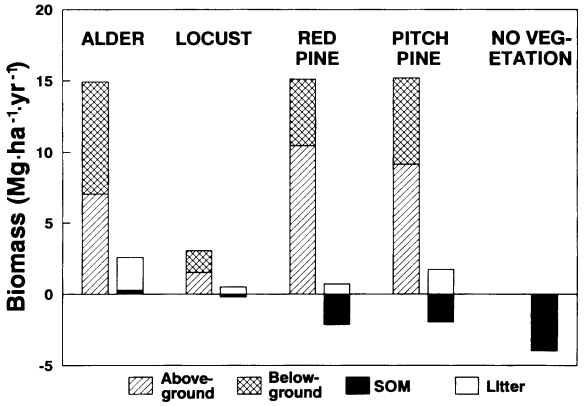

FIG. 2. Annual change in living biomass (left bar of each pair) and dead biomass (right bar). SOM is mineral soil organic matter (0–20 cm); litter is the O horizon.

TABLE 1. Accumulation of biomass and total N from planting to harvest and percent N content of biomass. Numbers in parentheses are one-half of the 95% confidence interval. Mass and N content of planted seedlings are subtracted from the accumulation totals. ΔNveg is calculated by dividing totals by the measurement period (Eq. 5).

Sandbox	Planted seedlings	Biomass at harvest							
	Total	Leaves	Branch + repro. + dead	Stem	Stump	Large roots (>2 mm)	Small roots (<2 mm)	Nodules	Above-ground total
					Biomass (Mg/ha)				
Alder	0.3	4.7	10.5	20.7	6.5	5.6	16.8	1.2	36
Locust	0.0	2.1	2.9	4.0	2.3	1.2	5.3	0.1	9
Red pine	0.7	23.7	15.2	14.4	8.2	2.9	12.6	...*	53
Pitch pine	0.2	13.0	12.0	23.4	12.9	5.3	13.9	...	48
					Percent N (g/100 g)				
Alder	0.95	3.26	0.97	0.39	0.39	0.64	0.81	1.49	0.93
Locust	2.02	3.13	1.09	0.72	1.40	1.59	1.44	2.20	1.08
Red pine	1.57	1.02	0.33	0.25	0.25	0.35	0.36	...	0.61
Pitch pine	1.30	0.96	0.38	0.23	0.26	0.46	0.39	...	0.49
					Total N content (kg/ha)				
Alder	3	150	102	108	28	36	136	17	332
		(68)	(76)	(37)	(14)	(22)	(178)	(21)	(111)
Locust	0	66	32	29	33	19	76	3	98
		(42)	(34)	(31)	(18)	(6)	(14)	(1)	(123)
Red pine	11	241	51	55	20	10	46	...	328
		(211)	(46)	(26)	(27)	(18)	(22)	...	(277)
Pitch pine	3	131	45	94	34	24	54	...	236
		(84)	(33)	(42)	(33)	(20)	(57)	...	(155)

* Not present or not applicable.

$\Delta N_{soil(0-20)}$ measurements to be most reliable. The final condition of mineral soil (0–20 cm) was assessed by collecting soil from 4 to 8 entire 0.25-m^2 growing spaces. Initial conditions were assessed with 16–18 cores at the start of the experiment. The nonvegetated sandbox was assessed with five initial and six final cores. The coefficient of variation was very low in both initial and final N mass values, ranging from 3 to 7% in the vegetated sandboxes. The coefficient of variation was slightly higher in the nonvegetated sandbox (11–13%).

Deeper mineral soil (20–135 cm) was sampled less intensively. As mentioned previously, at the start of the experiment, initial deep cores were limited to five cores taken in 5-cm increments from the nonvegetated sandbox. Comparison of initial and final deep (20–135 cm) cores were based on final measurements made in all sandboxes and initial measurements made in only the nonvegetated sandbox. A further consideration in deep core measurements was that N concentrations were quite low (0.005–0.014%), with differences between initial and final concentrations of 0.001–0.009%. Nevertheless, these apparently small differences result in large changes in N mass when multiplied by the large soil mass of the 20–135 cm layer. Because the absolute concentration was near the detection limit of our micro-Kjeldahl technique, N mass estimates have a higher variance. For these reasons, we consider our 20–135 cm mineral soil estimates to be less precise. Even so, the uniform conditions created in the sandboxes give more precise estimates than typically obtained under field conditions.

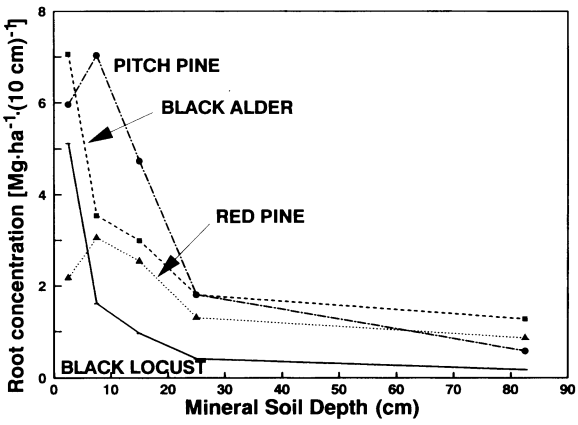

FIG. 3. Average root mass concentration in 10-cm soil layers, as function of depth.

Nitrogen accumulated rapidly in mineral soils [$\Delta N_{soil(0-20)}$] of alder and locust, declined slightly in pines, and declined sharply in the nonvegetated sandbox (Table 2). When averaged over the 3–5 yr of measurement, alder and locust accumulated N in soil (0–20 cm) at rates of 86 and 53 kg·ha^{-1}·yr^{-1}, respectively ($P < .05$). Pine soils (0–20 cm) had small nonsignificant losses of N: red pine $\Delta N_{soil(0-20)} = -17$ kg·ha^{-1}·yr^{-1} ($P = .22$); pitch pine $\Delta N_{soil(0-20)} = -19$ kg·ha^{-1}·yr^{-1} ($P = .17$). In pines, N accumulated ≈4 times faster in vegetation and litter than it was lost from soil layers.

Large unexplained N losses were observed in the nonvegetated sandbox on a 0–20 cm basis (−96 kg·ha^{-1}·yr^{-1}). Using the 0–135 cm basis in the non-

TABLE 1. Continued.

Biomass at harvest				
Below-ground total	Tree biomass	Herb biomass	Litter layer	Total
		Biomass (Mg/ha)		
30	66	0.01	11.7	77
9	18	0.71	3.1	22
24	77	...	3.7	80
32	80	0.00	6.8	87
		Percent N (g/100 g)		
0.81	0.88	1.34	2.90	...
1.78	1.43	1.36	2.33	...
0.40	0.55	...	0.75	...
0.46	0.47	1.54	0.65	...
		Total N content (kg/ha)		
244	576	0.2	340	913
(128)	(143)	(0.3)	(63)	
159	257	9.6	72	338
(17)	(124)	(1.8)	(16)	
95	423	...	28	440
(48)	(256)	...	(18)	
147	382	0.0	44	423
(19)	(176)	(0.0)	(8)	

vegetated sandbox seems more reasonable, however, because we have values for both initial and final deep-layer N mass. When this basis is used, $\Delta N_{soil(0-135)}$ is much less negative (-50 kg·ha^{-1}·yr^{-1}).

Measured input and output: N_{bp} *and* N_{dr}

Average N_{bp} for the period 1984–1989 is 5.2 kg·ha^{-1}·yr^{-1} (Table 3), more than an order of magnitude less than N accumulation in vegetation, litter, and soil (0–20 cm). Bulk precipitation has little influence on the N budget of these systems. Cloudwater deposition at the elevation of this site is unlikely and is assumed to be negligible (Weathers et al. 1988). Dry deposition and N_2 fixation, as sources of unexplained N, are considered later.

The pattern of discharge from the monitored pine and nonvegetated sandboxes was initially similar. After vegetation developed, however, discharge from the pine sandbox systematically declined. Large discrepancies did occur, especially in early spring, apparently resulting from differential melting of accumulated snow. In addition, considering that these discharge measurements are based on point samples, differences in discharge between two sandboxes might be large for short periods if, for example, the sample was taken during the rising limb of hydrographs that were slightly out of phase in the two sandboxes. Nevertheless, starting in 1985, discharge from the pine sandbox was clearly and systematically less than that from the nonvegetated sandbox (Table 4).

During the measurement period in the nonvegetated sandbox, a bimodal trend was found in NO_3^- concentration of leachate (Table 4), i.e., an initial flush in early spring followed by low but increasing NO_3^- export as temperatures of the soil surface increased.

Nitrate concentrations in leachate from the pine sandbox were low during the 1984 spring melt and lower during the 1984 and 1985 growing seasons. Nitrate was below detection limits in 1986 and remained below detection limits in samples taken during the 1990 field season. This pattern coincides with canopy closure in the pine sandbox and the systematic deviation in discharge between the pine and nonvegetated sandboxes.

Limited analyses of NH_4^+, dissolved organic N, NO_2^-, and ortho-phosphate indicated that none of these components are likely to have been important in the sandbox leachate.

Export was estimated from the product of the interpolated, seasonal, accumulated water yield and average seasonal concentration in the discharge (Table 4). Prior to planting in 1983, N export was high in both sandboxes and then fell sharply (Fig. 4). By the time of initial soil sampling, N export was low in the nonvegetated and nearly zero in the red pine sandbox. We attribute the lack of NO_3^- to uptake by vegetation.

To estimate N_{dr} for the alder, locust, red pine, and pitch pine sandboxes during the soil measurement period, we used the average drainage loss of N from the monitored red pine sandbox, <1 kg·ha^{-1}·yr^{-1}. If N_{dr} is higher than this in the alder or locust sandbox, then unexplained N would have to be higher. The nonvegetated sandbox average N_{dr} for this period was 7 kg·ha^{-1}·yr^{-1}.

Unexplained nitrogen accumulation (loss): $N_{unexplained}$

When the terms of mass-balance equation (Eq. 3) are assembled, we can calculate rates of $N_{unexplained}$ (Table 5; Fig. 5). Clearly the most important term in the equation is ΔN_{veg}, followed by $\Delta N_{soil(0-20)}$. Inputs in bulk precipitation and outputs via N_{dr} are not very important for these systems.

Adjusting for soil expansion and collapse

Soil expansion or collapse over time can influence calculation of changes in soil N storage when a shallow fixed-depth measure is used. Expansion of the upper 22.9-cm mineral soil layer over an 83-yr period at Rothamsted brought about a 22% decline in the mass of fine earth, ashed to remove organic matter (Jenkinson 1971). We measured significantly ($P < .05$) reduced soil bulk density and hence soil expansion in the 0–20 cm layer of alder (-4%), locust (-5%), and pitch pine (-5%). Collapse of the 0–20 cm layer was indicated by a significant ($P < .05$) increase in soil bulk density in the red pine ($+5\%$). No significant change in bulk density was observed in the nonvegetated sandbox.

Changes in mass of fine soil that we observed cannot

TABLE 2. Difference between initial and final conditions in planted and nonvegetated sandboxes. Numbers in parentheses are one-half of the 95% confidence interval. Average annual ΔNsoil is calculated by dividing the 0–20 cm and 0–135 cm sums by the duration of the measurement period.

Box	Year	Sampled soil depth zones (cm)							Calculated sums (cm depth)	
		0–10	10–20	20–30	30–40	40–60	60–80	80–135	0–20	0–135†
					Mass of fine (<2 mm) soil (Mg/ha)					
Alder	1984	1172	1215	...*	...	...	...	...	2387	16250
	1988	1086	1182	1196	1120	2269	2454	6602	2268	15908
Locust	1984	1148	1192	...	...	...	...	...	2340	16262
	1989	1077	1207	1211	1170	2378	2432	6728	2284	16204
Red pine	1984	1159	1203	...	...	...	...	...	2362	16224
	1988	1236	1215	1257	1142	2312	2588	6977	2451	16727
Pitch pine	1984	1155	1210	...	...	...	...	...	2365	16228
	1988	1094	1186	1209	1192	2427	2456	6851	2280	16415
No veg	1985	1085	1186	1197	1192	2438	2375	6661	2271	16134
	1988	1092	1199	1173	1146	2365	2365	6503	2291	15842
					Percent N (g/100 g)					
Alder	1984	0.053	0.030	...	...	...	...	...		
	1988	0.077	0.040	0.014	0.008	0.008	0.009	0.008		
Locust	1984	0.086	0.051	...	...	...	...	...		
	1989	0.114	0.048	0.014	0.014	0.010	0.014	0.013		
Red pine	1984	0.050	0.032	...	...	...	...	...		
	1988	0.049	0.023	0.012	0.009	0.009	0.008	0.008		
Pitch pine	1984	0.051	0.027	...	...	...	...	...		
	1988	0.049	0.026	0.011	0.007	0.008	0.007	0.008		
No veg	1985	0.049	0.023	0.005	0.005	0.006	0.005	0.006		
	1988	0.039	0.007	0.007	0.007	0.007	0.007	0.007		
					Total N content (kg/ha)					
Alder	1984	614	358	...	...	...	...	...	978	1754
	1988	826	476	167	95	191	213	528	1303	2496
Locust	1984	1000	608	...	...	...	...	...	1601	2556
	1989	1222	583	175	164	234	343	861	1806	3583
Red pine	1984	580	378	...	...	...	...	...	956	1732
	1988	610	282	153	101	204	202	530	891	2082
Pitch pine	1984	590	328	...	...	...	...	...	917	1693
	1988	529	313	135	88	184	177	557	842	1983
No veg	1985	529	271	66	65	153	114	378	801	1615
	1988	430	82	82	81	169	169	466	512	1479
					Difference in N content (kg/ha)					
Alder		212	118	...	...	...	...	...	325	742
		(122)	(175)	...	...	...	...	...	(198)	(480)
Locust		222	−25	...	...	...	...	...	205	1027
		(146)	(134)	...	...	...	...	...	(240)	(288)
Red pine		30	−96	...	...	...	...	...	−65	350
		(93)	(151)	...	...	...	...	...	(90)	(467)
Pitch pine		−61	−15	...	...	...	...	...	−76	290
		(128)	(80)	...	...	...	...	...	(98)	(386)
No veg		−100	−189	16	16	17	55	88	−289	−136
		(145)	(119)	(16)	(17)	(53)	(29)	(106)	(231)	(409)

* No initial samples were taken below 20 cm in these sandboxes.
† 1984 0–135 cm sums are based partly on 1985 nonvegetated 20–135 cm values.

be explained well by changes in organic matter because of the generally low organic matter concentrations (< 1.9% initially). We measured larger absolute changes in coarse fragment mass and root biomass. These changes in bulk density may also reflect the change in sampling procedure from coring to large-area sampling. These factors more likely influenced our measurements of bulk density in this layer more than did changes in organic matter content.

To account for changes in bulk density and organic matter additions and losses, we calculated new values for $\Delta N_{soil(0-20)}$ and $N_{unexplained}$ by correcting for fine-earth mass and organic matter (Table 5). The initial mass of fine earth (0–20), free of organic matter, was multiplied by the N concentration of both initial and final soil layers. This modification increased $N_{unexplained}$ by 16, 9, and 5 kg·ha^{-1}·yr^{-1} or 6, 10, and 10% in alder, locust, and pitch pine, respectively. $N_{unexplained}$ decreased for red pine by 8 kg·ha^{-1}·yr^{-1} or 15%, and the nonvegetated sandbox was unchanged. This adjustment does not fundamentally change the conclusion of large positive $N_{unexplained}$ in vegetated sandboxes and negative

TABLE 3. Annual input of N in bulk precipitation (N_{bp}) as ammonium and nitrate (kg·ha^{-1}·yr^{-1}; G. Likens, *personal communication*).

Year	NH_4	NO_3	Sum
1984	1.0	3.6	4.6
1985	1.1	4.1	5.2
1986	1.4	4.0	5.4
1987	1.2	3.6	4.8
1988	1.6	4.4	6.0
Average annual sum N_{bp}:			5.2

$N_{unexplained}$ in the nonvegetated sandbox, based on the 0–20 cm soil data. No adjustment is needed for the 0–135 cm calculations.

Direct measures of nitrogenase activity

We confirmed that rapid nitrogenase activity can occur in our red and pitch pine rhizospheres with measurements of acetylene reduction under low-O_2 conditions (Fig. 6). The highest rates of ethylene formation per unit mass of dry roots that we observed (0.7–1.8 nmol·g^{-1}·h^{-1}) are similar to the rapid rates reported for sugar cane (1.3–4.1 nmol·g^{-1}·h^{-1}; Dobereiner et al. 1972). Extrapolating our acetylene reduction measurements to a per-unit-area and annual or multiyear basis is not appropriate because of the limited extent of our sampling and the previously discussed problems including the uncertain ratio of C_2H_4 fixed to N_2 fixed and unknown O_2 concentrations in the rhizosphere.

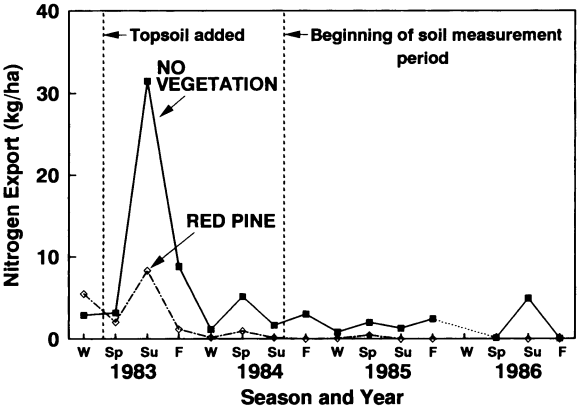

FIG. 4. Nitrogen export from monitored sandboxes.

Bacterial cultures were obtained from both red and pitch pine root surfaces in Rennie's N-free medium under microaerophilic conditions. Red and pitch pine cultures actively reduced acetylene (317 and 20 nmol·vial^{-1}·h^{-1}, respectively). The taxonomy of these bacteria remains unknown. Cultures were not obtained in Burk's or Dobereiner's N-free medium, suggesting that the bacteria cannot use malate or sucrose and thus are not *Azospirillum* or *Azotobacter*.

DISCUSSION

Unexplained N inputs into our sandboxes ranged from 54 kg·ha^{-1}·yr^{-1} in pine to 271 kg·ha^{-1}·yr^{-1} in

TABLE 4. Estimate of N loss in hydrologic export from monitored sandboxes based on hydrologic export times average concentration (ND indicates no data).

		Hydro. export (L)		Avg. NO_3-N flux (mg/L)		Avg. N flux (kg/ha)	
Season	Year	Red pine	No veg	Red pine	No veg	Red pine	No veg
Winter	1983	39300	26100	0.79	0.62	5.5	2.9
Spring	1983	24300	25200	0.48	0.71	2.0	3.2
			Topsoil added				
Summer	1983	42100	53400	1.11	3.31	8.3	31.5
Fall	1983	12300	12200	0.55	4.08	1.2	8.8
Winter	1984	6600	11500	0.14	0.57	0.2	1.2
Spring	1984	16100	22200	0.34	1.31	1.0	5.2
Summer	1984	12100	11500	0.07	0.82	0.2	1.7
			Beginning of soil measurements				
Fall	1984	6600	7700	0.03	2.23	0.0	3.0
Winter	1985	5500	4200	0.08	1.11	0.1	0.8
Spring	1985	11000	13600	0.23	0.83	0.5	2.0
Summer	1985	1300	3000	0.04	2.49	0.0	1.3
Fall	1985	5500	8200	0.03	1.68	0.0	2.4
Winter	1986	ND	ND	ND	ND	ND	ND
Spring	1986	600	1500	0.58	0.2	ND	ND
Summer	1986	5800	14700	0	1.89	0.0	4.9
Fall	1986	2000	5500	0	0.08	0.0	0.1
Winter	1987	ND	ND	ND	ND	ND	ND
Spring	1987	14200	17900	0.02	0.45	0.1	1.4
Summer	1987	200	12000	0.00	0.64	0.0	1.4
Fall	1987	500	4900	0.00	0.18	0.0	0.2
Avg. annual export						0.3	7.0

TABLE 5. Components of unexplained N accumulation or loss based on Eq. 3 and a 0–20 cm soil depth. Variation is shown as one-half of the 95% confidence interval. Adjusted $N_{unexplained}$ is corrected for soil expansion and collapse.

Sandbox	ΔN_{veg}		ΔN_{soil} (0–20)		N_{bp}	N_{dr}	$N_{unexplained}$		Adjusted $N_{unexplained}$
	$\bar{X}$	½ CI	$\bar{X}$	½ CI			$\bar{X}$	½ CI	
	Unexplained N: concentration changes (kg·ha^{-1}·yr^{-1})								
Alder	175	12	86	52	5	1	255	56	271
Locust	52	18	43	50	5	1	90	70	99
Red pine	83	8	−17	23	5	1	62	34	54
Pitch pine	70	40	−19	25	5	1	49	47	54
No veg	0	0	−96	77	5	7	−94	75	−94

alder sandboxes (Table 5). Unexplained N output of 94 kg·ha^{-1}·yr^{-1} was observed in the nonvegetated sandbox. Unexplained N changes might be explained by dry deposition (N_{dd}), volatilization (N_{vol}), and biological N_2 fixation (N_{bnf}; Eq. 4).

Dry deposition

Gaseous N may be added to ecosystems through physical–chemical dynamics in the atmosphere. This so-called "dry deposition" enters ecosystems in the form of nitric oxide, nitrogen dioxide, nitric acid vapor, and other forms of organic and particulate N. Rates of N_{dd} measured in Europe (Ivens et al. 1987, Derwent et al. 1988, Goulding 1990) are substantially higher than measured rates in the eastern U.S. (Lindberg et al. 1986, Lovett and Lindberg 1986, Lovett, *in press*). Data most applicable to Hubbard Brook suggest that dry N deposition is probably less than the N deposited in bulk precipitation. Thus, we assume $N_{dd} = 5$ kg·ha·yr^{-1}. Dry deposition of N would have to be ≈10 times greater than this to balance the N budget in the pine sandboxes without N_2 fixation.

Volatilization

Nitrogen can volatilize as a result of biological processes or from chemical reactions in the soil. Chemical denitrification is unlikely because of the nearly neutral pH of these soils, and NH_3 volatilization is unlikely because NH_4^+ was not present in the leachate. Biological denitrification (including NO_X, N_2O, and N_2 emissions) might have occurred to some extent where NO_3^- was present in the nonvegetated sandbox or early in the experiment before vegetation exerted control over NO_3^- export. Without a concerted effort, using appropriate instrumentation and frequent sampling, this loss cannot be quantified. For vegetated sandboxes, we assume $N_{vol} = 0$. If denitrification occurred in the vegetated sandboxes, then Eq. 4 suggests that $N_{unexplained}$ must be higher, and, consequently, N_{bnf} or N_{dd} must be higher to balance the N budget.

The nonvegetated sandbox was characterized by substantial losses of N (−94 kg·ha^{-1}·yr^{-1}) from the 0–20 cm soil layer (Fig. 5, top panel). Over the 0–135 cm depth, $N_{unexplained}$ was less negative (−43 kg·ha^{-1}·yr^{-1}). Because the calculation of $N_{unexplained}$ includes drainage losses of N, our equation for $N_{unexplained}$ suggests that this large loss results from biological or chemical volatilization processes. Denitrification seems to be the most likely possibility. Had any N_{bnf} occurred, it would only increase calculated N_{vol}.

Biological N_2 fixation

The physiology and microbiology of symbiotic N_2-fixing bacteria that form root nodules (*Rhizobium* and

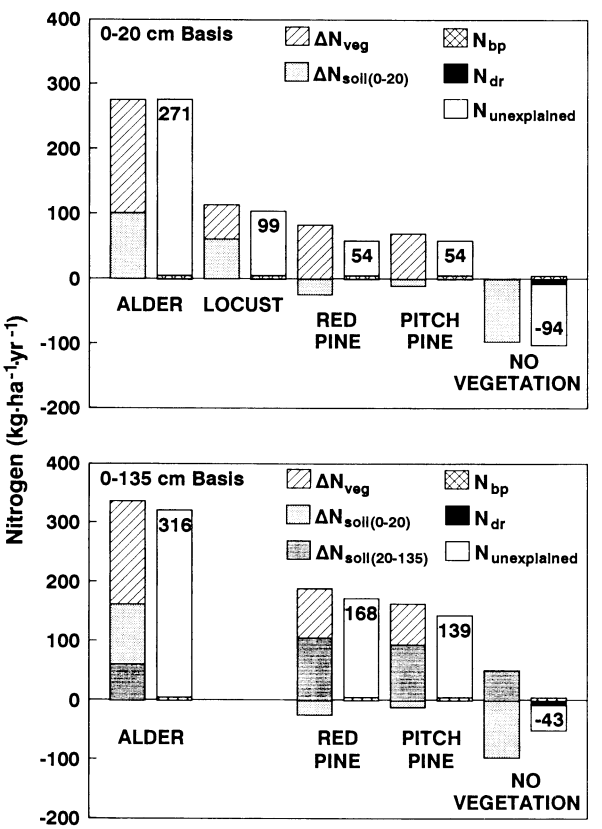

FIG. 5. Changes in N storage in the top 20 cm (top panel) and in the top 135 cm (bottom panel) in vegetation and soil (left bar for each species) and average annual inputs, outputs, and $N_{unexplained}$ (right bar). Numbers in the right bar are the values for $N_{unexplained}$.

Frankia) are well known. Free-living microbes that fix N_2, such as *Azotobacter* and *Clostridium,* have also been extensively studied. So-called associative N_2 fixers are less well known. These rhizosphere bacteria live on root surfaces, within mycorrhizal sheaths, or in the intercellular spaces within the root. Specialized facultative anaerobic bacteria such as *Azospirillum* have been implicated as the principal N_2-fixing agent in sugar cane (Dobereiner 1980) and in some conifers and shrubs (Li and Hung 1987, Amaranthus et al. 1990). Rapid N_2 fixation in the rhizosphere may result from an ample supply of photosynthate and a lower O_2 concentration because of intense respiration of roots and microbes. Evidence for biological N_2 fixation in our study is examined below.

Alder.—$N_{\text{unexplained}}$ adjusted for soil expansion is 271 kg·ha^{-1}·yr^{-1} (Fig. 5, top panel). Dry deposition and N_2 fixation are the input portions of $N_{\text{unexplained}}$. Thus, if we assume N_{dd} = 5 and N_{vol} = 0 kg·ha^{-1}·yr^{-1}, 266 kg·ha^{-1}·yr^{-1} can be ascribed to N_2 fixation by *Frankia* associated with the root nodules of *Alnus glutinosa.* Unexplained N increases to 316 kg·ha^{-1}·yr^{-1} when our less reliable deep soil change is taken into account (Fig. 5, bottom panel). For this reason, we consider the N accumulation rate of 266 kg·ha^{-1}·yr^{-1} to be conservative.

Rates of N_2 fixation are thought to range up to as much as 325 kg·ha^{-1}·yr^{-1} in alders (Cromack et al. 1979) and up to 500 kg·ha^{-1}·yr^{-1} in cultivated legume crops (Russell 1973). Rates for black alder in this study (266 kg·ha^{-1}·yr^{-1}) are higher than most values in the literature. The extraordinary nodule biomass (1200 kg/ha) and high rates of biomass accumulation (66 Mg/ha) we measured lend credibility to our estimates of high N_2 fixation rates. Akkermans (1971) found 444 kg/ha of nodules in a black alder stand in Denmark. Five-yr-old red alder plantations in Oregon had only 150 kg/ha (Bormann and Gordon 1984).

Locust.—Adjusted $N_{\text{unexplained}}$ is 99 kg·ha^{-1}·yr^{-1} (Fig. 5, top panel). With N_{dd} = 5 and N_{vol} = 0, N_{bnf} is 94 kg·ha^{-1}·yr^{-1}. This increment can be primarily ascribed to N_2 fixation by *Rhizobium* associated with the root nodules of *Robinia pseudoacacia.* This rate should also be considered conservative because of potential deeper soil N accumulation and volatilization. The ratios of unexplained N to biomass increment are similar in alder and locust, suggesting that N_2 fixation in locust is limited by low productivity. Black locust was not as well adapted as either alder or pine to the sandbox environment from a production perspective.

Red pine and pitch pine.—Adjusted $N_{\text{unexplained}}$ is 54 kg·ha^{-1}·yr^{1} for both pines (Fig. 5, top panel). The pattern of N accumulation for the pine systems was different from that of the symbiotic N_2 fixers in that pine systems lost N from the mineral soil. These losses, however, were not significant (P = .22 for red pine; P = .12 for pitch pine). If we assume N_{vol} = 0 and N_{dd} = 5 kg·ha^{-1}·yr^{-1}, N_{bnf} must be 49 kg·ha^{-1}·yr^{-1} for red and pitch pine in order to balance the N gain. When our less reliable change in deep soil is taken into account, $N_{\text{unexplained(0–135 cm)}}$ increases to 168 and 139 kg·ha^{-1}·yr^{-1} for red and pitch pine, respectively (Fig. 5, bottom panel). Any N volatilization would increase our estimates of N_{bnf} further. For these reasons, we consider the rates based on soil (0–20 cm) to be conservative.

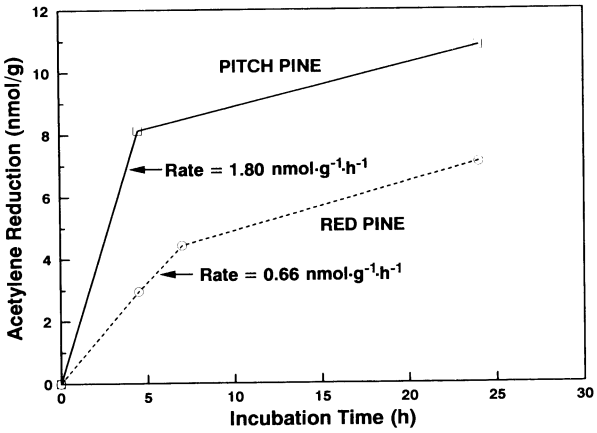

FIG. 6. Maximum observed ethylene production in pitch and red pine rhizospheres per unit of dry root mass.

Because free-living microbes are thought to be energetically limited, rhizosphere N_2 fixation becomes the most likely source of this unexplained N accumulation. N_2 fixation in pines is exceeded by locust and alder by a factor of 2–5, respectively. The ratio of N_{bnf} to biomass increment is 6–10 times higher in alder and locust than pines, indicating greater allocation, or efficiency in transfer, or photosynthate to N_2-fixing microbes.

Multiple lines of evidence support the conclusion that substantial associative N_2 fixation can occur in pines. Many observations of N gain have been reported (e.g., Stevenson 1959, Richards 1964, Moore 1966); various direct measures have been completed (e.g., Richards and Voigt 1964, Bevege et al. 1978, Delwiche et al. 1979); and microbial agents have been identified (Li and Hung 1987). Our more complete mass–balance study provides stronger evidence for substantial in situ N_2 fixation in pines, and this conclusion is supported by our observations of nitrogenase activity in pine rhizospheres and cultured bacteria.

Land restoration and sustainable forestry

Pines with rhizosphere microbes that fix N_2 at rates of ≈50 kg·ha^{-1}·yr^{-1} might be used for land restoration or to develop silvicultural systems that are N self sufficient. Nitrogen self sufficiency is achieved when the net N balance is positive after accounting for all N transfers including harvest removals (Bormann and Gordon 1989). We emphasize the need to better understand the conditions that lead to substantial N_2 fixation in pine sandboxes before such systems can be

developed for operational use, however. For example, the initial oxidation and mixing of topsoil with glacially ground outwash sands may have increased weathering and the supply of phosphorus, which is known to limit N_2 fixation. N_2 fixation may be suppressed in systems with higher N content. Beneficial rhizosphere organisms may have flourished because deeply buried outwash sand parent material lacked competing microbes and antagonistic compounds. Cultivars of sugar cane and rice are known to differ greatly in rates of rhizosphere fixation (App et al. 1980, Miranda and Boddey 1987). Thus, genetic control over N_2 fixation in pine-associated microflora systems may be important. Questions of this nature should form the basis for future investigation.

Conclusions

The sandbox approach combined with biogeochemical data provides an accurate method for evaluating N budgets for experimental ecosystems under field conditions. It also provides opportunities for studying the effects of plant–soil interactions. For the species and ecosystems reported here, N added in precipitation and lost in drainage was unimportant relative to biological N_2 fixation. Preventing vegetation from becoming established in a sandbox resulted in substantial losses of N in drainage water. Mass-balance analysis suggests that, in the nonvegetated sandbox, volatilization was greater than drainage losses. Ecosystems with symbiotic N_2 fixers, *Alnus* and *Robinia,* were conservatively estimated to fix 266 and 94 $kg \cdot ha^{-1} \cdot yr^{-1}$, respectively. The rate of fixation was apparently related to the rate of biomass accumulation. Associative N_2 fixation by *Pinus rigida* and *P. resinosa* was conservatively estimated to be ≈ 50 $kg \cdot ha^{-1} \cdot yr^{-1}$. This rate is consistent with our measurements of nitrogenase activity and is comparable to rates of associative N_2 fixation in grasses. Symbiotic N_2 fixation was 2–5 times more efficient than associative N_2 fixation. This difference suggests a greater allocation or more efficient transfer of photosynthate to symbiotic N_2-fixing microbes. The pattern of N accumulation for pine ecosystems was different from the symbiotic N_2 fixers. *Alnus* and *Robinia* ecosystems accumulated N in biomass, litter, and soil layers, and pine systems accumulated N biomass and litter but lost N from soil layers. These findings indicate the need for accelerated research on associative N_2 fixation in other pines and other conifers. If substantial associative N_2 fixation is possible on a wide array of sites, new approaches to land restoration and sustainable forestry will be possible.

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