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Biological nitrogen fixation by alternative nitrogenases in boreal cyanolichens: importance of molybdenum availability and implications for current biological nitrogen fixation estimates

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Summary

- Cryptogamic species and their associated cyanobacteria have attracted the attention of biogeochemists due to their critical roles in the nitrogen cycle through symbiotic and asymbiotic biological fixation of nitrogen (BNF). Biological nitrogen fixation is mediated by the nitrogenase enzyme, which, in its most common form, requires molybdenum at its active site. Molybdenum (Mo) has been reported as a limiting nutrient for BNF in many ecosystems, including tropical and temperate forests. Recent studies have suggested that alternative nitrogenases, which use vanadium or iron in place of molybdenum at their active site, might play a more prominent role in natural ecosystems than previously recognized.
- Here we studied the occurrence of vanadium, the role of molybdenum availability on vanadium acquisition, and alternative nitrogenases contribution to BNF in the ubiquitous cyanolichen *Peltigera aphthosa* s.l. **[Author, please confirm the text 's.l.' given after the species under investigation is correct here and elsewhere in the paper]**.
- We confirmed the use of the alternative vanadium-based nitrogenase in the *Nostoc* cyanobiont of these lichens and its substantial contribution to BNF in this organism. We also show that the acquisition of vanadium is strongly regulated by the abundance of molybdenum.
- These findings show that alternative nitrogenase can no longer be neglected in natural ecosystems, particularly in Mo-limited habitat.

Key words: alternative nitrogenases, biological nitrogen fixation, boreal forest, cyanolichens, molybdenum (Mo), *Peltigera aphthosa* s.l., vanadium (V).

Introduction

The boreal biome covers 17% of the terrestrial landscape and represent one of the highest carbon storage in terrestrial ecosystem (Dixon *et al.*, 1994; IPCC, 2000; Kasischke, 2000; DeLuca & Boisvenue, 2012). Over the course of the next century, boreal forests will undergo changes at an unprecedented rate due to global climate change (IPCC, 2013, 2014). Nitrogen (N) is the most frequently reported limiting nutrient for primary production in boreal forests (LeBauer & Treseder, 2008; Wang *et al.*, 2010). The availability of N to plants is expected to play a critical role in the response of boreal forests to global climate change (Heimann & Reichstein, 2008; Sigurdsson *et al.*, 2013; Yuan & Chen, 2015). Biological nitrogen fixation

(BNF) by cyanobacteria associated with bryophytes and lichen-forming fungi is a critical source of new N for unmanaged boreal ecosystems (DeLuca *et al.*, 2002).

Biological nitrogen fixation is catalyzed by the enzyme nitrogenase (Nase). Three iso-enzymes of the Nase have been identified so far (Eady, 1996; Reed *et al.*, 2011): the canonical molybdenum (Mo)-Nase and two alternative Mo-independent isozymes, that is, the vanadium (V) dependent Nase (V-Nase) and the iron (Fe)-only Nase (Fe-Nase). Until very recently, the contribution of alternative Nases to N inputs in natural habitats was very difficult to measure, and BNF was thus usually assumed to be carried out exclusively by the Mo-Nase. Yet, several studies carried out in tropical, temperate and cold temperate ecosystems, have reported limitation of BNF by Mo (Silvester, 1989; Barron *et al.*, 2008; Wurzburger *et al.*, 2012; Jean *et al.*, 2013). Vanadium, which is *c*. 50–200 times more abundant than Mo in the Earth's crust (Wedepohl, 1995), might present a suitable alternative to Mo to sustain BNF in these environments. This is consistent with several studies reporting the presence of the genes coding for alternative Nases in a wide range of ecosystems (Betancourt *et al.*, 2008) and in several cyanobacteria, including *Nostoc* spp. associated with five bi-membered (i.e. two symbionts) cyanolichens of the genus *Peltigera* (Hodkinson *et al.*, 2014). Another report, based on an exhaustive analysis of published acetylene reduction to ^{15}N incorporation conversion ratio concluded that both Mo-Nase and alternative Nases (V- and Fe-Nase) likely contribute to BNF in soils (Bellenger *et al.*, 2014). Finally a recent study on metal homeostasis in the ubiquitous tri-membered (i.e. three symbionts) cyanolichen *Peltigera aphthosa* s.l. also suggested that V very likely plays a significant role in BNF by boreal cyanolichens (Darnajoux *et al.*, 2014).

While these studies suggest that alternative Nases are widespread and active in a wide range of ecosystems, the definitive demonstration of their importance remains to be made. This goal is now within reach because new methods allow the assessment of the activity of alternative Nases in environmental samples. Recently, Zhang *et al*. (2014) demonstrated that alternative N fixation produces organic matter with lower $\delta^{15}N$ (i.e. ^{15}N isotopic fractionation for Mo-Nase is ε *c*. + 2‰, whereas for alternative Nases ε *c*. + 6 to + 7‰). More recently, another isotope fractionation-based technique, isotopic acetylene reduction assay (ISARA), has been developed to efficiently and reliably assess alternative Nase activity in environmental samples (Zhang *et al.*, 2016).

Here we make use of these new tools together with more conventional approaches to examine the contribution of alternative Nases to BNF in *Peltigera aphthosa* s.l. and the environmental factors influencing this activity. *Peltigera aphthosa* s.l. is a symbiotic

association between a fungus (*P. aphthosa*), a green alga (*Coccomyxa* sp.), and a N-fixing cyanobacterium (*Nostoc* sp.) confined to a specific structure called cephalodia which may contribute to as much as 87% of potential BNF in boreal forest habitat in Sweden (Gavazov *et al.*, 2010; Rousk *et al.*, 2015). Since only the gene for Mo-Nase and V-Nase has been found in *Nostoc* of *Peltigera* lichens to date (Hodkinson *et al*., 2014), and that unlike V, Fe has a broad role in metabolism, we restricted our study to Mo- and V-Nase.

We hypothesized that the recently observed accumulation of V in *P. aphthosa* cephalodia (Darnajoux *et al*., 2014) is widely distributed in the boreal biome. To test this hypothesis we performed a discriminant analysis on the metal contents of *P. aphthosa* samples collected in several sites within the boreal biome. Considering the reported limitation of BNF by Mo and the known regulation of alternative Nases by Mo in many bacteria we also hypothesized that Mo availability to cyanolichens is a critical environmental factor modulating V acquisition and use for BNF in cyanolichens. This hypothesis was tested on a fire chronosequence in Sweden. Since, aerial depositions (e.g. particles, canopy flow-through) are the main sources of metals (e.g. V and Mo) to lichens (Darnajoux *et al.*, 2015; Marks *et al*., 2015), we postulated that along a vegetative succession the amount of Mo and V as well as the relative Mo : V ratio might significantly vary due to different canopy openness and vegetation composition. A fire chronosequence might thus provide samples growing under similar climatic and geological conditions but with contrasted Mo and V exposures. In addition, BNF was shown to increase with stand age (Zackrisson *et al.*, 2004) on the selected fire chronosequence, offering a unique opportunity to determine whether or not Nase metal cofactor contents in cephalodia are influenced by stand age. Finally, we hypothesized that alternative nitrogenase are present and actually contribute to BNF in *P. aphthosa* cyanolichens. This hypothesis was tested on *P. aphthosa* samples from the fire chronosequence using DNA, ISARA and $\delta^{15}N$ analyses.

Our results show that V is a metal of biological importance to *P. aphthosa* throughout the boreal biome and provide direct evidence confirming the hypothesis that V-Nase is present and significantly contributes to BNF by cyanolichens in boreal forest (Darnajoux *et al*., 2014; Zhang *et al*., 2016). We also demonstrate that Mo availability is a critical factor for the regulation of V inside the N fixing symbiont of *Peltigera aphthosa*. Finally, we discuss to what extent the contribution of alternative Nases to BNF may affect the reliability of current BNF estimates reported in the literature.

Materials and Methods

Sampling

Specimens of *Peltigera aphthosa* (L.) Willd. s.l., were collected in Alberta (Wood Buffalo National Park, August 2013, n = 6) and from a 369-yr-old fire chronosequence in Sweden (September 2013, n = 16, details on the fire chronosequence can be found in Zackrisson *et al.*, 2004). Data from samples in northern Québec, Alaska (Fairbanks), Russia (Stolby Reserve, Krasnoyarsk) and Fjord du Saguenay (FdS, South Québec) (n = 27, 5, 6 and 9, respectively; Darnajoux *et al.*, 2014) were also used in the statistical analyses. Additional samples (S1–S6, individual thalli) were collected for ISARA experiment in August 2014 in four sites along the Swedish fire chronosequence (S1, 115 yr; S2, 282 yr; S3, 304 yr; S4–S6, 369 yr). All thalli were manually cleaned from foreign materials (e.g. bryophytes and leaves) with forceps and then kept dry in opaque paper bags at room temperature until further processing.

Thallus preparation and elemental analysis

Lichens were washed to remove metals present at the surface of the thallus (nonspecific absorption sites and particles). Algae and cephalodia were separated from the thallus of *P. aphthosa* s.l. and elemental contents for unwashed thallus, washed thallus, algae and cephalodia were determined by inductively coupled plasma mass spectrometry (ICP-MS) as described in Darnajoux *et al.* (2014). Metal contents are reported either mol^{-1} phosphorus ($mol_{metal}\ mol_{P}^{-1}$) or g^{-1} of thallus biomass ($\mu g_{metal}\ g_{thallus}^{-1}$).

$\delta^{15}N$ experiments

MilliQ-washed ground lichen thalli were analyzed for their $\delta^{15}N$ at the 'Laboratoire d'analyses des isotopes stables et légers' at GEOTOP-UQAM. Data are expressed as ‰ vs air (± 0.2‰ for 1σ).

Isotopic acetylene reduction assay

Acetylene was produced by reacting CaC_2 (Acros Organic, NJ, USA) with water and collecting the resulting gas in a Tedlar bag. Samples from Sweden (S1–S6), collected in 2014 2 months before analysis, were sprayed with deionised water, then left to acclimate at 10°C in an incubator (Infors HT multitron pro), with 12 h : 12 h, light : dark cycle for at least 5 d, with regular spraying (every 2 d) to maintain optimal humidity. Samples were then incubated in 250 ml Mason jars, with 10% headspace removed and replaced with acetylene. Lichens were left for 1–6 d under 12 h : 12 h, light : dark at 10°C, with regular sampling of the

headspace. Ethylene production and background concentration of ethylene in each acetylene batch was determined by gas chromatography as described in Jean *et al.* (2013). Linearity was confirmed up to 90 h in all samples. All gas samples were then sent for ISARA analyses at Princeton University (for more information on the method, see Zhang *et al.* (2016).

Results are presented as the ^{13}C fractionation of the reduction of acetylene to ethylene ($^{13}\varepsilon_{AR}$ (‰) = $\delta^{13}C_{acetylene}$-$\delta^{13}C_{ethylene}$, at low ethylene yield). The fraction of alternative fixation in acetylene reduction assay (ARA) was determined assuming a linear model from $^{13}\varepsilon_{AR,Mo}$ and $^{13}\varepsilon_{AR,V}$, and the percentage of alternative BNF activity was calculated as fraction of alternative fixation in ARA corrected with C_2H_4 : N_2 R values (R_{Mo} = 3, R_V = 1). Calculation details can be found in Supporting Information Methods S1 and in Zhang *et al.* (2016).

DNA extractions and PCR amplifications

DNA was extracted from dissected cephalodia of samples S1–S6 using the QuickExtract Plant DNA Extraction Solution (Epicentre, Madison WI, USA), according to the manufacturer's instructions (65°C for 6 min, 98°C for 2 min, chill at 4°C). DNA extracts were diluted 1 : 25 in nuclease-free water in order to avoid PCR inhibition. Degenerate *vnfD* PCR primers were designed to correspond roughly to the region amplified by Bellenger *et al.* (2014) with bases adapted to *Nostoc vnfD* sequences from Hodkinson *et al.* (2014). Primer sequences were as follows: F: 5′-TYA ACA TGG GGT GGA TGA-3′, R: 5′-CCA TTG TGG TAG CCA TG-3′. PCR reactions of 25 µl were conducted using 2U Expand High Fidelity DNA Polymerase (Roche Applied Science, Indianapolis IN, USA) per reaction, 200 µM dNTPs, 500 nM forward and reverse primer and 2 µl of DNA template per reaction. PCR conditions for all reactions were: 94°C for 2 min, followed by 38 cycles of 94°C for 15 s, 50°C for 30 s, and 72°C for 2 min, with final elongation at 72°C for 7 min. PCR products were gel purified using the QIAquick gel extraction kit, (Qiagen, Valencia CA, USA), ligated into the TOPO 4.0 vector (Invitrogen, Life Technologies, Grand Island, NY, USA), and cloned into One-Shot TOP10 (Invitrogen, Life Technologies) chemically competent *Escherichia coli*. Plasmid DNA was purified with the QIAprep Miniprep kit (Qiagen) and unidirectional Sanger DNA sequencing was conducted at Macrogen (Macrogen USA, New York, NY, USA) using the M13F(-47) primer: 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'. Eight and five *vnfD* sequences were analyzed from specimens collected at S1 (GenBank accession number: KX502814) and S4 (GenBank accession number: KX502813), respectively.

Statistical analyses

Data for regression and multivariate discriminate analyses were log transformed in order to avoid spurious correlation or to achieve normality. Canonical discriminant analysis (CDA) was performed using R software version 3.1.2 (Rstudio 0.98.501, package FactoMineR 1.24, Candisc 0.6-5, and Nortest 1.0-2, R Development Core Team, 2008). All regressions were performed with R using a linear model (lm and glm, Stat 3.1.2), with selection of variables based on the significance of regression ANOVA (function Anova, Car 2.0–22) and on the minimization of the Akaike information criterion (AIC). All residuals were tested for normality. Figures were drawn using either R or Sigmaplot 11.2.0.5 (Systat Software) and were edited using Inkscape version 0.48.4.

Results

Discriminant analysis on the metal contents in the symbiotic partners of *Peltigera aphthosa* across all sampling sites

To evaluate the biological relevance of V for boreal cyanolichens, that is *P. aphthosa* (Darnajoux *et al,* 2014), at a broad scale, we performed a discriminant analysis on metal contents of 63 specimens of *P. aphthosa* s.l. collected in boreal forests in pristine areas (northern Alberta and Québec), low contamination area (Sweden) and higher contamination areas (Alaska and Russia) (Fig. S1). Metal contents were analyzed in four different ways; unwashed thallus, washed thallus, algal layer and cephalodium. The discriminant analysis was able to correctly separate all compartments (error rate 0.031, Table S1). The first canonical axis mostly separates unwashed thalli from cephalodia, while the second canonical axis separates the algal layer from everything else (Fig. 1). The washed thallus group have a central position in the canonical score plot. Arrows indicate loading of variables on the canonical axes; the longer an arrow toward a group, the higher its participation to discriminate this group from others. The analysis identified manganese (Mn), zinc (Zn) and lead (Pb) as discriminants for unwashed thallus; magnesium (Mg) and titanium (Ti) as discriminant for algae; and Fe, Mo, V, chromium (Cr), copper (Cu) and cobalt (Co) as discriminant for cephalodia (Fig. 1). Similar results were obtained for samples from contaminated and uncontaminated sites were considered (compare Fig. 1a and b).

Relationship between atmospheric deposition and Mo and V contents in lichen cephalodia across pristine and low metal contamination sites

In order to evaluate the impact of atmospheric deposition on metal contents in the cephalodia, we analyzed Mo and V contents in unwashed thalli (in $\mu g_{metal}.g_{thallus}^{-1}$) and cephalodia (in $mol_{metal}\ mol_P^{-1}$) in samples collected in pristine and low metal contamination sites (n = 58). Metal contents in unwashed thalli are assumed to be closely correlated to atmospheric deposition fluxes (Darnajoux *et al.*, 2015). Samples from Alaska and Russia were not considered since they are characterized by higher metal deposition that can interfere with natural metal management (homeostasis) (Fig. S1). As shown in Fig. 2(a), Mo cellular quotas in cephalodia are positively correlated to Mo exposures (i.e. Mo content in unwashed thalli, $P < 0.001$; details about regression analyses can be found in Table S2). In contrast to Mo, V cellular quotas in cephalodia are relatively constant in all samples and are not correlated to V exposure (Fig. 2b), a clear indication that V content in cephalodia, the site of N fixation, is tightly regulated. Further analysis of the data revealed that Mo and V cellular quotas are correlated in cephalodia (Fig. 2c; quadratic correlation, $P = 0.003$; Table S1), with Mo and V being positively correlated until Mo concentrations reach 1–2 × 10^{-4} $mol_{Mo}\ mol_P^{-1}$, and negatively correlated afterward.

Mo and V content in *Peltigera aphthosa* samples collected along a fire chronosequence in Sweden

We analyzed Mo and V contents in unwashed thallus, washed thallus, algal layer and cephalodium of *P. aphthosa* samples collected at five sites along a fire chronosequence (Fig. 3). In unwashed and washed thalli, Mo is present at lower concentrations than V. Mo and V covary across sites (Fig. 3a,b), with higher concentrations at Reivo, Tjadnes and Vaksliden (50, 282 and 304 yr) than at Guorbaive and Ruttjeheden (185 and 369 yr). In the algal layer, Mo and V have similar concentrations and do not vary significantly across sites (Fig. 3c). In the cephalodia, V and Mo concentrations are in the same order of concentration but are clearly anticorrelated (Fig. 3d). Further analysis of the data revealed that V concentration is positively correlated with Mo concentration in unwashed thallus but is negatively correlated to Mo concentration in cephalodia (Fig. 3e; Table S2).

ISARA measurement on *Peltigera aphthosa* samples from Sweden

We used ISARA to determine the contribution of V-Nase to BNF on freshly collected *P. aphthosa* s.l. samples from northern Sweden (S1–S6) characterized by low to moderate metal

depositions (Fig. S1a). Results show that four of the six *Peltigera* samples tested have a lower $^{13}\varepsilon_{AR}$ value than what can be expected for Mo-only Nase activity (Fig. 4a). Estimated percentage of alternative BNF (Methods S1) show that alternative Nases contribute to BNF (mean ± SD: 35 ± 12%, range 20–60%) in all samples (Fig. 4b; see later Table 2). The presence of *vnfD* genes was confirmed in samples S1 (GenBank accession number KX502814) and S4 (GenBank accession number KX502813). BLASTN searches to GenBank (April 2015) showed highest identity to sequences from lichens deposited by (Hodkinson *et al.*, 2014) (accessions: KF662359, KF662360, KF662361, KF662362).

Characterization of $\delta^{15}N$ in *Peltigera aphthosa* samples from Sweden

We determined the $\delta^{15}N$ for 14 of the 16 thalli of *P. aphthosa* from Fig. 3 (Fig. 5). The $\delta^{15}N$ averaged value ranged from 0.8 to 1.7‰ (individual ranged from -1.4 to 3.2‰), with the highest averaged value measured in Tjadnes and the lowest averaged value measured in Guorbaive (individual extremes are found in Ruttjeheden). Reivo, Vaksliden and Ruttjeheden achieved intermediate values (Fig. 5a). The comparison of the $\delta^{15}N$ and the V concentration in the cephalodia showed a negative correlation between these two data sets (Fig. 5b), with lower $\delta^{15}N$ corresponding to higher V : P ratios in the cephalodia.

Discussion

Occurrence of V in *Peltigera aphthosa* cephalodia

Discriminant analysis performed on *P. aphthosa* s.l. collected along a natural gradient of metal deposition (Alberta, Quebec, Sweden, Alaska and Russia, Fig. S1a), identified Fe, Mo and V as discriminators for cephalodia (Fig. 1). This is consistent with Nases being important Fe and Mo reservoirs in N_2 fixers (Kustka *et al.*, 2003; Bellenger *et al.*, 2011). This also suggests that the presence of V in cyanolichen cephalodia is widely distributed in the boreal biome, from pristine to contaminated areas.

Mo and V accumulation in *Peltigera aphthosa* cephalodia as a function of deposition

Considering that atmospheric deposition is an important source of metal to lichens, we evaluated whether or not the presence of Mo and V in cephalodia reflects atmospheric deposition. The strong correlation between Mo concentrations in cephalodia and atmospheric Mo deposition (Fig. 2a), in pristine and low metal contamination sites, suggests that Mo deposition primarily controls Mo availability to cephalodia. In contrast to Mo, V cellular quotas in cephalodia are not correlated with V deposition (Fig. 2b; Table S2). Instead, V

cellular quotas in cephalodia in all samples are kept at a fairly constant level ($2.0 \times 10^{-4} \pm 0.3$ mol_V mol_P^{-1}), which is close to the optimum V cellular quotas (V : P) required to sustain diazotrophic growth through V-Nase in cyanobacteria ($2–5 \times 10^{-4}$ mol_V mol_P^{-1}, Darnajoux *et al.*, 2014). This suggests a tight regulation of V content in cephalodia independent of V exposure, possibly related to BNF. The correlation between V and Mo cellular quotas in cephalodia (quadratic correlation, $P = 0.003$; Table S2) suggests that Mo availability might control V accumulation in cephalodia. For Mo cellular quotas (Mo : P) lower than $1–2 \times 10^{-4}$ mol_{Mo} mol_P^{-1}, Mo and V are positively correlated in the cephalodia reflecting their correlation in metal exposure (Fig. S1b). This trend is observed primarily among the samples collected in the pristine areas of Québec and Alberta. We hypothesize that metal deposition in these sites is so low that cyanolichens use both Mo and V to sustain BNF. For Mo cellular quotas in cephalodia higher than $1–2 \times 10^{-4}$ mol_{Mo} mol_P^{-1}, Mo and V are negatively correlated, suggesting that Mo availability regulates, or at least impact, V accumulation in cephalodia.

Role of Mo on V accumulation in *Peltigera aphthosa* cephalodia

In order to further investigate the role of Mo availability on V accumulation in cephalodia, we focused on *P. aphthosa* s.l. samples collected along a fire chronosequence in Sweden. Samples collected along this fire chronosequence show a range of Mo and V deposition as illustrated by the unwashed and washed thalli data (Fig. 3a,b). Contrary to our initial hypothesis, Mo and V concentrations in the thalli do not show systematic variations with stand age. Variation in Mo and V exposure appears to be site specific. The covariation of Mo and V concentration in unwashed thalli suggest common sources for both Mo and V all along the chronosequence. The higher abundance of V compared with Mo in unwashed thalli reflects the relative abundance of Mo and V in the environment. Mo content in cephalodia followed the same trend as metal deposition, confirming that Mo deposition strongly influence Mo availability to cephalodia (compare Fig. 3a and d). However, V cellular quotas in cephalodia do not reflect V deposition. Instead, V cellular quotas in cephalodia follow an opposite trend when compared with Mo, and are low when Mo deposition and Mo in the cephalodia is high (Fig. 3d). Data from the fire chronosequence (local metal gradient) are consistent with data from metal surveys across pristine and contaminated sites (large scale metal gradient), and further support our hypothesis that V accumulation in cephalodia is primarily controlled by Mo abundance in cephalodia (Fig. 3e; Table S2) rather than V

deposition. This regulation of V by Mo is not observed in the algal symbiont (Fig. 3c), suggesting that it is related to BNF in the cephalodia.

Our data suggest that the presence of V in *P. aphthosa*'s cephalodia is ubiquitous in the boreal biome and that Mo availability is an important factor controlling V accumulation. This is consistent with the hypothesis that alternative Nases can support BNF in Mo limited ecosystems. The presence of V in the cephalodia (minimum *c*. 4.10^{-5} mol_V mol_P^{-1}) even when Mo is abundant, suggests that V can play a role in BNF even when Mo is not limiting. The effect of temperature comes to mind since experiments on purified enzymes have demonstrated that the V-Nase is less affected by low temperature than the Mo-Nase (Miller & Eady, 1988). In addition, at low temperature (<14°C) the expression of V-Nase genes is no longer repressed by Mo (Walmsley & Kennedy, 1991). Thus, the low temperature in boreal ecosystems is likely to favor alternative Nases and might explain in part the occurrence of V in cephalodia, even at high Mo content.

Contribution of alternative nitrogenases to BNF by *Peltigera aphthosa* s.l.

The presence of V in cephalodia and its strong correlation with Mo availability suggest an active contribution of this alternative Nase to BNF by *P. aphthosa*. Since Mo availability significantly impacts V acquisition, we further evaluated whether or not Mo : V abundances in cephalodia (Fig. 3d) reflects the contribution of alternative Nases to BNF in the field using $\delta^{15}N$ data, which provide an integrated view of the various N sources over the lifetime of the lichen. As reported by Zhang *et al.* (2014), N fractionation can be used to evaluate the contribution of alternative Nases ($\delta^{15}N$ *c*. -6 to 8‰ **[Author, please confirm amended text 'to' is correct]**) vs Mo Nase ($\delta^{15}N$ *c*. -2‰) in organic matter.

Variations in $\delta^{15}N$ in *P. aphthosa* s.l. from the fire chronosequence sites in Sweden are consistent with metal content measured in the cephalodia (Fig. 5a). $\delta^{15}N$ significantly decreases when V concentration in the cephalodia increases (Fig. 5b; $P = 0.023$; Table S2), which is in accordance with a higher contribution of alternative Nases to BNF. While these data have to be interpreted with caution, as other factors such as N deposition could also contribute to variations in the $\delta^{15}N$ data, they suggest that alternative Nases might contribute to a greater extent to BNF in Mo limited sites.

The presence of *vnfD* genes in samples from the Swedish chronosequence was established in two of the six samples tested (S1 and S4), corroborating previous publication on the presence of alternative Nases in cyanolichens from the genus *Peltigera* (Hodkinson *et al.*, 2014). In addition, ISARA data collected on a subset of lichen samples from the Swedish

chronosequence showed that alternative Nases contribute significantly to BNF (mean ± SD: 35 ± 12%, range 20–60%) in lab incubations at low temperature (10°C) (Fig. 4).

ISARA results and $\delta^{15}N$ data are consistent and suggest that alternative Nases are not only active in short-term laboratory experiments (ISARA data) but are also likely to contribute to BNF by these cyanolichens in the field ($\delta^{15}N$ data), especially in sites characterized by low Mo availability and low temperatures. Considering that most cyanolichens are physiologically active during cold periods of the year (MacFarlane & Kershaw, 1977; Muir *et al.*, 1997), further research is required to fully characterize the effect of temperature on the relative contribution of alternative Nases to BNF by boreal cyanolichens in Mo rich and Mo poor ecosystems.

Reevaluation of BNF estimates

Our results, along with others (Zhang *et al.*, 2016), imply that alternative Nases contribute to BNF in several boreal forested ecosystems, which could significantly change our understanding of the metal requirements of, and enzymes involved in, BNF. These findings also raise questions regarding the reliability of current BNF estimates. The canonical method used to assess BNF in field samples, that is, ARA (Hardy *et al.*, 1968), requires the use of a conversion ratio (ARA : N_2) in order to convert ARA data into BNF (N inputs). This ratio can be affected by many factors, such as growth conditions, but primarily by the type of Nase in use; ARA : N_2 = 3 : 1, 1–2 : 1 and <1 : 1 for the Mo-, V- and Fe-only Nases, respectively (Bellenger *et al.*, 2014). However, because the calibration of ARA data by ^{15}N incorporation experiments is problematic for many environmental samples, the theoretical Mo-Nase conversion ratio of ARA : N_2 = 3, is often assumed. A survey of >40 reports on BNF by cryptogamic covers, which could possibly account for nearly half of BNF on unmanaged land (Elbert *et al.*, 2012), highlights that ARA : ^{15}N conversion ratios were effectively measured in less than half of the studies (Tables 1, S3). Another potential bias comes from the experimental determination of ARA : N_2 ratios. Due to low BNF activity, ^{15}N incorporation data are often acquired under laboratory conditions that are poorly representative of the field (i.e. increased temperature, CO_2 and humidity, Table S3). Recent reports on the presence of easily absorbed forms of ^{15}N in commercial $^{15}N_2$ gas stocks (Dabundo *et al.*, 2014), further emphasize the importance of interpreting past reports on BNF with great caution.

Interestingly, studies that performed both ARA and ^{15}N incubations in the field on the same samples reveal that soil, lichens and mosses samples often yield intermediate ratios, indicating simultaneous N fixation by the Mo-Nase and alternative Nases (Menge & Hedin,

2009; Bellenger *et al.*, 2014; Vile *et al.*, 2014). Thus, current BNF estimates likely include large errors, and the magnitude of these errors remains to be fully determined. However, the assumption of the predominance of Mo-Nase, without the proper validations, could result in a significant underestimation of N inputs (16–46%, Table 2). Considering that alternative Nases have been virtually found everywhere in terrestrial ecosystems, with the exception of symbiotic associations with higher plants, it is urgent to better characterize the enzymatic diversity of BNF in various compartments of the boreal forest and other ecosystems in order to re-evaluate BNF estimates. One crucial step is to deploy more rigorous protocols allowing scientists to properly convert field measurements of Nases activity into N input estimates by implementing, for instance, new methods and tools allowing better evaluations of the contribution of alternative Nases to BNF (such as ISARA and $\delta^{15}N$).

Conclusions

Our data confirm that alternative Nases are not only present in boreal cyanolichens but they are also active and likely contribute to BNF by cyanolichens throughout the boreal biome. While Mo availability and temperature seem to be important factors controlling Nases activity, significant work remains to be done to fully understand the conditions under which alternative Nases come into play. It is also crucial to develop and implement methods, such as a combination of ARA, ISARA, and $\delta^{15}N$ to measure BNF and the relative contributions of the Mo and alternative Nases in field samples. A better characterization of Nase activity, including alternative Nases, in boreal forests and other ecosystems, will significantly improve our understanding of the role of micronutrients on ecosystem function, improve our estimates of BNF and may help clarify N budgets.

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Author contributions

J-P.B. and R.D. conceived the study; A.M.L.K. and X.Z. designed the ISARA experiments; F.L., J.M., J-P.B. and R.D. collected lichen specimens, F.L. and J.M. identified the lichens. R.D., X.Z. and D.L.M. performed the experiments and analyzed the data. R.D. wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Unwashed thallus and cephalodia content for V and Mo.

Table S1 Discrimination of lichen compartment with respect to metals

Table S2 Regression parameters for linear regression in Figs 2, 3(e) and 5(b)

Table S3 Literature survey of BNF estimation and measurement conditions for di-nitrogen fixing species in various ecosystems around the world

Methods S1 Contribution of alternative nitrogenase to acetylene reduction and to N_2 fixation.

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Fig. 1 Metals in tri-membered lichen *Peltigera aphthosa* s.l. Canonical (Can) discriminant analysis of all thalli of *P. aphthosa* s.l. (n = 63) with regard to compartments in (a) all samples, and (b) nonheavily contaminated boreal sites (Alberta, North Québec and Sweden) (see Supporting Information Fig. S1a). UWT, unwashed thalli (black triangles); WT, oxalate-EDTA washed thalli (brown circles); ALG, algae (green crosses); CEP, Cephalodia (blue plus symbol). All data in $mol_{metal}\ mol_P^{-1}$ were log-transformed before the analysis. Data from Fjord du Saguenay (South Québec) were not included as alga data were not available.

Fig. 2 Linear regression of molybdenum (Mo) and vanadium (V) in cephalodia (CEP) of *Peltigera aphthosa* s.l. thalli from nonheavily contaminated boreal sites (Northern Alberta, Québec and Sweden). All data were log-transformed (axes are in log_{10} scale) to achieve normality of residues and to avoid spurious correlation, (a) Mo : phosphorus (P) ($mol_{Mo}\ mol_P^{-1}$) in cephalodia as a function of Mo exposure ($\mu g_{Mo}\ g_{thallus}^{-1}$), (b) V : P ($mol_V\ mol_P^{-1}$) in cephalodia as a function of V exposure ($\mu g_V\ g_{thallus}^{-1}$), (c) V : P ($mol_V\ mol_P^{-1}$) in cephalodia as a function of Mo:P ($mol_{Mo}\ mol_P^{-1}$) in cephalodia. n = 58. Dotted lines show 95% prediction intervals and dashed lines show 95% confidence intervals.

Fig. 3 Nases metal cofactors in cyanolichens collected along a 369-yr fire chronosequence. Molybdenum (Mo) and vanadium (V) content in (a) unwashed thalli (UWT), (b) washed thalli (WT), (c) green algae (ALG), and (d) cephalodia (CEP). (e) Linear regression of Mo : phosphorus (P) ratio vs V : P ratio in cephalodia (CEP) and unwashed thalli (UWT). Data were log-transformed to avoid spurious correlation. Error bars are ± SE, n = 3, 2, 3, 3, 5 for

the 50- (Reivo burn, Rvo), 185- (Guorbåive, Gbv), 282- (Tjadnes, Tjd), 304- (Vaksliden, Vkd), and 369- (Ruttjeheden, Rjd) yr-old sites, respectively.

Fig. 4 Nases utilization in *Peltigera aphthosa* s.l. collected in Sweden based on Isotopic Acetylene Reduction Assay (ISARA) analysis. (a) $^{13}\varepsilon_{AR}$ data from pure culture of various diazotrophs for molybdenum (Mo)-Nase only (closed circles), vanadium (V)-Nase only (white circles) and from *P. aphthosa* s.l. (grey circles, samples S1–S6). Grey box represents the range of values obtained for Mo-Nase (13.1–14.7‰). Error bars represented estimated ± SE for a single ethylene measurement. (b) Estimated proportions of biological fixation of nitrogen (BNF) by alternative Nases for *P. aphthosa* s.l. (more details on calculations can be found in Zhang *et al.*, 2016, and in Supporting Information Methods S1). Samples are individual thalli collected along a 369-yr fire chronosequence S1 (115 yr), S2 (282 yr), S3 (304 yr), S4–S6 (369 yr). Error bars have been calculated using error propagation.

Fig. 5 Nitrogen isotopic content in *Peltigera aphthosa* s.l. collected along a 369-yr fire chronosequence. (a) $\delta^{15}N$ of washed thallus (WT); (b) regression (solid line) of $\delta^{15}N$ in washed thallus vs vanadium (V) content in cephalodia (CEP, mol_{metal} mol_P^{-1}). Data are averaged for each fire chronosequence site. Error bars are ± SE; n = 3, 2, 3, 3, 5 for the 50- (Reivo burn, Rvo), 185- (Guorbåive, Gbv), 282- (Tjadnes, Tjd), 304- (Vaksliden, Vdk), and 369- (Ruttjeheden, Rjd) yr-old sites, respectively. Dotted lines show 95% prediction intervals and dashed lines show 95% confidence intervals

Table 1 Summary of literature survey (Supporting Information Table S3) on biological nitrogen fixation by cryptogamic cover (N_2 fixing bacteria associated with bryophytes and lichens)

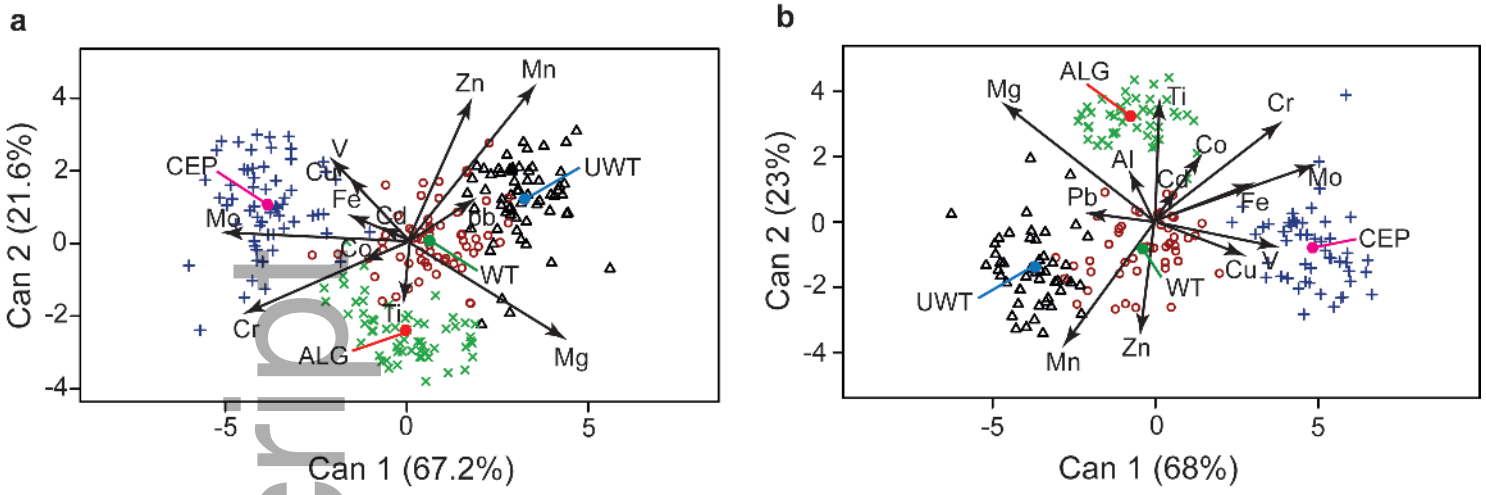
	No. of studies	Acetylene reduction assay based studies	^{15}N calibration made	Percentage of ^{15}N calibration
Arctic	2	1	0	0
Boreal	11	10	6	55
Continenta	6	5	1	17

l				
Temperate	12	12	6	50
Tropical	7	6	3	43
Dry	7	7	1	14
Total	45	41	17	38

Table 2 Estimation of nitrogene fixation ($\mu g N\ g_{lichens}^{-1}\ d^{-1}$) capacity of *Peltigera aphthosa* s.l. when considering molybdenum (Mo)-only contribution ($C_2H_4 : {}^{15}N_{Mo} = 3 : 1$) or estimation of alternative Nases activity with isotopic acetylene reduction assay ($C_2H_4 : {}^{15}N_{alt} = 1 : 1$)

Sample no.	C_2H_4 reduction ($\mu mol C_2H_4\ g_{lichens}^{-1}\ d^{-1}$)	N fixation Mo-only ($\mu gN\ g_{lichens}^{-1}\ d^{-1}$)	N fixation ISARA ($\mu gN\ g_{lichens}^{-1}\ d^{-1}$)	Percentage error/ISARA estimation
1	1.6	15	22	46
2	3.5	32	40	23
3	3.4	31	41	30
4	1.7	16	18	16
5	2.2	21	26	26
6	2.1	20	27	34

Percentage error represents the difference between estimates based on $C_2H_4 : {}^{15}N_{Mo} = 3 : 1$ and $C_2H_4 : {}^{15}N_{alt} = 1 : 1$.



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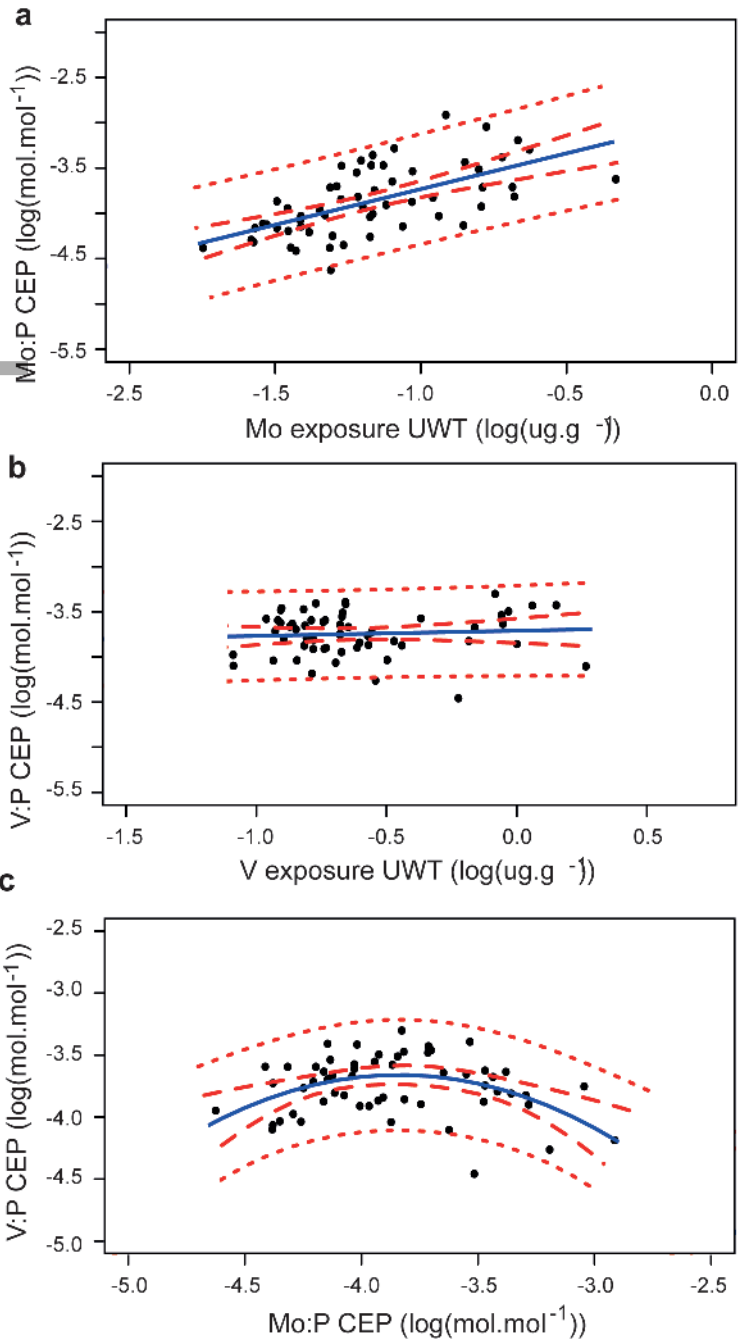

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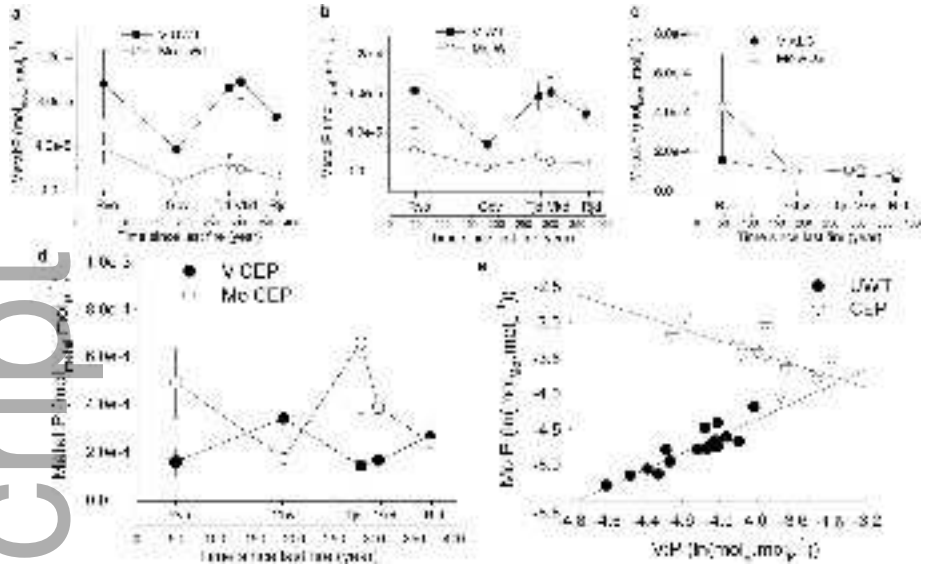

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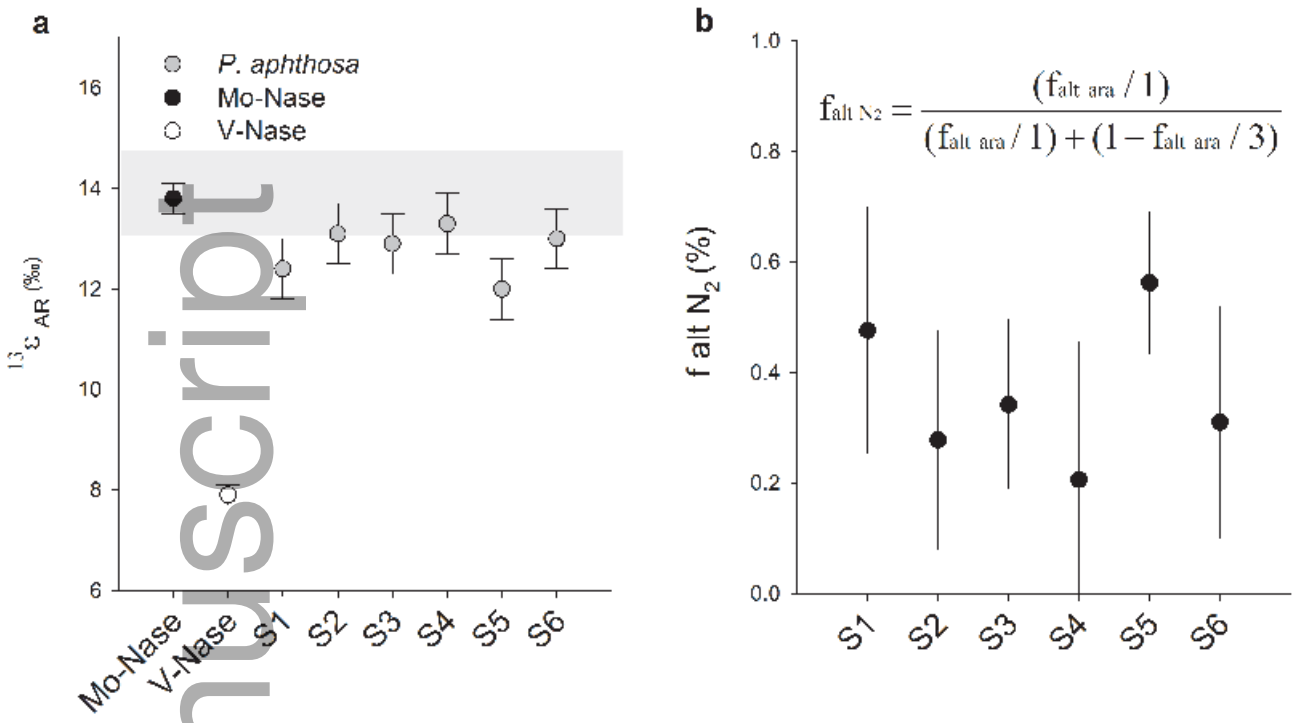

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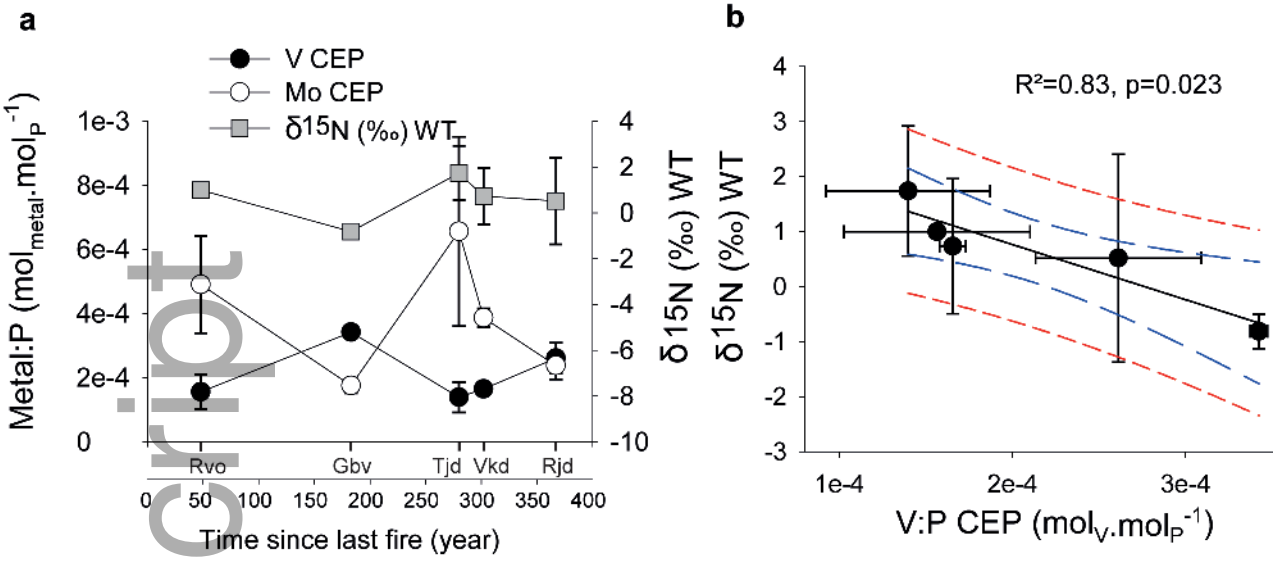

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