Estimating \( N_2 \) fixation in two species of \textit{Alnus} in interior Alaska using acetylene reduction and \( ^{15}N_2 \) uptake\textsuperscript{1}

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Abstract: In interior Alaskan boreal forests two species of alder, \textit{Alnus tenuifolia} and \textit{Alnus crispa}, represent keystone species in floodplain and upland landscapes, respectively, due to the ability of these plants to form symbiotic associations with the nitrogen-fixing actinomycete, \textit{Frankia}. It is believed that as much as 70\% of the nitrogen (N) accumulated during the 200-y successional development of these forests is derived through atmospheric fixation by these species. Estimates of gross N inputs in these and many other ecosystems have traditionally utilized the acetylene reduction assay (ARA), which requires a conversion factor of the ratio of acetylene to \( N_2 \) reduced by nitrogenase, the primary enzyme. Despite the fact that small variations in the reduction ratio can substantially influence estimates of N inputs, few studies have investigated how it varies spatially and temporally. The present study sought to 1) determine this conversion factor for both species of alder in situ by calibration of the ARA against a \( ^{15}N_2 \) uptake method we developed for field use and 2) determine whether the conversion factor varied with the successional stage in which the alders occurred. Averaged across all plants, the ratio of acetylene to \( N_2 \) reduced was significantly greater in \textit{A. crispa}. Significant differences in the value of the conversion factor were observed between early succession and later (mid and late) successional sites for both species. Such differences were also observed among replicate sites within and among stages. However, these stage and site differences may also be due to seasonal effects, which could not be controlled for with our design. Specific acetylene reductase activity (SARA) was only correlated with \( ^{15}N_2 \) uptake for early successional sites measured early in the growing season, when \( N_2 \)-fixation rates were lowest and the conversion factor was closest to the theoretical value of 4. A significant negative correlation was found between the conversion factor value and the rate of enzyme activity as determined by the \( ^{15}N_2 \) uptake method. Two hypotheses are proposed to explain this result: 1) that it is due to changes in the kinetic properties of nitrogenase at high levels of enzyme activity, resulting in an increased affinity of the enzyme for \( N_2 \) relative to \( C_2H_2 \), and 2) that the concentration of \( C_2H_2 \) used in our ARA was insufficient to saturate nitrogenase.

Keywords: acetylene reduction, ARA, Alaska, alder, boreal, \( C_2H_2:N_2 \) conversion factor, \textit{Frankia}, \( ^{15}N_2 \) uptake, nitrogen fixation, nitrogenase, Tanana River.

Résumé : Dans les forêts boréales du centre de l’Alaska, \textit{Alnus tenuifolia} et \textit{Alnus crispa} représentent les espèces-clés respectivement des plaines inondables et des hautes terres, en raison de leur habileté à former des associations symbiotiques avec le champignon actinomycète fixateur d’azote (N) \textit{Frankia}. On croit qu’au moins 70\% de l’azote accumulé au cours de la succession forestière, soit pendant 200 ans, proviendrait de source atmosphérique et serait fixé par ces champignons. Les estimés des apports en azote dans ces écosystèmes sont généralement établis à partir du dosage de la réduction de l’acétylène (DRA). Cette méthode nécessite l’utilisation d’un facteur de conversion du rapport entre l’acétylène et le \( N_2 \) réduit par la nitrogénase, l’enzyme primaire. Malgré le fait que de petites variations dans le rapport de réduction puissent modifier de façon substantielle les estimés produits, peu d’études se sont penchées sur les variations spatiales et temporelles de ce problème. Dans ce travail, nous avons tenté : 1) d’établir \textit{in situ} ce facteur de conversion pour les deux espèces d’aules par la calibration du DRA en tenant compte d’une méthode de captation de \( ^{15}N_2 \) qui nous avons développée pour utilisation sur le terrain et 2) de déterminer si le facteur de conversion varie selon le stade successional où se trouvent les aules. Le rapport moyen entre l’acétylène et le \( N_2 \) réduit était significativement plus élevé chez \textit{A. crispa}. Nous avons trouvé des différences significatives pour la valeur du facteur de conversion entre les stades successionnels chez les deux espèces. De telles différences sont aussi observées entre les réplicats pour un même stade successional ou entre les stades de succession. Ces différences peuvent être dues à des effets saisonniers, qui ne pouvaient être contrôlés pendant l’étude. Au début de la saison de croissance, l’activité de la réductase spécifique de l’acétylène (ARSA) n’est corréllée qu’avec la captation de \( ^{15}N_2 \) dans les sites en début de succession. Les taux de fixation de \( N_2 \) sont alors particulièrement bas et le facteur de conversion est très près de la valeur théorique de 4. Nous avons trouvé une corrélation négative significative entre la valeur du facteur de conversion et le taux de l’activité enzymatique déterminé par la méthode de captation du \( ^{15}N_2 \). Nous proposons deux hypothèses pour expliquer ce résultat : 1) les propriétés cinétiques de la nitrogénase changeraient à de hauts niveaux d’activité enzymatique, ce qui entraînerait une plus grande affinité de l’enzyme pour le \( N_2 \) par rapport au \( C_2H_2 \), et 2) la concentration de \( C_2H_2 \) utilisée lors de nos DRA était trop basse pour saturer la nitrogénase.

Mots-clés : Alaska, aulne, boréal, captation de \( ^{15}N_2 \), DRA, facteur de conversion du \( C_2H_2:N_2 \), fixation de l’azote, \textit{Frankia}, nitrogénase, réduction de l’acétylène, rivière Tanana.


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Introduction

Biologically available nitrogen (N) widely limits primary production in terrestrial ecosystems (Vitousek & Field, 1999). The largest non-anthropogenic input of N to the global N cycle is provided by N₂-fixing microbes in symbiotic associations with plants (Paul, 1988; Tate, 1995). These associations are of particular importance to the successional development of interior Alaska boreal forests, where as much as 70% of the N accumulated over the 200-y primary successional sequence on the Tanana River floodplain enters during the first 20-30 y via N₂ fixation by actinomycete bacteria of the genus Frankia in association with Alnus tenuifolia (Van Cleve et al., 1991; Viereck, Dymnss & Foote, 1993; Uliassi & Russ, 2002). The N fixed during this stage has been found to influence the productivity of subsequent succession on the Tanana River floodplain (Van Cleve et al., 1991; 1996). Similarly, it appears that the invasion of alder into interior Alaska during the early Holocene contributed to both the productivity of aquatic systems and N-cycling rates of the Alask River during the early Holocene contributed to both the

Rate-based estimates of N inputs due to N₂ fixation by interior Alaskan alder have relied on the use of the acetylene reduction assay (ARA) as an estimator of nitrogenase activity (Klingensmith & Van Cleve, 1993; Uliassi & Russ, 2002). This technique is based on the assumption that the N₂-fixing enzyme complex, nitrogenase, preferentially reduces acetylene (C₂H₂) to ethylene (C₂H₄) over N₂ to ammonia under an atmosphere containing more than 10% C₂H₂ (Hardy et al., 1968; Rivera-Ortiz & Burris, 1975; Shah, Davis & Brill, 1975). The incubation atmosphere is analyzed for C₂H₄ at varying intervals, providing a simple and low-cost assay of nitrogenase activity (Hardy et al., 1968). A major limitation of the method is acetylene-induced decline, or inhibition of nitrogenase activity by C₂H₄ within a few minutes of initial exposure (Minchin et al., 1983). However, this effect can be minimized or eliminated using short (2-6 min) incubation times and taking the maximum value of acetylene reduction as representative of pre-assay nitrogenase activity (Minchin et al., 1983; Schiwntzer & Tjepkema, 1994).

A second limitation of the ARA as a quantitative indicator of nitrogenase activity is the lack of stoichiometric equivalence between the two reactions catalyzed by the enzyme; i.e., the molar amounts of C₂H₂ converted to C₂H₄ and of N₂ converted to NH₃ are not equal. A ratio of 3 moles of C₂H₂ to 1 mole of N₂ is commonly assumed based on the observation that nitrogenase requires one pair of electrons to reduce C₂H₂ to C₂H₄, but three electron pairs to reduce N₂ to two molecules of NH₃ (Hardy et al., 1968). However, based on the discovery that nitrogenase reduces at least one pair of protons to H₂ gas per molecule of N₂, but none to reduce C₂H₂, some authors have proposed a value of 4:1 for this ratio (Simpson & Burris, 1984; Miller, 1991). By calibration against ¹⁵N₂ uptake methods, the ratio has been experimentally determined for a wide variety of species and found to vary by three orders of magnitude, depending on the nature of the system and the conditions under which the assay is performed (Seitzinger & Garber, 1987; Liengen, 1999). This has led several authors to recommend independent determination of the ratio for each biological system on which the ARA is to be used for quantifying N₂ fixation rates (Saito, Matsui & Salati, 1980; Nohrstedt, 1983; 1985; Seitzinger & Garber, 1987; Schwintzer & Tjepkema, 1994; Montoya et al., 1996). However, such determinations have only been reported for a handful of actinorhizal species in temperate (Fessenden, Knowles & Brouzes, 1973), boreal (Sellstedt, 1986), and tropical ecosystems (Vitousek & Walker, 1989).

Ecosystem level estimates of N inputs based on the ARA are sensitive to the value chosen for the conversion between acetylene reduction (AR) and N₂ fixation. Using a short-term assay to minimize C₂H₂ inhibition and a conversion factor of 3:1, Uliassi and Ruess (2002) estimated N₂ fixation inputs from A. tenuifolia of 59 ± 11 kg N·ha⁻¹·y⁻¹ and 39 ± 11 kg N·ha⁻¹·y⁻¹ in early-successional (alder canopy) and mid-successional (mature balsam poplar canopy) interior Alaskan floodplain forests, respectively (mean ± SE). Comparing these values to previously published whole-stand N accumulation data from the same area, they estimated annual losses of 29% (17 kg N·ha⁻¹·y⁻¹) and 74% (29 kg N·ha⁻¹·y⁻¹), respectively, from the two forest types, but acknowledged the strong dependence of their conclusions on the value of the C₂H₂ to N₂ reduction ratio.

The objectives of this study were 1) to determine the C₂H₂ to N₂ reduction ratio for thinleaf alder (Alnus tenuifolia) occurring in primary successional series of the Tanana River floodplain and green alder (Alnus crispa) occurring in secondary series in upland areas adjacent to the Tanana River floodplain and 2) to evaluate the variability of this ratio within and between the two Alnus species in their respective habitats. To accomplish this, we simultaneously measured AR and ¹⁵N₂ uptake by excised nodules in situ for both plant species growing in replicate early-, mid-, and late-successional floodplain and upland forests in interior Alaska.

Methods

Study sites

Sites for this study included early, mid-, and late-successional boreal forests located within the Bonanza Creek Experimental Forest, approximately 30 km southwest of Fairbanks, Alaska (64° 48' N, 147° 52' W). The general restriction of green alder to upland forests and thinleaf alder to floodplain forests, while not absolute, is thought to be related to soil moisture content, which is typically greater in floodplain stands (Hultén, 1968; Viereck & Little, 1986; Van Cleve et al., 1991). However, there may also be species-specific differences in response to soil phosphorus and pH, which differ between the two landscapes (Van Cleve et al., 1993; Viereck et al., 1993). Both species propagate by seed, but vegetative propagation may be more important in explaining the persistence of alder throughout all stages of succession.

Half of our study sites were located in forests along the floodplain of the Tanana River, where A. tenuifolia is common. These sites included early-successional stands dominated by A. tenuifolia and balsam poplar (Populus balsamifera) (15-25 y), mid-successional stands dominated
by balsam poplar with a near-continuous understory of *A. tenuifolia* (75-100 y), and late-successional stands of mature white spruce (*Picea glauca*) with a dense understory of *A. tenuifolia* (100-175 y) (Viereck, Dyrness & Foote, 1993). The other half of our study sites were located in upland areas adjacent to the Tanana River floodplain, in which the dominant alder species is *A. crispa*. These sites included early successional post-burn stands dominated by a mixture of *A. crispa*, paper birch (*Betula papyrifera*), and trembling aspen (*Populus tremuloides*) (15-25 y), mid-successional stands dominated by paper birch and/or aspen, with a sub-canopy of *A. crispa* (50-85 y), and late-successional stands of mature white spruce with a mixed sub-canopy of *A. crispa* and paper birch (75-200 y).

For each successional stage within both upland and floodplain forests, three replicate stands, separated by up to 5 km, were selected, for a total of 18 sites. Our sites are included within a larger network of permanent sites under study by the Bonanza Creek Long Term Ecological Research Program (BNZ LTER). Further information concerning these sites can be found on the BNZ LTER website (http://www.lter.uaf.edu/).

The climate of the region is characterized by an intensely cold snow period averaging 214 d and annual temperature extremes ranging from -50 °C to 35 °C. Average daily temperatures range from -24.9 °C in January to 16.4 °C in July, with an average annual temperature of -3.3 °C. Potential evapotranspiration (466 mm) exceeds average annual precipitation (269 mm), 65% of which falls during the growing season, which typically extends from mid-May to early September (Viereck, Dyrness & Foote, 1993).

Striking differences in soil climate accompany successional development in both upland (elevation = 270-375 m) and floodplain landscapes (elevation = 135 m), where canopy closure and the development of a continuous moss layer in later successional stages reduce average soil temperatures. Landscape differences in soil climate are also strongly influenced by topography. For example, north-facing slopes are usually underlain by permafrost and contrast sharply with the warm, well-drained soils of south-facing slopes. Poorly-drained black spruce lowlands are also largely underlain by permafrost. Parent material, slope, aspect, time, and climate have been used to describe the mosaic of soils found throughout interior Alaska. Soils are uniformly immature, reflecting the characteristics of three parent material categories: bedrock composed of Precambrian schist, thick loess deposits originating from glacial periods, and alluvial deposits in floodplain areas. Soils range from poorly-drained cold soils with shallow permafrost to warm well-drained soils in the uplands that support mature white spruce communities. Upland south-facing forests can experience drought stress throughout the growing season, a phenomenon that has become more prevalent during warming of the Alaskan interior over the past 75 y (Barber, Juday & Finney, 2000).

**EXPERIMENTAL DESIGN**

This study was conducted from late June to mid-August, 2001, during the peak of the season for *N₂* fixation activity (Uliassi & Ruess, 2002). At each of the three replicate stands within each successional stage in both floodplain and upland forests, 10 plants (*A. tenuifolia* on the floodplain and *A. crispa* in the uplands) were randomly selected for measurement of AR and [¹⁵N]₉ uptake. Sampling dates for each site are given in Table I. All measurements were taken between 0900 and 1400 Alaska Daylight Time. ARA and [¹⁵N]₂ uptake were run simultaneously on sub-samples of nodules harvested from each plant, as described below. Most nodules of both species occur within the fibric layer close to the soil surface, and a nodule sample adequate for both assays could be harvested within 10 min for each plant.

**ACETYLENE REDUCTION ASSAY**

We used a closed-system field assay with a short incubation time (2.5 min) to minimize the potential for acetylene-induced decline in nitrogenase activity (Uliassi & Ruess, 2002). Acetylene was generated on site by hydration of CaC₂ in a Bliss generator equipped with a rubber septum for convenient gas withdrawal. Approximately 2.5 g (fresh weight) of nodule clusters with subtending fine roots were harvested and placed in a 60-mL polyethylene syringe fitted with a septum to facilitate gas additions and withdrawals. Nodule harvests generally consisted of removal of the root to which the nodule clusters were attached, in order to minimize nodule disturbance. When large root sizes precluded this method, however, clusters were removed by breaking them off at the base along their natural planes of separation.

Nodules were maintained at ambient soil temperature, which was measured with a hand-held digital thermometer (Taylor Thermometers, Oak Brook, Illinois, U.S.A.), by placing the incubation syringe within the forest floor until the assay was performed. Within 5-10 min of initial nodule harvest, 6 mL of C₂H₂ were added to the syringe, producing an incubation atmosphere of approximately 10% v/v C₂H₂. This was designated as time zero (T₀). Gas samples were withdrawn from the incubation syringe at 30 s (T₁) and 150 s (T₂) from the time of initial C₂H₂

<table>
<thead>
<tr>
<th>Site type</th>
<th>Successional stage</th>
<th>Plant species</th>
<th>Replicate site</th>
<th>Sampling date</th>
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<tbody>
<tr>
<td>I</td>
<td>Early</td>
<td><em>A. tenuifolia</em></td>
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<td>25 June</td>
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<td><em>A. crispa</em></td>
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<td>II</td>
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<td><em>A. crispa</em></td>
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<td><em>A. tenuifolia</em></td>
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<td>III</td>
<td>Late</td>
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<td><em>A. crispa</em></td>
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injection. These samples were transported to the laboratory in Fairbanks within 8 h of sampling and immediately analyzed for C\textsubscript{2}H\textsubscript{4} content using a Shimadzu 14A gas chromatograph equipped with a 2 m Porapak N column and a back-flush valve to vent C\textsubscript{2}H\textsubscript{2} (Shimadzu Scientific, Houston, Texas, U.S.A.). Total C\textsubscript{2}H\textsubscript{2} reduction was calculated by dividing the increase in molar C\textsubscript{2}H\textsubscript{4} concentration from T\textsubscript{1} to T\textsubscript{2} by the time elapsed between the two samples. The result was expressed as specific acetylene reduction activity (SARA = µmol C\textsubscript{2}H\textsubscript{4}·g\textsubscript{DWT nodule}\textsuperscript{-1}·h\textsuperscript{-1}) by dividing the result by nodule dry weight of the sample in grams (Equation [1]). Nodule dry weight was determined by thoroughly rinsing each nodule sample, removing the roots, drying the nodules for 48 h at 65 °C, and weighing to the nearest 0.01 mg.

\[ SARA = \frac{\text{moles C}_2\text{H}_4 \text{ at } T_2 - \text{moles C}_2\text{H}_4 \text{ at } T_1}{\text{incubation time (h) \times nodule dry wt (g)}} \]  

\[ \text{15N}_2 \text{ UPTAKE} \]

Approximately 2.5 g of fresh nodule with attached fine root were harvested simultaneously with the sample used for SARA determination, placed in a second 60-mL polyethylene syringe fitted with a septum, and placed within the forest floor to maintain the sample at ambient soil temperature, as described above. Ten mL of 99 atom% 15N\textsubscript{2} (Isotec Inc., Miamisburg, Ohio, U.S.A.) was then added to the syringe to produce an incubation atmosphere of approximately 15% 15N\textsubscript{2}. Immediately after the addition of the 15N\textsubscript{2}, a 15-mL sample of the incubation atmosphere was removed to provide a quantitative measure of atom percent enrichment (APE) of 15N\textsubscript{2} at T\textsubscript{0}. These samples were stored in 10-mL exetainers (Labco, High Wycombe, Buckinghamshire, U.K.) and transported to Fairbanks for analysis using a dual-inlet isotope ratio mass spectrometer (PDZ Europa Scientific Instruments, Crewe, Cheshire, U.K.). After the removal of the incubation atmosphere sample, the syringe was immediately returned to the forest floor for 10 min. Nodules were then removed from the syringe and immediately frozen in liquid N\textsubscript{2}. In the laboratory, nodules were thoroughly rinsed through a fine sieve of all adhering soil and organic material, dried for 48 h at 65 °C, and ground using a Wig-L-Bug ball mill (Reflex Analytical, Ridgewood, New Jersey, U.S.A.) in preparation for mass spectrometry analysis. The dried nodule samples from the SARA on each plant were used as controls for the determination of APE for each nodule sample according to the following equation:

\[ \text{APE}_{\text{nodule}} = 15\text{N}_{\text{enriched nodule}} - 15\text{N}_{\text{control nodules}} \]  

where both 15N content measures are in atom%. By combining APE with total nodule N content, dividing by incubation time, and correcting for the composition of the initial incubation atmosphere as determined by mass spectrometry, we calculated the specific N\textsubscript{2} fixation activity of the nodule samples (SNF = µmol N assimilated·g\textsubscript{DWT nodule}\textsuperscript{-1}·h\textsuperscript{-1}) as follows:

\[ \text{SNF} = \frac{(\text{APE}_{\text{nodule}} \times \%N_{\text{nodule}})}{\text{incubation time (h) \times %^{15}N_{\text{atmosphere}}}} \]  

C\textsubscript{2}H\textsubscript{2} INHIBITION EXPERIMENT

To determine whether N\textsubscript{2} fixation was inhibited in the presence of C\textsubscript{2}H\textsubscript{2} under our assay conditions, we exposed nodules from 12 greenhouse-grown A. tenuifolia seedlings to both substrates simultaneously, using the assays and sample preparation methods employed in our field study. Three sets of nodules were excised from each plant; one was exposed to approximately 15% v/v 15N\textsubscript{2}, another was exposed to 15% v/v 15N\textsubscript{2} plus 10% v/v C\textsubscript{2}H\textsubscript{2}, and the final sample was used to determine 15N natural abundance for the 15N\textsubscript{2} uptake assay.

STATISTICAL ANALYSIS

Differences in parameters related to N\textsubscript{2} fixation were analyzed by ANOVA (PROC GLM; SAS Institute, 2001) using a cross-nested model (Neter et al., 1996). The full model tested for the effects of species, successional stage, ecosystem replicate within successional stage, and plant within replicate and stage. Species, successional stage, ecosystem replicate, and plant number were included as class variables. Ecosystem replicate within successional stage, and plant number within stage and replicate were included as random effects.

Successional stage and sampling date were confounded, due to the fact that it was not possible to sample both species within all three successional stages at the same time. These stage/seasonality effects often accounted for the largest proportion of explained variance in our data. While both successional stage and seasonality have been demonstrated to have strong effects on N\textsubscript{2}-fixation rates as measured by ARA in these sites (Schimel, Cates & Ruess, 1998; Uliassi et al., 2000; Uliassi & Ruess, 2002), we were unable to investigate these effects independently in this study. Therefore, to avoid confusion, early succession sites of both species, which were all sampled early in the season, are referred to as Type I sites, rather than as early succession or early season sites. Similarly, mid and late succession/season sites are referred to as Type II and Type III sites, respectively. These site types are defined in Table I.

Because both species were sampled in each of the three successional stages at approximately the same time, we tested for species differences for each successional stage/sampling period by removing successional stage from the model and running each stage separately. In several cases, apparent differences between species in N\textsubscript{2} fixation seemed to be a function of differences in soil temperature. To account for this, ANCOVA was run using soil temperature as a covariate. All species differences reported seemed to be a function of differences in soil temperature. To account for this, ANCOVA was run using soil temperature as a covariate. All species differences reported seemed to be a function of differences in soil temperature. To account for this, ANCOVA was run using soil temperature as a covariate. All species differences reported seemed to be a function of differences in soil temperature. To account for this, ANCOVA was run using soil temperature as a covariate. 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a paired *t*-test (SAS Institute, 2001). Unless otherwise stated, data presented are means (± 1 SE) of untransformed data.

**Results**

**15N2 UPTAKE AND SARA**

Specific N fixation (SNF), as measured by 15N2 uptake, was 34% greater in *A. tenuifolia* (36.9 ± 3.4 µmol N·gDWT nodule⁻¹·h⁻¹) compared with *A. crispa* (27.5 ± 3.8 µmol N·gDWT nodule⁻¹·h⁻¹) when averaged across all measurements (*P* = 0.02; Table II). Part of this difference could be attributed to slightly warmer soils in floodplain stands dominated by *A. tenuifolia* (11.9 ± 0.3°C) compared with upland stands dominated by *A. crispa* (11.2 ± 0.2°C; *P* = 0.06), reducing species differences in SNF (*F*₁,₈₈ = 3.50, *P* = 0.06). Across all sampling dates, SNF rates were positively correlated with soil temperature for both species, but more closely for *A. crispa* (*r² = 0.28, *P* < 0.0001) than for *A. tenuifolia* (*r² = 0.04, *P* < 0.05). The largest proportion of explained variation in SNF was accounted for by site type (*F*₂,₈₈ = 3.04, *P* < 0.05) and replicate within site type (*F*₆,₈₈ = 14.05, *P* < 0.0001). For reasons explained earlier, we are uncertain as to whether the apparent successional pattern in SNF (early: 8.2 ± 1.1 µmol N·gDWT nodule⁻¹·h⁻¹; mid: 47.3 ± 5.6 µmol N·gDWT nodule⁻¹·h⁻¹; late: 41.2 ± 3.5 µmol N·gDWT nodule⁻¹·h⁻¹) is mainly a function of a seasonal pattern, known to be pronounced in these forests (Ulissi & Ruess, 2002), given that these stages were sampled around 1 July, 17 July, and 3 August, respectively. The least squares means test indicated significant differences (*P* < 0.0005) in SNF between Type I sites and both Type II and Type III sites, but not between Type II and Type III sites in both *A. tenuifolia* and *A. crispa* (Table III).

Species differences in SARA varied to the same degree as SNF, with values for *A. tenuifolia* (15.6 ± 1.6 µmol C₂H₄·gDWT nodule⁻¹·h⁻¹) being approximately 37% greater than those measured for *A. crispa* (11.3 ± 1.6 µmol C₂H₄·gDWT nodule⁻¹·h⁻¹) when averaged across all measurements (*F*₁,₂₈ = 5.28, *P* < 0.05). Again, these species differences were less pronounced when controlled for differences in soil temperature (*F*₁,₂₈ = 2.37, *P* = 0.13). The overall model explained approximately 59% of the variation in SARA (*F*₉₁,₁₇₉ = 1.37, *P* = 0.07), which was less than the overall explained variation in SNF (69%, *F*₉₀,₁₇₉ = 2.18, *P* < 0.0001). Although variation among replicates within successional stages accounted for a large percentage of explained variation (*F*₆,₈₈ = 5.09, *P* < 0.001), no apparent differences in SARA among site types were

**Table II.** Site type averages (mean ± 1 SE, *n* = 30) for data collected during measurements of nitrogen fixation, listing soil temperature (°C), specific leaf area (SLA = cm²·g⁻¹), leaf natural 15N abundance ([δ]¹⁵N, ‰), specific N assimilation rate determined from 15N₂ uptake, was 34% greater in *A. tenuifolia* (36.9 ± 3.4 µmol N·gDWT nodule⁻¹·h⁻¹) compared with *A. crispa* (27.5 ± 3.8 µmol N·gDWT nodule⁻¹·h⁻¹) when averaged across all measurements (*P* = 0.02; Table II). Part of this difference could be attributed to slightly warmer soils in floodplain stands dominated by *A. tenuifolia* (11.9 ± 0.3°C) compared with upland stands dominated by *A. crispa* (11.2 ± 0.2°C; *P* = 0.06), reducing species differences in SNF (*F*₁,₈₈ = 3.50, *P* = 0.06). Across all sampling dates, SNF rates were positively correlated with soil temperature for both species, but more closely for *A. crispa* (*r² = 0.28, *P* < 0.0001) than for *A. tenuifolia* (*r² = 0.04, *P* < 0.05). The largest proportion of explained variation in SNF was accounted for by site type (*F*₂,₈₈ = 3.04, *P* < 0.05) and replicate within site type (*F*₆,₈₈ = 14.05, *P* < 0.0001). For reasons explained earlier, we are uncertain as to whether the apparent successional pattern in SNF (early: 8.2 ± 1.1 µmol N·gDWT nodule⁻¹·h⁻¹; mid: 47.3 ± 5.6 µmol N·gDWT nodule⁻¹·h⁻¹; late: 41.2 ± 3.5 µmol N·gDWT nodule⁻¹·h⁻¹) is mainly a function of a seasonal pattern, known to be pronounced in these forests (Ulissi & Ruess, 2002), given that these stages were sampled around 1 July, 17 July, and 3 August, respectively. The least squares means test indicated significant differences (*P* < 0.0005) in SNF between Type I sites and both Type II and Type III sites, but not between Type II and Type III sites in both *A. tenuifolia* and *A. crispa* (Table III).

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**Table III.** Results of pair-wise least-square means comparisons of SARA, SNF, and ratio values between all species and site-type combinations. Values given are *P*-values of pair-wise *t*-tests (*n* = 30). NS indicates no significant difference between the mean values compared (marginally-significant *P*-values are presented with full precision).
detected ($F_{2.88} = 1.14, P = 0.33$). SARA was positively, but weakly, correlated with soil temperature for both *A. crispa* ($r^2 = 0.06, P < 0.05$) and *A. tenuifolia* ($r^2 = 0.04, P < 0.05$).

**C$_2$H$_2$ to N$_2$ REDUCTION RATIO**

Averaged across all plants, the ratio of C$_2$H$_2$ to N$_2$ reduced was 1.92 ± 0.15 and was significantly greater in *A. crispa* (2.0 ± 0.2) compared with *A. tenuifolia* (1.81 ± 0.24; $F_{1.88} = 5.21, P < 0.05$). This ratio was greatest in Type I stands (3.47 ± 0.33), but significantly less in Type II (1.27 ± 0.14) and Type III stands (1.01 ± 0.13; $F_{2.6} = 10.11, P = 0.01$; Table III). In Type I *A. tenuifolia* stands the average ratio was 3.82 and was not significantly different from either 3 ($P = 0.1349$) or 4 ($P = 0.7332$). In Type I *A. crispa* stands the average ratio was 3.13, which was significantly different from 4 ($P = 0.0354$), but not from 3 ($P = 0.7559$). In all other stands for both species, the ratio was significantly different from both theoretical values ($P < 0.0001$). The ratio of C$_2$H$_2$ to N$_2$ reduced also varied significantly among replicates within site type within species ($F_{6.99} = 9.03, P < 0.0001$).

Rates of SNF and SARA were positively correlated for *A. crispa* ($r^2 = 0.11, P < 0.01$), but not for *A. tenuifolia* ($P = 0.23$) across all sites. In both species this correlation was strong and highly significant in Type I sites ($r^2 = 0.73, P < 0.0001$ for *A. tenuifolia*; $r^2 = 0.49, P < 0.0001$ for *A. crispa*), but not in Type II or Type III sites (Figure 1).

Our inhibition experiment indicated no significant difference in $^{15}$N enrichment of nodule tissue between sites (Figure 1).

**Leaf $\delta^{15}$N**

Leaf $\delta^{15}$N was significantly more enriched in *A. tenuifolia* (0.02 ± 0.17 ‰) compared with *A. crispa* (-0.55 ± 0.19 ‰; $P < 0.02$). This difference was primarily driven by strong species differences in Type II sites, in which leaf $\delta^{15}$N was significantly more depleted in *A. crispa* (-1.38 ± 0.16 ‰) relative to *A. tenuifolia* (1.09 ± 0.20 ‰; $P < 0.0001$; Figure 2). This result is consistent with higher N fixation rates in *A. tenuifolia* compared to *A. crispa*. These species differences were not found in Type I sites, where values for both species were most depleted and were nearly identical (*A. tenuifolia* = -1.68 ± 0.05 ‰, *A. crispa* = -1.63 ± 0.03 ‰; $P = 0.19$), but were again significantly different in Type III sites (*A. tenuifolia* = 1.01 ± 0.22 ‰, *A. crispa* = 1.38 ± 0.36 ‰; $P = 0.02$). Across all plants, $\delta^{15}$N and SNF were positively correlated for both *A. tenuifolia* ($r^2 = 0.19, P < 0.0001$) and *A. crispa* ($r^2 = 0.08, P < 0.01$). However, leaf $\delta^{15}$N was not correlated with SARA in either species.

**Discussion**

**Reduction ratio**

In this experiment, the C$_2$H$_2$ to N$_2$ reduction ratio was determined by dividing the rate of C$_2$H$_2$ reduction determined for a random sample of nodules from an individual plant by the rate of N$_2$ reduction determined using $^{15}$N$_2$ uptake on a second random sample of nodules from the same plant. However, the incubation times for the two assays used were not equal; the ARA was carried out for 2.5 min while the $^{15}$N$_2$ uptake was carried out for 10 min. We are aware of the possibility of methodological bias this creates in our calculation of the ratio due to the decline in nitrogenase activity after excision of nodules from the source plant (Mague & Burris, 1972). We tested this possibility in a small preliminary greenhouse experiment with 16 *A. tenuifolia* seedlings and found no difference between mean ARA of nodules assayed immediately after excision and those assayed 10 min after excision (data not shown).

The C$_2$H$_2$ to N$_2$ reduction ratio determined in this study was highly variable; site averages ranged from 0.37 ± 0.09 to 5.59 ± 0.76 (n = 10), and individual values ranged from 0.02 to 14.00. Previous studies have reported similar variation in the ratio, with values ranging from as low as 0.11 ± 0.01 (Liengen, 1999) to as high as 94 (Seitzinger & Garber, 1987). These upper and lower extremes may be more characteristic of free-living than symbiotic systems, as the above two studies investigated free-living soil and marine cyanobacterial systems, respectively. Previous studies with symbiotic systems have found somewhat less variability. At the low end, van Kessel and Burris (1983) reported a ratio of 0.65 for *Trifolium pratense*, while Saito, Matsui, and Salati (1980) found a ratio of 8.30 ± 0.07 for *Phaseolus vulgaris*, both of which are *Rhizobium*-infected species. However, the sample size for both studies was small and may have inadequately captured the full range of variation in the reduction ratio across broad environmental conditions for the host species.

The few reduction ratios measured for actinorhizal species demonstrate less variability than those for legumes. But, again, these studies have been conducted principally on greenhouse-grown plants with low replication. Using hydroponically grown *Myrica gale* seedlings, Schwintzer and Tjepkema (1994) found the reduction ratio ranged from 2.54 ± 0.33 in light-stressed seedlings to 4.32 ± 0.10 in water-stressed seedlings (n = 8). Vitousek and Walker (1989) used a $^{15}$N$_2$ uptake method on naturally occurring *Myrica faya* in Hawaii to calibrate their use of the ARA. They reported a value of 3.5 ± 0.7, but only six plants were included in their determination, so the full range of variation in the reduction ratio of this species at their site may not have been captured. Sellstedt (1986) used Kjeldahl-N accumulation, $^{15}$N$_2$ uptake, and H$_2$ evolution to calibrate the ARA and reported reduction ratios for 21 growth-chamber-cultivated *Alnus incana* seedlings ranging from 2.04 to 3.94. Fessenden, Knowles, and Brouzes (1973) reported a ratio of 3.14 ± 0.30 for excised nodule samples from 24 naturally occurring *Myrica asplenifolia* individuals from the northeastern United States and southeastern Canada. However, because they used a long-term acetylene incubation, it is possible their results underestimated the ratio due to acetylene-induced decline in nitrogenase activity (Minchin et al., 1983).

Within-species variation in the reduction ratio of C$_2$H$_2$ to N$_2$ has been correlated with several environmen-
tal and physiological variables for both free-living and symbiotic systems. Among non-symbiotic systems, these variables include soil parent material and vegetation cover (Zechmeister-Boltenstern & Kinzel, 1990), time of season (Liengen, 1999), soil water content (Nohrstedt, 1983), and uptake hydrogenase activity (Paerl, 1982). Among symbiotic systems these variables include SNF (Gibson & Alston, 1984), efficiency of symbiotic nitrogenase (van Kessel & Burris, 1983), and $pN_2$ (Peters, Toia & Lough, 1977) in non-actinorhizal systems, and host plant water and light stresses (Schwintzer & Tjepkema, 1994) in Myrica gale, an actinorhizal plant. Mechanisms proposed to explain the correlation of soil physical/chemical characteristics with variation in the reduction ratio in non-

![FIGURE 1. Linear regressions between SARA and SNF for *A. tenuifolia* (a, c, e) and *A. crispa* (b, d, f) measured in Type I (a, b), Type II (c, d), and Type III (e, f) sites. Each point represents an individual plant. Relationships are significant for Type I sites only (*A. tenuifolia*, $r^2 = 0.73$, $P < 0.0001$; *A. crispa*, $r^2 = 0.49$, $P < 0.0001$).](image-url)
symbiotic systems, such as differential adsorption of the two substrates to soil minerals (Rennie, Rennie & Fried, 1978; Nohrstedt, 1985) and differential diffusion rates of the two substrates in aqueous solution (Rice & Paul, 1971; Nohrstedt, 1983; Zechmeister-Boltenstern & Kinzel, 1990), may not be relevant to our study which utilized excised root nodules, since these explanations rely on direct effects of soil properties exerted during the assay.

In the present study, significant intra-specific differences in the value of the $C_2H_2:N_2$ reduction ratio were observed among replicate sites both within a site type and among site types. The site type effect probably contains components of both successional stage and seasonality. Due to the inherent confounding of these two factors in our experimental design, successional stage effects cannot be separated from seasonality effects in our analysis. Schwintzer and Tjepkema (1994) reported significant effects of both light and water availability on $C_2H_2:N_2$ reduction ratio in Myrica gale seedlings. Because these factors vary considerably both spatially and temporally in interior Alaskan boreal forests, it is possible that both site and site-type variations in the value of the $C_2H_2:N_2$ reduction ratio observed in our study are related to variation in one or both of these factors. In particular, Schwintzer and Tjepkema (1994) found that the $C_2H_2:N_2$ reduction ratio was significantly lower in light-stressed plants, which is consistent with our finding that the value of the ratio decreased significantly between Type I sites, where alder dominated the canopy, and both of the other site types, where alder was limited to the understory. However, the degree to which variation in light and/or water availability was associated with variation in the $C_2H_2:N_2$ reduction ratio in our study cannot be directly assessed because no data were collected for either of these variables.

The pattern of variation in the $C_2H_2:N_2$ reduction ratio observed in our study suggests this variation might be largely accounted for by differences in $N_2$-fixation rate among sites. Both SNF and the $C_2H_2:N_2$ reduction ratio varied significantly between Type I sites and both other site types, but not between Type II and Type III sites (Table III). Similarly, the relationship between ARA and SNF was most significant in Type I stands of both A. tenuifolia ($r^2 = 0.73, P < 0.0001$) and A. crispa ($r^2 = 0.49, P < 0.0001$), but was not significant in either species in Type II or Type III sites (Figure 1). Apparent rate-dependence of the $C_2H_2:N_2$ reduction ratio has been previously reported in Australian Lupinus angustifolius crops (Gibson & Alston, 1984). These authors suggested this observation could be accounted for by a change in kinetics of nitrogenase at high specific $N_2$ reduction rates, resulting in the preferential reduction of $N_2$ over $C_2H_2$. This hypothesis is supported by a number of studies of nitrogenase in vitro, in which the enzyme appeared to favour $N_2$ as a substrate over $C_2H_2$ at high or non-limiting levels of electron flux (Davis, Shah & Brill, 1975; Shah, Davis & Brill, 1975; Sadkov & Likhtenshtein, 1990) or simply to decrease affinity for $C_2H_2$ at relatively high levels of electron flux (Eady & Postgate, 1974; Lowe, Fisher & Thornley, 1990).

The apparent rate-dependence of the $C_2H_2:N_2$ ratio can be explained by the operating mechanisms of the nitrogenase complex. The complex is composed of two enzymes: nitrogenase, which binds either $N_2$ or $C_2H_2$, and nitrogenase reductase, which passes electrons one at a time to the nitrogenase enzyme/substrate complex. Each one-electron transfer from nitrogenase reductase to nitrogenase/substrate is followed by a dissociation of the two enzymes, after which the now-oxidized reductase is again reduced while the nitrogenase associates with another reduced molecule of reductase. Thus, the reductase exists in two oxidation states, while the nitrogenase must pass through several oxidation states before the bound substrate is completely reduced (Burgess & Lowe, 1996).

Some oxidation states of nitrogenase have been found to have differing affinities for the enzyme’s various substrates. For example, $N_2$ cannot bind to nitrogenase until the enzyme has been reduced at least three electrons above its ground state, while $C_2H_2$ can bind to nitrogenase in a less reduced state (Lowe; Fisher & Thornley, 1990; Burgess & Lowe, 1996). Thus, at high $N$ fixation rates, and presumably high levels of electron flux, a relatively high amount of nitrogenase may exist at a sufficiently reduced level to bind $N_2$, leading to a preferential reduction of this substrate relative to $C_2H_2$. At low fixation rates, the opposite substrate preference would be expected. This hypothesis relies on the assumption that high rates of fixation are largely due to high rates of electron flux through the nitrogenase complex.

A second hypothesis that may account for the negative correlation between the $C_2H_2:N_2$ reduction ratio and $N_2$-fixation observed in the present study is that a saturating level of $C_2H_2$ may not have been achieved during the assay. Hardy et al. (1968) proposed that 3-10% $C_2H_2$ would be sufficient to saturate the enzyme. This concentration is consistent with the apparent Michaelis constants of nitrogenase for the two substrates, which suggest an approximately 10- to 20-fold greater affinity of the enzyme for $C_2H_2$ relative to $N_2$ (Hardy et al., 1968; Davis, Shah & Brill, 1975; Christiansen, Seefeldt and Dean, 2000).
Several authors report a near total transfer of electron flux to the reduction of C₂H₂ under a 10-20% C₂H₂ atmosphere, as indicated by the inhibition of H₂ evolution normally accompanying N₂ fixation (Rivera-Ortiz & Burris, 1975; Schubert & Evans, 1976; Paerl, 1982). This is why an incubation atmosphere of 10-20% C₂H₂ is generally recommended for the ARA, and why it was used in the present study. Zuckermann et al. (1997), however, using a highly sensitive real-time method for monitoring C₂H₂ production, failed to achieve complete saturation of in vivo nitrogenase in cultures of the cyanobacterium Nodularia spumigena under an atmosphere of 25% C₂H₂. Considering the greater diffusion barrier presented by excised nodules compared to heterocystous cyanobacteria, it is possible that even higher C₂H₂ concentrations might be necessary to achieve enzyme saturation in these systems. At sufficiently high levels of nitrogenase activity the reduction of C₂H₂ might become diffusion-rather than enzyme-limited, causing the concentration of C₂H₂ in the immediate vicinity of nitrogenase to drop to levels insufficient to inhibit electron allocation to N₂. Thus, electron reallocation to C₂H₂ might be minimized at high levels of enzyme activity, allowing nitrogenase to reduce more N₂ relative to C₂H₂ than would be predicted from electron allocation stoichiometry. Such a diffusion limitation would be expected to be less pronounced for N₂, given the much higher concentration of this substrate compared to C₂H₂.

The failure of C₂H₂ to inhibit N₂ fixation under our assay conditions, as indicated by our greenhouse experiment, could be explained by the rate-dependence of the ratio; i.e., that a saturating level of C₂H₂ was not achieved during the assay or, given the high fixation rates observed in these greenhouse-grown plants (57.9 ± 9.5 μmol N·g DWT nodule⁻¹·h⁻¹), that nitrogenase favours nitrogen as a substrate at high levels of enzyme activity. Our greenhouse study demonstrated that ¹⁵N₂ uptake was not inhibited by C₂H₂ in the assay, but it is not clear which of our hypotheses, if either, explains this result.

It is interesting that the strongest correlation between the two methods for measuring N₂ fixation in the field occurred at a time when fixation rates were lowest and the C₂H₂:N₂ reduction ratio was closest to the theoretical value (Figure 1 and Table II). To our knowledge similar results have not been reported. This decoupling of the two assays can be accounted for by either of the two mechanisms proposed to explain the rate-dependence of the C₂H₂:N₂ ratio and is probably a condition necessary for the observed rate-dependence. If the two assay methods were not decoupled at high enzyme activities, that is, if all electron flux through the nitrogenase complex were allocated to one substrate or the other at all rates of enzyme activity, it would not be possible for the ratio to be rate-dependent.

IMPLICATIONS FOR ECOSYSTEM N CYCLING

Because of the simplicity and cost-effectiveness of the method, ARA has been widely used for estimating N₂-fixation rates across a broad spectrum of terrestrial systems, from boreal (Klingensmith & Van Cleve, 1993; Uliassi & Ruess, 2002), temperate (Tripp, Bezdicek & Heilman, 1979; Huss-Danell, Lundquist & Ohlsson, 1991; Hurd, Raynal & Schwintzer, 2001), and tropical forests (Vitousek & Walker, 1989; Pearson & Vitousek, 2001) to arctic and alpine tundra (Holzmann & Haselwandter, 1988; Kay & Virginia, 1989; Liangen & Olsen, 1997). The primary uncertainties in scaling ARA-based data to the stand level include 1) adequate characterization of the seasonal variation in nitrogenase activity, 2) accurate values for the C₂H₂:N₂ reduction ratio, and 3) reliable estimates of live nodule biomass.

Our finding that there is likely variation in the C₂H₂:N₂ reduction ratio at both temporal and spatial scales within and among conspecific hosts suggests that assessments of ecosystem N cycling based on ARA may require re-examination. For example, Pearson and Vitousek (2001) found that rates of N accumulation in vegetation biomass exceeded ARA-based N₂-fixation inputs in Hawaiian Acacia koa plantations and concluded that the largest proportion of plant N uptake was derived from soil organic matter turnover. A much different conclusion was presented by Uliassi and Ruess (2002), who found that ARA-based N₂-fixation inputs to interior Alaskan floodplain forests by A. tenuifolia significantly exceeded rates of biomass N accumulation and suggested that these forests were not retaining a large proportion of fixed N.

Even though the reduction ratio for A. tenuifolia derived from the current study (1.81 ± 0.24) is significantly less than the 3.0 used by Uliassi and Ruess (2002), we hesitate to conclude that these previous estimates of N₂ fixation and ecosystem N retention were conservative. The apparent rate-dependence of the C₂H₂:N₂ reduction ratio suggests that it may be impossible to rely on any one C₂H₂:N₂ reduction ratio value for A. tenuifolia, and perhaps any species. Moreover, the apparent uncoupling of SNF and SARA observed at high rates of SNF in the present study suggests the ARA may not be well suited for even relative comparisons of N₂-fixation rates between organisms. Further studies duplicating the scale of the present one with other N₂-fixing species and/or in other ecosystems will be necessary to determine whether the pattern we observed is typical. Despite the greater cost of the ¹⁵N₂ uptake method compared to the ARA, our results call for a reassessment of N₂-fixation inputs, using ¹⁵N₂ uptake for all ecosystems where N₂-fixing organisms contribute a significant amount to net primary production.

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Literature cited


