



Seasonal changes in nitrate assimilation of boreal woody species: importance of the leaf-expansion period

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Abstract

Key message Nitrate served as an important nitrogen source for dominant deciduous tree species, especially during their leaf expansion period, even in boreal forests, where nitrate availability was assumed to be low.

Abstract Temporal changes in leaf nitrate assimilation with leaf growth were intensively investigated in boreal tree species to demonstrate the contribution of nitrate as a N source and to determine temporal changes in the contribution of nitrate during leaf ontogeny. Leaf area, mass, nitrate reductase activity (NRA), N concentration, and $\delta^{15}\text{N}$ were repeatedly measured in developing leaves of naturally grown *Alnus crispa*, *Betula neoalaskana*, and *Populus tremuloides* during their leaf expansion period. *Alnus crispa* and *B. neoalaskana* showed distinct peaks in NRA during leaf expansion, whereas *P. tremuloides* did not. The highest peak in NRA occurred for *A. crispa*, whereas it had low NRA during the summer. Peak NRA in *B. neoalaskana* was lower than that of *A. crispa* ($p < 0.01$, ANOVA), although it showed higher NRA during summer ($p < 0.01$, ANOVA). All species showed clear decrease in N concentration through the leaf expansion period, but total N content per leaf increased. Only the N-fixing species *A. crispa* showed a rapid change in $\delta^{15}\text{N}$ during the leaf expansion, and the decline indicated the changes in N source during the leaf development. The results indicate leaves of target species assimilated nitrate during the leaf expansion period, consuming immense energy, although leaves were considered a carbon sink during the early leaf expansion period. We suggest the early onset of leaf growth due to climate warming could influence plant nutrition via asynchrony between supply and demand for energy during spring.

Keywords Boreal forest · Bud break · Green up · Leaf expansion period · Nitrate reductase

Introduction

The leaf expansion period is a spectacular season, not only from the view of the rapid increase in leaf biomass but also considering invisible physiological changes that occur in the rapidly growing leaves. Leaves are carbon sinks which receive carbon translocated from other tissues during the first half of their expansion period. They become carbon sources during the latter half of the expansion period as their

photosynthetic capacity increases with their growth (Šesták et al. 1985). Nitrogen concentration very quickly increases in breaking buds and decreases relatively slowly during the leaf expansion, followed by a steady state in fully expanded leaves (Millard 1994; Kielland et al. 1998; Koyama et al. 2008).

The changes in nutrient acquisition and translocation during the leaf expansion period have been investigated in less detail than carbon balance with leaf ontogeny. With respect to N sources for newly developing leaves, N remobilization has been investigated using ^{15}N tracers and intensive leaf and xylem sap sample collection during leaf expansion in seedlings or grafted nursery plants of species, such as apple (*Malus domestica* Borkh.), cherry (*Prunus avium* L.), walnut (*Juglans nigra* × *regia*), and birch (*Betula pendula* Roth.) (Millard et al. 1998; Frak et al. 2002; Grassi et al. 2002; Guak et al. 2003). In general, these studies showed that N remobilized to leaves from other tissues contributed greatly to leaf expansion in the early stage. In total, the dependence

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of newly developing shoots on N translocated from the other tissues ranged from 14 to 87% in these deciduous tree species. However, it remains to be shown how the remainder of the necessary N was acquired by new leaves; i.e., what form of N was used and how foliar N acquisition activity changed with the development of leaves. In a temperate region, two deciduous and an evergreen species were investigated at intervals of 2–3 days at the most, to observe the temporal changes in nitrate assimilation during leaf expansion (Koyama et al. 2008). All three of the studied species showed apparent peaks in nitrate reductase activity (NRA), the activity of an enzyme that catalyzes the rate-limiting process in nitrate assimilation, during the leaf expansion period. In boreal regions, where both the growing season and leaf expansion periods are shorter than in temperate regions, there have been no high-temporal resolution studies of the physiological changes in leaves during leaf expansion, such as in the study by Koyama et al. (2008). Climate change influences the foliation in boreal regions more readily than in temperate regions, and the spring phenology of leaves is advanced in boreal forests (Linkosalo et al. 2009). This suggests that elucidation of the physiological progress during leaf expansion and how it is altered under warmer climate conditions is urgent. Nevertheless, the relationship between leaf ontogeny and other physiological changes is not fully understood.

Nitrate is an important N source for terrestrial plants, and nitrate assimilation has been investigated in a variety of plant species using in vivo NRA as an index (e.g., Smirnov et al. 1984; Gebauer et al. 1988; Koyama et al. 2020). The advantage of NRA as an index for nitrate assimilation is that plant nitrate use can be estimated without disturbing the soil N condition, which is not the case for experimental manipulations, such as the application of ^{15}N tracers. Previous studies revealed significant variability in the capacity to use nitrate as a N source among plant species, and some species lacked the capacity to produce nitrate reductase (NR), an essential enzyme to assimilate nitrate. In boreal regions, nitrate is not considered a major N source for plants (Valentine et al. 2006), because of low microbial activity caused by low temperature and acidic soils. However, recent studies on the capacity of plants to use nitrate suggest it can be important in certain northern ecosystems, such as in riparian forests and non-acidic arctic tundra (Liu et al. 2018; Koyama and Kielland 2011).

Species dependence on nitrate or the role of nitrate as a N source for a species should be evaluated not only based on momentary NRA but also the time course of NRA; i.e., the potential of the species to use nitrate and the continuity of high NRA after leaf expansion. In the present study, we investigated the changes in NRA, N content, and $\delta^{15}\text{N}$ in the leaves of boreal tree species during leaf expansion. Our research questions were: (1) Does nitrate serve as a N

source for boreal tree species during leaf expansion? (2) Does nitrate assimilation (and the roles of nitrate as a source of N) change temporally during leaf expansion of the boreal species? (3) Does the pattern of plant nitrate assimilation exhibit a different time course between the boreal species and the temperate species studied by Koyama et al. (2008)? To address these questions, we repeatedly collected leaf samples from boreal species in the interior of Alaska, USA, throughout the leaf expansion period. Three deciduous species that are dominant in the interior of Alaska, *Alnus crispa*, *Betula neoalaskana*, and *Populus tremuloides*, were selected as target species. Based on the results, we discuss the possible influences of climate change on boreal forests focusing on the altered timing of leaf expansion and physiological activities during leaf growth.

Materials and methods

Study site

The study was conducted in a mixed forest adjacent to the campus of the University of Alaska, Fairbanks, USA (64° 51' 29.7" N, 147° 51' 15.4" W). The climate is strongly continental, and the area lies within a rain shadow created by the Alaska Range approximately 100 km to the south. Temperature extremes range from $-40\text{ }^{\circ}\text{C}$ in winter to $\geq 30\text{ }^{\circ}\text{C}$ during the summer, with an average of $-3.3\text{ }^{\circ}\text{C}$. The average annual precipitation is 269 mm, 37% of which falls as snow. Snow covers the ground for 6–7 months of the year, from mid-October until early or mid-April. Soil pH in the nearby US LTER site were 7.2 ± 0.4 , 5.6 ± 0.7 and 5.9 ± 0.6 in the sites with *A. crispa*, *B. neoalaskana* and *P. tremuloides*, respectively (Yarie 1998).

The mixed forest was dominated by some deciduous tree species, such as *A. crispa*, *B. neoalaskana*, and *P. tremuloides*, and an evergreen coniferous species, black spruce (*Picea mariana*). Dominant understory species included *Vaccinium vitis-idaea*, *Vaccinium uliginosum*, *Rubus chamaemorus*, *Rosa acicularis*, and *Epilobium angustifolium*.

Study species and sample collection

Five mature trees from each of three deciduous tree species, *A. crispa*, *B. neoalaskana*, and *P. tremuloides*, were sampled regularly about 10 times during the leaf expansion period. Prior to the investigation of the leaf expansion period, we collected leaf samples during summer (for comparison to newly expanded leaves) on July 22, 2009. In the following year, leaf samples were collected from the same individuals to investigate their size (area and mass), N concentration, $\delta^{15}\text{N}$, and NRA during the leaf expansion period. The

sample collection began just after the bud break, which occurred from early- to mid-May and ended June 7, 2010. Samples were collected at intervals of 3–4 days throughout this period. At each sampling day, 10 leaves were collected for leaf size measurement regardless of leaf growth stage, and a minimum of 5–10 leaf samples were collected and bulked for assay of N concentration, $\delta^{15}\text{N}$, and NRA from the various part of canopy to reduce the effects of within-individual variation. The samples were collected from the surface of the canopy at various heights from 10:00 to 14:00, and the sampled leaves were exposed to adequate light because of low canopy density. The samples were stored in dark until assay on ice and in 4 °C in the field and the lab, respectively. The storage time of all samples before analysis was about 1 h; thus, storage effects were similar for all samples, since the change in NRA is very slow after an initial decline in the first 30 min after collection (Högberg et al. 1986).

Assays

Leaf area and mass were measured to determine the degree of leaf expansion. To determine NRA, N concentration, and $\delta^{15}\text{N}$ of the leaves, aliquots of the sampled leaves were then assayed.

Ten leaves collected from a tree were weighed and scanned to determine leaf area using the image analysis software ImageJ (version 1.43 m; <https://imagej.nih.gov/ij/index.html>; Schneider et al. 2012). The average area and mass of the 10 leaves from each tree were used as an indicator of leaf area and mass for each tree, respectively. To clarify the growth stage of leaves on each sampling day, leaf growth was fitted to a logistic curve (Koyama et al. 2008):

$$L_e = \frac{L_f \cdot L_0 \cdot \exp(r \cdot d)}{L_f - L_0 + L_0 \cdot \exp(r \cdot d)},$$

We measured two modes of activities of NR, NRA(+NO₃) and NRA(−NO₃), as indices of plant NO₃[−]–N assimilation. NRA(+NO₃) is a measure of the nitrate reduction capacity with a non-limiting nitrate supply, whereas NRA(−NO₃) is the reduction rate of nitrate absorbed by plants, which is considered to be the closest approximation of the in situ NO₃[−]–N assimilation rate (Thomas and Hilker 2000). Both NRA assays were conducted with modified versions of the Jaworski procedure (Jaworski 1971; Koyama and Kielland 2018). NRA(+NO₃) was measured as the rate of nitrite (NO₂[−]–N) production in an incubation buffer containing a non-limiting concentration of NO₃[−]–N. NRA(−NO₃) was determined with parallel measurements using an incubation buffer without additional NO₃[−]–N, which allowed us to examine the relative magnitude of in situ NO₃[−]–N assimilation. Approximately 100 mg (fresh weight)

of leaves were cut into small discs ($D = 2.5$ mm) and transferred to test tubes. The incubation buffer (5 mL) was added to the leaves, and the tube contents were vacuum infiltrated. The composition of the incubation buffer for NRA(+NO₃) was as follows: 0.1 mol L^{−1} KNO₃, 0.1 mol L^{−1} KH₂PO₄, and 1.5% 1-propanol. The pH was adjusted to ca. 7.5 using a NaOH solution. The incubation buffer for NRA(−NO₃) contained all of the reagents other than KNO₃. The samples were incubated at 30 °C for 1 h in darkness. Enzyme activity was terminated by placing the sample vials in hot water (> 80 °C). The concentration of NO₂[−]–N in the incubation buffer was determined colorimetrically following diazotization and azo coupling (Keeney and Nelson 1982), by measuring absorbance at the wavelength 545 nm (Lambda 25, PerkinElmer Inc., MA, USA). The confounding effects of plant pigments were accounted for by subtracting the absorbance of controls to which *N*-naphthylethylene diamine dihydrochloride was not added (Gebauer et al. 1998). A fraction of each leaf sample was oven-dried at 60 °C and then weighed to calculate activity per unit dry weight. Leaf NRA was calculated per unit leaf, too.

Leaf samples were dried at 60 °C for longer than 48 h and ground for $\delta^{15}\text{N}$ analysis. The stable N isotope ratio of samples was analyzed using an isotope ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany) coupled with an elemental analyzer (EA1108, Fisons, Milan, Italy) via Conflo II as an interface. The stable isotope ratios were expressed in δ notation as the differences in parts per thousand (‰) of the ¹⁵N/¹⁴N ratio of the samples from the ¹⁵N/¹⁴N ratio of the standard (atmospheric nitrogen). The precision of the on-line procedure was better than $\pm 0.2\text{‰}$. The amount of N in the standard reagent, DL-Alanine, calculated by the weight and N concentration were regressed with the Thermal Conductivity Detector (TCD) area ($R^2 > 0.999$, $n = 23$) obtained by the elemental analyzer. Hence, N concentration in the samples was calculated based on the TCD area, and N content per leaf were also calculated.

Statistical analysis

Pearson's correlation coefficients for estimated leaf area, estimated leaf area growth rate, leaf N content (per dry weight and per leaf), leaf NRA(+NO₃) (per dry weight and per leaf), and $\delta^{15}\text{N}$ were calculated for each tree sample. The leaf growth curve was fitted for each tree to avoid the influence of individual differences in leaf growth stages at each sampling date, and the leaf area growth rate was calculated as the difference between leaf area on a certain day and that on the next day, as calculated for the leaf growth curve. Since the timings of bud breaks differed among species, it was unlikely to be appropriate to compare species at the same date especially to discuss the species potential for each trait. Therefore, we extracted

peak timing for each of plant traits (leaf area, mass, leaf mass per area, NRA (+NO₃) and NRA(−NO₃) per dry weight and per leaf, N concentration, N content per leaf, and δ¹⁵N), and compared the peak values among species. To determine peak timing of plant traits during leaf expansion, repeated measures ANOVA was applied for each species. Then, peak values were compared among species with one-way ANOVA with post hoc Tukey HSD test. In addition, the values during summer were also compared among species with one-way ANOVA with post hoc Tukey HSD test. All statistical analyses were conducted using

the statistical platform R (ver. 3.5.3; <http://www.R-project.org>).

Results

Bud break and leaf expansion of *B. neoalaskana* and *P. tremuloides* began and ended earlier than that of *A. crispa* (Table 1, Fig. 1 and S1). Leaf mass increased for a longer period than did leaf area for all three species, and the peak rate of leaf mass increase occurred later than that of leaf expansion rate, except for *A. crispa* (Fig. S1j–l). This

Table 1 Beginning and end dates, and duration of leaf expansion in the three target species in 2010

	Bud break	Full expansion in area	Duration for area growth (days)	Full expansion in leaf mass	Duration for mass growth (days)
<i>Alnus crispa</i>	May 14	June 6	23	June 11	28
<i>Betula neoalaskana</i>	May 3	May 30	27	June 7	35
<i>Populus tremuloides</i>	May 6	May 28	22	June 6	31

The day of full leaf expansion was defined as the day on which the estimated leaf area or mass reached to 99% of maximum based on their growth curve

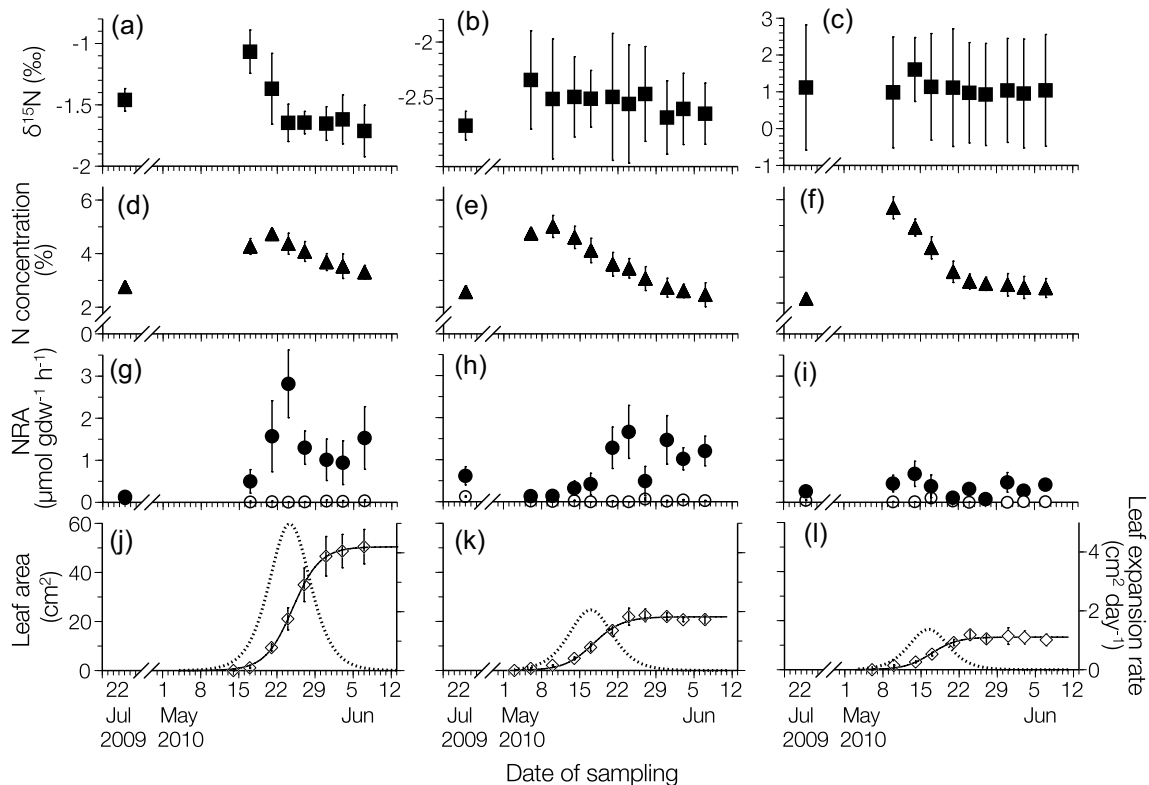


Fig. 1 Temporal changes in leaf traits regarding N acquisition and growth for *Alnus crispa* (left; **a**, **d**, **g**, and **j**), *Betula neoalaskana* (middle; **b**, **e**, **h**, and **k**), and *Populus tremuloides* (right; **c**, **f**, **i**, and **l**) during the greening season 2010 in comparison with that of summer 2009. **a–c** Leaf δ¹⁵N; **d–f** leaf N concentration; **g–i** leaf NRA(+NO₃)

(closed circle) and leaf NRA(−NO₃) (open circle); and **j–l** leaf area (open diamond) with estimated growth curve of leaf area (solid line; left axis) and leaf expansion rate (broken line; right axis). Average ± s.d. are shown for five trees

indicated that leaf mass per area (LMA) decreased with leaf expansion in the early expansion stage, and increased during the latter stage (Fig. S1g–i).

Leaf $\text{NRA}(+\text{NO}_3)$ rapidly increased after bud break and declined after the peaks in *A. crispa* and *B. neoalaskana* (Fig. 1g–i, Table S4), indicating that these species were capable of assimilating nitrate in leaves during leaf development. However, the relationship of $\text{NRA}(+\text{NO}_3)$ to leaf area or area growth rate differed among species (Table 2). A positive correlation between $\text{NRA}(+\text{NO}_3)$ and area growth rate was observed for *A. crispa*, whereas *B. papyrifera* showed a positive correlation between $\text{NRA}(+\text{NO}_3)$ and leaf area. On the other hand, the increase after bud break was not clear in *P. tremuloides* leaf $\text{NRA}(+\text{NO}_3)$, which was relatively low throughout the study period (Table S5), suggesting low dependence on nitrate for this species during this time period. $\text{NRA}(-\text{NO}_3)$ was lower than $\text{NRA}(+\text{NO}_3)$ by as much as an order of two or was not detected in all three species.

Leaf N concentration showed a peak at the beginning of the leaf expansion period, and then monotonically decreased in all three species (Fig. 1d–f, Table S4), which reflected a typical growth dilution of initial N concentration in leaves. On the other hand, N content per leaf at the end of leaf growth was 8.2, 9.2, and 5.9 times larger than that at the beginning of leaf expansion for *A. crispa*, *B. neoalaskana*, and *P. tremuloides*, respectively. These were equivalent to the increase of 5.7 ± 1.2 mg N per leaf in 23 days, 2.2 ± 0.5 mg N per leaf in 27 days, and 2.3 ± 0.8 mg N per leaf in 22 days for *A. crispa*, *B. neoalaskana* and *P. tremuloides*, respectively, indicating that newly acquired N was more than the offset by the diluted N concentration (Figs. 2d–f, S1a–c). All three species showed a strong negative correlation between leaf N concentration and leaf area (Table 2; See also Table S1 for leaf mass). N concentration in *P. tremuloides* leaves apparently reached a steady state after the decline, while in the two other species, the concentration of N continued to decrease until the end of the study period (Fig. 1d–f). However, the comparison between leaf N concentrations in the previous summer and at the end of study period showed that leaf N concentrations very likely reached steady states around the end of study period in both *A. crispa* and *B. neoalaskana*. Relations between leaf NRA and leaf N concentration or N contents per leaf differed among species (Table S2; Fig. S2), reflecting species difference in temporal changes of NRA.

Leaf $\delta^{15}\text{N}$ was stable throughout the study period in *B. neoalaskana* and *P. tremuloides* (Fig. 1b, c; Table S4), indicating that the composition of N sources did not change over time. On the other hand, $\delta^{15}\text{N}$ in *A. crispa* leaves exhibited the highest value which is compatible with reliance on N-fixation just after bud break, then rapidly decreased to a steady state (Fig. 1a, Table S4), implying a shift in N

sources during leaf expansion. $\delta^{15}\text{N}$ and other leaf traits did not show consistent responses among individuals (Table S3; Fig. S2a–c).

There was a positive relationship between N content per leaf and the degree of leaf expansion; i.e., the percentage of the estimated leaf area at the day of sampling (L_e) to the maximum leaf area (L_f) based on their growth curve, that showed leaf N contents monotonically increased with the growth of leaf area in all three species (Fig. 2d–f). On the other hand, leaf N concentration almost monotonically decreased with the increase in the degree of leaf expansion, indicating N dilution with leaf expansion (Fig. 2a–c). Following full leaf expansion then the N content per leaf reached a steady state (see also Fig. S1a–c).

Changes in $\text{NRA}(+\text{NO}_3)$ per weight and $\text{NRA}(+\text{NO}_3)$ per leaf with the degree of leaf expansion also showed different patterns among species (Fig. 2g–i). *Alnus crispa* exhibited peaks of $\text{NRA}(+\text{NO}_3)$ per weight and $\text{NRA}(+\text{NO}_3)$ per leaf, when the leaves reached approximately half of the full expansion. By contrast, *B. neoalaskana* did not peak until the leaves were nearly (ca. 90%) fully expanded. $\text{NRA}(+\text{NO}_3)$ per leaf showed a pattern different from that shown by $\text{NRA}(+\text{NO}_3)$ per weight in *P. tremuloides*. $\text{NRA}(+\text{NO}_3)$ per leaf increased when leaves approached full expansion in *P. tremuloides*, whereas $\text{NRA}(+\text{NO}_3)$ per weight generally decreased with leaf expansion. Increased $\text{NRA}(+\text{NO}_3)$ per leaf after full leaf expansion in *P. tremuloides* implied that nitrate played a more important role as the N source later in summer after leaves were fully developed, although a relatively low $\text{NRA}(+\text{NO}_3)$ in this species suggested low overall dependence on nitrate as a N source.

Discussion

Role of nitrate as a N source for the target species

Nitrate reductase is a substrate inducible enzyme, and the capacity to induce NR markedly differs among species (Smirnoff et al. 1984; Gebauer et al. 1988; Koyama et al. 2020). The target species in the current study also possessed NRA during the study period (Fig. 1g–i), indicating that all three of these boreal species had the capacity to induce NR and use nitrate as a source of N. Moreover, even though previous studies have indicated that woody species generally assimilate nitrate in roots especially under low N availability (Andrews 1986), the results of current study indicate that leaves assimilated nitrate in boreal tree species. The results suggested that nitrate was an important N source in boreal forests, as well as in arctic tundra, where recently, Liu et al. (2018) demonstrated the contribution of nitrate as a plant N source. The highest enzyme activity was detected in *A.*

Table 2 Relationship between leaf N traits and leaf area or leaf growth rate

Tree	<i>A. crispa</i>						<i>B. neolaskana</i>						<i>P. tremuloides</i>						
	Estimated leaf area (cm ²)			Area growth rate (cm ² day ⁻¹)			Estimated leaf area (cm ²)			Area growth rate (cm ² day ⁻¹)			Estimated leaf area (cm ²)			Area growth rate (cm ² day ⁻¹)			
	<i>R</i>	<i>P</i>	<i>n</i>	<i>R</i>	<i>P</i>	<i>n</i>	<i>R</i>	<i>P</i>	<i>n</i>	<i>R</i>	<i>P</i>	<i>n</i>	<i>R</i>	<i>P</i>	<i>n</i>	<i>R</i>	<i>P</i>	<i>n</i>	
N concentration (%)	1	-0.817	0.01	8	0.831	0.01	8	-0.966	<0.01	11	0.532	0.09	11	-0.991	<0.01	10	0.633	0.05	10
	2	-0.870	<0.01	8	0.682	0.06	8	-0.943	<0.01	11	0.53	0.09	11	-0.990	<0.01	10	0.506	0.14	10
	3	-0.960	<0.01	8	0.617	0.10	8	-0.869	<0.01	11	0.552	0.08	11	-0.972	<0.01	9	0.678	0.05	9
	4	-0.914	<0.01	8	0.641	0.09	8	-0.973	<0.01	11	0.406	0.22	11	-0.986	<0.01	10	0.592	0.07	10
	5	-0.493	0.22	8	0.832	0.01	8	-0.965	<0.01	11	0.503	0.12	11	-0.952	<0.01	10	0.636	0.05	10
N content per leaf (mg-N leaf ⁻¹)	1	0.974	<0.01	8	-0.330	0.42	8	0.934	<0.01	11	-0.249	0.46	11	0.979	<0.01	10	-0.768	<0.01	10
	2	0.944	<0.01	8	-0.268	0.52	8	0.909	<0.01	11	-0.363	0.27	11	0.964	<0.01	10	-0.676	0.03	10
	3	0.983	<0.01	8	-0.365	0.37	8	0.903	<0.01	11	-0.233	0.49	11	0.934	<0.01	9	-0.519	0.15	9
	4	0.958	<0.01	8	-0.378	0.36	8	0.963	<0.01	11	-0.362	0.27	11	0.958	<0.01	10	-0.717	0.02	10
	5	0.968	<0.01	8	-0.389	0.34	8	0.981	<0.01	11	-0.355	0.28	11	0.879	<0.01	10	-0.682	0.03	10
$\delta^{15}\text{N}$ (‰)	1	0.005	0.99	8	-0.687	0.06	8	-0.103	0.76	11	0.121	0.72	11	0.353	0.32	10	-0.415	0.23	10
	2	-0.693	0.06	8	-0.234	0.58	8	-0.500	0.12	11	0.234	0.49	11	-0.694	0.03	10	0.831	<0.01	10
	3	-0.796	0.02	8	0.026	0.95	8	-0.465	0.15	11	0.171	0.62	11	0.153	0.69	9	0.431	0.25	9
	4	-0.410	0.31	8	-0.259	0.54	8	-0.724	0.01	11	0.28	0.41	11	-0.016	0.96	10	0.343	0.33	10
	5	-0.811	0.02	8	0.186	0.66	8	-0.286	0.39	11	0.009	0.98	11	0.208	0.57	10	-0.100	0.78	10
NRA (+NO ₃) per dry weight (μmol gdw ⁻¹ h ⁻¹)	1	-0.277	0.51	8	0.854	<0.01	8	0.585	0.06	11	-0.088	0.80	11	-0.570	0.09	10	0.225	0.53	10
	2	-0.137	0.75	8	0.689	0.06	8	0.716	0.01	11	-0.105	0.76	11	-0.340	0.34	10	-0.127	0.73	10
	3	-0.421	0.30	8	0.781	0.02	8	0.700	0.02	10	-0.444	0.20	10	-0.129	0.72	10	0.165	0.65	10
	4	-0.310	0.46	8	0.622	0.10	8	0.647	0.03	11	-0.021	0.95	11	-0.801	<0.01	10	0.751	0.01	10
	5	0.118	0.78	8	0.633	0.09	8	0.792	<0.01	11	-0.345	0.30	11	-0.425	0.22	10	0.242	0.50	10
NRA(+NO ₃) per leaf (μmol leaf ⁻¹ h ⁻¹)	1	0.357	0.39	8	0.441	0.27	8	0.761	<0.01	11	-0.350	0.29	11	0.561	0.09	10	-0.574	0.08	10
	2	0.512	0.20	8	0.105	0.80	8	0.840	<0.01	11	-0.401	0.22	11	0.696	0.03	10	-0.655	0.04	10
	3	0.123	0.77	8	0.413	0.31	8	0.717	0.02	10	-0.516	0.13	10	0.509	0.13	10	-0.459	0.18	10
	4	0.341	0.41	8	0.043	0.92	8	0.795	<0.01	11	-0.380	0.25	11	0.678	0.03	10	-0.453	0.19	10
	5	0.519	0.19	8	0.150	0.72	8	0.826	<0.01	11	-0.526	0.10	11	0.480	0.16	10	-0.481	0.16	10

Pearson's correlation coefficients were calculated between physiological traits regarding N use, such as N concentration, N content per area or leaf, $\delta^{15}\text{N}$, NRA (+NO₃) per dry weight, area, leaf or N content in leaves and leaf expansion traits; i.e., estimated leaf area or its growth rate representing leaf growth stage, during leaf expansion

R Pearson's correlation coefficient, *P* *p* value, *n* number of samples

^aEstimated leaf areas were calculated based on the logistic curves fitted to the number of days after bud break and leaf area for each tree to reduce the effect of sampled leaf sizes

^bArea growth rate per day was calculated based on the logistic curves to calculate the leaf areas, by subtracting estimated leaf area at a sampling day from the area at the next day

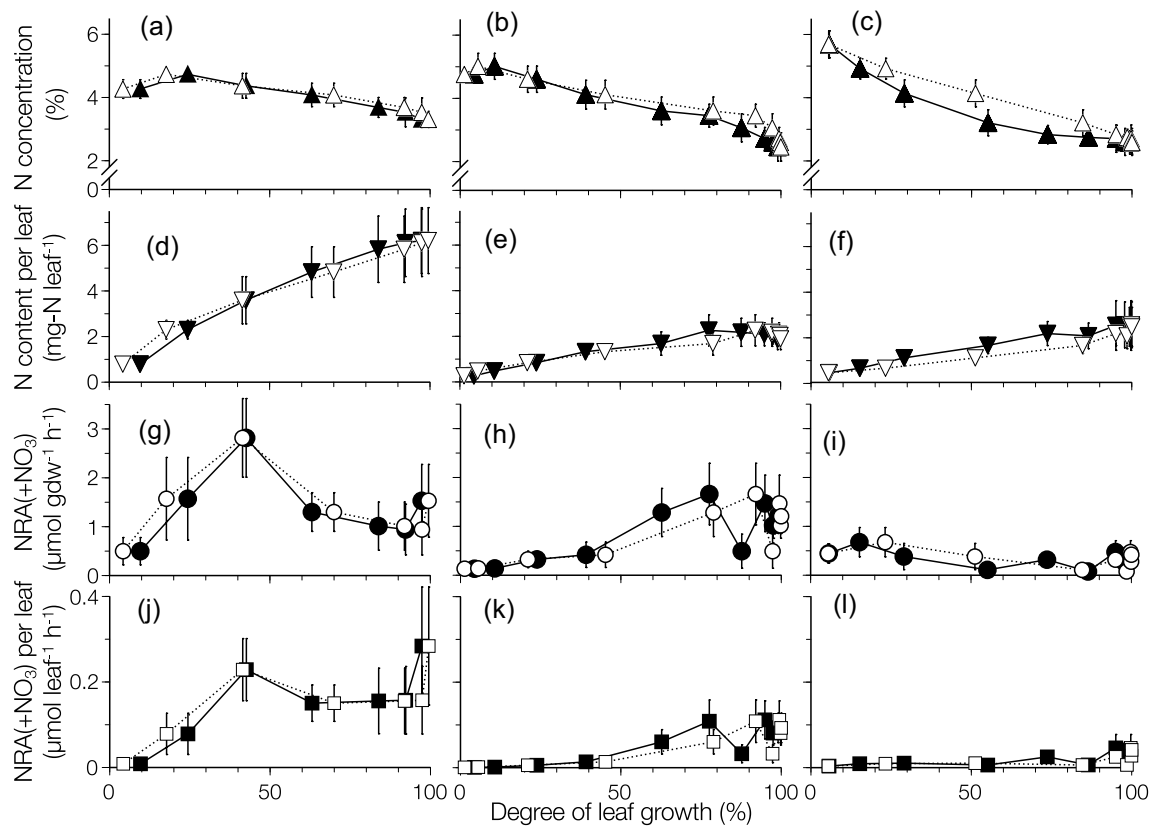


Fig. 2 Relationship between the degree of leaf expansion and leaf traits regarding N acquisition for *Alnus crispa* (left; **a**, **d**, **g** and **j**), *Betula neoalaskana* (middle; **b**, **e**, **h** and **k**), and *Populus tremuloides* (right; **c**, **f**, **i** and **l**) during the greening season 2010. **a–c** N concentration per weight; **d–f** N content per leaf; **g–i** NRA (+NO₃) per leaf

dry weight; and **j–l** NRA (+NO₃) per leaf. The degree of leaf expansion was defined as the percentage of the estimated leaf area (open symbols) or, mass (closed symbols) on the day of sampling relative to the maximum leaf area based on the growth curve. Average $\pm$ s.d. are shown for five trees

crispa, which exhibited higher NRA(+NO₃) than the species with the lowest activity, *P. tremuloides*, by a degree of magnitude ($p < 0.04$, ANOVA; Table S5). This observation is consistent with the observed higher rates of nitrification of alder stands in interior Alaska (Kielland et al. 2006).

The detection of NRA(+NO₃) in the study period indicated that the leaves of these species assimilated N themselves during the leaf expansion period in which N concentration drastically decreased due to growth dilution (Fig. 1). Increase in N content per leaf indicated that leaves acquired N during expansion (Fig. 2d–f, Fig. S1a–c). Because NRA values showed the potential activity but not the assimilated amount of N, we cannot quantitatively evaluate the contribution of nitrate assimilation in leaves to the increase of N content in leaves compared with N translocated from the other tissues. Regardless, our results indicated that leaves of boreal tree species assimilated nitrate themselves during expansion to at least partly compensate for the diluted N concentration and total increase in N content.

The patterns of temporal change in leaf NRA(+NO₃) differed among the three species (Fig. 1g–i, Table S4). The

highest NRA(+NO₃) per dry weight was detected when the leaf size reached approximately half of the full expansion in *A. crispa* (Fig. 2g), and it was consistent with the significant positive correlation between NRA(+NO₃) per dry weight and area growth rate (Table 2). Both NRA(+NO₃) per unit weight and that per unit leaf mostly increased with the degree of leaf expansion in *B. neoalaskana* (Fig. 2h, k). Furthermore, the significant positive correlation between leaf area and NRA(+NO₃), regardless of the unit (Table 2), suggested that during leaf expansion period, nitrate use increased with time in this species. By contrast, *A. crispa* may have relied on nitrate later in the season, perhaps in response to increased nitrification rates as soil temperatures increased over the summer.

The relative dependence of the species on variant N sources should be reflected in $\delta^{15}\text{N}$ values of plant tissues (Koba et al. 2003; Liu et al. 2018). The $\delta^{15}\text{N}$ values of soil nitrate were lower than that of soil ammonium, and thus plants with lower tissue $\delta^{15}\text{N}$ are very likely to depend more on soil nitrate as a N source than plants with higher $\delta^{15}\text{N}$ values. In the present study, *P. tremuloides* clearly had a higher

$\delta^{15}\text{N}$ value than that of the other species (Fig. 1a–c, Tables S5 and S6), suggesting its dependence on the N source with high $\delta^{15}\text{N}$, which supposedly was not nitrate. This is consistent with the results, wherein *P. tremuloides* showed a constantly low $\text{NRA}(+\text{NO}_3)$ throughout the study period (Fig. 1i, Table S5). However, the current results were inconsistent with early studies on nitrate use by *P. tremuloides* (Chapin et al. 1986; Kielland et al. 1998). Leaf $\delta^{15}\text{N}$ in *P. tremuloides* was much lower in the study of Kielland et al. (1998) than that in the current results, and were comparable to that in *B. neoalaskana*. It is still possible that soil nitrate availability differed between the sampling sites, and was reflected in leaf $\delta^{15}\text{N}$, although data regarding soil nitrate availability in the current sampling site were not available. Plant responsiveness to nitrate availability is species specific (Koyama et al. 2003), and it was observed that species that were less responsive and more flexible to nitrate availability had a wider distribution range with respect to nitrate availability (Koyama et al. 2013). Thus, we surmise that *P. tremuloides* are sufficiently physiologically flexible to change their N acquisition according to the soil conditions.

The depletion of $\delta^{15}\text{N}$ in *A. crispa* leaves in the early stage of leaf expansion (Fig. 1a, Table S4) suggest that the N source for leaves of *A. crispa* rapidly changed in the early leaf expansion season. *Alnus crispa* was the only symbiotic N-fixing species among the three target species, but $\delta^{15}\text{N}$ values in N-fixing plants vary in reliance on N_2 fixation. $\delta^{15}\text{N}$ values led to approximately 0‰ when the plants thoroughly depended on N_2 fixation (Craine et al. 2015). The $\delta^{15}\text{N}$ values of *A. crispa* were closer to 0‰ at the beginning of leaf expansion and deviated from 0‰ during the latter stage. The results suggested that leaves of *A. crispa* depended on N from N_2 fixation when they began to expand, and that the rapid increase in $\text{NRA}(+\text{NO}_3)$ synchronized with the decline of $\delta^{15}\text{N}$ suggest that nitrate was likely to increasingly contribute as N source in the early leaf expansion period (Fig. 1a, g, j).

The decrease in N concentration per weight showed that N with a high concentration in leaves at bud break was strongly diluted during leaf expansion (Figs. 1d–f, 2a–c; Table 2). On the other hand, the increase in N content per leaf represented that newly acquired N was more than the offset by the diluted N (Figs. 2d–f, S1a–c; Table 2), and that N was translocated to leaves from other tissues that had stored N beforehand or from roots that proximately absorbed N during the period, although the ratio of N origins was unidentified. Koyama et al. (2008) showed that N concentration was apparently reduced in 1-year-old leaves during leaf expansion, while N concentration in current buds increased in an evergreen temperate species, suggesting N was translocated from the 1-year leaves to the current buds. Previous researches on fruiting trees of orchard species showed generally low contribution of newly absorbed N in comparison

with stored N (orange: Feigenbaum et al. 1987; walnut: Deng et al. 1989; apple: Neilsen et al. 1997). Millard and Grelet (2010) summarized that the contribution of N remobilized to newly developing shoots from the storage tissues ranged from less than 10% to 80–100% in various deciduous woody species, although these estimates targeted relatively young individuals.

Temporal changes in nitrate use by boreal species compared with that of temperate species

In temperate region, it was shown that three broad-leaved species (including an evergreen species) had distinct peaks of NRA during leaf expansion period, and their NRA decreased after leaf expansion to a relatively steady state for each species, which continued throughout the following summer until the leaves fell (Koyama et al. 2008). The value of NRA in temperate species showed a positive correlation with leaf expansion rate, and it was concluded that the leaf growth stage was an important regulating factor of leaf nitrate assimilation. In addition, nitrate assimilated in the current leaves at least partly compensated for the decreased N concentrations in leaves during leaf expansion. In this study, we found similar increases of NRA in boreal broad-leaved species during their leaf expansion (Fig. 1g–i), although the relationship of NRA to the leaf growth stage varied among species (Table 2; Table S1). With this small number of examples, however, one cannot generalize that plant species have different temporal patterns of NRA according to climatic regions; i.e., that temperate species have relatively synchronized patterns, whereas boreal species have fluctuating patterns.

In temperate species, Koyama et al. (2008) revealed the positive correlations between $\text{NRA}(+\text{NO}_3)$ and leaf growth rate in individual trees of all three species with few exceptions. Likewise, $\text{NRA}(+\text{NO}_3)$ per unit dry weight showed a positive correlation with leaf area growth rate in *A. crispa* in this study (Table 2). On the other hand, $\text{NRA}(+\text{NO}_3)$ in *P. tremuloides* and *B. neoalaskana* showed different relationships with leaf growth. $\text{NRA}(+\text{NO}_3)$ in *P. tremuloides* was constantly low throughout the study period, and consequently, no clear relationship was observed in $\text{NRA}(+\text{NO}_3)$ with leaf area or growth rate. $\text{NRA}(+\text{NO}_3)$ of *B. neoalaskana* showed a positive correlation with leaf area but not with leaf growth rate, reflecting a peak $\text{NRA}(+\text{NO}_3)$ in the later stage of leaf expansion (Table 2; Figs. 1, 2g–i). Moreover, $\text{NRA}(+\text{NO}_3)$ per unit leaf in *B. neoalaskana* did not decrease even after the leaves fully expanded, and the summer reference showed a significantly higher $\text{NRA}(+\text{NO}_3)$ than *A. crispa* in summer (Figs. S1d–f, Table S6). On the other hand, $\text{NRA}(+\text{NO}_3)$ per unit leaf of *A. crispa* decreased after the peak in the middle of the growth stage, and the summer reference exhibited a very low $\text{NRA}(+\text{NO}_3)$. The

results suggested that the momentary NRA was not an adequate index to describe species characteristics regarding plant nitrate use.

Ecological implication—the influence of changing bud break timing

The results of this study revealed that the three boreal deciduous tree species had the capacity to assimilate nitrate as a source of N, and indeed assimilated nitrate during their leaf expansion period. However, questions remain regarding the uptake of nitrate from the soil, because, unlike ammonium, nitrate can be stored in plant tissues after uptake. Nitrate assimilation is a highly energy-consuming process, which consumes as much as 25% of the energy from photosynthesis (Solomonson and Barber 1990). In addition, the nitrite reduction process that occurs in chloroplast immediately after nitrate reduction requires reducing power through photosynthetically reduced ferredoxin, which is a part of the mechanism for light requirement in nitrate assimilation processes (Lillo 2008). Thus, leaves that assimilated nitrate during the period with high growth rate must invest the energy for nitrate assimilation and not only their growth; i.e., current carbon acquisition. Earlier studies have revealed that the rate of light-saturated net photosynthesis per unit leaf area reached its maximum at or slightly before full leaf area expansion, and leaves received carbon as a sink before net photosynthesis reached its maximum (Sesták et al. 1985). Considering that leaves are unlikely to have surplus carbon storage especially in the early leaf growth period, it must be cost effective for plants to have absorbed and incorporated N into organic N compounds consuming available energy in advance and translocate the organic N compounds to newly developing leaves. Nevertheless, the results of this study and that of Koyama et al. (2008) indicated that the leaves assimilated inorganic nitrate consuming energy during leaf growth, which suggested nitrate was proximately absorbed before assimilation.

The relationship between leaf development stage and leaf nitrate assimilation implies that leaf N acquisition is influenced by climate change in several ways. Global warming could directly influence soil N availability by changing snow cover and the soil freeze–thaw cycle (Templer 2012) and, accordingly, plant N use. Moreover, the spring leaf expansion period will be advanced by warmer temperatures, causing a mismatch between the leaf expansion period and solar energy status (Makoto et al. 2014). As stated above, nitrate assimilation is a highly energy consuming process, and the energy is directly provided by photosynthesis (Solomonson and Barber 1990; Yanagisawa 2014). This suggests the possibility of energy deficiency for nitrate assimilation during leaf expansion in the case, where leaves begin to grow earlier in lower light conditions and shorter daylight length,

especially in boreal forests in high latitudes. Linkosalo et al. (2009) evaluated the bud burst of some boreal forest species, and found them advanced by 7.6–8.0 days per century, corresponding with a temperature increase of 1.5 °C in the same period. The meta-analysis by Menzel et al. (2006) revealed that leaf unfolding in Europe was advanced by 2.5 days per decade on average over the last three decades. It was observed that a single but significantly extreme warm event during winter could advance the phenology of nearly half the tree and shrub species in 101 observed temperate species (Ladwig et al. 2019). The ecological implication of the shift in plant phenology caused by warming was discussed with respect to spring frost risk (Hanninen 2006; Linkosalo et al. 2000) and synchrony between pollinator emergence and flowering (Kudo et al. 2008; Kudo and Cooper 2019). However, we are unaware of any studies that have examined the effects of early onset of leaf expansion on leaf nutrition, especially from the viewpoint of energy sources. A limited number of previous studies have focused on temporal changes in physiological activities related to nutrient acquisition during leaf ontogeny. What is now required is an integrated discussion on the effects of warming on plant phenology, seasonal change in soil nutrient availability, and energy availability—with a focus on the leaf expansion period.

Author contribution statement LAK and KK planned and designed the research. LAK mainly conducted fieldwork, laboratory assay, and data analysis with help of KK. LAK and KK interpreted data, and LAK wrote the manuscript in consultation with KK.

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Availability of data and material (data transparency) Data will be made available in the Bonanza Creek LTER database.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts or competing interests associated with this manuscript.

References

- Andrews M (1986) The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant Cell Environ* 9:511–519. <https://doi.org/10.1111/1365-3040.ep11616228>
- Chapin FS, Van Cleve K, Tryon P (1986) Relationship of ion absorption to growth rate in taiga trees. *Oecologia* 69:238–242. <https://doi.org/10.1007/BF00377628>
- Craine JM, Brookshire ENJ, Cramer MD, Hasselquist NJ, Koba K, Marin-Spiotta E, Wang L (2015) Ecological interpretations of nitrogen isotope ratios of terrestrial plants and soils. *Plant Soil* 396:1–26. <https://doi.org/10.1007/s11104-015-2542-1>
- Deng X, Weinbaum SA, DeJong TM, Muraoka TT (1989) Utilization of nitrogen from storage and current-year uptake in walnut spurs during the spring flush of growth. *Physiol Plant* 75:492–498
- Feigenbaum S, Bielorai H, Erner Y, Dasberg S (1987) The fate of ^{15}N labeled nitrogen applied to mature citrus trees. *Plant Soil* 97:179–187
- Frak E, Millard P, Le Roux X, Guillaumie S, Wendler R (2002) Coupling sap flow velocity and amino acid concentrations as an alternative method to ^{15}N labeling for quantifying nitrogen remobilization by walnut trees. *Plant Physiol* 130:1043–1053. <https://doi.org/10.1104/pp.002139>
- Gebauer G, Rehder H, Wollenweber B (1988) Nitrate, nitrate reduction and organic nitrogen in plants from different ecological and taxonomic groups of Central Europe. *Oecologia* 75:371–385. <https://doi.org/10.1007/BF00376940>
- Gebauer G, Hahn G, Rodenkirchen H, Zuleger M (1998) Effects of acid irrigation and liming on nitrate reduction and nitrate content of *Picea abies* (L.) Karst. and *Oxalis acetosella* L. *Plant Soil* 199:59–70. <https://doi.org/10.1023/A:1004263223917>
- Grassi G, Millard P, Wendler R, Minotta G, Tagliavini M (2002) Measurement of xylem sap amino acid concentrations in conjunction with whole tree transpiration estimates spring N remobilization by cherry (*Prunus avium* L.) trees. *Plant Cell Environ* 25:1689–1699. <https://doi.org/10.1046/j.1365-3040.2002.00949.x>
- Guak S, Neilsen D, Millard P, Wendler R, Neilsen GH (2003) Determining the role of N remobilization for growth of apple (*Malus domestica* Borkh) trees by measuring xylem-sap N flux. *J Exp Bot* 54:2121–2131. <https://doi.org/10.1093/jxb/erg228>
- Hänninen H (2006) Climate warming and the risk of frost damage to boreal forest trees: identification of critical ecophysiological traits. *Tree Physiol* 26:889–898. <https://doi.org/10.1093/treephys/26.7.889>
- Höglberg P, Granström A, Johansson T, Lundmark-Thelin A, Näsholm T (1986) Plant nitrate reductase activity as an indicator of availability of nitrate in forest soils. *Can J for Res* 16:1165–1169. <https://doi.org/10.1139/x86-207>
- Jaworski E (1971) Nitrate reductase assay in intact plant tissues. *Biochem Biophys Res Commun* 43:1274–1279. [https://doi.org/10.1016/S0006-291X\(71\)80010-4](https://doi.org/10.1016/S0006-291X(71)80010-4)
- Keeney DR, Nelson DW (1982) Nitrogen—inorganic forms. In: Page AL, Miller RH, Keeney DR (eds) *Methods of soil analysis*. Part 2. ASA and SSSA, Madison, pp 643–698. <https://doi.org/10.2134/agronmonogr9.2.2ed.c33>
- Kielland K, Barnet B, Schell D (1998) Intraseasonal variation in the $\delta^{15}\text{N}$ signature of taiga trees and shrubs. *Can J for Res* 28:485–488. <https://doi.org/10.1139/x98-007>
- Kielland K, Ruess RW, Olson K, Boone RD (2006) Contribution of winter processes to soil nitrogen flux in taiga forest ecosystems. *Biogeochemistry* 81:340–360. <https://doi.org/10.1007/s10533-006-9045-3>
- Koba K, Hirobe M, Koyama L, Kohzu A, Tokuchi N, Nadelhoffer K, Wada E, Takeda H (2003) Natural ^{15}N abundance of plants and soil N in a temperate coniferous forest. *Ecosystems* 6:457–469. <https://doi.org/10.1007/s10021-002-0132-6>
- Koyama L, Kielland K (2011) Plant physiological responses to hydrologically mediated changes in nitrogen supply on a boreal forest floodplain: a mechanism explaining the discrepancy in nitrogen demand and supply. *Plant Soil* 342:129–139. <https://doi.org/10.1007/s11104-010-0676-8>
- Koyama LA, Kielland K (2018) Black spruce assimilates nitrate in boreal winter. *Tree Physiol* 39:536–543. <https://doi.org/10.1093/treephys/tpy109>
- Koyama L, Tokuchi N (2003) Effects of NO_3^- availability on NO_3^- use in seedlings of three woody shrub species. *Tree Physiol* 23:281–288. <https://doi.org/10.1093/treephys/23.4.281>
- Koyama L, Tokuchi N, Fukushima K, Terai M, Yamamoto Y (2008) Seasonal changes in nitrate use by three woody species: the importance of the leaf-expansion period. *Trees* 22:851–859. <https://doi.org/10.1007/s00468-008-0246-3>
- Koyama L, Hirobe M, Koba K, Tokuchi N (2013) Nitrate-use traits of understory plants as potential regulators of vegetation distribution on a slope in a Japanese cedar plantation. *Plant Soil* 362:119–134. <https://doi.org/10.1007/s11104-012-1257-9>
- Koyama LA, Terai M, Tokuchi N (2020) Nitrate reductase activities in plants from different ecological and taxonomic groups grown in Japan. *Ecol Res* 35:708–712. <https://doi.org/10.1111/1440-1703.12083>
- Kudo G, Cooper EJ (2019) When spring ephemerals fail to meet pollinators: mechanism of phenological mismatch and its impact on plant reproduction. *Proc Biol Sci* 286:20190573. <https://doi.org/10.1098/rspb.2019.0573>
- Kudo G, Ida TY, Tani T (2008) Linkages between phenology, pollination, photosynthesis, and reproduction in deciduous forest understory plants. *Ecology* 89:321–331. <https://doi.org/10.1890/06-2131.1>
- Ladwig LM, Chandler JL, Guiden PW, Henn JJ (2019) Extreme winter warm event causes exceptionally early bud break for many woody species. *Ecosphere* 10:e02542
- Lillo C (2008) Signalling cascades integrating light-enhanced nitrate metabolism. *Biochem J* 415:11–19. <https://doi.org/10.1042/BJ20081115>
- Linkosalo T, Carter TR, Hakkinen R, Hari P (2000) Predicting spring phenology and frost damage risk of *Betula* spp. under climatic warming: a comparison of two models. *Tree Physiol* 20:1175–1182. <https://doi.org/10.1093/treephys/20.17.1175>
- Linkosalo T, Häkkinen R, Terhivuo J, Tuomenvirta H, Hari P (2009) The time series of flowering and leaf bud burst of boreal trees (1846–2005) support the direct temperature observations of climatic warming. *Agric for Meteorol* 149:453–461. <https://doi.org/10.1016/j.agrformet.2008.09.006>
- Liu XY, Koba K, Koyama LA, Hobbie SE, Weiss MS, Inagaki Y, Shaver GR, Giblin AE, Hobara S, Nadelhoffer KJ, Sommerkorn M, Rastetter EB, Kling GW, Laundre JA, Yano Y, Makabe A, Yano M, Liu CQ (2018) Nitrate is an important nitrogen source for Arctic tundra plants. *Proc Natl Acad Sci USA* 115:3398–3403. <https://doi.org/10.1073/pnas.1715382115>
- Makoto K, Kajimoto T, Koyama L, Kudo G, Shibata H, Yanai Y, Cornelissen JHC (2014) Winter climate change in plant–soil systems: summary of recent findings and future perspectives. *Ecol Res* 29:593–606. <https://doi.org/10.1007/s11284-013-1115-0>
- Menzel A, Sparks TH, Estrella N, Koch E, Aasa A, Ahas R, Alm-Kübler K, Bissolli P, Braslavská OG, Briede A, Chmielewski FM, Crepinsek Z, Curnel Y, Dahl Å, Defila C, Donnelly A, Filella Y, Jatzcak K, Måge F, Mestre A, Nordli Ø, Peñuelas J, Pirinen P, Remišová V, Scheffinger H, Striz M, Susnik A, Van Vliet AJH, Wielgolaski F-E, Zach S, Züst ANA (2006) European phenological response to climate change matches the warming pattern. *Glob*

- Change Biol 12:1969–1976. <https://doi.org/10.1111/j.1365-2486.2006.01193.x>
- Millard P (1994) Measurement of the remobilization of nitrogen for spring leaf growth of trees under field conditions. *Tree Physiol* 14:1049–1054. <https://doi.org/10.1093/treephys/14.7-8-9.1049>
- Millard P, Grelet G-A (2010) Nitrogen storage and remobilization by trees: ecophysiological relevance in a changing world. *Tree Physiol* 30:1083–1095. <https://doi.org/10.1093/treephys/tpq042>
- Millard P, Wendler R, Hepburn A, Smith A (1998) Variations in the amino acid composition of xylem sap of *Betula pendula* Roth. trees due to remobilization of stored N in the spring. *Plant Cell Environ* 21:715–722. <https://doi.org/10.1046/j.1365-3040.1998.00313.x>
- Neilsen D, Millard P, Neilsen GH, Hogue EJ (1997) Sources of N for leaf growth in a high-density apple (*Malus domestica*) orchard irrigated with ammonium nitrate solution. *Tree Physiol* 17:733–739
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* 9:671–675. <https://doi.org/10.1038/nmeth.2089>
- Šesták Z, Tichá I, Čatský F, Solárová J, Pospíšilová J, Hodáňová D (1985) Integration of photosynthetic characteristics during leaf development. In: Šesták Z (ed) *Photosynthesis during leaf development*. Springer Science & Business Media, Berlin, pp 263–286. https://doi.org/10.1007/978-94-009-5530-1_11
- Smirnoff N, Todd P, Stewart G (1984) The occurrence of nitrate reduction in the leaves of woody plants. *Ann Bot* 54:363–374. <https://doi.org/10.1093/oxfordjournals.aob.a086806>
- Solomonson L, Barber M (1990) Assimilatory nitrate reductase: functional properties and regulation. *Annu Rev Plant Physiol Plant Mol Biol* 41:225–253. <https://doi.org/10.1146/annurev.pp.41.060190.001301>
- Templer PH (2012) Changes in winter climate: soil frost, root injury, and fungal communities. *Plant Soil* 353:15–17. <https://doi.org/10.1007/s11104-011-1064-8>
- Thomas F, Hilker C (2000) Nitrate reduction in leaves and roots of young pedunculate oaks (*Quercus robur*) growing on different nitrate concentrations. *Environ Exp Bot* 43:19–32. [https://doi.org/10.1016/S0098-8472\(99\)00040-4](https://doi.org/10.1016/S0098-8472(99)00040-4)
- Valentine DW, Kielland K, Chapin FS III, McGuire AD, Van Cleve K (2006) Patterns of biogeochemistry in alaskan boreal forests. In: Chapin FS III, Oswood MW, van Cleve K, Viereck LA, Verbyla DL (eds) *Alaska's changing boreal forest*. Oxford University Press, Oxford, pp 241–266. <https://doi.org/10.1093/oso/9780195154313.003.0021>
- Yanagisawa S (2014) Transcription factors involved in controlling the expression of nitrate reductase genes in higher plants. *Plant Sci* 229:167–171. <https://doi.org/10.1016/j.plantsci.2014.09.006>
- Yarie J, Bonanza Creek LTER (1998) Soil physical and chemical properties based on genetic horizon from 4 replicate pits placed around the replicate LTER control plots sampled in 1988 and 1989 Bonanza Creek LTER—University of Alaska Fairbanks. BNZ:134, <http://www.lter.uaf.edu/data/data-detail/id/134>. <https://doi.org/10.6073/pasta/475a1825dfa264822ed53ca3574bb8e6>

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