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

Romain Darnajoux1, Nicolas Magainb, Marie Renaudinc, François Lutzonib, Jean-Philippe Bellengerc, and Xinning Zhange,d

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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·glichen−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.

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The authors declare no competing interest.

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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 N2-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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1To whom correspondence may be addressed. Email: romaind@princeton.edu.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1913314116/-/DCSupplemental.
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**

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

*All tested samples showed presence of Mo-Nase (nifK) 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.

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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.

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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.
signatures. In the isotopic acetylene reduction assay (ISARA, ref. 27), the characteristically low $^{13}$C fractionations of acetylene reduction to ethylene ($^{13}e_{AV} = \delta^{13}C_{\text{acetylene}} - \delta^{13}C_{\text{ethylene}}$) by alternative Nase isoforms (e.g., V-Nase) compared to those by Mo-Nases ($^{13}e_{AV} = 13.2\text{--}14.7\%e$; ref. 27) can be used with measurements of sample $^{13}e_{AR}^M$ 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, from which specific BNF activity can be estimated (ref. 28 and SI Appendix, Table S1). Because 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 $^{13}e_{AR}^M$ (Fig. 2 A) from a Mo-Nase only signal at the southernmost site (site I) to $^{13}e_{AR}^M < 13.2\%e$, the lower bound of $^{13}e_{AR}^M$ 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 $\sim$0 to 71% of N$_2$ reduction) during the growing season are shown by a steady decrease in mean $^{13}e_{AR}^M$ values (from $\sim$14.5\%e to 11.5\%e, Fig. 2B). 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 $\sim$10-20% of BNF can be attributed to V-Nase in samples from northern sites III, IV, and V in May (Fig. 2C 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. 2D 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

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 (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 $^{13}e_{AR}^M$ (Fig. 2 A) from a Mo-Nase only signal at the southernmost site (site I) to $^{13}e_{AR}^M < 13.2\%e$, the lower bound of $^{13}e_{AR}^M$ 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 $\sim$0 to 71% of N$_2$ reduction) during the growing season are shown by a steady decrease in mean $^{13}e_{AR}^M$ values (from $\sim$14.5\%e to 11.5\%e, Fig. 2B). 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 $\sim$10-20% of BNF can be attributed to V-Nase in samples from northern sites III, IV, and V in May (Fig. 2C 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. 2D 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
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 Quebec 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 this 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 N2 fixation (N2 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 N2 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 ngMo·gthallus−1) then decreased northwards to 65 and 30 ngMo·gthallus−1 at site IV and V, respectively (Fig. 2G). 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 Quebec (29). Mo content also varied seasonally: At site IV, Peltigera thalli Mo levels (Fig. 2H) were found to be the highest in May, with a median value of 230 ngMo·gthallus−1, followed by a steady decrease down to a baseline of 50–100 ngMo·gthallus−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 N2 reduction activity) was only found at sites where the median Mo content was lower than ∼200 ngMo·gthallus−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 BNF as estimated using the ISARA (Fig. 3A; R2 = 0.26, P < 0.001, n = 58) and ethane methods (Fig. 3B; R2 = 0.41, P < 0.001, n = 72). The vast majority of lichen samples showing V-Nase activity contained less than ∼250 ngMo·gthallus−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 ngMo·gthallus−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 ngMo·gthallus−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.
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 (≤250 ngMo g\textit{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). Our literature survey of Mo contents in \textit{Peltigera} thalli across the circumboreal belt 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 ngMo g\textit{thallus}⁻¹. Even in sites that show higher Mo content due to their proximity to anthropogenic activity (median value from 250 to 500 ngMo g\textit{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 \textit{Peltigera} samples collected across the circumboreal belt, 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.

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 N₂ fixers, which cannot easily benefit from the nutrient mining potential of tree roots like symbiotic nodule associated N₂ fixers (16, 34, 35). \textit{Nostoc} cyanobacteria, the diazotrophic constituents of \textit{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 \textit{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., \textit{Proteobacteria}, \textit{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 \textit{Peltigera} cyanolichens as a model to study the environmental role of V-Nase, we show regional-scale alternative...
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 g\(^{-1}\) thallus 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 Quebec (site I at 46°09′7″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 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 1 or 2 P. fruticosa found in 4 of the 27 sites. Lichens sampled 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 Hodgkinson et al. (ref. 22 and **SI Appendix, Methods and Table S3**). For samples for which vnfN and vnfDG amplifications were negative, we attempted amplifications after designing primers specifically targeting vnfN and vnfDG sequences from lichenized Nostoc (**SI Appendix, Table S3**). Samples reported with missing vnfN (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 in 2.0 mL of glass vial, where 3 mL of the headspace (10% vol/vol) was replaced by pure acetylene made as described elsewhere (35). In 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).


