Symbiont nitrogenase, alder growth, and soil nitrate response to phosphorus addition in alder (*Alnus incana* ssp. *rugosa*) wetlands of the Adirondack Mountains, New York State, USA

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Abstract

Speckled alder (*Alnus incana* ssp. *rugosa*) is a characteristic species of scrub-shrub type wetlands, the second most common wetland type in major watersheds of the Adirondack Mountains in New York State. Speckled alder is an actinorhizal nitrogen fixer that relies heavily on N₂ over soil N and fixes substantial amounts of nitrogen in wetlands, resulting in little vegetation processing of anthropogenic N between alder-shrub wetlands and streams. Phosphorus (P) is an element that limits nitrogen fixation and plant growth. However, studies testing this hypothesis in the field, especially for actinorhizal plants, are very few.

The objectives of this study were to evaluate the potential limitation of N fixation and growth in speckled alder by P, and to determine interactions between P fertilization and nitrate levels in riparian alder stands in a region that receives elevated N in atmospheric deposition. P fertilization significantly increased specific nitrogenase activity during the seasonal peak in early August. Nitrate concentrations were greater in reference plots compared to treatment plots, and phosphate concentrations were lower in reference plots compared to treatment plots over a period of 6 weeks in the growing season. There was a significant twig and foliar biomass response to P fertilization in the second year after fertilization, but no significant change in individual biomass or relative numbers of different sized nodules. Response of nitrogen fixation to P appears limited to a brief but significant increase in specific activity of nitrogenase late in the growing season, but P stimulated growth of above ground tissues 1 year following fertilizer application, and decreased resin-captured nitrate beneath riparian speckled alder. These results suggest that growth of alder and growth or activity of soil microbes, rather than nitrogen fixation, is P limited in riparian wetlands dominated by speckled alder, and that P controls nitrate leaching in these near-stream systems.

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1. Introduction

Nitrogen (N)-fixing plants are known to have an inherently higher phosphorus (P) demand than non-N fixers (Ingestad, 1981; Sprent, 1988). Several experimental studies have shown significant responses of nodulation, plant growth and fixation to P fertilizations in both leguminous and actinorhizal species (Gates, 1974; Israel, 1987; Reddell et al., 1988; Sangenga et al., 1989; Ekbland and Huss-Danell, 1995; Uliassi et al., 2000). The results of a field study by Uliassi and Ruess (2002) reveal that P limits N fixation primarily by limiting nodule production in thimble Alder (A. tenuifolia) in the successional forests of the Tanana River floodplain in interior Alaska. Vitousek (1999) has demonstrated that N fixation associated with liverworts and lichens (cyanolichen, Pseudocyphellaria crocata), the litters of the tree Metrodoros polymorpha and the fern Dicranopteris linearis increased significantly with additions of P on young volcanic substrates in Hawaii. Chapin et al. (1991) also found significant stimulation of cyanobacterial acetylene reduction activity by P additions to young soils in the high Arctic. Wall et al. (2000) found that the degree of N inhibition on nodulation at N/P ratios >7, but not at N/P ratios ≤7, in A. incana ssp. incana. Ekbland and Huss-Danell (1995) demonstrated experimentally an interaction of N and P nutrition on N fixation in seedlings of A. incana ssp. incana; N fixation decreased the most under high N addition when P was limiting. In a bioassay study, the addition of P and sulphur (S) to soils from a stand of red alder and Douglas fir on Mt. Benson, British Columbia resulted in doubling of red alder biomass and increased acetylene reduction activity per seedling by five-fold (Binkley et al., 1994).

Despite the general view that low P availability limits N fixation, research investigating phosphorus limitation on nitrogen fixation in the field, especially for actinorhizal plants, is very limited. In a recent study, Uliassi and Ruess (2002) applied a 2-year total of 215 kg P ha⁻¹ in several separate applications to A. tenuifolia in Alaska and found that fertilization increased total nitrogen inputs to 140 ± 41 kg ha⁻¹ yr⁻¹ compared to 59 ± 11 kg ha⁻¹ yr⁻¹ in unfertilized plots. The treatment appeared to have little effect on nitrogenase activity per unit nodule dry mass, compared with nodule production response. They concluded that multiple studies in a range of environments are necessary to generalize about the effects of P on N fixation in natural terrestrial ecosystems.

Hurd et al. (2001) estimated 37–43 kg N ha⁻¹ yr⁻¹ fixed in a dense stand of A. incana ssp. rugosa in the central Adirondack Mountains, a region that receives elevated N in atmospheric deposition (NADP/NTN, 2002). Atmospheric N deposition is a concern in the northeastern United States, due to nitrate influences on surface water acidity (Driscoll et al., 2001). Wetland processing of excess nitrate (biotic uptake or denitrification) may result in either decreased nitrate or acidity to surface waters, and/or increased N₂O (greenhouse gas) from denitrification. Dense A. incana ssp. rugosa stands in the Adirondacks are also associated with elevated nitrate in shallow groundwater (Kiernan et al., 2003; Hurd and Raynal, 2004), demonstrating the influence of this species on riparian N cycling and the need for further studies on what causes wetlands to be sources or sinks for nutrients (Busse and Gunkel, 2002). The objectives of this study were to evaluate the potential limitation of N fixation and growth in speckled alder by P, and to determine interactions between P fertilization and nitrate levels in riparian alder stands in shrub wetlands that receive N inputs from both actinorhizal fixation and anthropogenic N deposition.

2. Materials and methods

The study was designed to measure nitrogenase response of Frankia actinomycetes in root nodules of A. incana ssp. rugosa to P addition during weeks immediately following treatment at the beginning of the growing season. Presumably, nitrogenase activity would increase immediately upon increased P availability, if the process were P limited. Measurement of nitrate and phosphate concentrations in the soil using ion exchange resins was coupled to nitrogenase activity measurements over the same time period, following fertilizer addition in the 2001-growing season. The purpose of this assay was to examine the changes in nitrate and phosphate concentrations in the substrate, where the root nodules occur to better understand patterns of nutrient availability and processing in these riparian systems, where especially nitrate leaching is a concern for...
acid-sensitive streams (Driscoll et al., 2001). We measured twig elongation and twig, and foliage mass during the growing season of treatment (2001) and the subsequent season (2002) to determine response of alder growth to P addition. Nodules were harvested at the end of the study in September 2002.

2.1. Site description

The study was conducted in two alder-dominated riparian wetlands (scrub-shrub 1-type; Cowardin et al., 1979) along Fishing Brook and Adjidaumo Flow at the Huntington Wildlife Forest (HWF) located in the central Adirondack Mountains region within the Adirondack State Park of New York (Fig. 1). Fishing brook is a large-order inlet to a lake, while Adjidaumo flow is a headwater stream in the same flow system. Total annual precipitation averages 101 cm and mean annual temperature is 4.4 °C with a dormant season mean of −2.8 °C and a growing season mean of 14.3 °C (Shepard et al., 1989). Surrounding vegetation is composed of mixed northern hardwood forests on the upper slopes. The lower slopes are characterized by yellow birch (Betula alleghaniensis), balsam fir (Abies balsamea), eastern hemlock (Tsuga canadensis), and red spruce (Picea rubens) (Hurd et al., 1998).

Common wetland species include A. incana ssp. rugosa, Viburnum dentatum, Carex spp., Spiraea alba, S. tomentosa, Chamaedaphne calyculata, Osmunda regalis, Calamagrostis Canadensis, and Myrica gale. Type SS1 wetlands in the Upper Hudson watershed, in which the study wetlands are located, had an average of 10,979 speckled alder stems per hectare (Kiernan, 2000). Speckled alder foliage from HWF wetlands averages 2.7% N with a C:N ratio of 18.7 (Hurd, 1999), and 85–100% foliar N derived from fixation (Hurd et al., 2001). Greenwood mucky peats are present in valley bottom wetlands (Somers, 1986) and range from 1 to 5 m in thickness in an adjacent catchment at HWF, overlying glacial till (McHale et al., 2002). These soils are hydric, rich in organic matter, dark in color, and exhibit redoximorphic features (personal observation). HWF is situated in a region, where atmospheric deposition of N is elevated. Inorganic nitrogen inputs in the form of wet and dry deposition at HWF averaged 4.7 kg N ha⁻¹ yr⁻¹ between 1978 and 2001 (National Atmospheric Deposition Program/National Trends Network (NADP/NTN), 2002) and 2.6 kg N ha⁻¹ yr⁻¹ between 1995 and 2000 (Park et al., 2003). Despite these inputs, alders in the region continue to rely heavily on fixed N (Hurd et al., 2001), making uncertain the degree to which alder wetlands in the region...
control elemental nutrient flux between hillslopes and streams.

2.2. Experimental design and fertilizer addition

A randomized block design with two plots in each block was used. Seven blocks were established across both wetlands (four at Fishing Brook and three at Adjidaumo Flow), in order to maximize spatial inference across low and high order riparian systems. Reference (R) and treatment (P) plots were located systematically in each block so that the control plot was consistently upstream from the treatment plot, to minimize potential treatment effects on the reference plots. Each plot was 5 m × 5 m, with a ≥ 10 m buffer zone between plots to allow access and ensure independent treatment. Reference plots received no fertilizer addition, and treatment plots received a single application of Hoffman granular triple superphosphate fertilizer (0% N–46% P–0% K) in the form of P₂O₅ at 5 g P m⁻², or 50 kg P ha⁻¹ addition rate.

An annual uptake of 3 kg P ha⁻¹ yr⁻¹ by speckled alder at Fishing Brook, Huntington Wildlife Forest, New York in 1998 was calculated by assuming 0.15% P concentration as average in speckled alder tissue based on findings of Compton et al. (1997) for red alder foliage in Washington (0.12–0.17% P) and Hurd (unpublished data) for N fixing M. Gale in Maine (0.08–0.15% P) and multiplying that value by the total biomass increment estimated for speckled alders at Fishing Brook in 1998 by Hurd (1999). Because microorganisms and other plants also compete for P, the 5 g P m⁻², or 50 kg P ha⁻¹ addition rate was considered appropriate.

A single application of granular superphosphate fertilizer was broadcast evenly by hand over the substrate of treatment plots on July 12, 2001. A 1-m zone around each treatment plot was included in broadcast of superphosphate to ensure the even uptake of the fertilizer by alders and even effect of treatment on alders whose roots extended out of the original 5 m × 5 m plot borders.

Blocks were established in May and June 2001. Pre-treatment nitrogenase activity was measured on July 11–12, 2001. Following fertilizer addition on July 12, 2001, nitrogenase activity was measured at biweekly intervals on July 24–25, August 7–8, and August 20–21. There was no standing water in the plots during the treatment period.

2.3. Acetylene reduction assay

Nitrogenase activity was measured using an open, flow through acetylene reduction assay (ARA) technique that gives an estimate of nitrogenase activity using the initial peak rate of acetylene reduction (Schwintzer and Tjepkema, 1994). This system is the only reliable technique to measure nitrogenase activity because speckled alder nodules in the field are known to exhibit a pronounced acetylene-induced decline in nitrogenase activity (Schwintzer and Tjepkema, 1997).

Four to six nodule clusters with attached root segments ≥ 2 cm in length from two to four alder shrubs (depending on the availability) were excavated from each plot at a depth of 5–10 cm and immediately transferred to a central assay location. Nodules were then placed in a 70 ml barrel cuvette that was inserted into the root zone of alders, where the nodules are typically found. Cooled, humified air was sent over the nodules for approximately 10 min and then 10% by volume acetylene gas (C₂H₂) introduced with a portable, peristaltic pump operating on an internal lithium battery (Cole-Parmer Masterflex L/S). A dual probe thermometer (Cole-Parmer model 8402-20) was used to ensure the temperature difference between inside the cuvette and soil was less than 2 °C. Acetylene gas was generated by mixing approximately 8 g of calcium carbide (CaC₂) in 300 ml of water in a flask. To prevent heating, the cooler with the air and C₂H₂ bags, and attached tubing were kept shaded with a cloth. Flow rates of the gases were measured by a bubble flowmeter and values at least three times the volume of the cuvette (~230–240 ml/min) were used to ensure continuous, uninterrupted flow through the system. Ethylene gas (C₂H₄) produced from reduction of acetylene by nitrogenase was drawn from the outlet of the cuvette into glass, gas-tight, 2.5 ml syringes (Hamilton no. 1002) at the first, second, and third minutes after acetylene introduction. These sampling times were found to capture the initial peak of nitrogenase activity in speckled alder nodules growing in the field in Maine (Schwintzer and Tjepkema, 1997). Additionally, analysis of a no nodule control to determine potential ethylene production from sources other than nodules was performed. In this case, one sample was drawn at the outlet of the cuvette...
at the second minute following acetylene introduction over root segments without nodules.

Syringes were then transported to SUNY College of Environmental Science and Forestry, and analyzed for ethylene (C2H4) by gas chromatography (GC) (Varian Star 3400 cx) with flame ionization detector. The GC was first calibrated with 10 or 100 ppm C2H4 standards (Scott Specialty Gases) and then a sample volume of 0.4 ml was injected into the column. Small amounts of ethylene detected in no nodule control gas samples were subtracted from the assay sample concentrations.

Assayed nodules were transported in ice chests from the field, washed, sorted into live (yellow, firm) and dead (brown, soft) categories and dried to constant mass at 60°C. Peak rate of specific nitrogenase activity of nodules (nmol C2H4 min-1 g-1 nodules) was calculated by multiplying the amount of C2H4 in the sample (nmol/ml) by the flow rate of the gas in the system (ml/min), and dividing by live nodule dry mass (g). Then, a 4:1 acetylene reduction to N2 fixation conversion ratio (molar ratio of ethylene to NH₃ production is 2) was used to estimate the amount of nitrogen gas fixed per dry nodule weight (nmol N2 min⁻¹ g⁻¹ dry nodule weight (dnw), units) (Schwintzer and Tjepkema, 1994).

2.4. Nitrate and phosphate determination by ion exchange resins

Three ion exchange resin (IER) bags were located systematically along the diagonal of each plot starting from the upstream left corner. The IER bags were constructed from 4 cm × 4 cm nylon hosiery and filled with 7 g of IONAC A-554, Cl⁻-form anion exchange resin and 7 g of DOWEX HCR-W2, H⁺-form cation exchange resin. These two types of resins were mixed thoroughly and enclosed within the IER bags by stapling or tying with wire ties at the ends. Prior to installation, the IER bags were cleaned by soaking in 1N HCl for 1 h and then triple rinsed with deionized water. IER bags were then put in plastic sealable bags and kept moist with deionized water. These were transported to plots in a cooler. Substrate soil was slit with a knife and the IER bag was placed to a depth of 6-7 cm horizontally to the surface. New IER bags were replaced with the old ones every 2 weeks on July 24-25, August 7-8, and August 20-21, 2001 during a period of 6 weeks.

After recovery, IER bags were kept frozen until analysis (Giblin et al., 1994). The IER extracts were analyzed for nitrate (NO₃⁻) and phosphate (PO₄³⁻). Resin bags were rinsed with deionized water to remove soil particles prior to extraction and kept overnight to dry. Five grams of resin were weighed from the bags separately for nitrate and phosphate analysis and put into flasks. Then, 100 ml of 2 M KCl (Wyland and Jackson, 1993) and 0.5N HCl (Sibbesen, 1977; Lajtha, 1988) were added into the flasks for nitrate and phosphate extractions, respectively. The flasks were shaken on an oscillating shaker for 1 h (Zou et al., 1992). The extractant-resin bead mixture was filtered using Whatman 42 filter paper. Nitrate in the filtered extract was analyzed by the Bran + Luebbe Auto Analyzer 3 and for phosphate by Perkin-Elmer Optima 3300 DV inductively coupled plasma (ICP). It is possible that organic matter and associated P also bound to the resins, and that organically bound P influenced these measurements.

2.5. Alder tissue and nodule collection

Alder twig and leaf tissue was collected in September 2001 and 2002. Between 5 and 10 shrubs were subsampled in each plot; 4–5 twigs were cut off from each shrub. Twigs were cut off the branches using a pole pruner for taller shrubs and a hand pruner for smaller shrubs. Twigs were removed evenly from upper, lower and middle canopy of a shrub. Twigs with intact leaves were placed in paper bags and brought to Syracuse. The current year’s growth of each twig was determined based on location of terminal bud-scale scars (Raven et al., 1986). Leaves with petioles were stripped off the twigs and were dried at 60°C. After drying, twigs were measured for length and twigs and leaves were weighed separately. Alder root nodules (in the form of five to six nodule clusters with attached root segments) were subsampled at random locations in the plots in September 2002. Nodules were transported to Syracuse and processed as described earlier in acetylene reduction assay section. Following these procedures, nodules were sorted into size classes as size class I is single lobe, size class II is diameter (d) <1 cm, and size class III is 1 ≤ d ≤ 2 cm. This size class grouping was used because nodules in different size classes were not expected to exhibit the same growth rate, or same biomass accumulation, and the method was also used by Uliassi and Ruess (2002) in a similar study for A. tenuifolia in Alaska. Individual
nodules in each of these size classes were weighed and counted.

### 2.6. Statistical analysis

Specific nitrogenase activity, phosphate, and nitrate concentration data were analyzed by the general linear model (GLM) procedure of the statistical analysis system (SAS) version 8.2 (SAS Institute, 2002) according to randomized complete block design with repeated measures, where data of different dates were pooled. Repeated measures methods to estimate seasonal and other time series response are a common approach in forestry ecophysiological studies (Meredith and Stehman, 1991). A two-way analysis of variance (ANOVA) with factors being time and treatment was used to detect the main effect of treatment, and time-treatment interaction. The marginal means of fixed factor treatment and cell means of time-treatment interaction and the associated standard errors were calculated by the LSMEANS command of SAS. Comparisons of cell means between reference and treatment plots for specific nitrogenase activity, resin-captured nitrate and phosphate data on each sampling date were done by $t$-test. Values are means with plus one standard error. Asterisk denotes significant difference on that particular date at alpha $= 0.05$.

### 3. Results

#### 3.1. Specific nitrogenase activity

Phosphorus fertilization stimulated peak levels of specific nitrogenase activity significantly on the second sampling date (August 7–8, 2001) after pre-treatment measurement. The specific nitrogenase activity values on that date were $676 \pm 63 \text{ nmol N}_2 \text{ min}^{-1} \text{ g}^{-1} \text{ dw}^{-1}$ for reference and treatment plot nodules, respectively (Fig. 2; $p = 0.05$). The difference between the nitrogenase activities of the treatment and reference plot nodules did not differ significantly over the entire course of sampling period following the pre-treatment measurement (no time and treatment interaction; Fig. 2, $p = 0.8088$).

Phosphorus fertilization had a significant main effect on the mean of specific nitrogenase activity values measured biweekly over a 6-week sampling period following the pre-treatment measurement ($p = 0.0121$) following the pre-treatment measurement. The specific nitrogenase activity of nodules averaged $709 \pm 27 \text{ nmol N}_2 \text{ min}^{-1} \text{ g}^{-1} \text{ dw}$ in treatment plots, and $573 \pm 27 \text{ nmol N}_2 \text{ min}^{-1} \text{ g}^{-1} \text{ dw}$ in reference plots, respectively.

#### 3.2. Nitrate accumulation

The amount of nitrate captured in the reference plots was significantly higher than treatment plots over the second and third 2-week intervals. The nitrate amount captured on these 2-week intervals (July 24–25 to August 7–8, and August 7–8 to August 20–21) was $0.57 \pm 0.09 \text{ mg NO}_3 \text{ g}^{-1}$ versus $0.24 \pm 0.09 \text{ mg NO}_3 \text{ g}^{-1}$ ($p = 0.0146$), and $0.80 \pm 0.09 \text{ mg NO}_3 \text{ g}^{-1}$ versus $0.50 \pm 0.09 \text{ mg NO}_3 \text{ g}^{-1}$ resin ($p = 0.0195$) for treatment versus reference plots, respectively (Fig. 3). The difference between the amounts of nitrate captured by ion exchange resins in treatment and reference plots over three consecutive 2-week intervals did not differ over the sampling period.
Fig. 3. The amount of nitrate captured by ion exchange resins over three 2-week periods in the reference and treatment plots. \( n = 7 \) for each 2-week sampling interval. Values are means with plus one standard error. Asterisks denote significant difference between the means on that particular date at alpha = 0.05.

Phosphorus fertilization significantly reduced the 2-week mean amount of nitrate that accumulated on the ion exchange resins over the 6-week sampling period (\( p = 0.0137 \)). The 2-week mean amount of nitrate captured by the ion exchange resins averaged 0.56 ± 0.05 mg NO\(_3\) g\(^{-1}\) resin in the reference plots, and 0.31 ± 0.05 mg NO\(_3\) g\(^{-1}\) resin in the treatment plots.

3.3. Phosphate accumulation

The phosphate amount captured by the ion exchange resins over the first 2-week period in the treatment plots, 0.19 ± 0.05 mg PO\(_4\) g\(^{-1}\) resin, was significantly greater than in the reference plots, 0.03 ± 0.05 mg PO\(_4\) g\(^{-1}\) resin (\( p = 0.0212 \); Fig. 4). The difference between the amounts of phosphate captured over three consecutive 2-week intervals in the treatment and reference plots did not change significantly across sampling period (no time and treatment interaction; \( p = 0.8060 \)).

Phosphorus fertilization significantly increased the 2-week mean amount of phosphate accumulated on the ion exchange resins over the 6-week sampling period. The 2-week mean amount of phosphate captured by the ion exchange resins in the treatment plots was 0.14 ± 0.03 mg PO\(_4\) g\(^{-1}\) resin, whereas only 0.01 ± 0.03 mg PO\(_4\) g\(^{-1}\) resin was captured in the reference plots (\( p = 0.0250 \)) over three consecutive 2-week intervals.

3.4. Twig length

In 2001, the length of twigs in reference plots was not significantly greater than treatment plot twigs (18.22 ± 1.46 cm versus 15.35 ± 1.46 cm; \( p = 0.2126 \); Fig. 5). In 2002, treatment plot twigs were significantly longer than reference plot twigs (22.63 ± 1.64 cm versus 14.95 ± 1.64 cm; \( p = 0.0161 \); Fig. 5).

3.5. Twig biomass

The dry mass of annual twigs collected from reference plots was not significantly different than treatment plots in 2001 (0.69 ± 0.08 g versus 0.57 ± 0.08 g; \( p = 0.3374 \); Fig. 6). In 2002, dry mass of treatment
twigs was significantly greater than reference plot twigs (1.03 ± 0.11 g versus 0.62 ± 0.11 g; p = 0.0421; Fig. 6).

3.6. Foliar biomass

The dry mass of foliage collected from reference versus treatment plots did not differ in 2001 (1.18 ± 0.07 g versus 1.19 ± 0.07 g; p = 0.8713; Fig. 7). In 2002, dry mass of treatment foliage was significantly greater than reference foliage (1.54 ± 0.13 g versus 1.08 ± 0.13 g; p = 0.0412; Fig. 7).

3.7. Nodule biomass and number

In 2002, the biomass of single lobed nodules in the reference and treatment plots did not differ significantly (p = 0.9359; Fig. 8). The values were 2.43 ± 1.09 mg and 2.30 ± 0.94 mg for reference and treatment plot nodules, respectively. For size class II nodules (d < 1 cm), the biomass values were 16.37 ± 4.27 mg versus 11.88 ± 3.69 mg for reference versus treatment plot nodules, respectively (p = 0.4621; Fig. 8). For size class III (1 ≤ d ≤ 2 cm) dry mass of treatment nodules was not significantly greater than reference nodules (67.46 ± 7.90 mg versus 66.10 ± 9.13 mg; p = 0.9145; Fig. 8).

The relative number of nodules did not differ significantly between the reference and treatment for any of the size classes. In size class I, reference plot nodules comprised 7.4 ± 4.2% and treatment plot nodules comprised 14.1 ± 3.7% of total nodule number (p = 0.2831; Fig. 9). For size class II, the percentage of reference and
treatment plot nodule numbers were 75.3 ± 4.3% versus 75.7 ± 3.7% (p = 0.9443; Fig. 9). In size class III, reference plot nodules comprised 17.3 ± 3.7% of the total nodule number, whereas treatment plot nodules made 10.2 ± 3.2% of total nodule number (p = 0.2052; Fig. 9).

4. Discussion

4.1. Specific nitrogenase activity

Results of this study indicate that a single application of phosphorus at an input of 50 kg P ha⁻¹ stimulated peak levels of specific nitrogenase activity significantly in early August 2001, during a short but peak activity period. This stimulation of specific nitrogenase activity coincided with the time in the growing season when limiting effects of abiotic factors, such as soil temperature and moisture are minimal, thus rendering P availability a limiting factor. This finding is consistent with the findings of Uliassi and Ruess (2002) who found significant difference in nitrogenase activity of thinleaf alder in the Tanana River floodplain of Alaska only in late July of the first year of 2 years of sampling. They attributed this pattern to release from limitation by other factors mentioned previously, or high N demands during the period of maximum plant growth. Phosphorus fertilization significantly increased specific nitrogenase activity in liverworts and lichens, and in the litter of the tree M. polymorpha and the fern D. linearis on young volcanic sites in Hawaii (Vitousek, 1999). Chapin et al. (1991) found that phosphorus fertilization had a strong positive effect on specific nitrogenase activity of cyanobacteria on young soils in a high Arctic lowland ecosystem in Devon Island, Northwest Territories. Peak nitrogenase activity in this study occurred around late July to early August, after a sharp seasonal increase following increased soil temperatures and decreased precipitation, as found by Hurd et al. (2001), for the same species at the Fishing Brook site in 1998.

Specific nitrogenase activity values reported in this study may be underestimated because of disturbance caused by detachment of the excavated nodules from the host plant, and by the change in the composition of the atmosphere around the nodules, and mechanical damage caused, while transporting the nodules to the assay location (Schwintzer and Tjepkema, 1997). Well-aerated soils have partial oxygen pressure (pO₂) close to atmospheric levels and nodules from such soils would be less affected at atmospheric pO₂ levels, whereas nodules found in poorly aerated soils would be more sensitive to atmospheric pO₂ levels (Silvester et al., 1988). Nevertheless, seasonal specific activities were very similar to those of Hurd et al. (2001), who corroborated acetylene reduction assay estimates with natural abundance ¹⁵N estimates of N₂ fixation. During the sampling period, the wetland was moist but not saturated, compared with the flooded period from snowmelt to mid June. Indeed, none of the nodules had to be sampled from substrate that was flooded. Subtending root segments of at least 2 cm were left attached to nodules in order to minimize disturbance caused by detachment (Schwintzer and Tjepkema, 1997), and care was taken to avoid damage to the nodule during transport to the assay location.

4.2. Nitrate accumulation

Sundareshwar et al. (2003) demonstrated P limitation of the growth of the microbial community in a pristine coastal wetland. In that study the greatest loss of nitrogen via denitrification occurred in the coastal wetland under low P availability, showing that P limitation of microbial heterotrophs has the potential to increase the loss of N and to change the ecosystem level inputs and outputs of N. Phosphorus could therefore be particularly important in controlling N loss to surface waters in riparian systems dominated by alders. The lower nitrate amounts captured by the resins in the treatment plots may be attributed to greater immobilization of nitrogen through stimulated microbial growth, or perhaps increased denitrification. Both N₂-fixing and nearby non-fixing shrubs in alder-dominated wetlands of the Adirondacks appear to be utilizing primarily fixed N (Hurd et al., in press), and P treatment only briefly increased N₂-fixation during the season of peak activity, suggesting that lower nitrate in treatment plots was due to microbial, not plant response. Net nitriﬁcation or nitrate leaching from hillslopes normally is greater than denitrification in these systems, based on elevated nitrate in shallow ground water observed beneath dense speckled alder stands (Kiernan et al., 2003). Ohrui et al. (1999) also demonstrated elevated nitrification in similar riparian wetland soils with alder at HWF. Taken collectively, these studies suggest that
these systems are N-saturated with respect to biological N demand by woody plants, denitrifying bacteria, and microbial heterotrophs, and it is likely that P controls whether this N is lost to proximal surface waters.

4.3. Phosphate accumulation

The decreasing phosphate concentrations in both reference and treatment plots over time indicate either the uptake of phosphorus by alders and other vegetation, utilization by the microbial biota, adsorption of phosphate onto clay particles, organic peat, and ferric and aluminium hydroxides and oxides, or precipitation of insoluble phosphates with ferric iron, aluminium, and calcium, depending on pH (Mitsch and Gosselink, 2000; Brady and Weil, 1999). Super phosphate pellets gradually dissolved over the treatment period (personal observation), due to consistently moist but not saturated soil conditions, indicating that the added P was soluble but was not lost by surface flooding. Added P could have been fixed as aluminum and iron phosphates according to results of soil pH measurements (5–10 cm depth) in late July 2001, which averaged 4 ± 0.1 in reference and treatment plots (Reddy et al., 1999). Nevertheless, it is evident in the growth response of alder the following summer, and in lower nitrate concentrations, that added P was eventually made available to plants, perhaps after microbial immobilization along with N, or was taken up by plants but not utilized initially (luxury consumption).

4.4. Alder tissue and nodule biomass

The significant shifts in growth and biomass response of alder twigs to fertilization from 2001 to 2002 was striking and reveals a delayed response of growth to treatment. The foliar biomass response to treatment was also significant in 2002. It is very likely that the 2001 sampling was too early to detect a response given the fact that the fertilizer was added in early July, which is past the peak growth activity. Phosphorus added in summer 2001 was probably taken up, translocated and stored in alder tissue, and utilized in biomass growth in the summer of 2002. Bayberry (Myrica pensylvanica Loisel), another actinorhizal plant of eastern North America, demonstrated luxury consumption and storage of P under experimental conditions, with foliar P concentration doubling without added plant growth (Hurd and Schwintzer, 1997).

The results of this study on nodule biomass differ from those of Uliassi and Ruess (2002), who found strong effects of P fertilization on nodule biomass at more than four times the application rate used in this study. There was no significant difference either in nodule biomass or in percentage of nodule number between the treatment and reference plots. It is likely that N:P ratios differed in the two studies, potentially influencing nodule growth response (Wall et al., 2000), with N:P less in the Uliassi and Ruess (2002) study due to very high P addition rates. Base N inputs from fixation appear slightly greater (59 ± 11 kg ha⁻¹ yr⁻¹) in the Alaskan pure A. tenuifolia stands, due to greater nodule biomass (27 ± 5.3 g m⁻²) in non-fertilized plots; Uliassi and Ruess, 2002) than in our Fishing Brook site (6.5 ± 1.7 g m⁻²) or in several other species of alder shrubs (Hurd et al., 2001). Peak rates of nitrogenase activity in control plots however appear lower in Uliassi and Ruess (2002) (<25 μmol versus 40 μmol C₂H₄ g nodule dry mass⁻¹ h⁻¹), and these authors suggest that high latitude alders may adjust N₂-fixation more strongly through changes in nodule biomass rather than changes in nitrogenase activity, due to limitation by soil temperature. Nevertheless, it is interesting to note that Uliassi and Ruess (2002) estimated net annual N increment in the Alaskan A. tenuifolia as 43 kg ha⁻¹ yr⁻¹, nearly identical to N fixation estimated at the Fishing Brook site (Hurd et al., 2001), suggesting similar N inputs from fixation. Nitrification is also elevated under both species (Ohrui et al., 1999; Van Cleve et al., 1993), yet the Adirondack sites also receive elevated N in atmospheric deposition that could also increase N:P ratios, particularly by increasing nitrate in soil solution.

5. Conclusions

Phosphorus limits specific activity of nitrogenase in actinorhizal speckled alder for a limited period during the growing season, invokes a delayed growth response in above ground tissues of speckled alder, and decreases nitrate leaching in near-stream wetlands dominated by speckled alder in the Adirondack region of the eastern North America, where excess N leaching to surface waters is a recent concern. Very low phosphate concentrations in the reference plots suggest that P availability
is low in these riparian systems, while nitrate is elevated due to presence of actinorhizal alder (Kiernan et al., 2003) and loss of anthropogenic N from surrounding forested watersheds (Driscoll et al., 2001). Greater phosphate concentrations in treatment plots indicate effective increase of P availability to the plant and microbial community and the decreasing concentrations of phosphate in both fertilized and reference plots over time suggest uptake of P by plants and the microbial community, or fixation by elements such as Fe, Al, Ca, and Mg depending on pH. Decreased nitrate in the rooting zone of P fertilized alder suggests that the microbial uptake increased relative to nitrification, or that alders were taking up more soil N. The latter is not likely however, as P did not decrease specific activity of nitrogenase, or nodule biomass. The significant increases in the length and biomass of alder twigs and significant increase in the biomass of alder foliage in 2002 are strong evidence of P limitation of alder growth in this systems. It is unlikely that extra N fixed in 2001 was retranslocated and utilized in 2002 because alders do not retranslocate N extensively before leaf abscission (Dawson, 1990; Bischoff et al., 2001), the increase in specific nitrogenase activity was relatively small, and nodule growth did not increase. Phosphorus fertilization in this study had no detectable effect on nodule biomass or on percentage of nodule number in different size classes after 2 years. Taken together with the small seasonal increase in nitrogenase activity and decreased nitrate availability indicated by IER, these results suggest that growth of alder and growth or activity of soil microbes, rather than nitrogen fixation, is P limited in riparian wetlands dominated by speckled alder, although delayed P-response in foliar mass could have been associated with an increase in nitrogenase activity the following year. Moreover, P-controls concentrations of mobile nitrate in these riparian systems, probably due to P limitation of heterotrophic microbes, and so likely controls loss of wetland-derived, actinorhizal fixed N, as well as anthropogenic N, across the terrestrial–aquatic ecotone.

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