Canopy-dependent environmental factors impact on nitrogen fixation in *Shepherdia canadensis* in the boreal forest and tundra

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Abstract

Actinorhizal shrubs, plants that symbiotically associate with nitrogen-fixing bacteria and do not depend on soil nitrogen, can increase productivity and facilitate successional changes. High-latitude environments are characterized by harsh abiotic climatic conditions that limit nitrogen availability to plants. However, actinorhizal shrubs are less prevalent at higher latitudes, even though those environments are generally nitrogen-limited. Reduced year-round temperatures, water availability, and light availability are all thought to limit nitrogen fixation due to its energetic costs, making nitrogen fixation unfavourable at higher latitudes. *Shepherdia canadensis* (buffalo berry) is an actinorhizal shrub that is found in higher-latitude environments across Canada. We wanted to determine, within the northern edge of the boreal forest and tundra, how much nitrogen fixation is occurring in buffalo berry, and how it varies across naturally occurring habitat and canopy-dependent environmental factors. The natural abundance stable isotope technique was used to quantify nitrogen fixation, while canopy-dependent variables were measured using hemispherical image analysis. We found that buffalo berry shrubs were getting a majority of their nitrogen content through nitrogen fixation (55.32% - 94.74%) and that on average, fixation occurred at a higher rate in the forest (74.67%) compared to the tundra (68.00%). Notably, foliar δ13C values varied with canopy openness. Specifically, shrubs in more open areas and the tundra had higher δ13C values, which were interpreted to be a result of stomatal closure in response to reduced water availability. Nitrogen fixation was found to be sensitive to water availability, decreasing with canopy openness (17.99% – 99.79%). This limitation could reduce the productivity of nitrogen-fixing plants in response to warmer climate conditions.

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Introduction

Nitrogen is an essential element for plant growth, survival, and function (Vitousek and Howarth 1991; Kuypers et al., 2018). Plants rely on nitrogen for photosynthetic processes and functioning, so environmental nitrogen limitations can negatively impact overall plant production (Vitousek and Howarth 1991). Specifically, essential proteins and pigments like chlorophyll which are necessary for photosynthesis require a large allocation of plant nitrogen (Evans 1989). Nitrogen limitation is defined as an increase in plant biomass via growth in response to an addition of nitrogen fertilizer (LeBauer and Treseder 2008). Nitrogen is thought to be the main limiting factor to primary production, particularly in temperate, arctic, and boreal, middle to high-latitude ecosystems (Vitousek and Howarth 1991). For example, increases in primary productivity in response to nitrogen deposition have been observed to be greatest in descending order for the tundra, boreal and temperate forest ecosystems compared to tropical forests in North America (McGuire et al. 1992). However, this is not to say that nitrogen limitation is not occurring at lower latitudes as overall, nitrogen appears to be a limiting factor globally, occurring in tropical environments also (Vitousek and Howarth 1991; LeBauer and Treseder 2008). The effect nitrogen can have on limiting primary production intertwines available nitrogen with the carbon cycle and an ecosystem's ability to sequester carbon (McGuire et al. 1992; Hungate et al. 2003), which is why in the context of climate change, it is essential to understand mechanisms that can alleviate environmental nitrogen limitations, like symbiotic nitrogen fixation.

Nitrogen is abundantly available in the form of N_2 gas, yet plants cannot utilize nitrogen in this form directly, instead requires nitrogen to be fixed into usable forms for uptake (Vitousek et al. 1997; Santi et al. 2013). Examples of usable forms of nitrogen, which are products of fixation are inorganic compounds like ammonium (NH_4^+) and nitrates (NO_3^-) (Santi et al. 2013). However, only certain forms of prokaryotic life can fix atmospheric nitrogen into these usable forms (Franche et al. 2009; Santi et al. 2013). A major source of terrestrial nitrogen fixation occurs through a symbiosis between vascular plants and nitrogen-fixing bacteria, where carbon from the plant is exchanged for nitrogen from the nitrogen fixer, which is housed in the roots (Vitousek and Howarth 1991; Vitousek et al. 2002). Actinorhizal plants are nitrogen-fixing perennial plants that can notably colonize nitrogen-limited areas during early succession or after a disturbance and, in turn, increase nitrogen levels in the soil through symbioses with nitrogen-fixing bacteria (Visser et al. 1991). Different species of actinorhizal shrubs, specifically *Alnus spp.* have been observed increasing environmental nitrogen during primary succession, which results in facilitating other non-nitrogen fixer plant species growth as a part of further successional stages (Rhoades et al. 2001; Uliassi and Ruess 2002; Mitchell and Reuss 2009). These environmental nitrogen inputs from actinorhizal shrubs come from their leaf litter deposits, along with root/nodule senescence which introduce organic nitrogen into the previously nitrogen-limited environment (Freund et al. 2018).

In higher latitude environments like the boreal forest and tundra, primary production is limited by nitrogen availability due to reduced decomposition associated with harsher climatic conditions, notably lower soil temperatures (Chapin et al. 1991; Allison and Treseder 2011). Many studies have demonstrated that lower temperatures, reduced

moisture availability, and lower litter quality (high C:N ratio and high lignin content) all contribute to reducing litter decomposition rates in high latitude ecosystems (Hobbie et al. 2000; Cornwell et al. 2008; Prescott 2010). At high latitudes, conditions create a negative feedback loop, where reduced decomposition leads to a buildup of organic matter that insulates the soil, further lowering temperatures and promoting permafrost formation, ultimately restricting potential inorganic nitrogen availability in these environments (Chapin et al. 1991; Allison and Treseder 2011). Due to factors limiting environmental nitrogen inputs, it is then presumed that nitrogen fixers would be more prevalent in these nitrogen-limited environments (Vitousek and Howarth 1991; Vitousek et al. 2002; Lundquist 2005; Anderson and Markham 2021). However, most symbiotic nitrogen-fixing plants are located and more prevalent at lower latitudes, especially in the tropics where nitrogen is less of a limiting factor (McGuire et al. 1992; Houlton et al. 2008). Of the nitrogen-fixing vascular plants present at higher latitudes, most are found in early successional habitats and are restricted to shrub-like forms (Menge et al. 2010; Anderson and Markham, 2021).

The lack of abundance of nitrogen-fixing plants in northern latitude and nitrogen-limited habitats can be further attributed to the harsh environmental factors associated with higher latitude environments. For example, colder temperatures can inhibit plant growth and nitrogen fixation, specifically the nitrogen-fixing enzyme nitrogenase (Uliassi and Ruess 2002; Houlton et al. 2008 Gundale et al. 2012; Rousk et al. 2017; Anderson and Markham 2021). Particularly at high latitudes, where cold soil temperatures can persist into the early growing season and significantly contrast with warmer air temperatures, promoting photosynthesis while nitrogen fixation is inhibited (Anderson

and Markham 2021). Also, moisture availability can be restricted at higher latitudes due to colder year-round temperatures associated with shorter growing seasons and the consistent presence of permafrost in the soil, with certain *Alnus* spp. demonstrating reductions in nitrogen fixation in response to less moisture availability and drought conditions (Uliassi and Ruess 2002; Mitchell and Ruess 2009; Markham and Anderson 2021). Reduced soil moisture availability can inhibit nitrogen fixation by directly inhibiting the function of the nitrogenase enzyme (Rousk et al. 2018) or by limiting photosynthesis and its products as a physiological response to water stress (Farquhar et al. 1989), which are required to drive nitrogen fixation. Furthermore, restricted light access due to a shorter growing season and shading reduces the total energy budget available to the plant, further increasing the costs of photosynthetic products required in the symbiotic relationship to fix nitrogen as opposed to taking up inorganic nitrogen from the soil and ultimately reducing the plant's competitive success (Vitousek and Field 1999; Vitousek et al 2002; Houlton et al. 2008; Gundale et al. 2012). Nitrogen fixation can also be affected by the presence of large amounts of usable available nitrogen in an environment, where the amount of nitrogen fixed by the plant is reduced through negative feedback as it is energetically more favourable to uptake available soil nitrogen, compared to fixing it (Markham and Zekveld 2007; DeLuca et al. 2008; Ruess et al. 2013). Although it is important to note that these studies typically demonstrated this effect in response to high soil nitrogen levels that are unlikely to be found under natural conditions (Markham and Zekveld 2007; DeLuca et al. 2008; Ruess et al. 2013). Overall, reduced temperature, moisture, and light availability associated with higher latitudes seem to restrict nitrogen-fixing plants and counter the advantage they possess even in nitrogen-limited environments (Rastetter et al. 2001; Houlton et al. 2008; Gundale et al. 2012; Anderson

and Markham 2021). Due to the interlinked and cyclic nature of higher latitude environmental factor interactions like reduced soil temperatures, reduced light availability, reduced growing season lengths, and reduced decomposition, it can be difficult to pinpoint which environmental factor effect ranks highest in its effect on nitrogen fixation.

In northern environments like the boreal forest and tundra, shifts in the composition of plant species have been observed and attributed to climate change, where warming is occurring at a higher rate compared to other biomes (Walker et al. 2006; Myers-Smith et al., 2011; Scheffer et al. 2012). Increased northern warming due to climate change suggests that northern primary production will increase, leading to an increase in plant biomass and carbon collection, which in turn can increase the demand of available nitrogen as these northern environments are already nitrogen-limited (Hungate et al. 2003; Walker et al. 2006). Furthermore, although general warming has the potential to increase northern decomposition and increase soil nitrogen (Allison and Treseder 2011), increased carbon content in leaves and their litter due to increased atmospheric carbon dioxide levels will further reduce soil nitrogen content, favouring nitrogen fixers (Anderson and Markham 2021). Regardless, the observed increase in plant biomass in high-latitude areas that has been attributed to warming has mainly been associated with increased shrub growth, range expansion, dominance, and canopy height (Walker et al. 2006; Mitchell and Ruess, 2009; Myers-Smith et al. 2011; Macander et al. 2022). Larger and more abundant shrubs will accumulate more wind-dispersed snow, particularly depth hoar, a type of snow with better insulating properties, at greater depths compared to smaller and less dense shrub patches (Sturm et al. 2001). As a result, these taller, denser shrub patches better

insulate the soil, leading to higher than usual winter temperatures, but also reduce summer soil temperatures due to increased shading from higher shrub abundance and increased litter insulation (Sturm et al. 2001; Myers-Smith et al. 2011). The resulting consequences where shrub increases have been observed corresponded with reductions in shade-intolerant plants, lichens, bryophytes and tundra diversity, resulting in a shift to a more woody tundra overall (Walker et al. 2006; Myers-Smith et al 2011; Pajunen et al. 2011).

Although many shrubs are increasing in abundance, the degree of nitrogen fixation occurring by actinorhizal shrubs across changing environmental conditions has not been of concern, due to most studies focusing on the shift in overall species composition as opposed to specific side effects like species-specific nitrogen fixation. This is why, in the context of climate change, it is essential to understand how nitrogen-fixing plants respond to warming due to the impact they have on high-latitude nitrogen-limited environments regarding nitrogen deposition (Vitousek et al. 2002; Mitchell and Ruess 2009). Most studies on northern latitude actinorhizal shrubs regarding nitrogen fixation have focused on *Alnus spp*. which has been the model organism due to its involvement in inputting environmental nitrogen and facilitating other species during primary and secondary succession and also being one of the most abundant high latitude actinorhizal plants across North America (Chapin et al. 1994; Rhoades et al. 2001; Uliassi and Ruess 2002; Anderson et al. 2004; Anderson et al. 2009; Shaftel et al. 2021; Anderson and Markham 2021). Some studies have focused on northern shrub range expansion and abundance that involve alder, like Myers-Smith et al. (2011), although quantifying nitrogen fixation and its impacts within those studies was overlooked. Minimal work in regards to range expansion and fixation has been conducted on the widespread buffalo berry (*Shepherdia*

canadensis) which has been documented to be a nitrogen-fixing plant used as a control for non-nitrogen fixing related studies, as well as a potential plant viable in land reclamation practices regarding mine tailings (Visser et al. 1991; Boonstra et al. 2017). Notably, buffalo berry has been identified as contributing to the snowshoe hare population cycle as a form of primary winter sustenance via browsing and is uniquely widespread and restricted to North America (Boonstra et al. 2017). It has been observed to possess the capacity to be browsed on by a variety of ungulates (*Artyodactyla* spp.), specifically different members of the *Cervidae* family, as it is generally considered of low palatability and only consumed in the absence of other optimal sustenance (Walkup 1991). In contrast, the berries are in much higher demand in the late fall for grouse (*Tetraoninae* spp.**)** and bears (*Ursidae* spp.) (Walkup 1991). The most notable influence of buffalo berry fruit, which most research has focused on, is grizzly bears (*Ursus arctos*) of which the fruit can make up a large proportion of their diet in preparation for their winter recluse (Hamer 1996; Denny et al. 2018). However, any research that has been conducted using buffalo berry related to fruit and sustenance or into range expansion and abundance has not fully investigated the variation in nitrogen fixation that can occur across different environmental conditions. In short, gaining insight into how a less-studied boreal actinorhizal shrub species like buffalo berry fixes nitrogen under varying environmental conditions can contribute to a greater understanding of how nitrogen-fixing plants within nitrogen-limited high-latitude ecosystems will respond to changing climatic factors.

Nitrogen fixation can be measured by the natural stable isotope abundance technique (Bergersen 1980). This technique is ideal for field experiments because no pretreatment of sampled plants is necessary, although obtaining reference plants that are precisely

accessing the same local soil conditions, specifically the same available soil nitrogen content as the plant fixing nitrogen, can be more logistically challenging (Bergersen 1980). Fixed nitrogen is measured via isotopic fractionation between ^{15}N and ^{14}N, which occurs when an alteration involving nitrogen (in this case, conversion of atmospheric nitrogen to usable nitrogen) occurs (Bergersen 1980). This then creates a different pool of nitrogen that can be observed as a distinct ratio of ^{14}N: ^{15}N in the sampled plant tissue, which is then compared to a reference plant which is not capable of fixing nitrogen (Bergersen 1980). This is because environmental nitrogen is taken up to a similar degree between test and reference plant which provides an accurate reference for measuring nitrogen fixation (Boddey et al. 2000). Although reference values may not always be representative of available environmental nitrogen levels, as they are susceptible to local natural increases in ^{15}N, like for example fertilizer (Boddey et al. 2000). Collected plant tissue samples are processed by a mass spectrometer where the isotopic ratio of nitrogen is measured and via the ratio of ^{15}N to ^{14}N and the calculated resulting $\delta^{15}N$ values from each sample are obtained. These values represent the contribution to growth by fixed nitrogen and can be calculated as the percent of nitrogen derived from the atmosphere (Ndfa), meaning this is nitrogen was biologically fixed by the plant's nitrogen-fixing symbiont and not taken up from the environment (Boddey et al. 2000). The percent of fixed N calculate as:

$$\%Ndfa = 100\frac{(\delta 15\mathrm{N}_{reference} - \delta 15\mathrm{N}_{fixing\ plant})}{(\delta 15\mathrm{N}_{reference} - B)}$$

(Boddey et al. 2000)

The $\delta^{15}N$ reference value is obtained from the control plant tissue, the $\delta^{15}N$ fixing plant value is obtained from nitrogen-fixing plant tissue, and the B value represents the ^{15}N obtained from a sample plant that achieves all its nitrogen from fixation. It is assumed the reference plants ^{15}N will represent the available soil N because they are non-nitrogen fixers, the soil nitrogen pool for reference plants is assumed to be the same as the tested plant, and the greater difference between δ15N values between soil and air the greater the accuracy of %Ndfa values (Boddey et al. 2000). Other leaf isotope components can infer plant physiological status also. $\delta^{13}C$ is the isotopic ratio of C^{12} and C^{13}, and higher values of $\delta^{13}C$ can reflect stomatal closure in response to drought conditions (Farquhar et al. 1982; Bchir et al. 2016). Rubisco discriminates against $^{13}CO_2$ under ideal conditions for photosynthesizing however, when water loss via transpiration is too high, stomatal closure can occur, leading to a reduction in available $^{12}CO_2$ and an alleviation of the rubiscos discrimination against $^{13}CO_2$, leading to an increase in the C^{13} isotope within plant tissue (Farquhar et al. 1982; Farquhar et al. 1989 Bchir et al. 2016). In turn, increases in $\delta^{13}C$ values can reflect higher rates of stomatal closure due to drought conditions increasing the amount of water lost via transpiration, leading to an overall reduction in plant photosynthesis (Farquhar et al. 1989; Birchir et al. 2016).

Objectives

The objective of this experiment is to evaluate the proportion of nitrogen that the widespread actinorhizal shrub Canadian buffalo berry (*Shepherdia canadensis*) receives across naturally varying light availability, soil temperature, soil nitrogen, and plant productivity levels that occur within the boreal forest woodland and tundra. This study

will help to better grasp the potential changes in abundance and productivity of the shrub in response to climate change, as it has the potential to have a significant impact on the amount of nitrogen deposition in these nitrogen-limited environments. Within the forest, variation in light availability will be a more relevant effect variable as there is no canopy presence on the tundra, whereas the forest can have a gradient of low to high shading. Not only does this study aim to understand how nitrogen fixation of buffalo berry varies under varying environmental conditions, but also to examine the differences of fixation between the shrub in the forest vs. tundra to better understand the impact the shrub has in contributing to the nitrogen cycle in the respective habitats. It is hypothesized that nitrogen fixation rates will be limited by environmental factors like shading, reduced soil temperatures, and soil nitrogen due to the high energy costs associated with fixing nitrogen compared to acquiring available nitrogen from the soil.

Predictions

It is expected that nitrogen fixation rates will vary between shrubs found in the forest and those in the tundra due to differences in environmental factors that are habitat-dependent. In the forest, for example, reduced levels of light availability and soil temperatures due to canopy shading should reduce nitrogen fixation. However, buffalo berry may still fix more nitrogen in the forest compared to the tundra since the tundra shrubs are generally kept smaller and prostrate, whereas, in the understory of the boreal forest, shrubs can grow up to 2 meters tall (Boonstra et al. 2017). The shrubs within the forest can more readily reach greater sizes due to protection from wind abrasion and desiccation by the forest canopy structure, which is not available to the shrubs on the open tundra (Payette et al. 2001; Harper et al. 2011). However, there are specific tundra

conditions where shrubs can grow to larger than prostrate sizes due to protection via favourable microtopographic conditions and shrub aggregation (Holtmeier and Broll 2005; Harper et al. 2011). It is predicted that more productive shrubs, for example, taller, longer shoots and the presence of fruit, will likely be fixing more nitrogen overall.

Soil temperature within the forest is expected to be cooler than the tundra due to solar energy transmission within the forest occurring mainly within the upper canopy, this differs in comparison to the tundra where most of the solar transmission occurs closer to the surface or directly on the soil (Holtmeier and Broll 2005; Harper et al. 2011). Cooler forest soil temperatures are further modified by lichens and bryophyte understory plants which form dense mats within the boreal forest and can further insulate the soil keeping soil temperatures cooler compared to the tundra (Van Zuijlen et al. 2020). In turn, lower soil temperature should lead to reduced nitrogen fixation in actinorhizal shrubs within the boreal forest (Anderson and Markham 2021).

Another trade-off actinorhizal shrubs make within the forest compared to the tundra relates to light accessibility, where the tundra shrubs experience little to no canopy shading and the forest experiences varying degrees of shading. Nitrogen fixation can be limited by available energy, which is directly linked to photosynthesis through available sunlight and hence, more nitrogen will be fixed when it is energetically available to do so (Vitousek and Field 1999; Rastetter et al. 2001; Markham and Anderson 2021). This leads to the prediction that where there is less upper canopy shading and increased light available within the understory forest, more photosynthetic energy will be available, leading to more nitrogen fixed overall. In contrast, higher $\delta^{13}C$ values are expected to be observed in shrubs located in more open areas within the forest and in the tundra, where there is minimal natural feature protection. Plants exposed to prolonged solar exposure

and increased desiccation via wind on the tundra will likely lead to higher rates of transpiration, resulting in stomatal closure and potentially even water stress. So, higher $\delta^{13}C$ values could influence the amount of overall nitrogen fixation occurring if the result of increased stomatal closure results in a significant decrease in photosynthesis, in turn reducing those products to go towards fixation.

Soil nitrogen is also expected to have a minimal role within the forest and tundra due to these environments being nitrogen-limited with little decomposition. Increases in usable nitrogen within the environment have demonstrated decreases in nitrogen fixation in plants when the associated costs of fixation are high (Markham and Zekveld 2007; Ruess et al. 2013). Due to natural variation in soil nitrogen content across the study sites, which can affect the amount of nitrogen fixed by sampled plants, soil nitrogen will be accounted for.

Shrub specific leaf area should differ in shrubs located within the forest in shaded conditions compared to more open forests and tundra shrubs. Forest shrub SLA should be greater overall as lower SLA is associated with higher exposure to solar radiation (Burton et al. 2017). A similar experiment found that *Alnus crispa* made larger and thinner leaves, which are more photosynthetically efficient and cost-effective to compensate for shading under the forest canopy, negating the shading effect, which did not limit nitrogen fixation (Markham and Anderson 2021). Considering the harsher environmental conditions associated with this study near Churchill, Manitoba, compared to the latter study location in southeastern Manitoba, it is difficult to predict how much compensatory variation in SLA will occur. However, lower SLA is generally associated with plants found in more N-limited soils (He et al. 2018) and has been observed to correlate with nutrient availability, where higher SLA values are associated with alleviation in nutrient

limitations like nitrogen and phosphorus (Heim et al. 2023). We then expect nitrogen fixation and available soil nitrogen to also exhibit a positive relationship with increases in SLA.

We also expect foliar C:N ratios and %N content to demonstrate a significant relationship with nitrogen fixation. Generally, foliar C:N ratios will decrease in response to nitrogen additions (Sardans et al. 2012) and positing that where more nitrogen fixation is occurring (an equivalent to nitrogen supplementation) which we predict to be on the tundra, plants should have lower C:N ratios and higher foliar N content compared to forest shrubs. However, harsher abiotic tundra conditions like increased desiccation and drier conditions could offset differences in C:N ratios from greater overall nitrogen fixation resulting in thicker leaves with higher C content and C:N ratio (Zhang et al. 2020).

Photosynthesis and nitrogen content generally demonstrate a strong underlying relationship across species, where increases in nitrogen content correspond with an increase in photosynthesis (Evans 1989). While also understanding that a large allocation of nitrogen goes toward the photosynthetic process, specifically chlorophyll (Evans and Clarke 2019), we would expect plants fixing more nitrogen to also possess higher overall chlorophyll content. We would also expect there to be increased chlorophyll content in tundra plants where there are presumably warmer soils, as Anderson and Markham (2021) found that reduced soil temperatures also reduced leaf chlorophyll content of *A.crispa*.

Methods and materials

Study Area

The study took place within the Hudson's Bay lowlands near Churchill, Manitoba (Figure 1), and data collection occurred from mid-July to mid-August 2024, with most of the data collection occurring across the second week (9th-12th) of August. We selected three general sites, one tundra site North of the Churchill Northern Studies Center (CNSC, N58° 45.928' W93° 48.120') and two forest locations, one around twin lakes road south of the CNSC (N58° 42.121' W93° 50.836') and one east near Ramsey Lake (N58° 43.670' W93° 46.554'), all of which are within a larger vicinity of one another and where the plant buffalo berry (*Shepherdia canadensis*) is present (Figure 2). Within each larger area, we pre-selected sub-groups of 2-6 plants randomly, with a minimum distance of 6 meters between each plant and collected data on 50 plants in total (Table 1). Plants were selected with the intent to collect a variety of shading within forest plots to ensure a viable distribution of canopy shading. The twin lakes site has four distinct subgroups of plants, while the larger area has a great degree of environmental heterogeneity from dry mineral soil to thicker organic layers as well as denser sections of canopy interspersed with more open areas. To summarize, the Twin Lakes site has the most diversity between subgroup's environmental characteristics, and as a result, the most plants of any group were collected there. The Ramsey Lake site is higher in elevation and less heterogeneous, with a more consistent canopy dispersion and organic layering with plants being collected in three subgroups. Lastly, the tundra site has four subgroups, each one located on the crests of beach ridge's where there is a thin organic layer on top of mineral soil (Table 1).

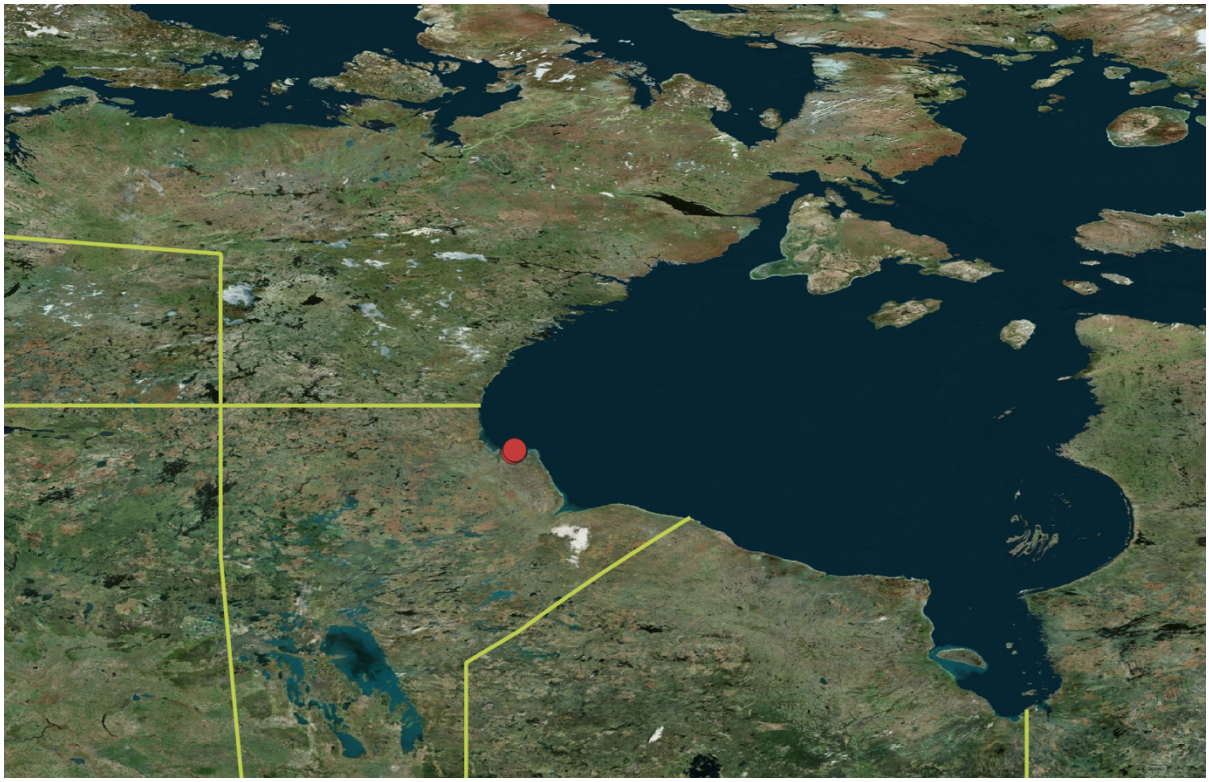

Figure 1. Churchill, Manitoba, Canada. The approximate location of sites from a greater perspective, encapsulating the main body of water to the east being Hudson's Bay.

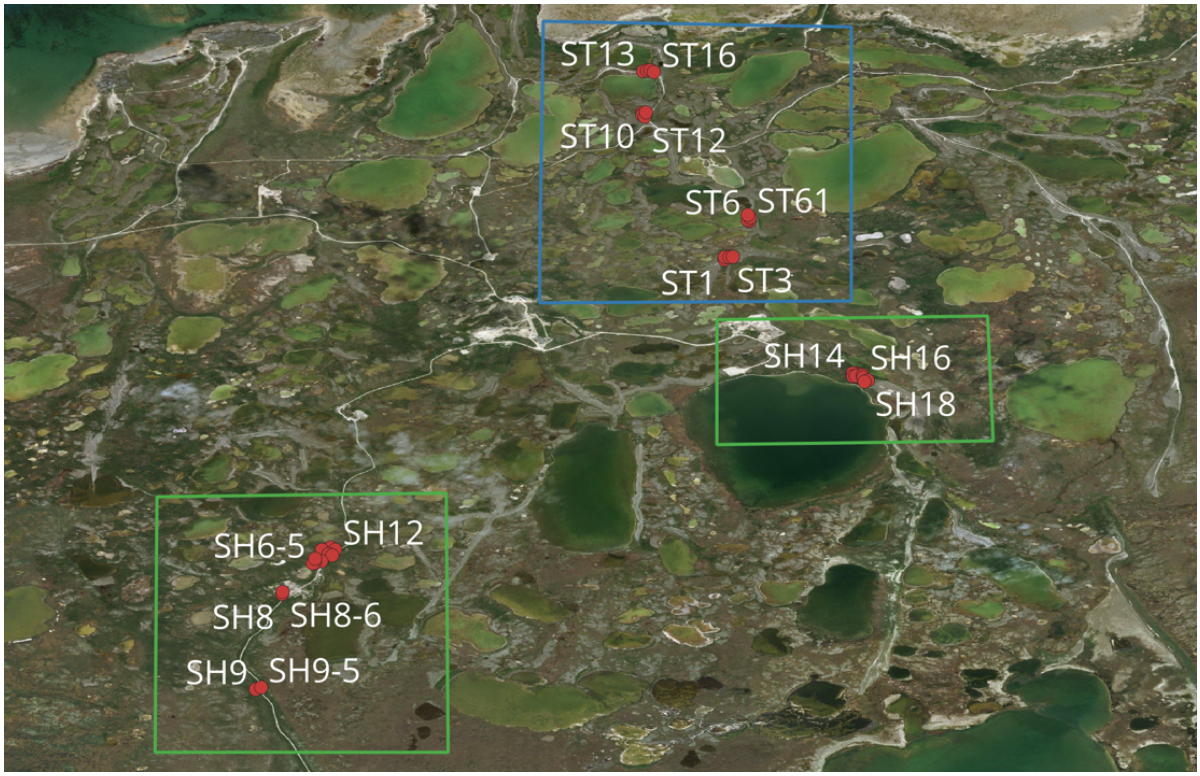

Figure 2. Satellite imagery of the distribution of plants across the general study area east of Churchill (Scale 1:16,818), with Hudson's Bay out of view but immediately north of the area. Points in Red represent a mixture of locations representing subgroups and individual buffalo berry plants. The green squares as well as labels coded "SH" represent buffalo berry located within the forest with the western square representing the Twin Lakes site and the eastern square representing the Ramsey Lake site. The blue square and label code "ST" represent each individual tundra plant.

Table 1. Summary of the number of plants data was collected from at each site with the forest containing two locations, while the tundra represents itself.

Forest		Tundra
Twin lakes	Ramsey Lake	
20	15	15

Experimental Protocol

At each designated plant, we collected the necessary data to determine nitrogen fixation, soil temperature, light availability, soil pH, soil nitrogen, specific leaf area, shrub productivity (via height and growth), $\delta^{13}C$, C:N ratio, N content, C content, total chlorophyll content, and shrub productivity (via height and growth). Control plant leaves from willow (*Salix athabascensis*) for N fixation reference were collected in correspondence with each local subgroup of plants (three buffalo berry per larger location, one willow per subgroup) to use as a reference for measuring nitrogen fixation for buffalo berry plants in the area. Across the second week (9th-12th) of August, at each designated shrub (reference included), 8-18 leaves (depending on size) were collected from different individual shoots. Before drying, individual leaves of each shrub were laid out and photographed at a constant frame of reference to maintain consistency and later determine specific leaf area using ImageJ. The leaves were then placed within a drying oven at 65°C and held at the CNSC until transport back to Winnipeg, where they were then dried again on the University of Manitoba campus and weighed prior to grinding. Photographs were analyzed using ImageJ software to determine the area (cm^2), and then the specific leaf area could be determined by dividing the area of each value by the total dry mass.

To obtain plant C and N isotope values, the dried leaf tissue was initially ground and mixed using a ball mill and then placed into individual 3-milligram tinfoil containers. Then, samples were sent to the University of Windsor stable isotope facility, where the isotopic ratios of nitrogen were measured via the ratio of ^{15}N to ^{14}N. The resulting $\delta^{15}N$ values from each sample were obtained from the isotope-ratio mass spectrometer which is able to determine the difference between the ratio of ^{14}N and the heavy ^{15}N isotope

(Boddy et al. 2000). Where Ndfa which is the amount of fixed nitrogen, can then be calculated in comparison to a reference value which cannot fix any nitrogen (Boddy et al. 2000). Prior to calculating Ndfa which uses the $\delta^{15}N$ from the fixing plant and compares it to the reference plant, an outlier value could be determined based on values which far exceeded the general trend range of values due to naturally occurring nitrogen depositions, which can skew the reference value away from the natural environmental nitrogen level. After obtaining values within the subgroups, two clear reference $\delta^{15}N$ plant outliers were obtained, one at Ramsey Lake and one at Twin Lakes, and although the original intention was to use each corresponding willow to each corresponding local plant subgroup, a change in procedure was made. The willow $\delta^{15}N$ outlier at Twin Lakes was located on a fox den, which is known to be an area of high nutrient deposition, which justified its removal as the corresponding buffalo berry in the area would be fixing negative or no nitrogen (Lang et al. 2022). For the Ramsey Lake area, we were aware it was frequent to local human activity, where small amounts of waste or refuse were the likely cause for such a high $\delta^{15}N$ value. Initial attempts were made using means reference values between locations as well as just corresponding reference values with outliers eliminated, which reduced the sample size to calculate Ndfa. Eventually, with the ten remaining δ15N reference values, we decided the best course of action was to take the mean of the values in correspondence to their habitats, which were six forest and four tundra willows. These two values were then used to calculate Ndfa depending on the location of the buffalo berry in question. From previous experiments in the lab, a value of 0.65 was able to be used as the B constant in the Ndfa equation, which is a plant that acquires all its nitrogen from fixation.

Foliar C:N ratio, C content, N content, and $\delta^{13}C$ were also obtained from the mass spectrometer. $\delta^{13}C$ is the isotopic ratio of C^{12} and C^{13}, C and N content are determined by the percent mass of each sample, and C:N ratio was calculated by the mass ratio of each sample. This ratio was analyzed as an indication of plant nitrogen content to correlate nitrogen fixation with plant quality and growth, as lower plant nitrogen levels are associated with a higher C:N ratio, leading to increased plant nitrogen stress and, consequently, poor growth (Chen et al. 2019). Nitrogen-fixing plants have higher overall nitrogen content within their leaf tissue compared to non-nitrogen fixers, leading to a lower overall carbon: nitrogen ratio (Wright et al. 2005). In turn, a lower leaf C:N ratio is presumed correlate with a higher fixation output or available soil nitrogen compared to higher leaf C:N ratios.

Soil temperature was measured via a standard thermometer placed 10 cm into the organic layer at the base of each plant and sampled at the time of tissue collection. Readings were taken across 2-3 days of consistent weather in August, and it is expected that the depth of the organic layer the readings were taken from disregards the effect of variation in changing air temperature that can occur throughout the day.

Light availability was recorded using a hemispherical canopy photography camera, which is a 180-degree fisheye lens that captures the entire visible area around the camera's location (Frazer et al. 1999). Images were taken during periods of cloud overcast during the evenings to maintain precision during analysis. This is because when sunlight illuminates parts of the canopy, it can be difficult for the Gap Light Analyzer (GLA) program to distinguish between parts of the canopy and the open sky, whereas it is easier to distinguish the difference between canopy and a uniformly cloud covered sky. The

camera used a small tripod that was aligned North and placed above the most central point of the main stem. The Gap Light Analyzer (GLA) program was then used to analyze the images to calculate canopy openness and infer the total solar radiation that the plant receives in the specific area (Frazer et al. 1999). Analyses with the GLA software were performed using an adequate black-and-white threshold, which was unique to each image depending on the natural light levels (Frazer et al. 1999). With the parameter of the geographic location set, the program was able to calculate canopy openness, direct solar radiation, diffuse solar radiation, and total solar radiation for each image. Canopy openness is the proportion of area around the plant that is not obstructed by the canopy or viewed as an open sky (Frazer et al. 1999). Direct solar radiation is radiation in which the energy is unaffected and unfiltered by atmospheric effects, whereas diffuse solar radiation is radiation that is dispersed and absorbed by the atmosphere, total solar radiation is a combination of the two (Frazer et al. 1999). Notably, most of the solar radiation that reaches the surface on a cloudy day is diffuse radiation (Frazer et al. 1999).

At each buffalo berry plant, four individual samples of soil were collected in a pattern resembling the cardinal directions, half a meter from the base of the plant to achieve a homogenous sample, where the top litter layer was removed, and then a handful of soil approximately 20-30 cm deep was collected. Samples were then placed in a drying oven at 65°C at the CNSC until travel back to Winnipeg for processing. We determined inorganic soil nitrogen (ammonia and nitrate) through a micro diffusion assay. Prior to the assay, samples were sieved through a 2 mm screen, and 10-20 grams (depending on how much was available) of soil was weighed and mixed with 40-80 ml of 2M KCL solution (depending on soil mass), shaken to mix and left overnight where the following day the individual sample volume was measured, filtered, and stored in a freezer. The

microdiffusion assay and subsequent titration were conducted on the thawed samples the following week as outlined in Kahn et al. (2000), where 10 ml of sample extract were placed in a mason jar along with 0.2g MgO and 0.2g Devarda's alloy and sealed in the jar along with an indicator solution (Kahn et al. 2000). After 24 hours, an acid is used to return the indicator solution that was sealed in each sample to neutral, and its volume can be used to calculate inorganic soil nitrogen (Kahn et al. 2000).

Shrub productivity and quality were assessed by measuring the plant's height from its tallest point to the base of the stem and note was taken of whether the shrub is producing fruit or not. Five random shoot measurements were taken from each plant were taken and the mean, max, and minimum, lengths were calculated from these values. The length of new shoot formation can be determined by identifying the previous year's bud scar on the shoot of the plant and measuring the length from that bud scar to the tip of the new shoot (Urza and Sibold 2013). This indicates growth for the shoot during the current growing season indicating plant productivity (Urza and Sibold 2013).

Methods described by MacKinney (1941) were us to measure chlorophyll content of leaves. 25 mg of dried leaf tissue is weighed and placed into 10 mL tubes. The pigments were then extracted using 95% ethanol, and the samples were left in the dark for 24 hours and after extraction, the samples were then vortexed and centrifuged. The spectrophotometer is zeroed using the ethanol extracting solution, and the absorbance of the extract was measured at the specified wavelengths 649nm and 665nm. Chlorophyll a and chlorophyll b values were then calculated using these equations: Chl a = 13.95(A665) – 6.68(A649), Chl b = 24.96(A649) – 7.2(A665) and combined as a measure of total chlorophyll content.

Statistical Analysis

Initial statistical tests were performed on each variable comparing the differences between habitats. Either a two-sample t-test, welch's unequal variance test, or Wilcoxon ranked sum test was performed based on the ability to meet assumptions of normality and homoscedasticity. Based on the initial failure to meet these assumptions and after examining visual distributions of the data different transformations were made. The subsequent Welch's unequal variance test was conducted if no amendment could be made to the variance, while the Wilcoxon ranked sum test was performed if no amendment to the normality or variance was found through transformations indicated by the W statistic within the results section. The means were compared between habitats for the variables Ndfa, soil temperature, soil nitrogen content, shrub height, mean, max, and minimum new shoot growth, specific leaf area, $\delta^{13}C$, C:N ratio, N content, C content, and total chlorophyll content. A Fisher exact test was conducted to compare differences between the binary observation of fruiting between shrubs across forest and tundra.

Multiple linear regression models were conducted with the dependent variables being Ndfa and $\delta^{13}C$. The main explanatory variables tested were canopy-dependent variables like canopy openness, total solar radiation, and soil temperature. A variety of other explanatory variables predictors were tested in combination: soil nitrogen, specific leaf area, productivity measurements, $\delta^{13}C$, and total chlorophyll when theoretically appropriate as an explanatory variable. A Spearman's correlation matrix was conducted (Table 2) to outline which variables could be included together in combination as explanatory variables while avoiding multicollinearity. The correlation matrix included (Table 2) shows variables that are of direct interest like those relating to soil temperature and canopy openness, along with variables of less direct interest like C:N ratio and specific leaf area. Certain variables have been omitted from the table for practical reasons,

in actual testing, up to 20 variables were compared to be able to test all linear model possibilities. The threshold value determined to be a cut off for multicollinearity was 0.6, due to the conservative nature of Spearman's correlation test as it is nonparametric. Due to the high correlation of many of the direct variables of interest, multiple independent linear models were created, including a variety of non-correlated variables, to determine the best linear model or predictor of nitrogen fixation. An AIC (Akaike Information Criteria) test was conducted comparing the models and the best model corresponding with a Δ AIC = 0. All assumptions of a linear regression were met for linear models regarding the normality of residuals using a Shapiro-Wilks test, the homoscedasticity of residuals was tested using a Breusch-Pagan test**,** the degree of multicollinearity was tested by examination the variance inflation factor of the model. The Durbin-Watson statistic was calculated for each linear model to determine if there was spatial autocorrelation occurring. A majority of tested model values were just outside of the range of 1.5-2.5, at the 1.3-1.4 value, indicating a small degree of spatial autocorrelation.

Results

Table 2. An example of the Spearman's correlation matrix conducted prior to linear model testing to avoid multicollinearity; not all variables tested in the study are present in the table.

	Specific leaf area	Soil temp ºC	Canopy openness %	Direct radiation %	Diffuse radiation %	Total radiation %	Soil N µg N g-1	$\delta^{13}C$	C:N ratio	Ndfa
Specific leaf area	1.00									
Soil temp ºC	-0.45	1.00								
Canopy openness %	-0.49	0.70	1.00							
Direct radiation %	-0.52	0.72	0.93	1.00						
Diffuse radiation %	-0.46	0.69	0.98	0.92	1.00					
Total radiation %	-0.51	0.72	0.96	0.98	0.97	1.00				
Soil N µg N g-1	0.12	-0.42	-0.42	-0.35	-0.44	-0.39	1.00			
$\delta^{13}C$	-0.43	0.67	0.79	0.85	0.77	0.82	-0.40	1.00		
C:N ratio	-0.24	0.18	0.53	0.54	0.53	0.53	-0.30	0.48	1.00	
%Ndfa	0.35	-0.29	-0.42	-0.41	-0.46	-0.44	0.39	-0.43	-0.38	1.00

Table 3. Mean ± standard deviation calculated for variables from the forest (n = 35) and tundra (n = 15) habitats, as well as the corresponding p-value where significant differences in mean values have a **bold** p-value.

Variable	Forest	Tundra	p-value
%Ndfa	74.67 ± 8.18	68.00 ± 5.34	**0.0056**
Soil nitrogen (µg N g-1)	205.91 ± 112.58	110.02 ± 52.22	**0.0056**
Shrub height (cm)	38.71 ± 13.91	12.24 ± 3.06	**< 0.0001**
Mean shoot length (cm)	3.63 ± 1.80	5.20 ± 1.41	**0.0043**
Maximum shoot length (cm)	6.33 ± 3.67	7.69 ± 2.37	0.1126
Minimum shoot length (cm)	1.87 ± 1.17	3.16 ± 1.19	**0.0009**
Specific leaf area (cm^2/g)	46.51 ± 2.16	31.35 ± 1.73	**< 0.0001**
δ13C	-29.47 ± 1.24	-27.35 ± 0.62	**< 0.0001**
C:N Ratio	15.71 ± 2.07	16.82 ± 1.18	**0.0180**
%N Content	3.26 ± 0.42	2.94 ± 0.32	**0.0135**
%C Content	50.39 ± 1.23	49.33 ± 4.17	0.1746
Chlorophyll (µg/mL)	8.23 ± 1.99	6.48 ± 0.96	**0.0001**
Soil temperature ºC	10.65 ± 1.75	16.73 ± 2.26	**< 0.0001**
% Canopy openness	37.83 ± 12.42	98.14 ± 0.80	**< 0.0001**
% Direct radiation	52.52 ± 20.54	100.00 ± 0.00	**< 0.0001**
% Diffuse radiation	47.86 ± 16.25	99.64 ± 0.12	**0.0012**
% Total radiation	50.29 ± 17.40	99.82 ± 0.06	**< 0.0001**

Nitrogen Fixation

Shrubs in both habitats acquired a majority of their nitrogen through fixation (Table 3). Tundra shrubs fixed 8.93% less nitrogen than forest shrubs, which was significantly different between habitats ($t_{48} = 2.98$, $p = 0.0056$) (Table 3). The p-value was marginally

> 0.05 when testing for homogeneity of variance, so Welch's unequal variance test was also conducted to confirm the result ($t_{40} = 3.42$, $p = 0.0015$) (Table 3).

Canopy-Dependent Variables

Mean canopy-dependent variables like soil temperature ($t_{48} = -9.57$, $p < 0.0001$), direct ($W = 0$, $p < 0.0001$), diffuse ($W = 0$, $p < 0.0001$), and total solar radiation ($W = 0$, $p < 0.0001$), were approximately two times greater in the tundra compared to the forest (Table 3). Variables related to forest canopy and soil temperatures also demonstrated a significant linear relationship with nitrogen fixation. Reductions in canopy shading, more overall solar radiation, and higher soil temperatures all had a negative effect on Ndfa (Table 4). The best model, according to AIC (Table 4) used diffuse solar radiation (Figure 3C) as its predictor: Ndfa = 80.8966 − 0.1298 × diffuse solar radiation ($F_{1,48} = 11.92$, $p = 0.0012$). The model explained 19.90% of the variation in Ndfa, and for each increase in diffuse solar radiation, Ndfa decreased by -0.1298%. Canopy openness (Figure 3B) had substantial support (Table 4) as a predictor of nitrogen fixation: Ndfa = 92.94 − 0.086 × canopy openness −0.964 ×C:N ratio ($F_{2,47} = 6.417$, $p = 0.003436$). The model explained 21.45% of the variation in Ndfa and notably, canopy openness was the only significant coefficient ($p = 0.0329$), where for each 1 % increase in openness, Ndfa decreased by -0.086%. There was insufficient evidence to suggest the C:N ratio had a significant effect on Ndfa ($p = 0.1365$), although it was a part of the second-best model. The relationship for both canopy openness and diffuse radiation was not significant within the forest when tundra plants were excluded ($p>0.05$). Soil temperature similarly, had a significant linear relationship with Ndfa (Figure 3A) ($F_{2,47} = 6.417$, $p = 0.0040$), where increases in soil temperature corresponded with a reduction in nitrogen fixation. The best model containing soil temperature (Ndfa = 102.98 − 0.660 × soil temperature ºC −1.376 ×C:N

ratio) also contained C:N ratio as the other coefficient and explaining 20.92% (R^2 = 0.2145) of the variance in Ndfa. The AIC value (Table 4) for the best temperature model indicates the model has considerable support, as well as increases in both temperature ($p = 0.0395$) and C:N ratio ($p = 0.0214$) demonstrated a significant negative effect on nitrogen fixation. Direct solar radiation demonstrated a significant linear relationship with Ndfa ($F_{1,48} = 8.647$, $p = 0.00503$) and explained 15.26% of its variation. The best model (Ndfa = 80.162 − 0.11 × direct solar radiation) also had good but not strong AIC support (Table 4). Total solar radiation exhibited a significant linear relationship with Ndfa ($F_{1,48} = 8.647$, $p = 0.002125$) and explained 18.02% of its variation (Figure 3D). The best model only contained total solar radiation as its coefficient (Ndfa = 80.83 − 0.1253 × total solar radiation) with its AIC value giving it substantial support (Table 4), where increases in solar radiation corresponded with a decrease in nitrogen fixation. Notably, when tundra plants were excluded all mentioned linear relationships were insignificant ($p > 0.05$) when tested with forest shrubs only.

Table 4. Comparison of the most significant models with Ndfa, as well as the AIC analysis in determining the most significant model. Model terms are defined as Ndfa (nitrogen derived from fixation), canopy openness (openness), C:N ratio (ratio), total solar radiation (total), soil temperature (temp), direct solar radiation (direct), and diffuse (diffuse solar radiation).

Model	P-value	Adjusted R^2	Multiple R^2	AIC	ΔAIC	Weight
$Ndfa = \beta0+\beta1\cdot Openess + \beta1\cdot Ratio + \epsilon$	0.0034	0.181	0.215	344.84	1.023	0.064
$Ndfa = \beta0+\beta1\cdot Total + \epsilon$	0.0021	0.163	0.180	344.98	1.161	0.060
$Ndfa = \beta0+\beta1\cdot Temp+ \beta1\cdot Ratio + \epsilon$	0.0040	0.176	0.209	345.18	1.358	0.054
$Ndfa = \beta0+\beta1\cdot Direct + \epsilon$	0.0050	0.135	0.153	346.63	2.812	0.026
$Ndfa = \beta0+\beta1\cdot Diffuse + \epsilon$	0.0027	0.190	0.223	343.82	0.000	0.107

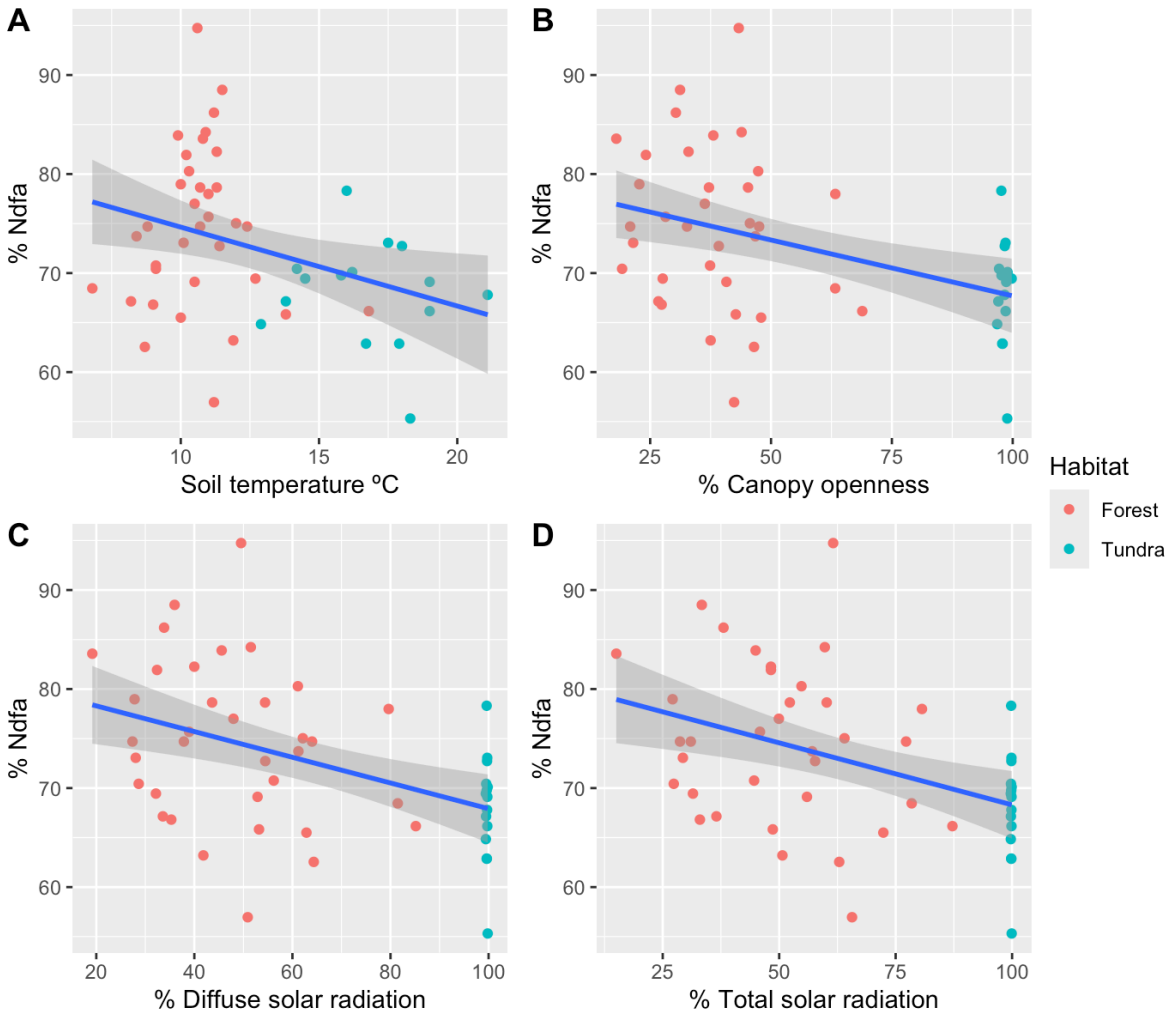

Figure 3. Scatterplots of the linear relationship between %Ndfa and (A) Soil temperature ºC, (B) % Canopy openness, (C) % Diffuse solar radiation, (D) % Total solar radiation. The dark grey area around each regression line represents a 95% confidence interval. All the linear regression models demonstrated substantial support by Δ AIC being less than 2 with plot B being the best overall.

Soil Nitrogen

The level of inorganic soil nitrogen significantly differed between the forest and tundra ($t_{48} = 2.98$, $p = 0.0056$) (Figure 4A), with the test of mean differences performed on square root transformed values. There was approximately twice as much soil nitrogen available in the forest sites compared to tundra (Table 3). An increase in available soil nitrogen (after square root transformation) corresponded with an increase in nitrogen fixation, demonstrating a significant linear relationship with %Ndfa (Figure 4B) ($F_{1,48} = 4.437$, $p = 0.0404$), with the model explaining 8.46 % of %Ndfa's variance. Although, this relationship presented the weakest support in AIC comparison with other models ($\Delta AIC = 6.67$) and was similar to previous results this relationship was insignificant ($p = 0.51$) when tundra shrubs data was excluded. The coefficient of the model indicated that for each unit increase in the square root of available soil nitrogen (after transformation), the expected Ndfa value would increase by 0.6096, although the rate of increase decreases as soil nitrogen rises. Ultimately, soil nitrogen was insignificant as an effect on Ndfa in combination as an additive effect or as an interaction term in other linear models tested and did not improve any metrics either. However, soil nitrogen did exhibit a notable linear relationship when considering soil temperature as a predictor ($F_{1,48} = 4.437$, $p = 0.0404$), where increases in soil temperature lead to a significant decrease in available soil nitrogen. The model explained 21.39% of the variance, with each increase in soil temperature corresponding to a -0.5211 decrease in the square root of soil nitrogen, indicating that the effect diminishes as soil nitrogen increases.

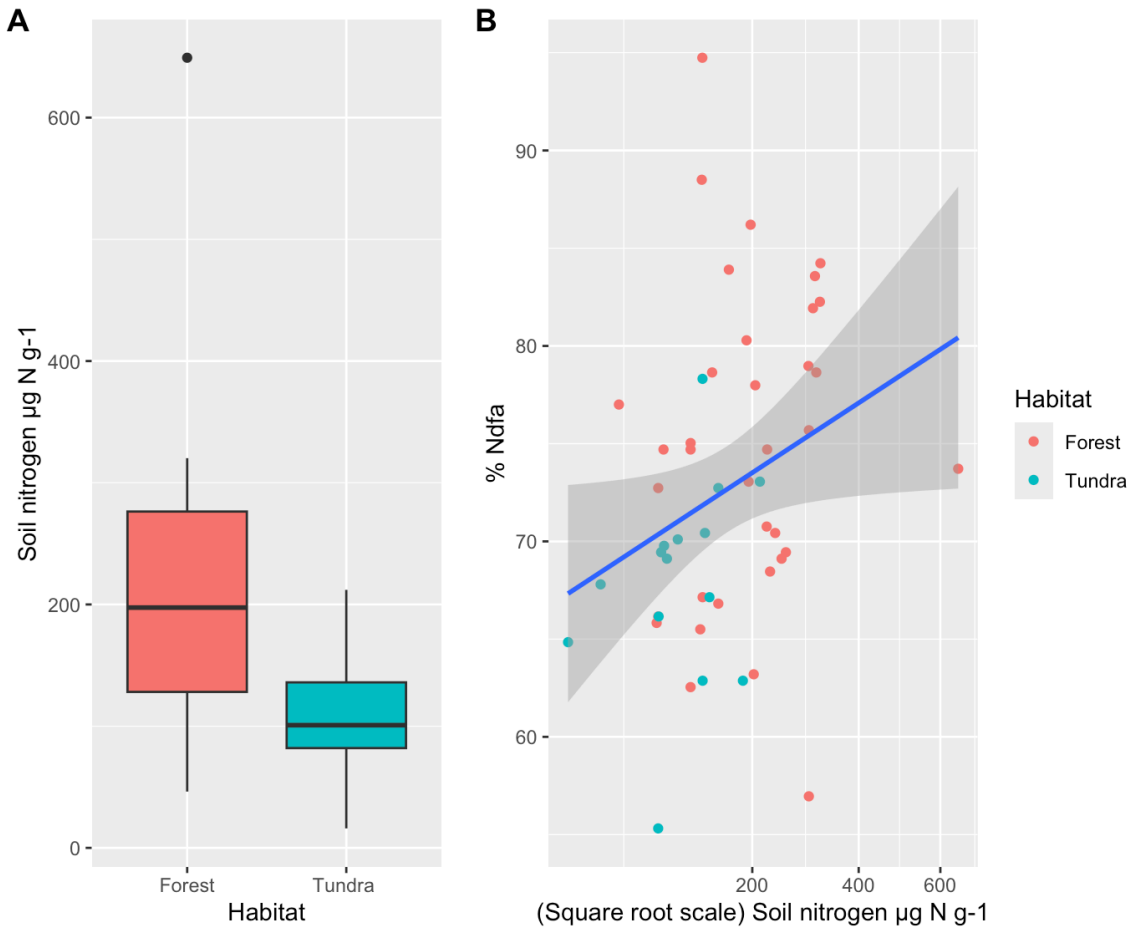

Figure 4. (A) Comparison of the mean inorganic soil nitrogen µg N g-1 between forest (n = 35) and tundra (n = 15) buffalo berry shrubs. (B) Representation of the linear relationship between (square root transformed) inorganic soil nitrogen µg N g-1 and %Ndfa where the dark shading around the regression represents a 95 percent confidence interval.

Productivity

Shrub characteristics like shrub height, mean, minimum, and max shoot length, as well as the presence of fruit were evaluated across habitats and variation in canopy openness. Comparisons of the mean shrub heights between habitats concluded that they were significantly different in their means ($t_{40.85} = 10.67$, $p < 0.001$) and that forest shrub height

was approximately three times greater than tundra shrubs on average (Table 3). A non-parametric equivalent was performed due to violating the assumption of normality and also found a significant difference in shrub height between habitats ($W = 510$, $p < 0.0001$), with a further one-sided ranked sum test showing shrub height was greater in forest shrubs than tundra shrubs ($W = 510$, $p < 0.0001$). Shrub height had an insignificant effect on Ndfa in any of the tested linear models and showed no effect of improving any either. Mean shoot length significantly differed between forest and tundra shrubs ($t_{48} = -2.99$, $p = 0.0043$), with tundra shrubs being 30.77% longer than forest shrubs on average (Table 3). However, the maximum shoot length observed did not significantly differ between habitats ($t_{48} = -1.62$, $p = 0.1126$) (Table 3). The minimum observed shoot length did significantly differ between forest and tundra ($t_{48} = -3.54$, $p = 0.0009$) and was shorter in forest shrubs by 40.8% compared to tundra shrubs (Table 3). A Fischer's exact test determined there was a significant relationship between habitat and the presence of fruiting as all observations of fruiting occurred in forest shrubs ($\chi^2 = 7.26$, $df = 1$ $p = 0.0019$). Overall, there were insignificant trends when including measures of shrub productivity in any of the tested linear models as an additive effect or as an interaction term regarding Ndfa. A low degree of correlation was found in the overall correlation matrix for each of height (0.16), mean (-0.09), maximum (0.04), minimum (-0.13), and fruiting (0.10) in relation to Ndfa. A similar lack of correlation was found between each productivity variable with foliar C, N, and chlorophyll values, as no significant results were found, and all correlations between each were generally low ($\rho < 0.2$).

Specific Leaf Area

Specific leaf area was 32.60% greater in shrubs found within the forest compared to shrubs found in the tundra (Table 3). While mean SLA significantly differed between the

forest and tundra ($t_{48} = 4.67$, $p < 0.0001$) and was performed on log-transformed values (Figure 5A). Log-transformed SLA also demonstrated a significant linear relationship with %Ndfa (Figure 5B) ($F_{1,48} = 5.19$, $p = 0.0272$), with the linear model explaining 9.76 %) of %Ndfa's variance. The parameter of the model indicated that for each increase in log-transformed SLA, the expected %Ndfa value would increase by 7.95%, with the effect diminishing as SLA increases, suggesting that larger, thinner leaves correspond with a modest increase in nitrogen fixation. Furthermore, this relationship had lower support in AIC model selection when compared with other models ($\Delta AIC = 5.96$) and the relationship was insignificant within the forest when tundra shrubs were excluded ($p > 0.05$). Also including SLA as an additive or interaction variable with one of the more significant predictor variables in Table 4 generally reduced the strength and significance of those models. Notably, both direct solar radiation and plant height were strong predictors of log-transformed SLA, with increases in direct radiation corresponding with a negative dwindling effect on SLA, while increases in plant height were associated with a diminishing, yet positive, effect on SLA (Figure 6) ($F_{2,47} = 14.38$, $p < 0.0001$) and explained 37.97% of its variance. Each increase in direct solar radiation corresponds with a -0.0034 decrease in log-transformed SLA, while each increase in shrub height corresponds with a 0.0075 increase in log-transformed SLA. Both were insignificant within the forest with tundra shrubs removed ($p > 0.05$).

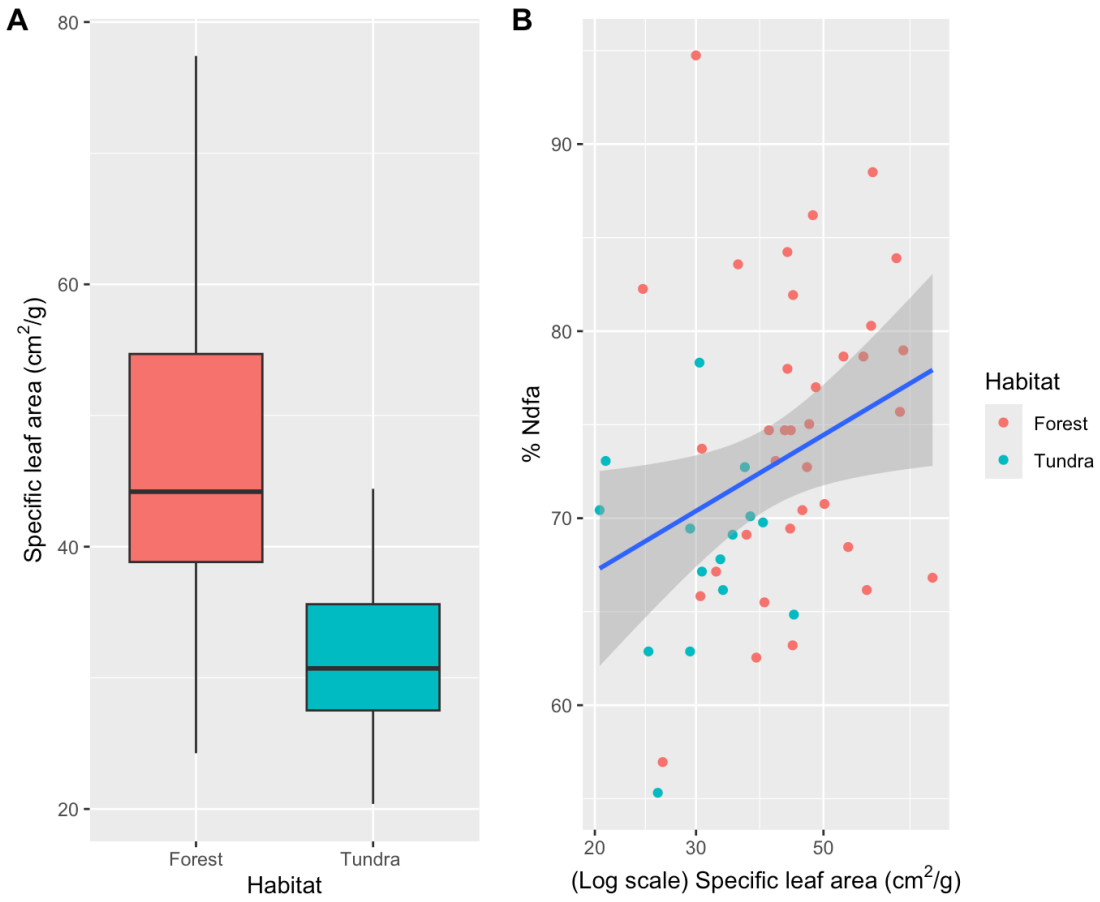

Figure 5. (A) Mean specific leaf area (cm^2/g) compared between forest and tundra buffalo berry shrubs. (B) Representation of the relationship between (log scale) SLA cm^2/g and %Ndfa where the dark shading around the regression represents a 95 percent confidence interval.

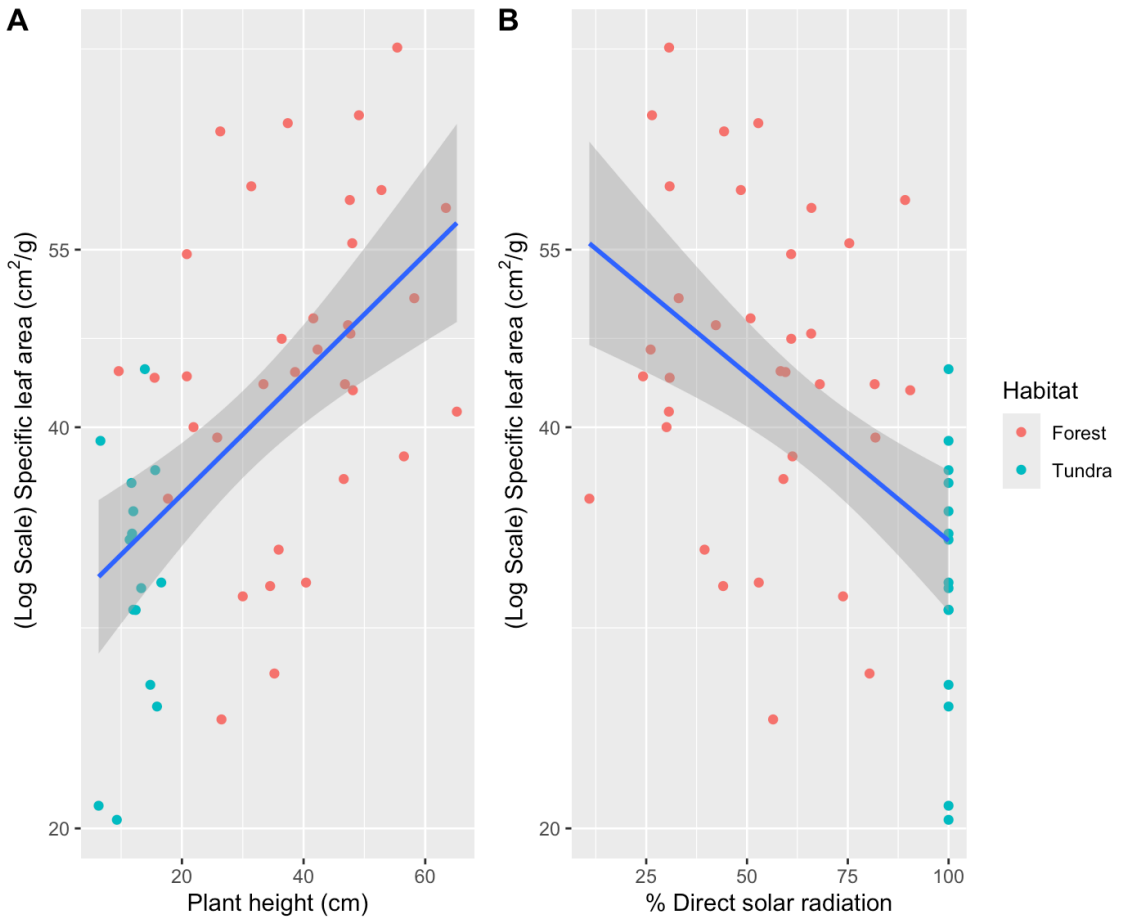

Figure 6. Representation of the relationship between the (log scale) specific leaf area (cm^2/g) and (A) plant height (cm), (B) % direct solar radiation. Both in combination are the strongest predictors of specific leaf area (cm^2/g): log SLA = 3.6865 − 0.0034 × direct solar radiation + 0.0075 × plant height (cm)

$\delta^{13}C$

Tundra shrubs had 7.19% higher levels of foliar $\delta^{13}C$ compared to forest shrubs, as the mean values significantly differed between the forest and the tundra ($t_{46.59}$ = -8.04, p <0.0001) (Table 3). Canopy-dependent variables (soil temperature, canopy openness, direct, diffuse, and total solar radiation) each demonstrated a significant linear relationship with $\delta^{13}C$ (Table 5). Increases in canopy openness, solar radiation, and soil

temperature corresponded with increases in foliar $\delta^{13}C$ across the forest and tundra. Models were constructed regarding the correlation values from Table 2 to avoid multicollinearity. From the best model according to a second AIC model selection, log-transformed total solar radiation significantly explained 74.60 % of the variation in $\delta^{13}C$ ($F_{1,48} = 144.9$, $p = <0.0001$). The best model ($\delta^{13}C = -39.76 + 2.68$ * log-transformed total solar radiation) demonstrates a strong linear relationship (Figure 7A), with the log transformation revealing a diminishing effect of total solar radiation on $\delta^{13}C$ as solar radiation increases. The second best model according to AIC (Table 5), used square root transformed direct solar radiation which significantly explained 74.00 % ($R^2 = 0.7400$) of the variation in $\delta^{13}C$ ($F_{1,48} = 140.2$, $p = <0.0001$). The second-best model ($\delta^{13}C = -34.33 + 0.690$ * square root-transformed direct solar radiation) also demonstrates a smaller probability than the best overall model (Table 5), with the square root transformation revealing a diminishing effect of direct solar radiation on $\delta^{13}C$ as solar radiation increases, and a similar slope of the line (Figure 7B). Both models notably remained significant when tundra plots were excluded ($p > 0.05$), demonstrating a significant linear relationship within the forest also. $\delta^{13}C$ had a significant linear relationship with Ndfa ($F_{1,48} = 8.276$, $p = 0.00598$), where increase in foliar $\delta^{13}C$ corresponded with a reduction in %Ndfa. The model explained 14.71% of the variation in Ndfa ($R^2 = 0.1471$) and received good but not substantial support in AIC model comparison ($\Delta AIC = 3.38$), although was insignificant within the forest when tundra shrubs were excluded ($p > 0.05$).

Table 5. Comparison of all the significant models concerning $\delta^{13}C$, as well as the AIC analysis in determining the most significant model. Model terms are defined as canopy openness (openness), total solar radiation (total), soil temperature (temp), direct solar radiation (direct), and diffuse (diffuse solar radiation).

Model	P-value	Adjusted R^2	Multiple R^2	AIC	ΔAIC	Weight
$\delta^{13}C = \beta0+\beta1\cdot Direct +\epsilon$	<0.0001	0.740	0.745	116.73	1.25	0.3481
$\delta^{13}C = \beta0+\beta1\cdot Diffuse +\epsilon$	<0.0001	0.677	0.683	127.53	12.05	0.0016
$\delta^{13}C = \beta0+\beta1\cdot Total +\epsilon$	<0.0001	0.746	0.751	115.49	0.00	0.6490
$\delta^{13}C = \beta0+\beta1\cdot Temp+\epsilon$	<0.0001	0.330	0.344	164.00	48.51	<0.0001
$\delta^{13}C = \beta0+\beta1\cdot Openness +\epsilon$	<0.0001	0.682	0.675	127.81	12.05	0.0003

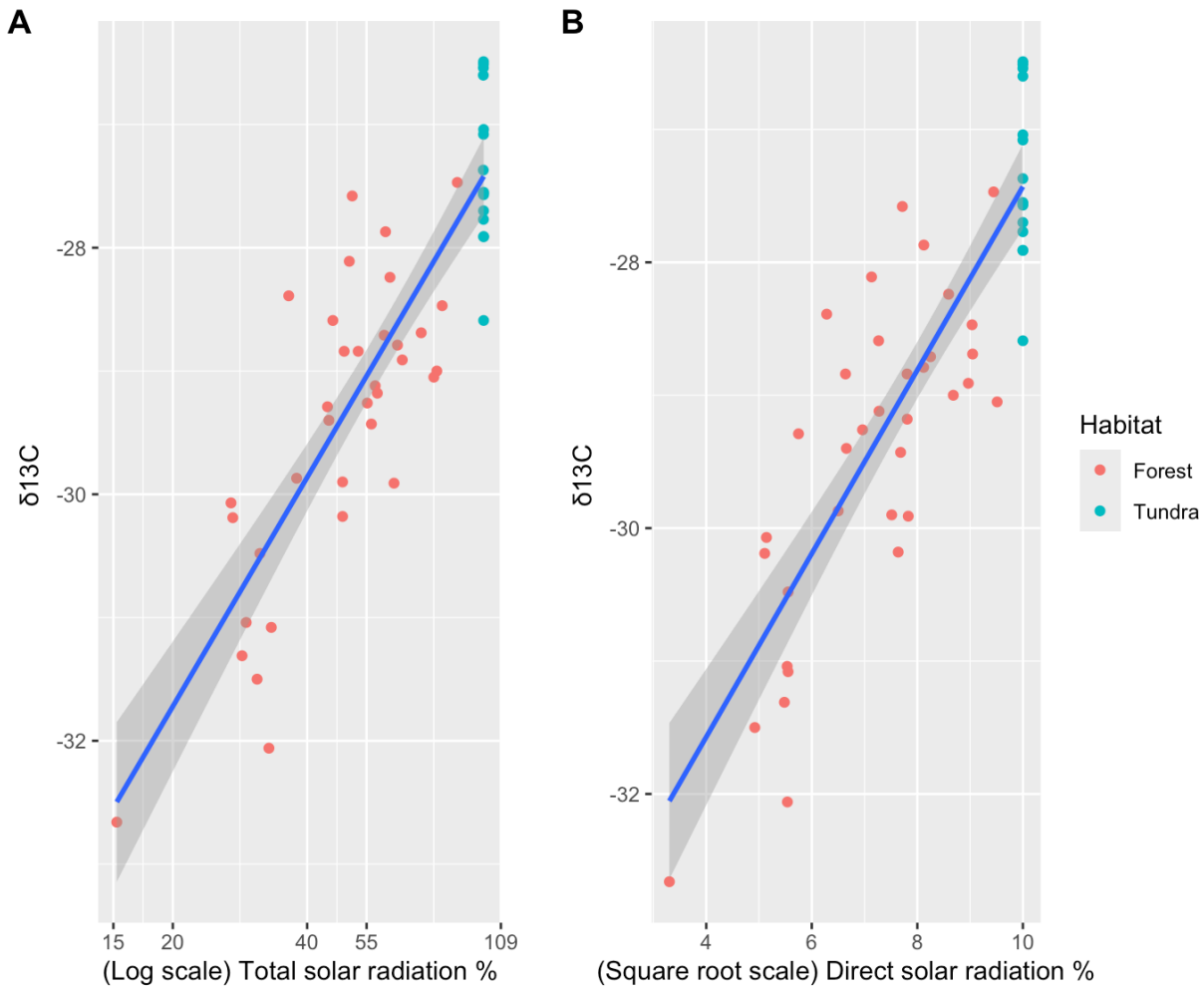

Figure 7. Plot of the two models with the most substantial support in explaining $\delta^{13}C$, (A) Log-transformed total solar radiation is the model with the strongest support while (B) is square root transformed direct solar radiation which is the second model with less, but still substantial support. The shaded area around the line represents a 95% confidence interval.

Foliar C and N

The C:N ratio, N content, and C content of collected leaves all should represent or indicate a degree of plant productivity and overall nutrient availability to the plant. Tundra shrubs had 6.60% higher foliar C:N ratios compared to forest shrubs and the difference in mean values was significant between the forest and the tundra ($t_{42.433} = -2.46$, $p = 0.0180$)

(Table 3). C:N ratio also demonstrated a significant linear relationship with Ndfa ($F_{1,48}$ = 7.413, p = 0.009), with the linear model explaining 13.38%) of Ndfa's variance and received good but not substantial support in AIC model comparison (ΔAIC = 3.91)(Figure 8A). The parameter of the model indicated a negative relationship, as for each increase in C:N ratio, the expected %Ndfa value would decrease by 1.60%. Furthermore, even if not significant C:N ratio improved other models with notable substantial support in AIC model comparison (Table 4) as an additive effect. Mean N content significantly differed between the forest and the tundra (t_{48} = 2.57, p = 0.0135) (Table 3), with 9.81% higher overall N content in forest shrubs comparatively. N content also had a significant linear relationship with Ndfa ($F_{1,48}$ = 7.5, p = 0.0084), with the linear model explaining 13.6% of Ndfa's variance, with good but not substantial support in AIC model comparison (ΔAIC = 3.79) (Figure 8B). The positive parameter of the model indicated that for each individual increase in N content, the expected Ndfa's value would increase by 7.07%. Notably both C:N ratio and N content linear relationships with Ndfa remained significant within the forest when tundra plots were excluded (p > 0.05). The mean C content did not significantly differ between the forest and the tundra (t_{48} = 1.38, p = 0.1746)(Table 3). Using a separate test, the Wilcoxon ranked sum test also demonstrated no significant difference in leaf C content between the two habitats (W = 302.5, p = 0.2015). Leaf C content did not demonstrate a significant linear relationship with Ndfa either ($F_{1,48}$ = 0.67, p = 0.4154).

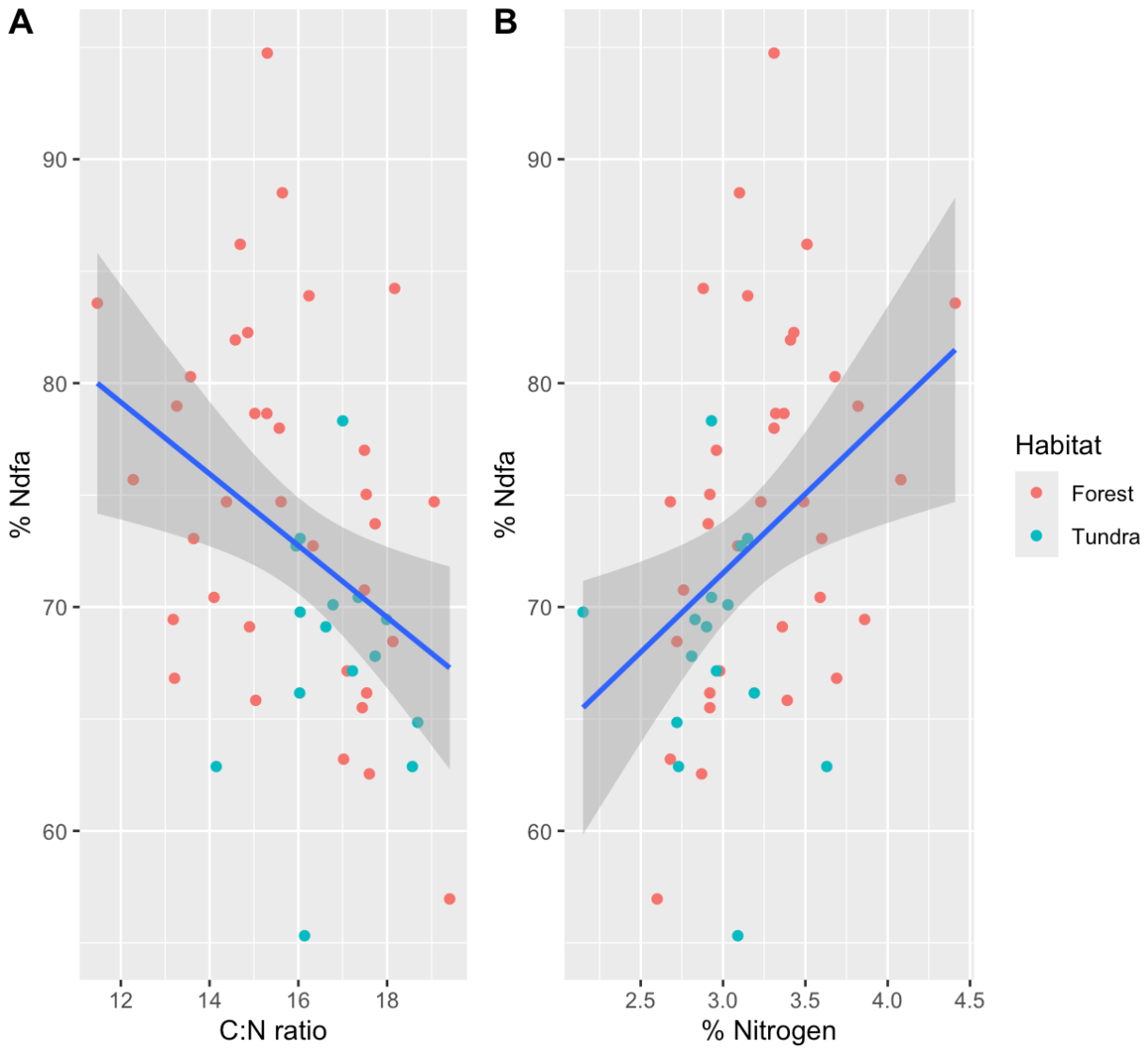

Figure 8. Linear relationships of Ndfa and: (A) C:N ratio. Ndfa = 98.3698 − 1.6018 × C:N ratio, (R^2 = 0.1338) and (B) leaf N content. %Ndfa = 91.8724 + 7.070 × N content, (R^2 = 0.136).

Chlorophyll

Chlorophyll content was evaluated to reflect plant photosynthetic capacity and nutrient content, specifically nitrogen. The mean chlorophyll content was significantly higher in forest shrubs by 21.26% compared to tundra shrubs ($t_{47.16}$ = 4.19, p = 0.0001) (Table 3)

and was determined using Welch's unequal variance t-test. Chlorophyll content also demonstrated a significant positive linear relationship as a predictor of Ndfa ($F_{1,48} = 8.72$, $p = 0.0048$) and received good but not substantial support in AIC model comparison ($\Delta AIC = 2.74$). The linear model explained 15.38% of the variance in Ndfa, with the positive coefficient indicating for each increase in total chlorophyll content, the expected Ndfa value would increase by 1.64%. However, this relationship was insignificant within the forest when tundra plants were removed ($p > 0.05$). Notably, C:N ratio (Figure 9B) and direct solar radiation (Figure 9A) in combination were both the strongest significant predictors of total foliar chlorophyll content ($F_{2,47} = 57.24$, $p < 0.0001$) and explained 70.89% ($R^2 = 0.7089$) of its variation. The best model (chlorophyll content = 18.6354 − 0.5650 × C:N ratio − 0.0279 × direct solar radiation) reflects that as both buffalo berry C:N ratio and direct solar radiation increases, total foliar chlorophyll content will decrease in a linear fashion (Figure 9). Furthermore, N content was positively correlated with Chlorophyll content (0.71), while other canopy-related factors like total(-0.73), direct (-0.71), and diffuse (-0.70) solar radiation were negatively correlated to a similar scale.

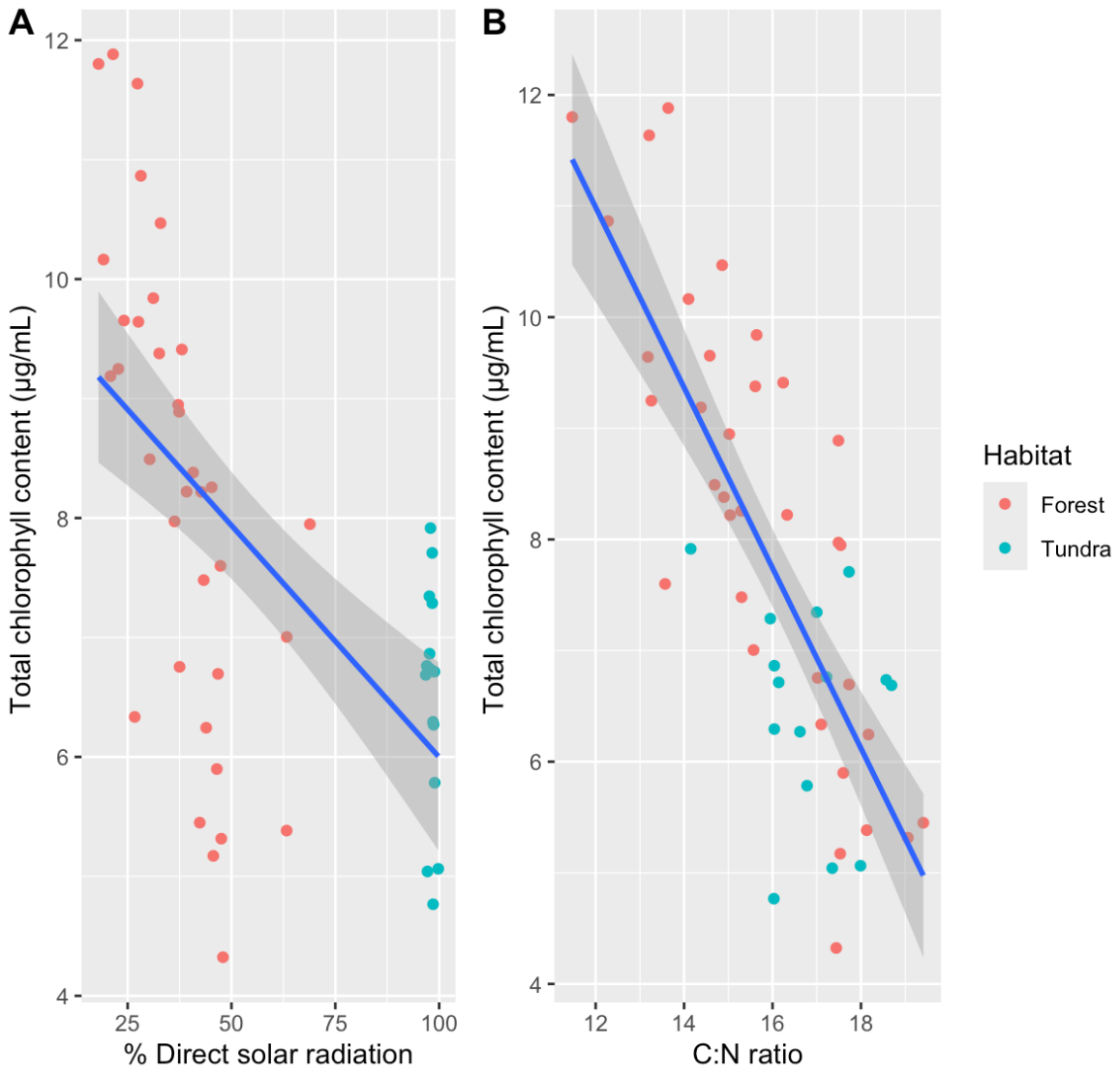

Figure 9. Linear relationships of total chlorophyll content (µg/mL) and (A) direct solar radiation and (B) C:N ratio, as both combined as strong predictors of total chlorophyll content (µg/mL) with the best model: Chlorophyll content (µg/mL) = 18.6354 − 0.5650 × C:N ratio − 0.0279 × % Direct solar radiation.

Discussion

Actinorhizal plants like buffalo berry can colonize nitrogen-limited, low-nutrient areas during early succession or after a disturbance, increasing nitrogen levels in the soil through symbioses with nitrogen-fixing bacteria and facilitating the succession of other

species (Visser et al. 1991). Comprehending how nitrogen fixation in the shrub buffalo berry changes across varying environmental conditions can contribute to a greater understanding of how understory nitrogen-fixing plants within nitrogen-limited high-latitude ecosystems will respond to a changing climate and influence the environments they occupy through nitrogen deposition.

The overall results regarding nitrogen fixation oppose the initial hypothesis that shrubs present in more open areas with more solar radiation and increased soil temperatures should fix more nitrogen compared to shaded shrubs with lower soil temperatures and reduced solar radiation. However, the linear relationship between canopy openness, increased solar radiation, and soil temperatures as predictors of nitrogen fixation was not significant, specifically within the forest when tundra plants were excluded, suggesting the variables' effect on fixation was primarily a result of habitat differences and not a linear relationship. This was because there was still greater overall nitrogen fixation in the forest, which had lower soil temperatures and less solar radiation due to canopy shading compared to the tundra. These results differ from expectations as lower environmental soil temperatures have demonstrated a reduction in nitrogenase activity and hence nitrogen fixation, in other actinorhizal shrubs (Uliassi and Ruess 2002; Mitchell and Ruess 2009; Anderson and Markham 2021). The effect of restricted light availability due to shading which in turn amplifies the cost of plant photosynthetic products to fulfill the symbiotic allocation for nitrogen fixation (Vitousek et al 2002; Houlton et al. 2008; Gundale et al. 2012) appeared to be completely mitigated for buffalo berry, demonstrating a relationship in the opposite direction than expected. However, this cost is likely reduced by the higher specific leaf area demonstrated by plants in the forest with more shaded and cooler areas, as leaves with higher SLA are more cost-effective, photosynthetically

efficient, and thinner than thicker lower SLA leaves (Dwyer et al. 2014; Markham and Anderson 2021). The results showed a decrease in nitrogen fixation with increasing canopy openness and forms of solar radiation as the predominant explanatory factor across tested linear models, while increased soil temperature also was highly correlated and produced similar conclusions which at best explained only 20% of the variation in fixation. This is in opposition to results found by Mitchell and Reuss (2009) which found reductions in fixation corresponded with reductions in soil temperature, although their best results similarly explained only about 20% of the overall variance which they also noted to be the common result among a few other studies.

One potential explanation for these contrasting results regarding fixation could be the amount of inorganic soil nitrogen present. Available soil nitrogen was lower around shrubs located in the tundra and demonstrated a significant positive linear relationship with nitrogen fixation. The relationship was not as strong in comparison to other models through model selection, but was complementary in its results to the best models, as higher available soil nitrogen was associated with forest shrubs that experienced increased canopy shading and less solar radiation. Notably, soil temperatures were cooler in the forest compared to the tundra as expected, although due to the short time of sampling, which was also a warmer-than-average week for the area, it is hard to be confident about how representative these temperature values are over the whole growing season. However, we can suggest that the observed difference in soil temperature between habitats is just as, if not more pronounced, during the spring, where the presence of canopy and thicker organic layers in the forest likely delays the soil warming process to a greater effect compared to the tundra (Anderson and Markham 2021). Furthermore, the opposite likely occurs in the fall or towards the end of the growing season, where forest

soils remain warmer and tundra soils become cooler more quickly in comparison. Regardless, if our soil temperature values are representative of a maximum peak in soil temperatures throughout the growing season, they are still in agreement with expectations, as open tundra areas have demonstrated higher mean, maximum, and minimum soil temperatures throughout the summer in comparison to the forest (Aalto et al. 2022). The decrease in observed available soil nitrogen collected around buffalo berry shrubs located in the tundra could be a result of increased decomposition due to increased soil temperatures associated with thinner organic layers and little to no canopy shading, as suggested in Markham and Anderson (2021). This could partially explain the reduction in observed fixation of tundra and more open forest shrubs, as there are many examples of reductions of fixation in response to increased available soil nitrogen which the plants will preferably take up instead of fixing nitrogen (Uliassi and Ruess 2002; Markham and Zekveld 2007; Ruess et al. 2013; Markham and Anderson 2021). In turn, this would explain the higher levels of available nitrogen found in forest soil locations, as there is less decomposition occurring, purely based on lower soil temperatures (Chapin et al. 1991; Allison and Treseder 2011). Although higher leaf C:N ratio, lower N content, and lower SLA can reduce decomposition rates (Cornwell et al. 2008; Prescott 2010), all traits were more pronounced on tundra leaves. This likely reduced the amount of nitrogen available in the soil, to begin with and potentially counteracted the effect of increased soil temperatures increasing decomposition, although it is hard to estimate the size of the effect on available soil nitrogen between the two habitats. Overall, lower-quality litter deposition, reduced nitrogen fixation, and increased decomposition due to higher soil temperatures all likely contributed to the reduced amount of available soil nitrogen found on the tundra.

Increases in shrub biomass in high-latitude areas that have been attributed to warming have mainly been associated with increased shrub growth, range expansion, dominance, and canopy height mainly in shrubs like *Betula*, *Salix* and *Alnus* spp. (Mitchell and Ruess, 2009; Myers-Smith et al.). Productivity and growth differences in buffalo berry appeared to be more plastic in response to their habitats with forest shrubs generally being taller with shorter shoots, while tundra shrubs appeared to be shorter compensating with longer shoots. However, it is hard to determine the relevance of the observed shrub habitat height difference, as increases in insulative properties based on the snow-holding capacity of shrubs are mainly related to shrub height and density (Strum et al. 2001). Further investigation is required to determine if changes in the snow holding capacity as a result of increased shrub height on the tundra are occurring with buffalo berry on the tundra in this area specifically. Changes in shrub density along with height are the key part of tundra shrubification, which has the potential to influence tundra soil insulative properties and species composition (Sturm et al. 2001; Myers-Smith et al. 2011). The only inference that can be made concerning changing abundance is that no plants were observed to be fruiting on the tundra, while a third of the observed plants possessed fruit in the forest. The strongest predictors of fruit production in buffalo berry can be attributed to the previous year's rainfall (Krebs et al. 2009), while another experiment by Hamer (1996) found a reduction in buffalo berry fruit production in response to increasing canopy cover which differed from the observations in this experiment. A later experiment by Boonstra et al. (2017) found that buffalo berry fruit production was not limited by available nitrogen, as their two-year fertilizer treatment experiment found no change in berry production and size across varying levels of environmental nitrogen levels. This implies that while tundra shrubs experience a slightly higher degree of nitrogen-related resource

limitation compared to forest shrubs, it does not appear to affect fruit production. Instead, the availability of moisture from the previous year is the most significant factor influencing buffalo berry fruit production, while lower soil temperatures and solar radiation in the forest may help retain moisture during peak summer temperatures, unlike the hotter, drier conditions of the tundra.

Leaf chlorophyll content was highly correlated with N content and demonstrated a significant linear relationship with Ndfa, as predicted. Generally, a majority of available nitrogen is allocated toward photosynthetic processes and pigments like chlorophyll (Evans and Clarke 2019). Our findings aligned with this principle as plants in the forest which were fixing more nitrogen had higher N content, lower C:N ratio, higher SLA, and greater height overall. This result in regards to leaf C:N ratio and nitrogen fixation corresponded with Anderson and Markham's (2021) findings, but also differed as their result was attributed to low water availability due to colder soils, whereas the results of this paper were due to warmer temperatures and available solar radiation. This highlights the importance of water availability for nitrogen fixation, which another variable, $\delta^{13}C$, has the potential to reflect.

$\delta^{13}C$ values closer to zero can reflect a higher incidence of stomatal closure as a result of decreased transpiration associated with drier conditions, in turn leading to a reduced photosynthetic capacity (Farquhar et al. 1982; Bchir et al. 2016). This effect could be seen by the highly significant linear relationship $\delta^{13}C$ demonstrated with total and direct solar radiation that was found in plants in the tundra and open forested areas. Considering the strength of this effect, where total sunlight accounted for about 75% of the variation in $\delta^{13}C$, along with variables related to solar radiation demonstrating the strongest explanatory effect for nitrogen fixation, we can summarize that water limitation is the

predominant limiting factor to nitrogen fixation in buffalo berry. This would correspond with several other findings of nitrogen fixation being limited by moisture variability in other actinorhizal shrubs (Uliassi and Ruess 2002; Mitchell and Reuss 2009; Markham and Anderson 2021). For example, Uliassi and Ruess (2002) found that moisture was the predominant limiting factor for nitrogen fixation in *A. tenuifolia,* with their best linear model explaining 29% of its variance. Mitchell and Reuss (2009) noted the sensitivity of fixation in *A. viridis* to precipitation, with fixation being notably lower during a drought year compared to wetter years. Anderson and Markham (2021) observed a reduction in C^{13} discrimination as a result of stomatal closure in response to colder soils reducing root water uptake in *A. viridis*, which differs in comparison to our result where increasing soil temperatures corresponded with a decrease in C^{13} discrimination. But, the outcome of reductions in C^{13} discrimination or more positive $\delta^{13}C$, resulted in a similar increase in leaf C:N ratio and reduction in Ndfa, highlighting the sensitivity of high-latitude actinorhizal plants to water availability (Anderson and Markham 2021). This supports Markham and Anderson's (2021) suggestion that most actinorhizal shrubs possess a lower drought tolerance in comparison to legumes, meaning that at higher latitudes where most actinorhizal shrubs are prevalent, drought conditions will likely limit nitrogen fixation.

Conclusion

Overall, buffalo berry shrubs in the forest and tundra near Churchill, Manitoba, appear to be getting most of their nitrogen from fixation. Moisture availability appears to be the strongest limiting factor to nitrogen fixation in buffalo berry, where open areas like the tundra and forested areas with less canopy shading result in higher amounts of solar radiation and soil temperature, which is not only reflected in a small decrease in Ndfa values but a strong linear relationship with $\delta^{13}C$. This result has larger implications

regarding climate change and the observed increase in shrub height and abundance with other shrub species in high-latitude nitrogen-limited areas (Myers-Smith et al. 2011). Suggesting that the influence of increased nitrogen deposition from changes in actinorhizal shrub growth and abundance should be given more attention in high-latitude shrubification studies than previously acknowledged. Furthermore, the capacity of actinorhizal shrubs to input nitrogen into the tundra in the context of increased warming due to climate change will also be linked to soil moisture availability (Mitchell and Reuss 2009). It appears that buffalo berry productivity, as well as nitrogen fixation across the boreal forest and tundra, will be dependent on changing environmental gradients in soil moisture availability too. Regardless, because buffalo berry shrubs in both the forest and tundra are fixing a majority of their available nitrogen, recognizing their contribution to environmental nitrogen deposition in high-latitude nitrogen-limited habitats should warrant further consideration.

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