

Short Communication

Stigonema associated with boreal *Stereocaulon* possesses the alternative vanadium nitrogenase

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Reindeer lichens from the genus *Cladonia* and snow lichens from the genus *Stereocaulon* are components of spruce lichen woodland, which is one of the most extensive ecosystems in Eastern Canada (Payette & Delwaide 2018). Unlike reindeer lichens, *Stereocaulon* species have a mutualistic association with cyanobacteria, mainly from the genus *Stigonema*, which are located in cephalodia (Huss-Danell 1977, 1979; Kershaw 1978; Kytöviita & Crittenden 2002; Lavoie *et al.* 2020). In Canada, acetylene reduction assays on *Stereocaulon* cyanobacteria have demonstrated their critical contribution to the nitrogen (N) budget (Crittenden & Kershaw 1978; Kershaw 1978). Cyanobacteria use the nitrogenase enzymatic complex to reduce atmospheric N₂ into bioavailable ammonium. Thus, understanding the biology and efficacy of the nitrogenase, which reduces atmospheric N₂ into bioavailable ammonium, is crucial to obtaining a clearer picture of nutrient flow in boreal forests.

Nitrogenase comes in three main isoforms, characterized by different metal cofactors in their active site (Eady & Leigh 1994): molybdenum (Mo)-nitrogenase, present in all N₂ fixers, and at least two ‘complementary nitrogenases’ in which vanadium (V) or iron (Fe) replace Mo in the cofactor (V- and Fe-nitrogenase) (Eady 1996). Recent studies on species of *Nostoc* associated with *Peltigera* revealed that V-nitrogenase is present and provides 15–50% of N fixed by these cyanolichens in boreal forests (Darnajoux *et al.* 2019). However, whether V-nitrogenase is present in other boreal or arctic cyanolichens remains unknown. Evaluating the presence of V-nitrogenase in other cyanolichens is essential to establish whether this trait is ubiquitous or limited to a small number of cyanobacterial clades.

The contribution of V-nitrogenase to N₂ fixation is strongly controlled by Mo availability. Darnajoux *et al.* (2019) reported that, in *Peltigera* species, V accumulation in cephalodia is controlled by Mo availability and identified a Mo threshold of ~250 pbb (ng_{Mo}·g⁻¹_{thallus}) below which V-nitrogenase also contributes to N₂ fixation (Darnajoux *et al.* 2017, 2019). Another potentially important environmental factor affecting V-nitrogenase contribution to N₂ fixation is temperature. *In vitro* studies showed

that the regulation of V-nitrogenase genes by Mo disappears at temperatures below 15 °C and at these lower temperatures V-nitrogenase, which is less efficient than its Mo-based counterpart at high temperatures (> ~20 °C), achieves similar or even higher activity to that of Mo-nitrogenase (Miller & Eady 1988; Walmsley & Kennedy 1991). This suggests that V-nitrogenase could significantly contribute to N₂ fixation in cold and Mo-poor habitats, such as the boreal forest (Bellenger *et al.* 2020). Indeed, Darnajoux *et al.* (2019) reported an increasing contribution of V-nitrogenase to N₂ fixation with latitude in *Peltigera* species in Eastern Canada, which was primarily attributed to increased Mo stress but could also reflect decrease in average temperature (Darnajoux *et al.* 2019).

The main objective of this study was to determine the presence of the complementary V-nitrogenase in *Stigonema*, from several *Stereocaulon* accessions in a latitudinal gradient in Eastern Canada (Fig. 1), using phylogenetic analyses. Additionally, using metal analyses (i.e. Mo and V) we also evaluated if V accumulation by *Stereocaulon* is indicative of a potential use of this metal to cope with Mo limitation of N₂ fixation as observed with *Peltigera* species. Sequence analyses targeting the *vnfD* region confirmed the presence of V-nitrogenase in *Nostoc* strains associated with *Peltigera* collected both in North America and Northern Europe (Hodkinson *et al.* 2014; Darnajoux *et al.* 2017), and here we report similar findings in *Stigonema* for the first time. Metal analyses (i.e. Mo and V) of *Stigonema*-containing cephalodia confirmed that V accumulation in *Stereocaulon* responds to Mo availability, strongly suggesting that V-nitrogenase is used by *Stereocaulon*, at least in part, to cope with Mo limitation as observed in *Peltigera* (Darnajoux *et al.* 2017, 2019).

Material and Methods

A molecular marker to test the presence of the alternative V-nitrogenase

To determine the presence of the alternative V-nitrogenase and the phylogenetic affinities of the cyanobionts found in *Stereocaulon*, six specimens representing four morphospecies from the boreal biome were used (*S. cf. dactylophyllum*, *S. cf. paschale*, *S. cf. saxatile*, *S. cf. tomentosum*) (Fig. 1, Table 1). Accessions were sampled from New Brunswick (Fundy National Park), southern Québec (Forêt Montmorency-Charlevoix,

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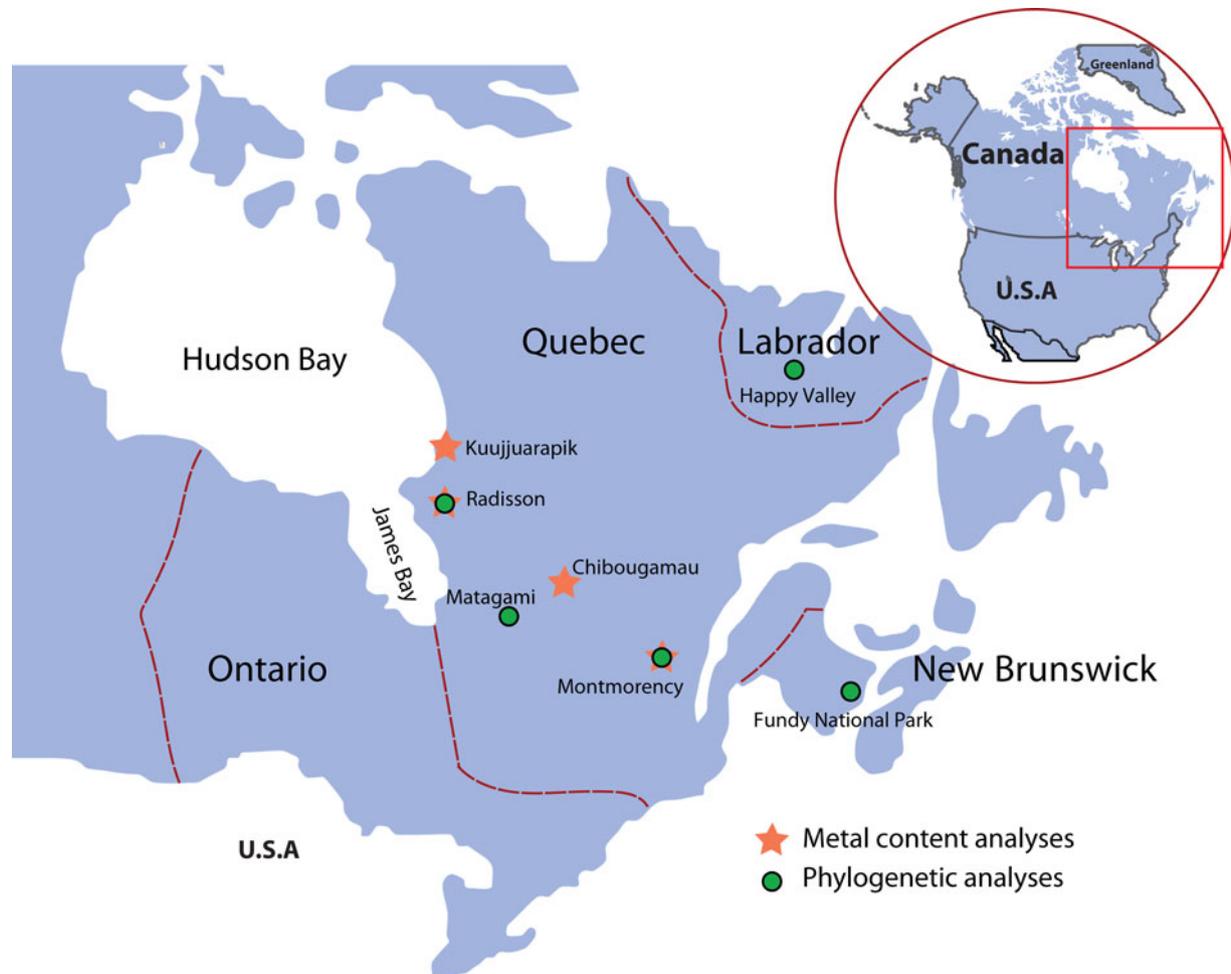


Fig. 1. Map of eastern North America showing collection points of *Stereocaulon* samples. Sites with samples used for the metal analyses (i.e. molybdenum and vanadium) of *Stigonema*-containing cephalodia, are indicated by stars; those sites sampled for phylogenetic analyses of *Stigonema* are indicated by filled circles. Note that sampling for both analyses is undertaken at some sites. In colour online.

hereafter referred to as ‘Montmorency’) and the Québec boreal zone (Happy Valley in Labrador, Matagami, Radisson at James Bay) (shown as filled circles in Fig. 1). The samples for the phylogenetic analyses differ from those of the metal analyses (see below), except those from the Montmorency Forest and Radisson (Table 1). Sanger sequencing was performed with new primers designed using Geneious 9.0.5 (Biomatters Ltd), targeting the coding region of the vanadium cluster (*vnf*), specifically the *vnfDG* region (Hodkinson *et al.* 2014). Cyanobiont DNA was extracted using the Qiagen DNeasy Plant Kit (Qiagen, Hilden, Germany). The *vnfDG* region was amplified by PCR using the new primers: VN 60F (forward), CGTTGT CCGAGAGAGGA TGC; VN 807R (reverse), CGCAATCCTTCTTTGGCATAGT. Each 25 μ l PCR reaction included 25 μ g BSA, 0.626 U Taq DNA polymerase (Qiagen, Hilden, Germany), 1.5 mM MgCl₂, dNTPs (0.2 mM each), 0.5 μ M forward and reverse primers and 1 \times PCR buffer, with 1 μ l of DNA template. The thermocycler temperature profile for the locus *vnfDG* was an annealing temperature set at 56 $^{\circ}$ C and a 95 $^{\circ}$ C denaturation temperature for 5 min, followed by 35 cycles at 95 $^{\circ}$ C for 45 s, 56 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 1 min 30 s, with a final extension of 72 $^{\circ}$ C for 10 min. Sequencing was carried by the Genomic Analysis Platform of the Institut de biologie integrative et des systems, Université Laval.

We analyzed a total of 24 accessions, including our six newly sequenced cyanobiont samples. The dataset used was obtained from Hodkinson *et al.* (2014) and includes 15 sequences from the *vnfD*-like region across Archaea, Bacteroidetes (Chlorobi), Proteobacteria, Firmicutes and Cyanobacteria, with four *Nostoc* sequences associated with *Peltigera* species. The outgroup sequences were three accessions of the *anfD* (Fe-nitrogenase) region from *Clostridium pasteurianum*, *Chloroherpeton thalassium* and *Rhodobacter capsulatum* (Hodkinson *et al.* 2014). We used Geneious 9.0.5 (Biomatters Ltd) to align the nucleotide sequences, with manual verification of the resulting alignment. Sequences have been deposited in GenBank (Table 1) and the alignment is available upon request.

Phylogenetic analyses

The *vnfDG* coding region was analyzed under the maximum likelihood (ML) criterion using the GTR-CAT model approximation implemented in RAXML (Stamatakis 2014) with 500 bootstrap replicates (MLB). Basic statistics on the dataset were retrieved using PAUP* (Swofford 2002) (Table 2). Bayesian analyses of the genomic region were conducted in MrBayes 3.2 (Ronquist *et al.* 2012), using the default two runs and four chains, with

Table 1. Specimens of *Stereocaulon* used in the present study, including area of collection, voucher information and GenBank Accession numbers for sequences of the vnfDG region from associated cyanobacteria.

Fungal morphospecies host species	Biome or ecoregion	Location	Sample information		
			GenBank Accession no.	Voucher accession no.	Collector
<i>Stereocaulon</i> cf. <i>dactylophyllum</i>	Boreal/Acadian Forest	Fundy National Park, New Brunswick, Canada	MK040915	QFA600843	C. Roy
<i>S. cf. paschale</i>	Boreal	Forêt Montmorency, Québec, Canada	MK040912	CA-17-1676	J. C. Villarreal A.
<i>S. cf. paschale</i>	Boreal	Happy Valley, Labrador, Canada	MK040914	17157	R. T. McMullin
<i>S. cf. paschale</i>	Taiga	Radisson, James Bay, Québec, Canada	MK040921	CA-17-1735	J. C. Villarreal A.
<i>S. cf. saxatile</i>	Boreal	Forêt Montmorency, Québec, Canada	MK040913	PA-CAN-029	P. A. Picord
<i>S. cf. tomentosum</i>	Boreal	Matagami, Québec, Canada	MK040916	CA-17-1677B	J. C. Villarreal A.

default priors on most parameters and a model chosen by MrModeltest (Nylander 2004). To assess burn-in and convergence, we compared bipartitions across the two runs and stationarity in the chain using Tracer (Rambaut *et al.* 2018). Convergence was achieved in MrBayes after 2 million generations, with trees sampled every 10 000th generation for a total length of 20 000 000 generations; 25% of each run were discarded and the runs were pooled. All analyses were carried out on the CIPRES platform (Miller *et al.* 2010).

Element analyses: molybdenum, vanadium and phosphorus

Samples of *Stereocaulon paschale* were collected from the southern Québec black spruce forest (Montmorency, $n = 3$), the northern Québec boreal forest (Chibougamau, $n = 2$ and Radisson, $n = 4$) and the forest tundra (Kuujuarapik, Hudson Bay, $n = 3$) (shown as stars in Fig. 1). Sampling was carried out at least 20 m from roads and buildings to avoid the influence of human activities. To determine Mo, V and phosphorus (P) concentrations in cephalodia, these structures were dissected under a stereomicroscope using needles and forceps. The cephalodia were macroscopically free of large visible fragments of the main fungal host, however, fungal hyphae are intrinsically part of the structure, along with bacteria. Therefore, it is possible that some epiphytic bacteria (even cyanobacteria) were included in the cephalodia dissected out from the pseudopodetia. The samples were oven dried (50 °C for 48 h) and weighed. Samples were digested for 4 h in 1 ml of nitric acid (trace metal grade, Fisher Scientific) mixed with 0.2 ml of hydrogen peroxide (trace metal grade, Sigma-Aldrich) using a heating block digestion system (DigiPREP Jr, SCP Sciences) as previously described (Darnajoux *et al.* 2014). Mo, V and P levels in the cephalodia were determined by inductively coupled mass spectrometry (ICP-MS, X-series II, Thermo Fisher Scientific) using the XSeries PlasmaLab 2.6 software. Results are presented as Mo:P and V:P (mol:mol) ratios and as element concentration in cephalodium in ppb ($\text{ng}_{\text{element}} \cdot \text{g}_{\text{dry weight}}^{-1}$). Statistical analyses were performed using R v. 3.4.3 (R Core Team 2017). Data normality was tested using the Shapiro test. The nonparametric Kruskal-Wallis test followed by the post hoc Dunn test, were used to test for

significant differences ($\alpha < 0.05$) among the Mo:P and V:P ratios at the different collecting sites.

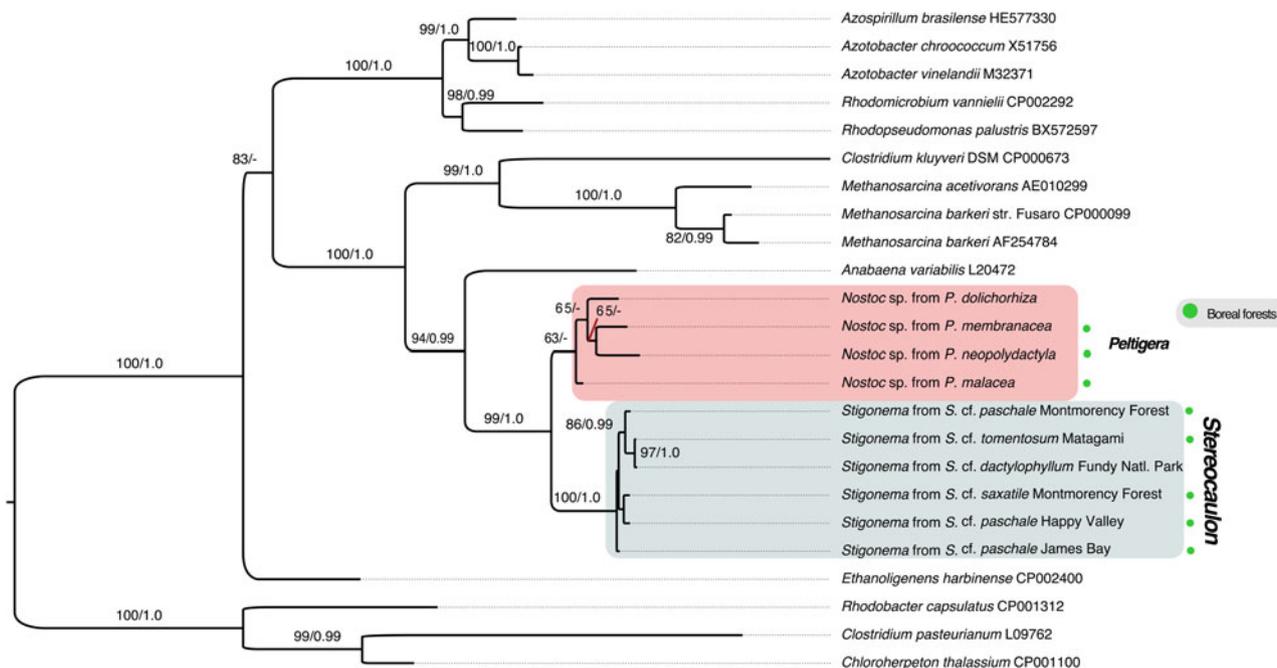
Results and Discussion

In this study, we confirm the presence of the alternative vanadium nitrogenase in *Stigonema*, as our newly designed primers successfully amplified a portion of the vnfDG region (Hodkinson *et al.* 2014). This suggests that cyanobionts associated with *Stereocaulon*, an important component of temperate, boreal and tundra ecosystems (Crittenden & Kershaw 1978; Kershaw 1978), are able to use both nitrogenases (Mo- and V- nitrogenases). Recent molecular analyses have shown an extremely low genetic diversity in *Stigonema* across temperate, boreal and tundra biomes (Lavoie *et al.* 2020), without mycobiont specificity. The vnfDG sequences from *Stigonema* form a monophyletic group (Fig. 2) sister to *Nostoc* from *Peltigera* species (*P. malacea*, *P. membranacea*, *P. neopolydactyla*). Although reported in *Peltigera* species (Hodkinson *et al.* 2014), the complementary V-nitrogenase is not ubiquitous across the bacterial and archaeal world (Harwood 2020). In *Peltigera*, it contributes significantly to N_2 fixation by allowing species to cope with Mo limitation (Darnajoux *et al.* 2019). The presence and activity of V-nitrogenase in other cyanolichen genera remains to be determined. The presence of the alternative V-nitrogenase in *Stigonema* associated with *Stereocaulon*, although not entirely surprising, is a major finding because *Stereocaulon* is widespread in boreal forests. Estimates from *Peltigera* species suggests that V-nitrogenase provides 15–50% of fixed N by *Peltigera* in boreal forests (Darnajoux *et al.* 2019). *Stereocaulon* species are more locally abundant than *Peltigera* in boreal forests and, therefore, we expect the contribution by *Stigonema* to the fixed N budget to be much higher, with a considerable input from V-nitrogenase.

Mo and V content as well as ratios (Mo:P and V:P) in cephalodia suggest not only that genes coding for V-nitrogenase are present in the genome of *Stigonema*, but that both Mo and V contribute to N_2 fixation by *Stereocaulon* species. Mo concentration in cephalodia ranged from 182 to 412 ppb ($\text{ng}_{\text{element}} \cdot \text{g}_{\text{dry weight}}^{-1}$) (Fig. 3A). Samples collected in the most southern site (Montmorency, near Québec City) had significantly higher Mo concentrations (412 ± 42 ppb) than other sites located in less densely

Table 2. Basic statistics and informative sites for the *vnfDG* locus analyzed using the GTR + Γ model chosen by MrModeltest (Nylander 2004).

Locus	No. of taxa	Total length	No. of excluded positions	No. of included positions	No. of uninformative characters	No. of informative characters
VnfDG region	24	876	0	876	54	518

**Fig. 2.** Majority-rule consensus tree (MJR) of the *vnfDG* region (including the *vnf*-like regions), displaying the posterior probabilities (PP) and maximum likelihood bootstrap (MLB) values from each clade indicated above the branches (MLB/PP). The analyses included six samples from the present study representing several species of *Stigonema* associated with *Stereocaulon* (see Table 1), as well as selected bacterial and archaeal strains, and cyanobionts associated with four species of *Peltigera* (Hodkinson et al. 2014). The outgroup sequences were three accessions of the *anfD* (Fe-nitrogenase) region from *Clostridium pasteurianum*, *Chloroherpeton thalassium* and *Rhodobacter capsulatus* (Hodkinson et al. 2014). In colour online.

populated areas (Chibougamau, Radisson and Kuujuarapik: 209 ± 37 ppb, 210 ± 46 ppb and 182 ± 6 ppb, respectively). This trend in Mo content in *Stereocaulon* is consistent with atmospheric metal deposition measured using *Peltigera* species in this region (Darnajoux et al. 2015). Darnajoux et al. (2015) reported a sharp decrease in atmospheric metal deposition with latitude. Metal depositions above the 48th parallel (slightly below the Chibougamau site) were low and comparable to deposition in remote area such as the Himalayas, Greenland and Antarctica. Mo:P ratios in cephalodia were similar among sites and maintained within a narrow range (0.76×10^{-4} to 1.76×10^{-4} mol_{Mo}.mol_P⁻¹; Fig. 3B). These ratios were close to, but below, the minimal cellular Mo requirement (between $2\text{--}6 \times 10^{-4}$ mol_{Mo}.mol_P⁻¹; grey area in Fig. 3B) for N₂ fixation reported in cyanobacteria and other cyanolichens (Darnajoux et al. 2014).

The consistency of Mo:P ratios in samples collected along the 1000 km latitudinal gradient, characterized by a sharp latitudinal drop in metal deposition (between the site below and the sites above the 48th parallel; Darnajoux et al. 2015), reflects a tight homeostatic control of Mo accumulation in *Stereocaulon*, a phenomenon common among N₂ fixers. However, data suggest that there might not be enough Mo accumulated in cephalodia to fully support N₂ fixation, especially at the most northern site (Kuujuarapik) where cephalodial Mo concentrations are

particularly low ($0.76 \pm 0.04 \times 10^{-4}$ mol_{Mo}.mol_P⁻¹; Fig. 3B). It appears that V can be used to cope with Mo limitation in N₂ fixers possessing the alternative V-nitrogenase. In *Peltigera* species, V accumulation in response to Mo availability and independent of atmospheric sources, has been shown to be a strong indicator of V use for N₂ fixation to cope with Mo limitation (Darnajoux et al. 2017).

In our study, V concentrations ranged from 313 to 1434 ppb (ng_{element}.g_{dry weight}⁻¹) with no clear latitudinal pattern, suggesting that atmospheric deposition is not the primary driver of V accumulation in *Stereocaulon*. The highest V concentrations were found at Chibougamau and Radisson (1434 ± 425 ppb and 482 ± 104 ppb, respectively), while concentrations at Montmorency (the southernmost site) and Kuujuarapik (the northernmost site) were low and comparable (337 ± 145 ppb and 313 ± 21 ppb, respectively) (Fig. 3A). V:P ratios in cephalodia of *Stereocaulon* specimens also varied significantly (by up to 7-fold, from 2.45×10^{-4} to 1.73×10^{-3}) among sites (Fig. 3B). These patterns in cephalodia V concentrations and V:P ratios along the latitudinal gradient are unlikely to be explained by passive accumulation, which would reflect deposition and thus result in lower V concentrations in Chibougamau, Radisson and Kuujuarapik (sites above the 48th parallel, where atmospheric metal deposition is low) than in Montmorency (Darnajoux

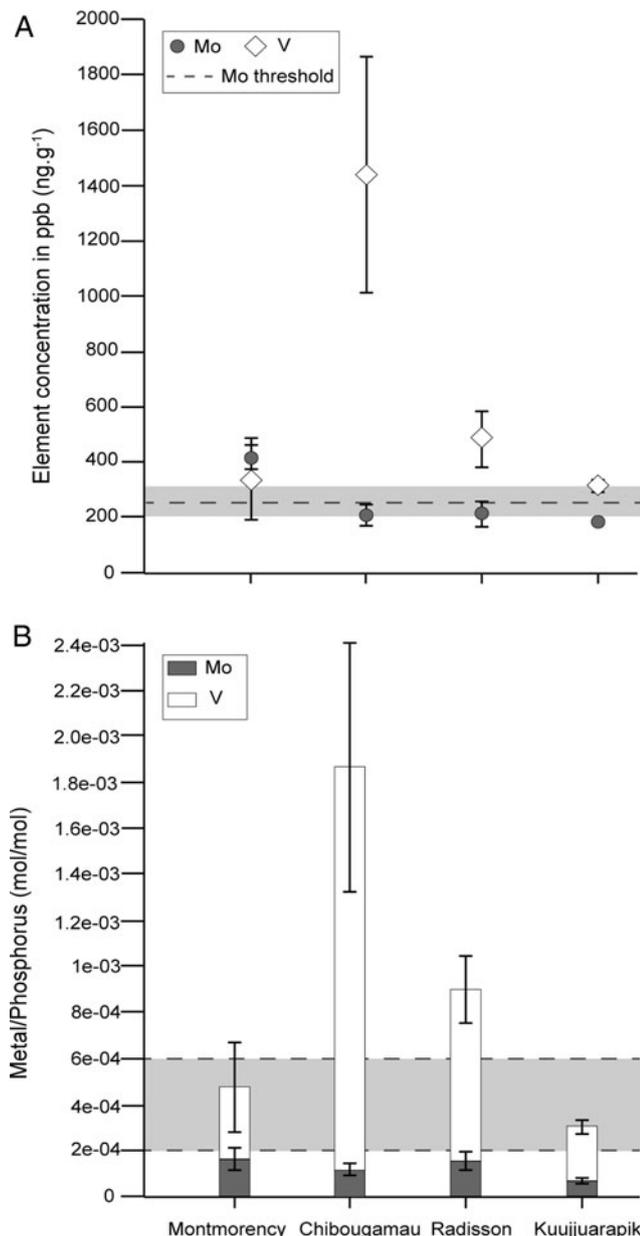


Fig. 3. Molybdenum (Mo) and vanadium (V) content in cephalodia of *Stereocaulon* in four different areas ranging from southern Québec (e.g. Montmorency, 47°N, 71°W) and northern Québec (Chibougamau (49°N, 74°W), Radisson (53°N, 77°W) and Kuujuarapik (55°N, 77°W)). A, molybdenum (grey circles) and vanadium (white diamonds) concentrations in ppb. The dashed grey line represents the average value and the grey shaded area represents the molybdenum threshold (250 ± 50 ppb) for the contribution of V-nitrogenase to N_2 fixation reported in *Peltigera* species by Darnajoux *et al.* (2019). B, molybdenum:phosphorous (grey bars) and vanadium:phosphorous (white bars) ratios (mol:mol). The grey shaded area represents the minimum Mo:P and V:P ratio for N_2 fixation reported in cyanobacteria and cyanolichen species (Darnajoux *et al.* 2014). Error bars in parts A and B represent standard errors.

et al. 2015). This suggests a biological control over V accumulation in cephalodia, possibly in response to Mo stress (i.e. Mo limitation of N_2 fixation) as reported in *Peltigera* species (Darnajoux *et al.* 2014, 2017, 2019). The minimum V cellular requirement for N_2 fixation reported for cyanobacteria is similar to the Mo cellular requirement ($2\text{--}6 \times 10^{-4} \text{ mol}_V \text{ mol}_P^{-1}$; Darnajoux *et al.* 2014). Thus, in all our sites, there was enough V in cephalodia to support N_2 fixation with the V-nitrogenase alone.

Since V can be toxic at high concentrations, this large accumulation of V in cephalodia probably reflects a role in N_2 fixation. Indeed, one of the best-known biological functions of V in terrestrial ecosystems is N_2 fixation (see also Rehder 2013). Considering the strong similarities in V and Mo homeostasis among *Stereocaulon* species (this study) and *Peltigera* species (Darnajoux *et al.* 2014, 2017, 2019), we hypothesize that the higher concentration of V in cephalodia of *Stereocaulon* with low Mo content (below 250 ppb) reflects a higher physiological V demand to cope with Mo limitation of N_2 fixation. The decrease in V content with latitude within sites located above the 48th parallel (Chibougamau, Radisson and Kuujuarapik) probably reflects the decrease in V availability (atmospheric deposition) with latitude reported in this area (Darnajoux *et al.* 2015). Further research is required to confirm V-nitrogenase activity and fully characterize the spatiotemporal contribution of V-nitrogenase to N_2 fixation by *Stereocaulon*. Nonetheless, our study provides evidence of the presence of a complementary nitrogenase in *Stigonema* associated with the lichen *Stereocaulon* and gives a new dimension to studies on nitrogen cycling in temperate, boreal and tundra ecosystems.

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