Molybdenum threshold for ecosystem scale alternative vanadium nitrogenase activity in boreal forests

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Biological nitrogen fixation (BNF) by microorganisms associated with cryptogamic covers, such as cyanolichens and bryophytes, is a primary source of fixed nitrogen in pristine, high-latitude ecosystems. On land, low molybdenum (Mo) availability has been shown to limit BNF by the most common form of nitrogenase (Nase), which requires Mo in its active site. Vanadium (V) and iron-only Nases have been suggested as viable alternatives to countering Mo limitation of BNF; however, field data supporting this long-standing hypothesis have been lacking. Here, we elucidate the contribution of vanadium nitrogenase (V-Nase) to BNF by cyanolichens across a 600-km latitudinal transect in eastern boreal forests of North America. Widespread V-Nase activity was detected (~15–50% of total BNF rates), with most of the activity found in the northern part of the transect. We observed a 3-fold increase of V-Nase contribution during the 20-wk growing season. By including the contribution of V-Nase to BNF, estimates of new N input by cyanolichens increase by up to 30%. We find that variability in V-based BNF is strongly related to Mo availability, and we identify a Mo threshold of ~250 ng·g_{lichen}^{-1} for the onset of V-based BNF. Our results provide compelling ecosystem-scale evidence for the use of the V-Nase as a surrogate enzyme that contributes to BNF when Mo is limiting. Given widespread findings of terrestrial Mo limitation, including the carbon-rich circumboreal belt where global change is most rapid, additional consideration of V-based BNF is required in experimental and modeling studies of terrestrial biogeochemistry.

alternative nitrogenases | biological nitrogen fixation | boreal forest | molybdenum limitation | *Peltigera* cyanolichens

Circumboreal forests cover ~20% of exposed land, host ~15% of terrestrial forest biomass, and store a substantial amount of carbon (C) in their soils (1, 2). The availability of nitrogen (N) will strongly constrain the response of this biome to human-driven increases in atmospheric CO_2 during the next 100 y (3, 4). Our ability to accurately predict future carbon dynamics in boreal and arctic ecosystem is thus intimately linked to our understanding of N input and cycling (5, 6).

In boreal and arctic ecosystems, most new N is supplied through biological nitrogen fixation (BNF), the process by which atmospheric dinitrogen (N_2) is reduced into bioavailable ammonia by the prokaryotic metalloenzyme nitrogenase (Nase) (7). In the face of the low abundance of symbiotic N_2 fixers associated with plant root nodules at high latitudes (8), BNF in northern ecosystems is carried out by a broad diversity of microorganisms, which live either asymbiotically on various substrates (e.g., in soil, leaf litter, and canopies), or symbiotically within autotrophic ground-associated and epiphytic communities, commonly referred to as cryptogamic covers (e.g., biocrusts, cyanolichens, bryophytes). Cryptogamic covers may account for nearly half of terrestrial BNF globally (~49 Tg N/yr) (9). Consistent with this estimate, BNF by moss-associated cyanobacteria have been reported to contribute up to 50% of N input to boreal forests (10) and arctic tundra (11), while cyanolichens (symbioses between fungi and cyanobacteria) can contribute over 85% of BNF in subarctic birch forest (12).

One important characteristic of BNF on land is that it relies on the scarcest micronutrient of the continental crust (13), molybdenum (Mo). Mo is present in the active site of the most common isoform of Nase, the Mo-Nase (7). While N_2 fixers associated with the root nodule of plant can profit from the foraging capacity of their host to acquire trace nutrients from deeper soil layers and even parent-rock materials (14), N_2 fixers associated with cryptogamic covers must meet their nutrient needs directly from their organic and mineral surroundings (e.g., soils, rocks), including atmospheric deposition, vegetation stemflow, and throughfall. Accordingly, Mo limitation of N_2 fixation in root nodules is rarely reported, and only under specific growing conditions (e.g., increased CO_2; ref. 15). In contrast, Mo limitation of N_2 fixation in soils, leaf litter, and cryptogams is a common feature of ecosystems ranging from the tropics to the Arctic (16).

Mo-independent, "alternative" vanadium (V), and iron (Fe)-only Nase isoforms (7) utilize the more abundant crustal-sourced trace metals V and Fe in place of Mo in the active site. These Nases are present in the genome of diverse N_2 fixers, always co-occurrent with

Significance

Biological nitrogen fixation (BNF) is responsible for large new N inputs to terrestrial ecosystems, particularly in pristine, high-latitude areas undergoing rapid global change. The most common form of nitrogenase requires molybdenum (Mo) and Mo limitation of BNF is ubiquitous. Mo-free alternative forms of nitrogenase exist, but their roles in environmental BNF have remained uncertain. This study on N_2-fixing cyanolichens provides extensive field evidence, at an ecosystem scale, that vanadium (V)-based nitrogenase greatly contributes to BNF when Mo availability is limited. Mo exposure data in circumboreal forests further suggest that V-based BNF is widespread. The results showcase the resilience of BNF to micronutrient limitation and reveal strong links between the biogeochemical cycle of macronutrients and micronutrients in terrestrial ecosystems.

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The authors declare no competing interest.

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Data deposition: *Nif*K, *vnf*DG, and *vnf*N sequences were deposited to GenBank, https://www.ncbi.nlm.nih.gov/genbank (accession nos. MN562797–MN562856).

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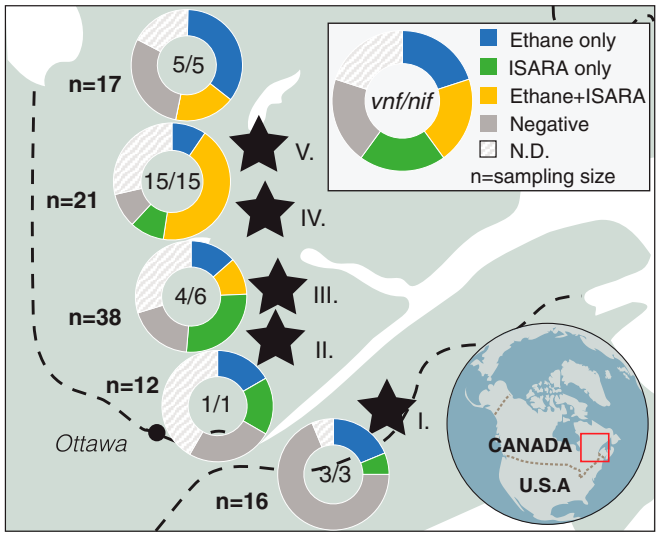

Fig. 1. Presence and activity of V-Nase in boreal cyanolichens along a 600-km transect in eastern Canada. The black stars show the approximate location of the 5 sampling sites (n = sample size per site). Ringed pie-charts summarize the number of samples showing positive ethane (blue), ISARA (green), or both ISARA and ethane signals (yellow), the number of samples showing no V-Nase activity (dark gray), and the number of samples below the detection limit of either method (N.D., light gray). Numbers within the pie charts indicate the number of individual thalli in which vanadium nitrogenase genes were detected over the total number of samples tested. Molybdenum nitrogenase genes were detected for all 30 samples tested.

the Mo-Nase (17). Several reports from pure culture studies demonstrating the onset of alternative Nase activity upon Mo depletion from the medium (18–20) have prompted the long-standing hypothesis that an environmental role of "alternative" Nases is to sustain BNF under low Mo availability. Consistent with this idea, environmental surveys have revealed the widespread presence of alternative Nase genes in multiple environments subject to Mo limitation, from tropical to boreal ecosystems (17, 21, 22). However, this hypothesis is still lacking an environmental validation.

Here, we investigate the ecosystem scale contribution of alternative Nases to boreal BNF and its potential control by Mo availability. We use the ubiquitous cyanolichen genus *Peltigera* and its N_2-fixing *Nostoc* partner, which is particularly abundant in boreal and arctic ecosystems (23, 24). Prior investigations (25–27) support the use of the *Peltigera-Nostoc* as a model system for this study, as both Mo-Nase and V-Nase genes co-occur in these *Nostoc* genomes (22), V contents are regulated by Mo availability, and preliminary evaluations of V-Nase activity in a limited number of samples show that it can contribute up to 60% of cyanolichen BNF (26, 27). For this study, we examined V-Nase activity (27, 28) in multiple *Peltigera* species collected along a 600-km latitudinal transect following a gradient of atmospheric metal deposition in a northeastern American boreal forest (29). To evaluate the role of Mo availability in controlling alternative Nase usage, we measured the Mo content of cyanolichen thalli (i.e., the vegetative structure of lichens). Finally, we discuss the relevance of our findings for the biogeochemistry of N and micronutrients in terrestrial ecosystems.

Result and Discussion

Widespread Presence and Activity of Alternative V-Nase in *Peltigera* Cyanolichens. We detected the presence and activity of alternative V-Nases in a majority of the 104 colonies of *Peltigera* cyanolichen (Fig. 1 and Table 1) collected from 5 sites across a 600-km latitudinal transect within the boreal biome. Genes for V-Nase (*vnf*DG and *vnf*N) in the *Nostoc* cyanobacterial partner were identified in 28 of 30 cyanolichens representative of the diversity of our sampling (Table 1). Every *Peltigera* species typical of boreal forests (e.g., *P. aphthosa, P. malacea, P. neopolydactyla s.l., P. scabrosa*; Table 1) showed the presence of *vnf* genes. The 2 lichens samples for which we did not detect *vnf* genes are from *Peltigera* species found mainly in temperate regions (i.e., *P. leucophlebia, P. canina*; Table 1). Mo-Nase genes were detected in all tested samples. These results, consistent with earlier reports (22, 26, 27, 30), indicate that alternative V-Nase genes are a common feature of *Nostoc* found in *Peltigera* thalli at high latitudes.

To elucidate the contribution of alternative Nases to total cyanolichen BNF, we used 2 independent methods (27, 28) that make use of the acetylene reduction assay (31) to detect isoform-specific

Table 1. Identification and activity of vanadium nitrogenase (V-Nase) in *Peltigera* cyanolichens by species

		Samples with V-Nase genes*/samples tested	Samples with V-Nase activity/valid samples†		
Species	n	*vnf*DG/N	ISARA	Ethane	Both methods
P. aphthosa	15	5/5	3/3	6/7	3/3
P. canina s.l.	28	3/4	6/21	4/22	0/17
P. degenii	5	1/1	2/2	2/3	1/1
P. didactyla	2	1/1	1/2	0/2	0/2
P. horizontalis s.l.	6	1/1	2/3	1/4	1/3
P. leucophlebia	1	0/1	0/0	0/0	0/0
P. malacea	1	1/1	0/0	0/0	0/0
P. neopolydactyla 1	5	2/2	4/4	2/4	2/4
P. neopolydactyla 4	18	4/4	6/8	6/8	2/5
P. occidentalis	3	2/2	2/2	2/2	2/2
P. scabrosa	11	7/7	4/7	6/9	4/7
Undetermined species‡	9	N/A	1/3	5/8	1/2
% samples with V-Nase activity (positive/valid)‡		93% (28/30)	56% (31/55)	49% (34/69)	35% (16/46)

*All tested samples showed presence of Mo-Nase (*nif*K) genes.
†Samples exhibiting sufficient ethylene or ethane production to be assessed by ISARA (27) or ethane (28) proxies for alternative Nase activity.
‡Specimens for which a definitive determination was not possible due to insufficient material.

signatures. In the isotopic acetylene reduction assay (ISARA, ref. 27), the characteristically low ^{13}C fractionations of acetylene reduction to ethylene ($^{13}\varepsilon_{AR} = \delta^{13}C_{acetylene} - \delta^{13}C_{ethylene}$) by alternative Nase isoforms (e.g., V-Nase) compared to those by Mo-Nases ($^{13}\varepsilon_{Mo}$ = 13.2–14.7‰, $^{13}\varepsilon_{V}$ = 7.5–8.8‰; ref. 27) can be used with measurements of sample $^{13}\varepsilon_{AR}$ to calculate the contribution of each isoform to total BNF activity. In the ethane formation method (28), alternative Nase activity is detected based on the production of ethane as a side product during reduction of acetylene by the enzyme. The ratio of ethane to ethylene (negligible for Mo-Nase) in a given sample thus enables isoform-specific BNF activity to be estimated (ref. 28 and *SI Appendix*, Fig. S1). Both methods require samples, such as cyanolichens, with high levels of BNF activity. For the ∼75% fraction of cyanolichen samples with sufficient BNF activity (Fig. 1 and Table 1), alternative Nase activity was detected in 56% (31 of 55) of these thalli based on a low ISARA $^{13}\varepsilon_{AR}$ value (<13.2‰; ref. 32), and in 49% (34 of 69) based on a high ethane ethylene ratio (i.e., >0.1%; *SI Appendix*, Fig. S1). More samples showed alternative Nase activity in the 3 northern sites (sites III–V, Fig. 1), with over 50% of the samples demonstrating a positive signal with at least one of the methods. Since Fe-only Nase genes have not been found in any cyanobacteria genomes to date (17), we attribute all alternative Nase activity in our study to the V-Nase isoform.

Ecosystem-Scale Contribution of Alternative V-Nase Activity in *Peltigera* Cyanolichens. We investigated the spatial distribution of V-Nase activity in *Peltigera* lichens by analyzing samples collected during the early growing season (May, June) from multiple sites (sites I–V) spanning a 600-km latitudinal transect (Fig. 1). To elucidate temporal changes in V-Nase BNF, we also collected and analyzed samples at site IV throughout the growing season (late May to late September). Both the ISARA and ethane methods showed an increase in alternative Nase activity northwards along the transect and during the growing season at site IV (Fig. 2 *A*–*F*). The spatial pattern is shown by decreases in the median value of $^{13}\varepsilon_{AR}$ (Fig. 2*A*) from a Mo-Nase only signal at the southernmost site (site I, $^{13}\varepsilon_{AR}$ = 13.8‰) to values indicative of alternative Nase activity at higher latitude sites (sites II–V, $^{13}\varepsilon_{AR}$ < 13.2‰, the lower bound of $^{13}\varepsilon_{AR}$ for Mo-Nases; ref. 27). Based on ISARA, we estimate that 25–30% of N_2 reduction activity is due to V-Nase across sites II, III, IV, and V in May (*SI Appendix*, Table S1). Increases in V-Nase contributions to BNF at site IV (from ∼0 to 71% of N_2 reduction) during the growing season are shown by a steady decrease in median $^{13}\varepsilon_{AR}$ values (from ∼14.5‰ to 11.5‰, Fig. 2*B*). Higher ratios of ethane to ethylene northwards along the transect provide further evidence of alternative Nase activity. Qualitatively consistent with ISARA estimates, we find that ∼10–20% of BNF can be attributed to V-Nase in samples from northern sites III, IV, and V in May (Fig. 2*C* and *SI Appendix*, Table S1) as well as a clear contribution of V-Nase to BNF at site IV from June to September (26–47%, Fig. 2*D* and *SI Appendix*, Table S1). Because ISARA and ethane methods are subject to different biases (*SI Appendix*, *Methods*), we use the average of the 2 proxies as the best estimate of V-Nase BNF. We find that alternative V-Nase accounted for 20–35% of total BNF by *Peltigera* cyanolichens collected in the northern part of the transect (beyond 48°N) in the early growing season (May, June). Given the increase in V-Nase activity during the summer at site IV (Fig. 2 *E* and *F* and *SI Appendix*, Table S1), our results suggest that

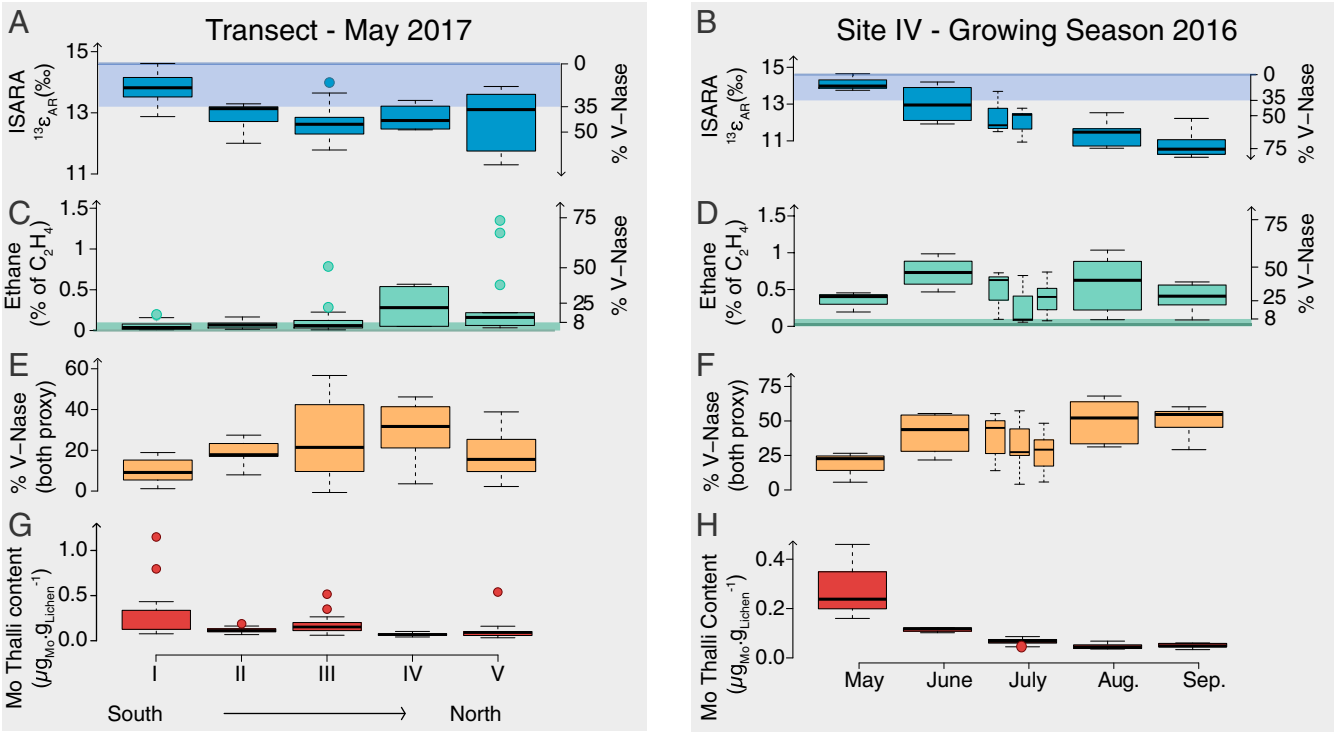

Fig. 2. Alternative V-Nase activity and Mo thalli content along the south–north transect described in Fig. 1 (*A*, *C*, *E*, and *G*) and growing season at site IV (*B*, *D*, *F*, and *H*). The boxplots show the ISARA carbon isotopic fractionations (*A* and *B*), ethane productions (*C* and *D*), and contributions of alternative V-Nase to the total BNF activity calculated as the average of both methods when available (*E* and *F*). Molybdenum contents of the thalli are shown for comparison (*G* and *H*). In *A*–*D*, the right axis represents the estimated contribution of V-Nase to BNF (note the direction of axis). Filled symbols represent values outside the whiskers, i.e., 1.5 interquartile range over the upper quartile or below the lower quartile. When filled symbols are absent, the whiskers represent the full range of the data. The horizontal colored boxes in *A*–*D* show the range of Mo-only signal for each proxy (ethane, <0.1% of C_2H_4; ISARA, $^{13}\varepsilon_{AR}$ > 13.2‰).

V-Nase contributions at northern sites have increased to 50% or more by the end of the growing season.

The measurement of significant and widespread contributions by alternative V-Nase to cyanolichen BNF confirms prior genomic (17, 22), isotopic (26, 27), R ratio (33), and micronutrient evidence (25, 26) indicating that alternative Nases are more important for environmental BNF than previously thought. Estimates of V-Nase contribution to BNF across all sites based on ISARA and ethane methods (individually or averaged) ranged from 0 to 74% (Fig. 2 and *SI Appendix*, Table S1). These results are consistent with previous estimates from individual cyanolichen thalli collected in southern Québec and northern Sweden (0–60%, refs. 26 and 27). Our findings that V-Nase contributions to BNF increase over the growing season are supported by recent literature suggesting Mo limitation is seasonal in cold temperate and boreal forests (34, 35).

Large contributions of V-Nase to BNF have important consequences for BNF rates assessed using acetylene reduction methods and, thus, for N budgets. The ratio of the acetylene reduction rate to N_2 fixation (N_2 reduction) rate is higher for Mo-Nase (R ratio = 3–4) than V-Nase (R ratio < 2; ref. 33). Our data indicate that the sole use of the acetylene reduction assay method to assess N_2 fixation rates, i.e., without considering alternative Nase activity, results in large underestimation of BNF rates (by up to ~30% during the late period of the growing season at site IV). This finding is particularly relevant to cold, N-limited high latitude ecosystems, such as boreal forest and arctic tundra, where BNF by cryptogamic covers sustains an important part of N input (11, 12).

Mo Threshold for Alternative V-Nase Contribution in the Boreal Forest. Because Mo levels have been identified as the primary factor controlling V-Nase activity in laboratory studies (18–20), we assessed the role of Mo availability as a driver of cyanolichen V-Nase activity. We found that the Mo content of *Peltigera* thalli varied across latitude and during the growing season (Fig. 2). Mo deposition on the forest floor, typically assessed with measurements of cryptogam elemental composition (36), was highest at site I (median content of Mo in *Peltigera* thalli = 300 $ng_{Mo} \cdot g_{thallus}^{-1}$) then decreased northwards to 65 and 90 $ng_{Mo} \cdot g_{thallus}^{-1}$ at site IV and V, respectively (Fig. 2*G*). These data indicate higher metal exposure in the southernmost portion of the boreal forest, in agreement with a previous large-scale study of metal deposition in northern Québec (29). Mo content also varied seasonally: At site IV, *Peltigera* thalli Mo levels (Fig. 2*H*) were found to be the highest in May, with a median value of 230 $ng_{Mo} \cdot g_{thallus}^{-1}$, followed by a steady decrease down to a baseline of 50–100 $ng_{Mo} \cdot g_{thallus}^{-1}$ from July to September.

As expected, average Mo contents of lichen thalli were inversely related to average V-Nase activities along the sampling transect and the growing season, suggesting that Mo drives spatial and temporal variation of V-Nase activity (Fig. 2 *E–H*). Sites with high median and large ranges in lichen Mo content (Fig. 2 *G* and *H*) exhibited the lowest level of V-Nase activity as measured by both ISARA and ethane methods (i.e., site I samples in May 2017, site IV samples in May 2016; Fig. 2 *A–F*). Conversely, site IV and V samples in 2017 exhibited low Mo and high V-Nase activity. Interestingly, substantial V-Nase activity (>15% of N_2 reduction activity) was only found at sites where the median Mo content was lower than ~200 $ng_{Mo} \cdot g_{thallus}^{-1}$ (Fig. 2), suggesting the existence of a Mo threshold for V-Nase activity.

Because both Mo thallus content and V-Nase contribution exhibited large intrasite variability (Fig. 2), we determined whether the relationship between Mo content and V-Nase BNF could be better explained at the level of individual thalli. We found a clear negative, nonlinear relationship (Fig. 3, log–log regression) between Mo content and V-Nase contribution to

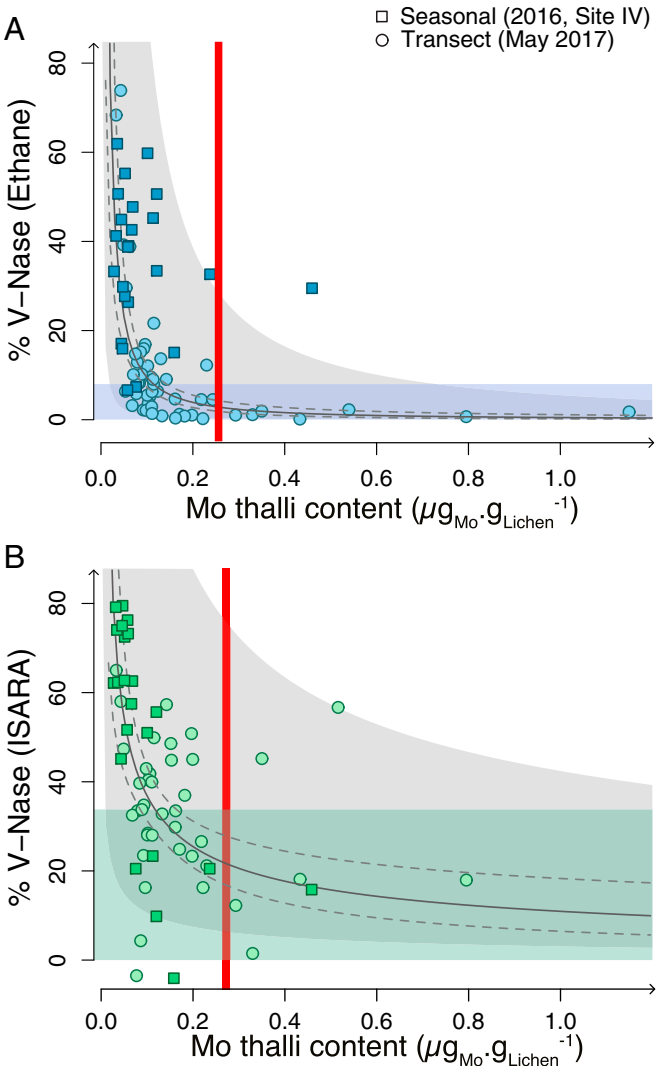

Fig. 3. Relationship between Mo content in *Peltigera* and contribution of V-Nase to BNF in individual thalli. Each point represents an individual thallus from either the 2017 (circle) and 2016 (square) field seasons. Vanadium nitrogenase contributions were separately estimated based on the ethane (*A*, $n = 58$ samples) and ISARA methods (*B*, $n = 72$ samples). Linear regression was performed on the log–log transformed data and results were back-transformed in the original space. Dashed lines show 95% confidence intervals and gray shadowed areas show 95% prediction intervals for this regression. Horizontal colored boxes derive from ethane and ISARA values for which V-Nase BNF contributions are uncertain (ethane, <8% V-Nase; ISARA, <34% V-Nase). Vertical red lines show the estimated Mo concentration threshold for the onset of V-Nase activity based on visual analysis ($Mo_{threshold}$ = 250 $ng_{Mo} \cdot g_{thallus}^{-1}$).

BNF as estimated using the ISARA (Fig. 3*A*; $R^2 = 0.26$, $P < 0.001$, $n = 58$) and ethane methods (Fig. 3*B*; $R^2 = 0.41$, $P < 0.001$, $n = 72$). The vast majority of lichen samples showing V-Nase activity contained less than ~250 $ng_{Mo} \cdot g_{thallus}^{-1}$ (see the vertical lines in Fig. 3), indicating the existence of a Mo threshold for V-Nase activity. Indeed, the highest V-Nase contributions to BNF (up to 80%) were identified in lichens with the lowest Mo content (30 $ng_{Mo} \cdot g_{thallus}^{-1}$). With a few exceptions, no substantial V-Nase contributions (i.e., within the margin of error as shown by horizontal colored boxes in Fig. 3) were found in lichens with high Mo content (400 $ng_{Mo} \cdot g_{thallus}^{-1}$).

These results provide compelling ecosystem-scale evidence for the use of alternative V-Nase as a supporting enzyme that sustains BNF under Mo-limited conditions.

Contribution of Alternative V-Nase to Terrestrial N Input. Interestingly, the 250 $ng_{Mo} \cdot g_{thallus}^{-1}$ threshold value reported here for the onset of alternative V-Nase activity in *Peltigera* thalli falls within the range of previous threshold estimates (200–300 $ng_{Mo} \cdot g_{leaf\ litter}^{-1}$) for Mo limitation of BNF in tropical leaf litter, as assessed with Mo amendment experiments (37). This striking agreement suggests a "global" threshold for Mo limitation of free-living BNF across organisms and biomes as well as similarities in the biogeochemical controls on Mo availability to these organisms across ecosystems.

The results imply that the Mo content of cyanolichens can be used to predict the contribution of V-Nase to BNF. Indeed, lichen Mo contents ($\leq$ 250 $ng_{Mo} \cdot g_{thallus}^{-1}$) correctly predicted V-Nase activity in 95% of the samples that showed V-Nase activity based on ISARA or ethane methods (n = 82; *SI Appendix*, Table S2). Our literature survey of Mo contents in *Peltigera* lichen thalli across the circumboreal belt (25, 26) confirms that most of the boreal area is exposed to relatively low levels of Mo through atmospheric deposition (Fig. 4). The average Mo concentration at sites within the Canadian–European boreal region (Alberta, northern Québec, and northern Sweden) falls below the threshold of 250 $ng_{Mo} \cdot g_{thallus}^{-1}$. Even in sites that show higher Mo content due to their proximity to anthropogenic activity (median value from 250 to 500 $ng_{Mo} \cdot g_{thallus}^{-1}$ in Russia, Alaska, southeastern Canada; Fig. 4), several individual thalli exhibited Mo levels below the threshold, indicating that V-Nase might still contribute to BNF in these areas (see histograms in Fig. 4). Indeed, we found the presence of V-Nase genes and evidence for V-Nase activity in *Peltigera* samples collected across the circumboreal belt (32, 37–39), including in human-impacted areas (32). These results imply that the reliance of boreal ecosystems on V-Nase N input would have been higher in preindustrial times when atmospheric deposition of metals and fixed N, which increase with anthropogenic activities (40, 41), were lower. Recent increases in anthropogenic pollutants enriched in Mo and/or V, such as tar sands (41, 42), could provide new fluxes of Mo and V to natural environments. Conversely, successful pollution control policies (e.g., the Clean Air Act in the United States), which have led to recent declines in atmospheric deposition of heavy metal pollutants and reactive N (43), may help to maintain Mo limitation of BNF in high latitude N-limited forests and increase the need for V-Nase BNF. Interestingly, previous studies on cyanolichens (25, 26) suggest that V might also be limiting BNF in areas with low levels of metal deposition, such as in northeastern Canada (29).

Our findings that V-Nase supplements cyanolichen BNF under Mo limitation in boreal forests may be an example of a general strategy of free-living and cryptogam associated N_2 fixers, which cannot easily benefit from the nutrient mining potential of tree roots like symbiotic nodule associated N_2 fixers (16, 34, 35). *Nostoc* cyanobacteria, the diazotrophic constituents of *Peltigera* cyanolichens, represent a globally distributed genus in terrestrial environments (e.g., biocrust, bryophytes, soils, freshwater; refs. 38 and 43–45). Genes for V-Nase have been found in the genome of *Nostoc* associated with cyanolichens and bryophytes from temperate to tropical areas (22, 46). In addition, taxonomically diverse V-Nase genes have also been identified in other substrata, including wood mulch, sediment from mangroves and salt marshes, and forest soils (17, 21, 33), suggesting that a variety of microorganisms (e.g., *Proteobacteria*, *Firmicutes*) rely on alternative BNF for N acquisition. Because Mo limitation of BNF is a common feature of terrestrial ecosystems (16), where cryptogamic covers are estimated to contribute half of terrestrial nitrogen fixation (9), our research calls for a broad reevaluation of the importance of alternative Nases to N cycles and budgets.

Conclusions

Using boreal *Peltigera* cyanolichens as a model to study the environmental role of V-Nase, we show regional-scale alternative

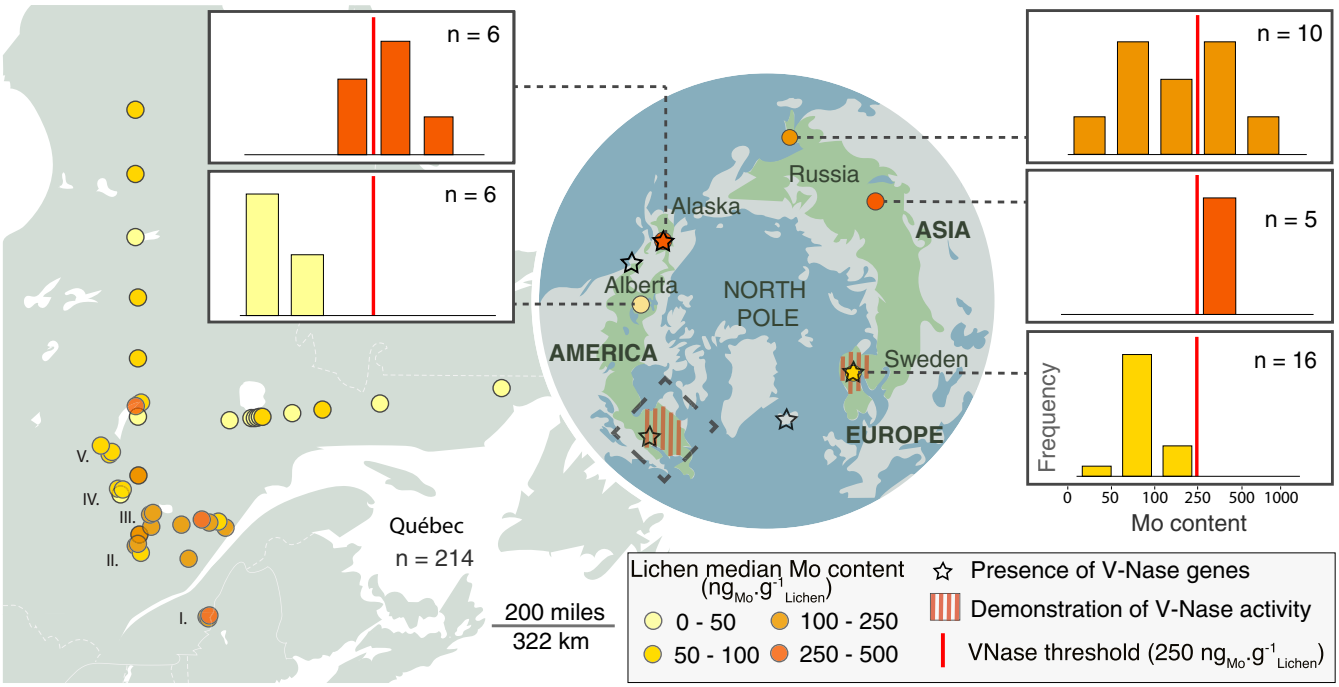

Fig. 4. Putative biome scale activity of vanadium nitrogenase in the circumboreal belt. Map of the circumboreal biome with the geographic position and Mo content of lichen thalli from available literature (25, 26, 29) and from this study. Light gray, close-up map (*Left*) of the circumboreal area outlined by dashed box shows the location and median Mo content of samples from northeastern North America (Québec). The histograms indicate the distribution of Mo content in lichen thalli at each location on the globe, with colors indicating median Mo content of thalli. The red line indicates the Mo content threshold for the onset of V-Nase activity.

biological nitrogen fixation activity in natural ecosystems. Contributions of V-Nase to cyanolichen BNF increased with latitude and during the growing season. V-Nase activity was strongly inversely correlated with the Mo content of *Peltigera* thalli, with a clear Mo threshold of 250 $ng_{Mo} \cdot g_{thallus}^{-1}$ for the onset of V-Nase BNF. This result validates the decades-old hypothesis that alternative Nases can help support environmental N input under Mo limited conditions, reveals an important biological role for vanadium in terrestrial ecosystems, and prompts additional research on alternative Nases in other Mo-limited natural environments.

Materials and Methods

Sample Collection. At the end of May 2017, 97 cyanolichen samples were collected at 5 locations along a 600-km latitudinal transect spanning southern Québec (site I at 46°09′42.1″N - 70°21′55.9″W to site V, 50°16′10.7″N - 74°14′11.1″W; Fig. 1). At each location, 3 sites within 50 m to 500 m of each other and showing reasonable abundance of *Peltigera* thalli were selected for sample collection. Additional sites 2 km to 20 km away from the primary sites were also visited, for a total of 27 distinct collection sites. At each site, we collected samples representing the biological variability of *Peltigera* cyanolichens within a 10-m radius. At least 6 different species of *Peltigera* were found at each site, with 1 large group of species (*P. canina s.l.*) found in all sites, and 4 other species found in 4 of the 5 sites. Lichens samples were air-dried in brown paper bags and stored for a maximum of 3 wk before further processing. In 2016, 7 distinct colonies (4 different species) of *Peltigera* lichens were collected in 1 site from location IV on 7 occasions during the growing season (late May–late September, 3 consecutive days in July), for a total of 47 samples. Data shown in Fig. 4 were collected during earlier studies (25, 26, 29).

Assessment of *nif* (Mo-Nase) and *vnf* (V-Nase) Genes Presence. *Peltigera* species were identified based on morphology (24, see *SI Appendix, Methods*). Presence of the *nif* genes (Mo-Nase) and the *vnf* genes (V-Nase) were established following Hodkinson et al. (ref. 22 and *SI Appendix, Methods* and Table S3). For samples for which *vnf*N and *vnf*DG amplifications were negative, we attempted amplifications after designing primers specifically targeting *vnf*N and *vnf*DG sequences from lichenized *Nostoc* (*SI Appendix*, Table S3). Samples reported with missing *vnf* (Fig. 1 and Table 1) had no PCR product amplification for any of the 6 primer combinations.

Total Nase Activity and Estimation of Alternative Nases Contribution. Rates of ethylene and ethane production were obtained using the acetylene reduction assay (31). Cyanolichens (0.35 ± 0.15 g) were rewetted overnight and incubated 24 h in a 23-mL glass vial, where 3 mL of the headspace (13% vol/vol) was replaced by pure acetylene made as described elsewhere (35). In 2016, incubations were conducted on-site (average daily temperature ranging from 8 to 26 °C) in a 250-mL glass vessel with 25 mL of acetylene (10% vol/vol). A gas aliquot of the headspace was collected after 24 h and analyzed by Isotope Ratio Mass Spectrometry (ISARA method, ref. 27) and gas chromatography (ethane method, ref. 35).

Data for ISARA are presented as the isotopic fractionation from acetylene to ethylene ($^{13}\varepsilon_{AR} = \delta^{13}C_{ethylene} - \delta^{13}C_{acetylene}$), and data for ethane are presented as the ratio of ethane to ethylene production rate. Conservative thresholds for a sample's signal to be considered different from pure Mo-Nase BNF have been taken as $^{13}\varepsilon_{AR} < 13.2‰$ for ISARA (27), and as an ethane to ethylene molar ratio > 0.1% for ethane production (*SI Appendix*, Fig. S1). Contributions of alternative Nases to acetylene reduction (AR) and nitrogen fixation (N_2) for both proxies were calculated according to Zhang et al. (27) using values of 14.6‰ and 7.9‰ (ISARA proxy) and 0.008 and 2.3% (ethane proxy) for 0 and 100% V-Nase activity (see also *SI Appendix, Methods*).

Elemental Analysis of Lichen Thallus. Concentrations of Mo and V in the lichen thalli were obtained according to Darnajoux et al. (29). Briefly, 150 ± 5 mg of lichen was cleaned, oven-dried (45 °C, 24 h), and mineralized with 10 mL of 67% HNO_3 (trace metal grade, Fisher) in Teflon vessels at 170 °C for 1 h using a microwave digesting system (Mars Xpress, CEM). Samples were then measured by ICP-MS (ThermoFisher XSeries II) (29).

Statistical Analysis. All statistics were performed using R v3.5.2 and R studio v1.1.463 software (39), using packages "stats" and "graphics." Linear regressions were performed using the lm function. Regressions between V-Nase contributions to BNF and Mo thallus content were performed on the log–log transformed data to achieve normality of residuals and presented in their original space for easier interpretation (47). All figures were edited using Adobe Illustrator v18.1 (2014). Additional statistical calculations can be found in *SI Appendix*.

Data Availability. All of the data used in this article can be found in Dataset S1. *Nif*K, *vnf*DG, and *vnf*N sequences were deposited to GenBank, https://www.ncbi.nlm.nih.gov/genbank (accession nos. MN562797–MN562856).

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