Supplementary information to

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

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Supplementary Methods:

Peltigera species identifications

To avoid potential misidentifications, members of section *Peltigera* (sensu Miadlikowska and Lutzoni 2000, 1) forming large tomentose thalli (*Peltigera canina, P. evansiana, P. praetextata, P. rufescens and P. "neocanina"*) were considered as *P. canina s.l.*, whereas members of section *Horizontales* (*P. elisabethae, P. horizontalis,* and *P. neckeri*) were considered as *P. horizontalis s.l.*

Nitrogenases genes presence determination:

The presence of the *nif* genes for molybdenum nitrogenase was determined by sequencing the *nif*K (β-subunit of the molybdenum–iron protein, dinitrogenase 1) locus using primers nifK_F and nifK_R (SI Appendix Table S3). Presence of the *vnf* genes for vanadium nitrogenase was verified by sequencing the *vnf*N (coding for the scaffolding protein of the FeVco) locus using primers vnfN2F and vnfN5R and two loci within *vnf*DG (coding for the vanadium–iron protein, dinitrogenase 2) using primers vnfDG1F and vnfDG4R and vnfDG4F and vnfDG9R (39, SI Appendix Table S3). For samples for which *vnf*N and *vnf*DG amplifications were negative, we attempted new amplifications after designing the following new primers targeting specifically *vnf*N and *vnf*DG sequences from lichenized *Nostoc* (Table S3).

PCR conditions for *vnf* and *nif* primers were: 94 °C for 30 s, 55 °C for 30 s (−0.4°/cycle), 72 °C for 1 min (+2 s/cycle) for 24 cycles; 94 °C for 30 s, 45 °C for 30 s, 72 °C for 2 min (+3 s/cycle) for 12 cycles; 72 °C for 10 min, followed by storage at 4 °C.

NifK, vnfDG, and vnfN sequences were deposited to Genbank under accession numbers MN562797-MN562856. Seven sequences that show clear signs of cross-contamination during the preparation step were not deposited in the database.

Contribution of alternative nitrogenase to acetylene reduction (f alt ara) and to N2 fixation (f alt N2).

Contributions of alternative V-Nase to acetylene reduction assessed with the ISARA method were calculated according to Zhang *et al*., 2016 (2):

$$\mathrm{f}_{\text{alt ISARA}} = \frac{\left({}^{13}\varepsilon_{\text{Mo}} - {}^{13}\varepsilon_{\text{AR,sample}}\right)}{\left({}^{13}\varepsilon_{\text{Mo}} - {}^{13}\varepsilon_{\text{alt}}\right)}$$

Contributions of V-Nase to acetylene reduction assessed using the ethane method (Dilworth et al., 1987) were calculated using the following equation:

$$\mathrm{f}_{\text{alt Ethane}} = \frac{\left(\text{Ethane}_{\text{Mo}} - \text{Ethane}_{\text{sample}}\right)}{\left(\text{Ethane}_{\text{Mo}} - \text{Ethane}_{\text{V}}\right)}$$

The V-nase contribution to total N_2 fixation rate was evaluated using V-nase contributions to AR and conservative values of the conversion factors ($R_V = 2$ and $R_{Mo} = 4$) to convert acetylene reduction rate to N_2 reduction rate (3).

$$\mathrm{f}_{\text{alt }N_2} = \frac{(\mathrm{f}_{\text{alt}}/2)}{\left(\frac{\mathrm{f}_{\text{alt}}}{2}\right) + \left(\frac{1-\mathrm{f}_{\text{alt}}}{4}\right)}$$

A Rayleigh correction was applied to all ${}^{13}\varepsilon_{\text{sample}}$ according to Hayes 2004 (4). A maximum of 5% conversion of acetylene to ethylene was found during ARA, and corrections were < 0.4‰. Isozyme specific ${}^{13}\varepsilon$ and ethane production rate for Mo and V were determined in deletion mutants CA 1.70 (Mo-Nase only) and CA 11.70 (V-Nase only) of *Azotobacter vinelandi*. Various levels of activity for alternative vanadium nitrogenase were obtained by mixing known volumes of two separates Mo-Nase and V-Nase strain cultures in various proportions (Figure S1).

Carbon isotopic analyses of ethylene and acetylene for ISARA analysis were obtained using a Thermo Scientific GC Isolink system containing a Trace GC Ultra interfaced to a Thermo Delta V Advantage Isotope Ratio Mass Spectrometer with a Conflo IV as described in Zhang et al. (2016). Ethane production was measured using a Shimadzu GC-FID A8, as referred in the main text. Conservative values of $^{13}\varepsilon_{Mo} = 14.6 \pm 0.3‰$ and $^{13}\varepsilon_{V} = 7.9 \pm 0.2‰$ for ISARA, and $Ethane_{Mo}=0.008\%$ and $Ethane_{V}=2.3\%$ for the ethane production methods were used in further calculation (Figure S1).

Re-classification of samples according to the threshold of 250 $ng_{Mo}.g_{lichen}^{-1}$*:*

All samples were re-classified according to the following rules: lichen thalli with Mo content > 250 $ng_{Mo}.g_{lichen}^{-1}$ were classified as negative for alternative V-Nase, and lichen thalli with Mo content < 250 $ng_{Mo}.g_{lichen}^{-1}$ were classified as positive. Any sample showing at least one proxy (ISARA or Ethane) positive were considered true positive. Similarly, samples with none of the proxy showing positive value were considered true negative.

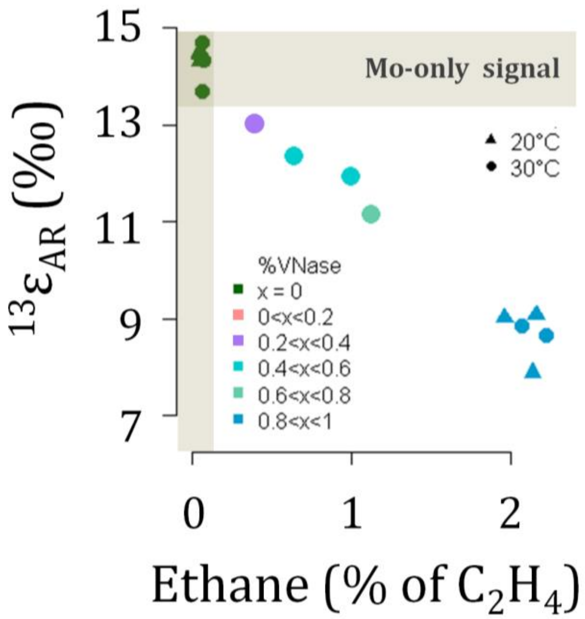

Figure S1. Cross-calibration of the ISARA and ethane production proxies of V-nase activity at 20°C (triangle) and 30°C (circle) using mutant strains of *Azotobacter vinelandii* CA1.70 (Mo-nitrogenase only) and CA11.70 (V-nitrogenase only).

Table S1. Summary of vanadium nitrogenase (V-Nase) contribution as percentages of total N_2 reduction (corrected for N_2 : acetylene reduction activity) along the transect and the growing season in boreal cyanolichens.

	Ethane : ethylene[a]	ISARA[1]	Mean[2]
Latitudinal transect (May 2017)	Estimate *(SD)*	Estimate *(SD)*	Estimate *(SE)*
I	5 *(5)*	17 *(13)*	10 *(2)*
II	5 *(4)*	37 *(10)*	21 *(1)*
III	8 *(12)*	40 *(13)*	22 *(2)*
IV	21 *(17)*	40 *(7)*	31 *(3)*
V	20 *(24)*	38 *(19)*	34 *(6)*
		Latitudinal transect	**21 *(1)***

Growing season (Site 4, 2016)	Estimate *(SD)*	Estimate *(SD)*	Estimate *(SE)*
May	26 *(9)*	11 *(13)*	18 *(6)*
June	47 *(11)*	35 *(22)*	41 *(8)*
July	34 *(17)*	47 *(23)*	40 *(10)*
August	38 *(22)*	63 *(10)*	50 *(6)*
September	28 *(13)*	71 *(10)*	50 *(4)*
		Growing season	**43 *(3)***

[1] *Estimates for each proxy were averaged per site or time of collection (standard deviation assesses the spread).*

[2] *Mean contribution is the average of both ISARA and ethane methods (standard errors assess uncertainty)*

Table S2. Decision matrix for alternative V-Nase activity using a Mo threshold of 250 $ng_{Mo}.g_{thallus}^{-1}$, showing the results of the re-classification for our characterized samples.

		Predicted[1]		
		YES	**NO**	**Total**
Actual[2]	**YES**	53	3	56
	NO	19	7	26
	Total	72	10	**82**

Precision	Good classification for YES	74%
Recall	Fraction of actual YES predicted YES	95%
Accuracy	Overall good classification	73%

[1] Samples are classified as positive when thallus content is inferior to the 250 $ng_{Mo}.g_{thallus}^{-1}$ threshold value.

[2] Samples are considered true positive if at least one of the proxy is positive for alternative V-Nase activity ($^{13}\varepsilon_{AR}$ <13.2‰ or Ethane ratio >0.1%).

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Table S3. List of primers used in this study.

Primer Name	Sequence	Reference
nifK_F	ACAGGTTCAGCACCCATTTC	(5)
nifK_R	AAGGGTACTGACGAGTTCTTGA	(5)
vnfN2F	AAAGATGTCAGTATTGT	(5)
vnfN5R	GGACTAAATACATCAAAA	(5)
vnfDG1F	TATTAAAGTGCGACGAAAC	(5)
vnfDG4R	ATACAGACTTTTTTGCC	(5)
vnfDG4F	GGCAAAAAAGTCTGTAT	(5)
vnfDG9R	AAACAMCGYTCTTGAAT	(5)
vnfDG1F_Pelt	TGTTTAAGTGCGACGAAWC	This study
vnfDG4F_Pelt	RBAARAARGTCTGTAT	This study
vnfDG4R_Pelt	ATACAGACYTTYTTVYC	This study
vnfDG9R_Pelt	AAACAVCGYTCTTGAAT	This study
vnfN2F_Pelt	AAAGATGTYAGTATTGT	This study
vnfN5R_Pelt	GGGCTAAATACATCAAAA	This study

SUPPLEMENTARY REFERENCES

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