

**Ecology of Arctic Legumes in Upland Tundra Heath near Rankin Inlet,
Nunavut**

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By

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Abstract

Restoring mining sites in Arctic regions poses significant challenges due to slow natural ecosystem recovery, which can take decades or even centuries given the harsh environmental conditions. Compounding this challenge is the scarcity of native seeds and the reliance on fast-growing non-native species, which can disrupt ecosystem processes and become invasive. These studies focus on understanding the ecology of four native Arctic legumes and provide recommendations for their use in targeted restoration strategies.

In the first study, nodules from disturbed gravel areas and adjacent intact tundra near Rankin Inlet, Nunavut, Canada, were collected from four native legume species — *Astragalus alpinus*, *Hedysarum americanum*, *Oxytropis arctica*, and *Oxytropis maydelliana*. Next-generation sequencing of the 16S and *nifH* regions was used to characterize the nodule bacterial community composition and diversity. Despite large differences in substrate characteristics, no significant effects of soil environment were found on bacterial community composition within plant nodules, and few differences were observed in nodule communities between the plant species. These results suggest that the essential microbial propagules required for successful nodulation are present in disturbed substrates. Thus, restoration efforts to expedite ecological succession by planting native legumes may succeed without the need for commercial inoculants.

The second study aims to understand the belowground ecology of four native legume species near Rankin Inlet, Nunavut, Canada – *Astragalus alpinus*, *Hedysarum americanum*, *Oxytropis arctica*, and *Oxytropis maydelliana*. Entire plants, including intact root systems and surrounding soil, were collected and imaged using a positron emission tomography/ X-ray computed tomography (PET/CT) instrument. Experiments were conducted to adapt and optimize [¹¹C]CO₂ dosing and imaging procedures from recommended agricultural crop procedures. *Oxytropis maydelliana* was successfully exposed to [¹¹C]CO₂ tracer, and root structures were imaged using the GNEXT PET/CT imaging system. Non-invasive imaging technologies like PET provide insights into intact plant processes associated with belowground ecology, aiding in recommending better reclamation plans for Arctic sites.

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Dedication

To my beloved parents,

Who, with immense courage and foresight, left behind their family, friends, and the life they knew. In the aftermath of the 1989 protests, you made the brave decision to seek a better future for your children, venturing into an unfamiliar world where you didn't speak the language all in pursuit of a life where freedom, education, and opportunity could flourish. Your sacrifices and resilience have shaped the path I walk today. It is because of you that I am able to stand as a scholar today.

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List of Abbreviations

ANOVA	Analysis of variance
ASV	Amplicon sequence variant
BIOM	Biological Observation Matrix
^{11}C	Carbon-11
CLR	Centered-log ratio
CO_2	Carbon dioxide
CT	Computed tomography
^{18}F	Fluorine-18
GBq	Giga-becquerel
GBM	Geometric Bayesian-multiplicative
IQR	Inter-quartile range
^{13}N	Nitrogen-13
^{15}O	Oxygen-15
PCA	Principle component analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PET	Positron emission tomography
PSI	Pounds per square inch
MRI	Magnetic resonance imaging
Tukey HSD	Tukey's Honestly Significant Difference test

1. Introduction

Mining activities commonly result in the widespread removal of vegetation, primarily through the establishment of gravel quarries and roads, resulting in soil that lacks an adequate amount of organic material (Miller et al., 2021; Naeth & Wilkinson, 2014). Restoring such landscapes poses significant challenges, especially in tundra regions characterized by low temperatures, limited precipitation, and brief growing seasons. These conditions can hinder essential ecosystem functions like decomposition, nutrient cycling, and vegetative growth rates (Forbes & Jefferies, 1999; Kearns et al., 2015; Mehlhoop et al., 2018; Naeth & Wilkinson, 2014). Given these conditions, the successful restoration of these disturbed soils into productive ecosystems may require decades or even centuries (Forbes & Jefferies, 1999; Hodkinson et al., 2003).

There is often little organic matter on disturbed substrates in the tundra that result from disturbances such as mining activity or cryoturbation. On such substrates, early plant colonizers are one of the first sources of carbon that can stimulate microbial activity in the soil and may subsequently drive the establishment and succession of secondary colonizers. These early vascular plants have great influence on nutrient limited environments; however rates of seedling establishment and vegetative growth rates are slow in tundra regions due to low annual temperatures, limited precipitation, and short growing seasons (Forbes & Jefferies, 1999; Kearns et al., 2015; Mehlhoop et al., 2018; Miller et al., 2021; Naeth & Wilkinson, 2014). There is a need for better understanding of the processes that take place during ecosystem regeneration following resource extraction. We know that soil microbial communities contribute to soil formation, improve plant growth and health, and affect the nutrient cycles through enrichment with nitrogen and carbon, or through processes such as denitrification that remove nutrients (Cicczazzo et al., 2016). Given that early colonizing vascular plants, including nodulated legumes, are an important component of the nitrogen cycle in many tundra plant communities (Bliss et al., 1973; Lawrence et al., 1967), a better understanding of the microbial communities associated with root nodules or mycorrhizae may facilitate improved ecosystem restoration by improving nutrient acquisition and plant growth.

Nutrient availability on the Arctic tundra is often limited, with a cycle of slow plant growth and low organic matter input affecting the microbial activity involved in nutrient cycling (Shrestha & Lal, 2006). Biological nitrogen fixation are the main sources of external nitrogen, with

cyanobacteria as primary contributors to the total nitrogen budget, but legumes are major players in these systems as well (Karagatzides et al., 1985; Stewart et al., 2011). Plants have been shown to influence the availability of different forms of soil nitrogen through microbial feedback (Reynolds et al., 2003), mediated by the production of plant exudates (Dakora & Phillips, 2002). Therefore, in addition to playing important roles as hosts to nitrogen-fixing bacteria, the composition and quantity of organic materials exuded from Arctic legume roots is thought to drive the development and structure of microbial communities (Paterson, 2003).

In this study, I compared the bacterial communities within nodules isolated from four common legume species found at the study site in two differing habitats: gravel substrate areas and adjacently located, intact tundra areas. These legume species (*Astragalus alpinus*, *Hedysarum americanum*, *Oxytropis arctica*, and *Oxytropis maydelliana*) appeared to be some of the first colonizers on the disturbed areas that had little or no organic matter. The objectives of this study were to explore the rhizobial relationships of these species by: (1) comparing the general bacterial and nitrogen-fixing bacterial communities among four legume species found in two different habitats – gravel substrate and intact tundra; (2) developing positron emission tomography techniques to visualize the source-sink relationships between legumes and rhizobia *in situ*.

This thesis is written in the manuscript format with five chapters. The first chapter presents a general introduction, followed by a literature review focusing on ecological restoration in Arctic environments, plant-microbe interactions in the Arctic, factors influencing nodule endophyte recruitment, and the use of positron emission tomography to study belowground processes. Two research chapters follow, with one investigating bacterial diversity and community structure in legume nodules collected from a low-arctic environment, and the other assessing the application of positron emission tomography techniques on entire legume plants collected from a low-arctic environment. The final chapter summarizes key findings and proposes future research directions regarding ecological restoration within the low-arctic. The manuscript also includes a list of citations and appendices.

2. Literature Review

2.1. Restoration in Arctic environments

Resource exploration and extraction activity, particularly surface mining, causes massive disturbances to the topography of the landscape by removing organic layers and eliminating vegetation (Miller et al., 2021). In northern environments, revegetation efforts are restricted by short growing seasons, low air and soil temperatures, and low levels of precipitation, which are associated with reduced plant productivity and slow rates of decomposition and nutrient cycling (Forbes & Jefferies, 1999; Mehlhoop et al., 2018; Naeth & Wilkinson, 2014). Additionally, carbon input from aboveground vegetation is low due to low rates of photosynthesis and small amounts of standing biomass. Nutrients present are often biologically unavailable in the slowly decomposing organic materials such as plant litter (Chu & Grogan, 2010; Jonasson et al., 1999). Under these conditions, decades to centuries may be required for natural revegetation and unassisted ecosystem recovery of disturbed tundra systems (Forbes et al., 2001; Hodkinson et al., 2003).

Restoration plantings commonly include nitrogen-fixing legumes due to their contributions to improved soil conditions from nitrogen fixation. Legumes host rhizobia within their nodules which fix atmospheric nitrogen, and as a result can produce nutrient rich plant exudates and litter that may influence microbial activity and contribute to soil organic matter through deposition of plant litter (Dakora & Phillips, 2002; S. E. Hobbie, 1996; Reynolds et al., 2003). Seeded Arctic plants grow slowly and commercially available native seed stock is largely unavailable, thus many restoration techniques involve the use of rapid-growing non-native species to quickly revegetate an area and control. These introduced species may prove to be invasive in the environment and reduce overall biodiversity or persistently dominate communities, leading to limited long-term recovery of ecosystem function to pre-disturbance conditions. Natural seeding or transplanting and spreading local tundra-sourced material may be more appropriate in many Arctic sites (Forbes & Jefferies, 1999; Hagen et al., 2014; Kearns et al., 2015; Rydgren et al., 2016; Vloon et al., 2022). For example, in a recent transplant and shredding study conducted at a mining-disturbed site in the Arctic, transplantation of locally sourced Fabaceae species was recommended along with other fast-growing forbs and graminoids (Hnatowich et al., 2023a).

The limited soil organic material on substrates left following resource extraction means that early colonizing nitrogen-fixing plants may be very important in influencing early successional ecosystems by modifying soil conditions through high quality organic litter deposits and root exudates (A. D. Bradshaw, 1993; Myrold & Huss-Danell, 2003). These contributions increase carbon and nitrogen soil availability and may influence microbial and plant community dynamics by serving as long-lasting nutrient sources for microbial communities. This promotes microbial nutrient mineralization, thus facilitating colonization by later successional species (Bardgett & Walker, 2004; Iversen et al., 2015; Knelman et al., 2012; Stewart & Siciliano, 2015).

2.2. Plant-soil microbe relationships in Arctic environments

2.2.1. Microbial activity effects on nutrient availability

In tundra ecosystems, nitrogen found in decomposing organic material comprise most of the total soil nitrogen, but plants predominantly use inorganic forms of nitrogen (D. L. Jones & Kielland, 2002; Persson et al., 2003). The soil microbial processes required for conversion of plant litter and decaying plant roots into inorganic forms are affected by short growing seasons in the Arctic and are a contributing factor to the low turnover and availability of mineral nutrients (Chapin, 1983; D. L. Jones & Kielland, 2002; Stark, 2007). There is evidence that some plants can uptake soluble free amino acids and peptides in soil (Henry & Jefferies, 2003; Persson et al., 2003). However, soil microbes have a higher affinity toward free amino acids compared to plant roots (Owen & Jones, 2001), which means that in general, many plants are dependent for their nitrogen on soil bacteria and fungal mycorrhizal associations capable of utilizing organic nitrogen sources or soil microbial conversion of organic nitrogen sources (E. A. Hobbie & Högberg, 2012).

As atmospheric deposition and weathering contribute negligible amounts of nitrogen into the ecosystem, particularly in remote Arctic sites, the primary source of nitrogen is biological fixation of atmospheric nitrogen. This is a nitrogen source directly accessible to free-living nitrogen-fixing bacteria (diazotrophs) and specialized biological interactions between these diazotrophs and leguminous plants, mosses, and lichens (Rousk et al., 2018; Stewart et al., 2011). Across the Arctic, different tundra ecosystems will have distinct microbial community composition and function with drier upland areas having higher microbial respiration rates (Zak & Kling, 2006). In addition to slow microbial activity due to cold temperatures, there is evidence

suggesting that microbial communities in some ecosystems may also be nitrogen limited (Lavoie et al., 2011). Shifts in nitrogen availability through litter deposition or changes in the nitrogen cycle may change soil respiration and decomposition processes. These changes may lead to changes in microbial community composition, but the specific responses may vary by ecosystem, season, and spatially (Wookey et al., 2009).

2.2.2. Plant litter, root exudates, and microbial nutrient cycling

The composition and quantity (C:N ratio) of both plant litter and organic materials exuded from plant roots is critical to soil development and the development and structure of microbial communities, particularly post-disturbance where there is little soil organic material (Paterson, 2003). In mining-disturbed areas where the organic matter has been removed, primary colonizers may be contributing the first sources of organic material in the area through litter deposition and decaying plant or roots (Bliss et al., 1973). The effects of plant litter on microbial cycling are dependent on variation in the quantity and quality of the litter, as different plant taxa and communities allocate carbon and nitrogen in different ratios among different litter types (e.g. roots, leaves, or stems) (S. E. Hobbie, 1996). Carbon turnover rates in organic material derived from woody tissues, for example, may be significantly slower than rates in soft tissues such as fine roots, potentially by decades (J. E. Hobbie et al., 1998). Additionally, differences in the quality of woody litter can reduce the net nitrogen mineralization rates in heathland soils associated with evergreen shrubs differed from soils associated with grasses, with greater amounts of nitrogen mineralized in heathlands dominated by grasses (Van Vuuren et al., 1992). Variations in allocation may also be observed within plant functional types as two woody Arctic shrubs, *Alnus viridis* and *Betula glandulosa*, exhibited similar above-ground features. However, it was observed that they allocate photosynthates into stems differently. *A. viridis* stems were found to comprise approximately double the total amount of carbon and nitrogen compared to *B. glandulosa* stem tissues (Street et al., 2018). The observed differences may stem from *A. viridis* exhibiting higher growth rates or allocating a greater proportion of photosynthates to stem tissues. Belowground, these same plants demonstrated that *A. viridis* primarily allocated photosynthates to root tissues, while *B. glandulosa* distributed photosynthates widely into root-free bulk soils. This is hypothesized to be because *A. viridis* forms associations with nitrogen-fixing endophytes, reducing the need for resources diverted to ectomycorrhizal associations. The absence of changes in nitrogen availability in either vegetation type indicates that the differences in the amount of

photosynthate exuded belowground is more likely influenced by vegetation type rather than nitrogen availability (Street et al., 2018). Regardless of the underlying cause, these variations in photosynthate allocation have an impact on litter deposition and decomposition dynamics.

In addition to influencing nutrient availability through differences in litter deposition, plants may also affect the availability of different forms of soil nutrients through microbial feedback (Reynolds et al., 2003). The presence of plant roots can induce a mechanism called rhizosphere priming through changes in soil disturbance or through addition of plant exudates into the soil. These effects can cause strong short-term changes in the turnover of soil organic matter by increasing carbon availability for microbial activity and result in changes in nutrient availability (Keuper et al., 2020). For example, a girdling experiment on birch and willow shrubs in the subarctic showed disproportionate reductions in soil CO₂ efflux under girdled shrubs relative to intact shrubs, indicating that the allocation of photosynthates to the rhizosphere can have a large priming effect (Parker et al., 2020). Such effects are not always seen as a similar girdling experiment on the ericaceous shrub *Calluna vulgaris* showed no significant change in soil CO₂ efflux, indicating that *C. vulgaris* roots and their associated fungi have only a small impact on overall soil microbial respiration (Kritzler et al., 2016). These contrasting results indicate that, like litter deposition, rhizospheric allocation can vary among plant species and as a result, species can differ in the strength of their influence on soil nutrient dynamics.

2.3. Nitrogen fixation by legumes

Legumes carry out biological nitrogen fixation by hosting nitrogen-fixing bacteria in nodules, or small organs on the root system (Peix et al., 2015). The nitrogen-fixing bacteria found within nodules are primarily α -proteobacteria such as *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*, along with two genera of β -proteobacteria, *Burkholderia*, and *Cupriavidus*, which are the most common rhizobia species (Andrews & Andrews, 2017; Martínez-Hidalgo & Hirsch, 2017). These rhizobia are characterized as endophytic nitrogen-fixing bacteria capable of inducing nodulation (Lu et al., 2017). Variation in rhizobia species within the nodules occurs due to factors such as host plant species, host life stage, and environmental conditions (Carvalhais et al., 2015). This carefully selected and managed microbiome within the nodule, comprising both rhizobia and non-rhizobia species, is the basis of nitrogen fixation in legumes (Sturz et al., 2000).

Some legume genera, such as *Macroptilium* or *Phaseolus*, act as more cosmopolitan hosts, allowing nodulation by several rhizobia species (Peix et al., 2015; Zurdo-Piñero et al., 2009). Other genera, such as *Cicer*, *Trifolium*, or *Vicia*, engage in more specific rhizobial interactions (Broughton & Perret, 1999; Peix et al., 2015; Zurdo-Piñero et al., 2009). Similarly, individual rhizobia taxa may exhibit broader or narrower host ranges (Peix et al., 2015). These differences between rhizobial taxa can be overcome through the lateral transfer of Nod genes between different rhizobial species. This transfer enables the recipient of the donor plasmid to colonize hosts beyond its natural range (Liu & Murray, 2016; L. Walker et al., 2020).

2.3.1. Recruitment of nodule occupants

In addition to organic compounds like carbohydrates and proteins released by plant roots, secondary metabolites are also released into the soil, often exerting very specific effects on soil organisms. These secondary metabolites can enable specific interactions between microbes and plants (Kumar et al., 2016; L. Walker et al., 2020; Weston & Mathesius, 2013). For example, flavonoids function as signaling molecules in the rhizosphere and extracellular root spaces and can activate rhizobial nod genes, attract rhizobia, and inhibit microbial activity (Liu & Murray, 2016; Weston & Mathesius, 2013). Notably, some flavonoids serve dual roles as both transcriptional activators and chemical attractants for rhizobia (Aguilar et al., 1988; Caetano-Anollés et al., 1988). The initial recruitment of specific rhizobia and the subsequent induction of Nod factors in different legume species are influenced by the combination and timing of flavonoid production and secretion. Moreover, specific flavonoids may act as phytoalexins, suppressing some less compatible bacteria and creating a favorable niche for desired symbiotic partners (Liu & Murray, 2016; L. Walker et al., 2020).

Rhizobial receptors recognize signals from root exudates, inducing the expression of signaling molecules known as Nod factors that are associated with nodulation and infection (Downie, 2010). While these Nod factors share a basic oligomer structure, there are extensive variations and combinations produced by different rhizobia species, with the recognition of these Nod factors by host plants representing an additional mechanism for host-range specificity (Downie, 2010; Long, 1996; L. Walker et al., 2020). In addition to diverse Nod factor combinations, rhizobia produce other polysaccharides that aid in infection facilitation or suppression of plant defense responses (Downie, 2010). Each Rhizobia taxon has a specific set of plant hosts it can

colonize, and although certain associations are preferred, a strict correlation in these relationships does not seem to exist (Masson-Boivin et al., 2009).

Aside from the nitrogen-fixing rhizobia that may be recruited into nodules, there are also non-rhizobial bacteria genera associated with nodulation, as well as bacteria genera that neither fix nitrogen nor induce nodulation. The presence of these nonpathogenic bacteria within nodules can enhance survival of their nitrogen-fixing counterparts by performing functions such as bolstering defense mechanisms, synthesizing plant growth hormones, or producing enzymes that promote nodulation (Martínez-Hidalgo & Hirsch, 2017).

The host plant's flavonoid production and the specific Nod factors induced during nodulation play a critical role in controlling the rhizobial host range. The composition of plant flavonoids varies depending on the plant species and environmental factors (Weston & Mathesius, 2013). Consequently, the induction of rhizobial Nod factors relies on specific combinations of flavonoids, and the recruitment of rhizobia is often unique to the timing of nodulation, host plant species, and environment. These distinct combinations result in the recruitment of diverse microbial communities into the nodules that can vary substantially between both plant species and within species, between environments (Carvalhais et al., 2015; Long, 1996; Martínez-Hidalgo & Hirsch, 2017). The composition of flavonoids can also be modified by the microbiome (Rao & Cooper, 1994, 1995), implying that specific interactions and feedbacks over time contribute to the formation of nodule communities. In addition, microbial community composition may also depend on soil composition and structure. For example, Acidobacteria appears more frequently in mineral soil with low carbon and nitrogen content, and neutral or alkaline pH, whereas Bacteroidetes seems to colonize acidic soil with organic matter supplied by mosses (Ciccazzo et al., 2016). Such differences in nodule communities may subsequently impact rates of nitrogen fixation, plant growth rates, and ultimately, plant deposition and soil nutrient cycling.

2.4. Positron Emission Tomography (PET) usage in plants

Interactions between plants and soil in natural environments are intricate, involving specific dynamics that impact plant survival and competitive strength. Despite the complexity of these belowground processes, their exact nature remains unclear. Plant-microbial interactions within the rhizosphere may play a crucial role in assisting in plant survival under challenging

environmental conditions by aiding in nutrient and water acquisition. The traits of plant roots are also vital for nutrient absorption, and in nutrient-deficient conditions, roots tend to distribute themselves differently across soil layers. For instance, in phosphorus-deficient environments, roots may be distributed more shallowly, while under water deficiency, roots tend to extend deeper into the soil (Jordan et al., 1983; Lynch, 1995). Additionally, lateral root growth might be stimulated to enhance nutrient uptake (Chun et al., 2005).

In instances of nitrogen deficiency, legume roots form root nodules through symbiotic interactions with nitrogen-fixing bacteria. As the nodules receive carbon from the plant, the regulation of nodule number is managed by plant-produced hormones like ethylene, abscisic acid, and jasmonic acid. Early nodulation events can also inhibit nodulation and nodules may become restricted into specific root areas through mechanisms that limit nodule number (Mortier et al., 2012). Given the complexity of these belowground interactions, further research is needed to explore the traits and mechanisms contributing to plant resilience in diverse environmental conditions. This is particularly important in ecosystems such as mining-disturbed tundra, where limited studies have been conducted on belowground interactions.

Methods employed for characterizing root architectural traits in the field, such as root length or the number of lateral roots, often rely on excavation approaches. However, these methods are limited to observing only a fraction of the data because, although root lengths can be accurately measured after sampling, any geometric information is lost in the process. Traditionally, the carbon dynamics between plants and belowground carbon sinks, such as nodules, were investigated using stable or long-lived carbon isotopes. These methods, requiring destructive processing for quantifying sample differences, do not facilitate dynamic observations or subsequent studies.

Positron emission tomography (PET) is a non-invasive imaging technique that relies on detecting gamma rays generated when a positron, emitted during the decay of a short-lived radionuclide tracer, collides with an electron. The radiation is captured by PET scanners, and specialized software processes the data to produce a 3D image. Commonly used radionuclides in biological research include ^{11}C , ^{13}N , and ^{18}F . In medical applications, ^{18}F , in the form of fluorodeoxyglucose, acts as a glucose substitute and is widely employed to identify organs with heightened metabolic activity, such as areas affected by cancer or with heightened brain activity

(Som et al., 1980; Wehrl et al., 2013). Interestingly, it can also be utilized as a tracer in studying plant sugar metabolism (Fatangare & Svatoš, 2016). An alternative approach involves introducing gaseous [^{11}C]CO₂ to plant leaves, enabling the real-time and in-situ visualization of the distribution of photoassimilates into active root tips, fruits, or storage organs, providing valuable insights into plant physiological processes (Fatangare & Svatoš, 2016; Hubeau & Steppe, 2015).

When combined with structural imaging methods like magnetic resonance imaging (MRI) or computerized tomography (CT), positron imaging presents a unique approach to visualize the belowground structure and activity of plant roots. Plant fixed [^{11}C]CO₂ enables tracking of the tracer from aboveground leaves and stems to the roots, where structural images from MRI/CT aid in identifying root regions involved in photoassimilate partitioning, identifying source-sink relationships. Root biomass differences may be detectable through MRI/CT imaging and allow us to identify plants that may differ in carbon allocation to root growth. This is particularly interesting in nutrient-limited environments like tundra ecosystems, as studies indicate that plants often respond to nutrient limitations by increasing their root biomass (Chun et al., 2005). Furthermore, research has demonstrated that nitrate availability plays a role in regulating nodule numbers (Mohd-Radzman et al., 2013). Given that nodules serve as carbon sinks, positron emission tomography methods may offer the potential to identify differences in nodule number, size, or location along the roots. This opens opportunities for studies combining root and nodule characteristics with environmental or other variables. PET-based approaches offer invaluable insights into further understanding the intricate relationship among plant host species, environmental variations, and the composition of nodule communities.

3. Bacterial diversity and community structure in native legume root nodules in intact and mining disturbed tundra

3.1. Abstract

Mining restoration in Arctic regions is challenging due to harsh environmental conditions with slow natural ecosystem recovery taking decades or even centuries. Identifying native species that can both colonize disturbed areas and contribute to soil development are critical to hasten restoration timelines. Nodules were collected from four native legume species (*Astragalus alpinus*, *Hedysarum americanum*, *Oxytropis arctica*, and *Oxytropis maydelliana*) observed to be naturally colonizing gravel quarries within a mine footprint near Rankin Inlet, Nunavut, Canada. Samples came from both gravel quarries and adjacent intact tundra. Next-generation sequencing of the 16S and *nifH* regions was used to characterize the nodule bacterial community composition and diversity. Despite the large differences in soil conditions between gravel substrates and intact tundra, no significant effects of soil environment were found on bacterial community composition within plant nodules. Further, few differences were observed in nodule communities between the plant species. Overall, the study implies that the essential microbial propagules required for successful nodulation are found in gravel quarries; restoration efforts to speed succession by planting native legumes may be successful without a need for commercial inoculants.

3.2. Introduction

Mining activities commonly result in the widespread removal of vegetation, primarily through the establishment of gravel quarries and roads, resulting in soil that lacks an adequate amount of organic material (Miller et al., 2021; Naeth & Wilkinson, 2014). Restoring such landscapes has significant challenges, especially in tundra regions characterized by low temperatures, limited precipitation, and short growing seasons. These conditions can hinder essential ecosystem functions like decomposition, nutrient cycling, and vegetative growth rates (Forbes & Jefferies, 1999; Kearns et al., 2015; Mehlhoop et al., 2018; Naeth & Wilkinson, 2014). Given these conditions, the successful restoration of disturbed soils into productive ecosystems may span decades or even centuries (Forbes & Jefferies, 1999; Hodkinson et al., 2003).

The slow growth of Arctic plants and the limited availability of commercially viable native seed stock pose challenges to restoration work in Arctic environments. Past restoration approaches

have turned to the use of fast-growing non-native species for quick revegetation and erosion control. However, this practice is increasingly unacceptable in northern mine site closure plans due to the risks of non-native species invasion, reductions in biodiversity, alteration of ecosystem function, and persistence of non-target plant communities. This can result in less successful long-term recovery of ecosystem function to pre-disturbance conditions (Forbes & Jefferies, 1999; Hagen et al., 2014; Kearns et al., 2015; Rydgren et al., 2016; Vloon et al., 2022). Adding to these challenges, limited infrastructure in remote Arctic locations necessitates more straightforward, cost-effective methods, with the transplantation and spreading of tundra-sourced material possibly proving more suitable.

Recent studies show that transplantation of plugs of intact tundra can be effective for restoration of upland-heath Arctic tundra, but the availability of material for transplantation is a serious limitation (Hnatowich et al., 2023a, 2023b). Anecdotal observations during fieldwork for the above studies suggest that two species of native legumes, *Astragalus alpinus* and *Oxytropis arctica*, are among the first natural colonizers of these gravel quarries. Given the scarcity of soil organic material after resource extraction, primary colonizing nitrogen-fixing plants may shape early successional ecosystems through the deposition of high-quality organic litter and root exudates (A. D. Bradshaw, 1993; Myrold & Huss-Danell, 2003; Stewart & Siciliano, 2015). Litter and exudates enhance the availability of carbon and nitrogen in the soil, influencing microbial and plant community dynamics by providing enduring nutrient sources for microbial communities. This, in turn, promotes microbial nutrient mineralization, facilitating the colonization of later successional species (Bardgett & Walker, 2004; Iversen et al., 2015; Knelman et al., 2012; Stewart & Siciliano, 2015).

Our understanding of the root nodule communities of Arctic legumes remains limited. Rhizobial symbionts often establish a close association with specific plant species, relying on signals exchanged between the host and symbiont (Oldroyd et al., 2011). Though certain associations are often preferred, these relationships do not seem to be strictly deterministic (Masson-Boivin et al., 2009). Rhizobial populations, for example, can be genetically distinct among legume populations of the same species, even when those populations are separated by only a few kilometers (Van Cauwenberghe et al., 2014). Significant changes in rhizobial composition within nodules have been observed along environmental gradients (Birnbaum et al., 2018). Additionally, the

distribution of propagules in the field may be patchy or scarce, leading to site-specific variation (Simonsen et al., 2017). Upon the death of nodules, rhizobial species may be released into the soil where they can persist for years without host plants (Denison & Kiers, 2011). Ultimately, variation in nodule microbial community assemblies among different legume species and soil types may play a role in the success rate of colonization and subsequent succession through differences in the deposition of soil resources, impacting soil nutrient cycling.

Here, our objectives were to compare the nodule microbial communities between four native Arctic legume species collected from both gravel substrates and intact tundra substrates in Nunavut, Canada. We used 16S and *nifH* amplicon sequencing to determine how variation in community structure, diversity, and abundance differ between substrate types and among various legume species.

3.3. Methods

3.3.1. Study Location

Our collection sites were located near the Agnico Eagle Mines Ltd. Meliadine mine, approximately 25 km northwest of Rankin Inlet, Nunavut, Canada (63°01'23"N 92°11'41"W). The predominant vegetation in the area consists of heath tundra, heath-lichen, and lichen-rock vegetation. Among the vascular plant species commonly found are dwarf shrubs such as *Cassiope tetragona* (Linnaeus) D. Don (Arctic Mountain Heather), *Dryas integrifolia* Vahl (White Mountain-avens), *Salix reticulata* Linnaeus (Net Veined Willow), and *Vaccinium uliginosum* Linnaeus (Bog Blueberry). Additionally, various forbs populate the region: *Cardamine digitata* Richardson (Richardson's Bittercress), *Pedicularis flammea* Linnaeus (Red-tipped Lousewort), and *Stellaria longipes* Goldie (Long-Stalked Starwort) (Hnatowich et al., 2023a, 2023b). Three abandoned gravel quarries and nearby undisturbed upland heath tundra, located within a 100-meter radius of the gravel quarries, were selected for habitat comparison. The substrate in these gravel quarries was predominantly composed of gravel and sand particles, with minimal organic matter content in the soil (Hnatowich et al., 2023a). Five collection sites were selected from the three gravel quarries and associated undisturbed heath tundra locations. Each collection site was separated by at least 300 meters. At each collection site, nodules from four native legume species found on both gravel quarry and intact tundra: *Astragalus alpinus* Linnaeus, *Hedysarum americanum* (Michaux ex Pursh) Britton, *Oxytropis arctica* R. Brown, and

Oxytropis maydelliana Trautvetter were collected (Figure 3.1; Table A1). All of the native legumes are widely distributed Arctic legume species (Flora of North America Editorial Committee, 1993, 1993; Porsild & Cody, 1980); *A. alpinus* and *O. arctica* were commonly found establishing on the gravel substrates, while the other two species were rarely observed colonizing the gravel substrates. All species were abundant in adjacent intact tundra at each location.

3.3.2. Vascular Plant Descriptions

The four perennial legume species are insect-pollinated, featuring colourful flowers and compound leaves. A brief description of each species is provided below:

Astragalus alpinus Linnaeus (Flora of North America Editorial Committee, 1993+) is a low creeping plant with branching thin stems. The leaves are strigose beneath, glabrous above, and pinnate with 15 – 23 leaflets. Its flowers are pale violet and arranged in a short raceme. This widely distributed alpine and arctic species is found in sandy or gravelly areas (Porsild & Cody, 1980).

Oxytropis arctica R. Brown (Flora of North America Editorial Committee, 1993+) often forms dense branching tufts 20 to 30 cm in diameter. It has 9 – 13 leaflets on its leaves and the underside of them are densely covered with fine thin hairs. Located at the end of a peduncle slightly longer than its leaves are 3 – 5 dark purple flowers. It is endemic to arctic North America and ubiquitous on dry, open tundra, though less common westward (Porsild & Cody, 1980).

Oxytropis maydelliana Trautvetter (Flora of North America Editorial Committee, 1993+) is distinguishable by the brown stipules that densely cover its branching caudex. Its leaves have 13 hairless or sparingly villous leaflets and its florescence has 5 – 7 pale yellow flowers arranged in a short spike. This species is found across arctic and alpine parts of East Asia to northern Labrador (Porsild & Cody, 1980).

Hedysarum americanum (Michaux ex Pursh) Britton (Elven, R (Ed), 2007+) has large fleshy rhizomes that can be half an inch in diameter and its stems grow up to 60 cm tall. Leaves are glabrous with 9 – 13 leaflets and brown stipules. Its inflorescence is large compared to the other three species, with 10 – 20 pink or pale purple flowers arranged in a raceme of 3 – 12 cm long at the top of a 5 – 10 cm long peduncle. Above the treeline, however, the plant may be lower and fewer-flowered. Additionally, this species has indehiscent pods composed of separable 1-seeded

sections. It has a general arctic and alpine distribution across North America (Porsild & Cody, 1980).

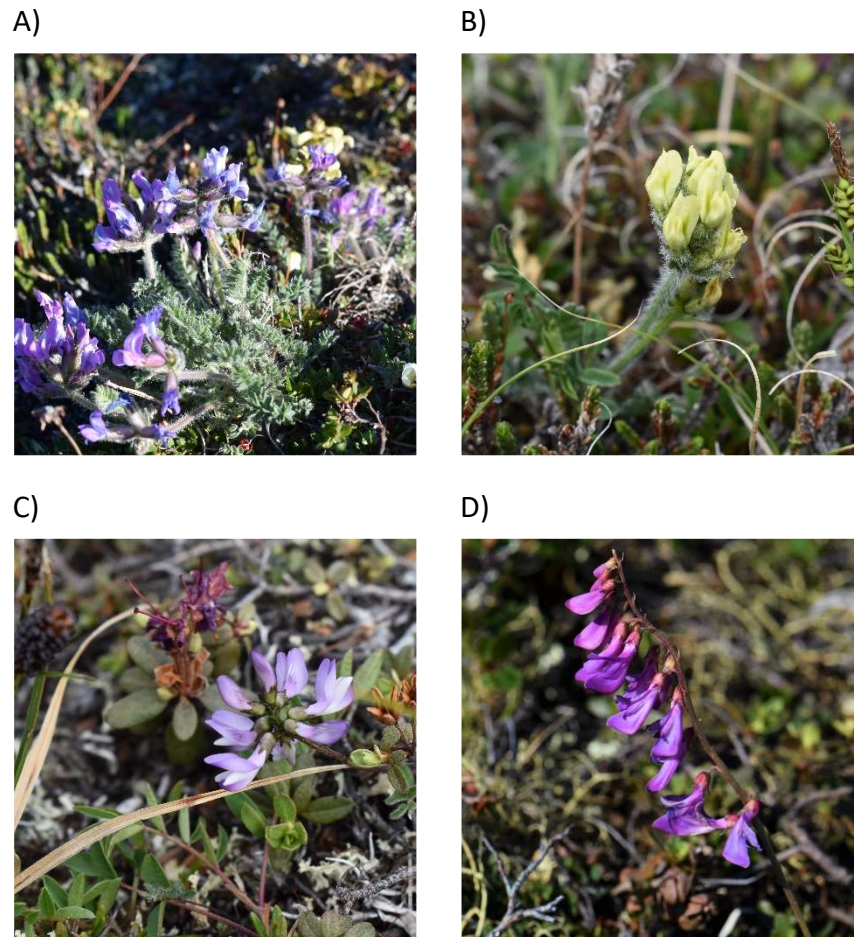


Figure 3.1. Photographs of A) *Oxytropis arctica*, B) *Oxytropis maydelliana*, C) *Astragalus alpinus*, and D) *Hedysarum americanum*; taken near Rankin Inlet, Nunavut by Dr. Eric Lamb, used with permission.

3.3.3. Sample Collection

Samples were collected from all four species on July 17 and July 18, 2021. These consisted of nodules that were pooled from individuals within a 30 m diameter for each species and substrate type. Nodules were collected by carefully digging around the plant to extract intact shoots and roots for each plant. Digging intact shoot-root systems was important, particularly in intact tundra sites, because plant roots were entangled with neighbouring plants and this strategy

ensured that the nodules collected were from the correct species. Nodules were placed in sterile plastic collection tubes and stored at 4 °C before being packed into a cooler with ice packs for transport to the University of Saskatchewan and subsequent storage at -20 °C.

3.3.4. Sample Preparation

Frozen nodules were separated from the root and weighed prior to DNA extraction. The nodule weights ranged from 5 to 91 mg, with an average weight of 41.7 mg. To ensure only DNA from nodule occupants was extracted, nodules were surface sterilized by shaking in a 0.6% w/v NaClO solution for 10 min, rinsed with sterile distilled water, and then shaken with 70% ethanol for 10 min. Finally, nodules were rinsed with sterile distilled water three times (Hakim et al., 2018). Nodules were kept on ice between steps. In total, 37 samples were processed (Table A1).

Each sample was spiked with an internal standard using 1.75 uL of 33 ng/uL *Aliivibrio fischeri* DNA before DNA extraction following the standard recommended DNeasy® Plant Pro Kit protocol (Qiagen, Cat. No. #69204). For tissue disruption, samples were placed in a Retsch Grinder and run at 25 Hz for 2 min. Samples were rotated and run again for 2 min. DNA samples were quantified following a standard protocol for the Qubit 2.0 Fluorometer (Invitrogen, Massachusetts, USA) with the Qubit HS assay kit (Invitrogen, Massachusetts, USA) before DNA samples were standardized to 1.5 ng DNA/uL in preparation for PCR amplification.

Primer sets used for PCR amplification were 342F/806R and *nifH*-F/*nifH*-R primers with Illumina sequencing adapters (342F: 5'-ACA CTG ACG ACA TGG TTC TAC ACT ACG GGG GGC AGC AG-3' and 806R: 5'-TAC GGT AGC AGA GAC TTG GTC TGG ACT ACC GGG GTA TCT-3'; *nifH*-F: 5'-AAA GGY GGW ATC GGY AAR TCC ACC AC-3' and *nifH*-R: 5'-TTG TTS GCS GCR TAC ATS GCC ATC AT-3') (Mori et al., 2014; Rösch et al., 2002).

Each 20 uL PCR reaction mix contained 0.25 uL 5 U/uLDreamTaq polymerase, 2.5 uL 10X DreamTaq reaction buffer, 2.5 uL 2mM dNTP (all from Thermo Fisher Scientific, Massachusetts, USA), 0.25 uL of each of the forward primer and reverse primers (10uM), 12.25 uL nuclease-free water, and 2 uL DNA template. Negative controls and PCR duplicates were included. The 16S PCR conditions were: 95°C for 5 min; 30 cycles of 95°C for 30 seconds, 54°C for 45 seconds, 72°C for 1 min; and a final extension at 72°C for 7 min. *nifH* PCR conditions were: 94°C for 5 min; 35 cycles of 94°C for 30 seconds, 52°C for 45 seconds, 72°C for 30 seconds;

and a final extension at 72°C for 10 min. PCR products were purified using Sera-Mag Select (Cytiva, Marlborough, USA) at a 1:1 ratio and diluted to 1.5 ng/uL for library preparation.

Library preparation used the Nextera XT Index Kit v2 (Illumina, San Diego, USA) to attach dual indices and sequencing adapters. Each 25 uL PCR reaction mix contained 0.25 µL DreamTaq polymerase, 2.5 µL DreamTaq reaction buffer, 2.5 µL 2mM dNTP (all from Thermo Fisher Scientific, Massachusetts, USA), 2.5 µL of each of the forward primer and reverse primers (10µM), 12.25µL nuclease-free water, and 2 µL DNA template. Cycling conditions were 95°C for 3 minutes; 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and a final extension at 72°C for 7 minutes. Indexed samples were purified with Sera-Mag Select (Cytiva, Marlborough, USA), standardized to 4 nM, and pooled.

Sequencing was performed at the Western College of Veterinary Medicine Molecular Microbiology Lab, University of Saskatchewan, Canada, on the Illumina MiSeq system using a 300-cycle MiSeq Reagent Kit v2 (Illumina, Cat. No. #MS-102-2002). A total of 41, 16S samples were sequenced, including two negative controls and two duplicates. For *nifH* primers, a total of 40 samples were sequenced, including one negative control and two duplicates.

3.3.5. Bioinformatics

16S sequences were classified using a RESCRIPt-processed SILVA reference database trained to the 342F/806R primers (Quast et al., 2013). A *nifH* reference database was created by querying the NCBI database for all classified nitrogenase reductase entries using RESCRIPt (Robeson II et al., 2021), which downloads the files and generates a 7-level taxonomy reference. Sequences with five or more ambiguous bases or with homopolymers that are eight or more base pairs were filtered out. Sequences and taxonomies were dereplicated to remove identical sequences with identical taxonomies before extracting the *nifH*-F/*nifH*-R regions and a final dereplication to generate the *nifH* reference database.

Sequenced 16S and *nifH* reads were separately imported and processed within the QIIME2 2022.8 environment (Bolyen et al., 2019). A total of 1 337 unique 16S reads, with an average sequence length of 420 bp, and 1 549 *nifH* reads, with an average sequence length of 331 bp, were imported for analysis. Primers were removed with *cutadapt* (Martin, 2011) before the DADA2 plugin was used to remove low quality read ends and join the denoised paired-end reads

(Callahan et al., 2016). Reads were truncated to a quality score of 30. A minimum quality score of 20 and an overlap of 12 bp is required for DADA2. To remove any sequencing errors, singletons and sequencing artifacts were filtered out, and low-quality samples were filtered out using a threshold for total read counts, determined by our negative controls. After filtering, 350 unique 16S reads and 313 *nifH* reads remained. Finally, the processed reads were classified to amplicon sequence variants (ASVs) and identified taxonomically using the trained 16S and *nifH* reference databases described above (Bokulich et al., 2018).

The classified sequencing data was extracted from the QIIME2 environment and imported into R for statistical analysis (version 4.2.2, R Core Team, 2022) using the Biological Observation Matrix (BIOM) format (McDonald et al., 2012) and the *biomformat* package (version 1.24.0, McMurdie & Paulson, 2022). Abundance data were combined with taxonomy and sample metadata using package *phyloseq* (version 1.40.0, McMurdie & Holmes, 2013) before further processing to remove 16S ASVs identified as chloroplasts and mitochondrial rRNA.

Contaminant ASVs were identified using *decontam* (version 1.16.0, Davis et al., 2017). Four ASVs were identified from the 16S sequences and removed from analysis. Sequence counts were standardized to account for differences in sequencing depth based on the *A. fischerii* internal standard before removing the *A. fischerii* sequences (Smets et al., 2016). 310 unique 16S reads and 306 *nifH* reads remained after removal of contaminant sequences.

The sequencing data was then transformed to capture the relationship between the ASVs in the dataset, as sequencing data are naturally described as proportions (Gloor et al., 2017). Firstly, the large number of zeros typical of sequencing datasets were replaced using zero-imputation with the recommended Geometric Bayesian Multiplicative (GBM) method in the *zCompositions* package (version 1.4.0-1, Palarea-Albaladejo & Martín-Fernández, 2015). For the 16S dataset, the GBM method corrected 2385 of 11 433 values, while for the *nifH* dataset, 6793 of 11 322 values were corrected. Finally, the GBM zero-imputed data was transformed into centered-log ratios using the *CoDaSeq* package (version 0.99.6, Gloor & Reid, 2016).

3.3.6. Statistical Analysis

Principle Component Analysis (PCA) was used to visualize the community composition data. After centered-log ratio transformation, Aitchison distance, which is equivalent to Euclidean distance between samples in CLR-transformed datasets, can be used for clustering (Gloor et al.,

2017). To identify statistically significant differences in the nodule communities between the four legume species and substrate types, Euclidean distances were calculated for both 16S and *nifH* datasets using wrapper functions in the package *phyloseq* before running permutational multivariate analysis of variance (PERMANOVA) and homogeneity of dispersion tests using functions in the *vegan* package (version 2.6-4, [Oksanen et al., 2022](#)). Pairwise comparisons between legume species were performed using package *pairwiseAdonis* (version 0.4, [Martinez Arbizu, 2017](#)).

Shannon diversity indices were calculated using modified functions adapted from the *microbiomutilities* package (version 1.00.17, [Shetty & Lahti, 2023](#)). Indices were compared using a two-way analysis of variance (ANOVA) to identify statistically significant differences in nodule diversity of the legume species or substrate types and species level differences were identified using Tukey Honest Significant Differences (Tukey HSD).

Common ASVs with strong loadings on the ordination axes were identified using species scores and selected for differential abundance analysis. Selection criteria were the ten ASVs with: 1) the most positive or negative loadings along PC 1; and 2) found in at least ten samples. PC 1 accounted for 46.8% of the variation in the data while PC 2 was omitted for this analysis as it only accounted for 7.2% of the variation. The *ALDEx2* tool (version 1.28.1, [Fernandes et al., 2013](#)) models a probability distribution and estimates effect size differences to identify significant differential abundances between groups. We used *ALDEx2* to identify differential abundances between substrates using a Welch's t-test with Benjamini-Hochberg procedure adjusted p-values. Differential abundances of these ASVs between legume species were identified using a two-way ANOVA and Tukey HSD.

3.4. Results

3.4.1. Nodule taxonomic characterization

The total number of 16S reads across 37 samples after data filtering and standardizing was 23,417,575 reads, ranging from 68,429 to 155,817 per sample. Of the total number of filtered and standardized 16S reads across our 37 samples, Proteobacteria (58.6%), Bacteroidota (22.3%), and Actinobacteriota (5.5%) were the most abundant of the 11 detected phyla. Bacteria classes Alphaproteobacteria and Gammaproteobacteria encompassed most of the 16S bacterial diversity at 29.8% and 28.8%, respectively. The total number of *nifH* reads across 37 samples was

3,505,639 reads, ranging from 14,917 to 145,585 per sample. The vast majority of the identified *nifH* reads classified to phylum Proteobacteria (98%) and, within the Proteobacteria, to genus *Rhizobium* (97.1%).

3.4.2. 16S composition and diversity of nodule bacterial communities

Nodule 16S bacterial communities were not significantly different between either substrate type ($p = 0.194$) or among legume species ($p = 0.444$) (**Error! Reference source not found.**) (

Table 3.1. Permutational Multivariate Analysis of Variation (PerMANOVA) analysis testing for differences in 16S and *nifH* nodule community composition and ANOVA testing for 16S and *nifH* Shannon diversity differences between legume species and two substrate types.

	16S Composition	16S Diversity	<i>nifH</i> Composition	<i>nifH</i> Diversity
Species	F3,29=0.949; p=0.446	F3,29=0.836; p=0.485	F3,29=1.889; p=0.045	F3,29=4.44; p=0.011
Substrate	F1,29=1.316; p=0.193	F1,29=0.256; p=0.624	F1,29=1.436; p=0.161	F1,29=0.961; p=0.335
Species x Substrate	F3,29=1.555; p=0.109	F3,29=3.285; p=0.035	F3,29=1.043; p=0.376	F3,29=1.091; p=0.369

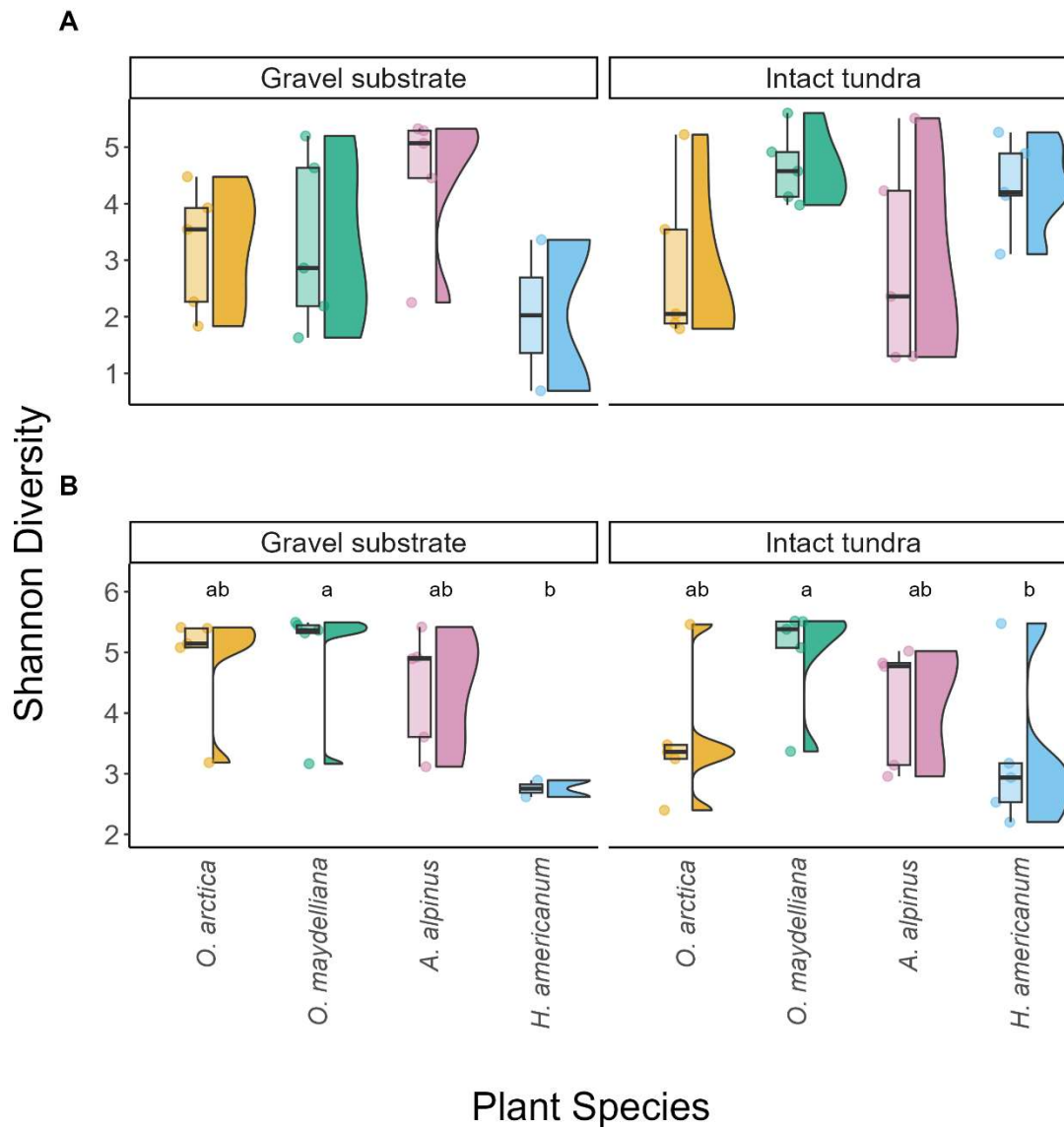
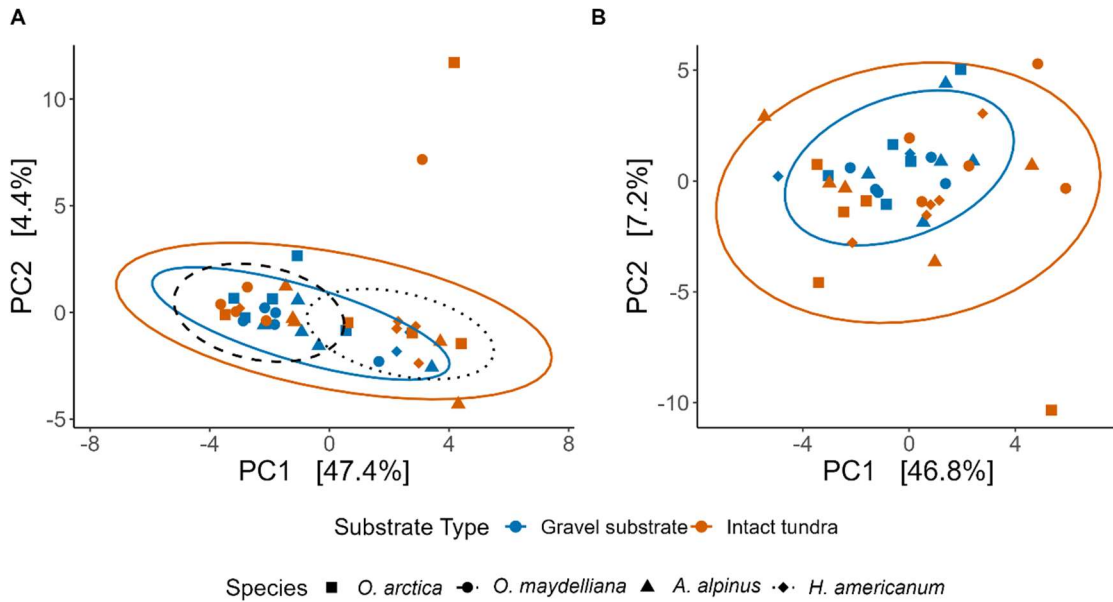


Figure 3.3. Boxplots and violin plots of A) 16S and B) *nifH* Shannon diversity in nodule communities of four Arctic legume species – *O. arctica*, *O. maydelliana*, *A. alpinus*, *H. americanum* from gravel substrate and intact tundra areas. Boxes encompass 25%- 75% percentiles of the data. The median is indicated by the horizontal black line, with each nodule sample represented by dots.

). The 95% confidence intervals of the gravel substrate and intact tundra communities overlap completely, and the gravel substrate communities seem to be subset within the broader intact

tundra communities. There was also no difference in Shannon diversity between substrate types ($p = 0.623$) or legume species ($p = 0.485$), although there was a significant interaction between species and substrate ($p = 0.035$) (**Error! Reference source not found.**) (Table 3.1) likely due to the difference in response by *H. americanum* nodule communities between substrates.



f

Figure 3.2. A) Principle components analysis (PCA) plot of sample scores for nodule samples from four legume species in two environment types using 16S as a marker gene. B) Principle components analysis (PCA) plot of sample scores for nodule samples from four legume species in two environment types using *nifH* as a marker gene. Points in blue represent samples taken from gravel substrate areas and points in orange represent samples taken from intact tundra areas. Samples taken from *O. arctica*, *O. maydelliana*, *A. alpinus*, and *H. americanum* are represented by squares, circles, triangles, diamonds, respectively. Ellipses represent 95% confidence intervals. *O. maydelliana* confidence interval is represented by a dashed ellipse and *H. americanum* confidence interval is represented by a dotted ellipse.

Table 3.1. Permutational Multivariate Analysis of Variation (PerMANOVA) analysis testing for differences in 16S and *nifH* nodule community composition and ANOVA testing for 16S and *nifH* Shannon diversity differences between legume species and two substrate types.

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Substrate	F _{1,29} =1.316; p=0.193	F _{1,29} =0.256; p=0.624	F _{1,29} =1.436; p=0.161	F _{1,29} =0.961; p=0.335
Species x Substrate	F _{3,29} =1.555; p=0.109	F_{3,29}=3.285; p=0.035	F _{3,29} =1.043; p=0.376	F _{3,29} =1.091; p=0.369

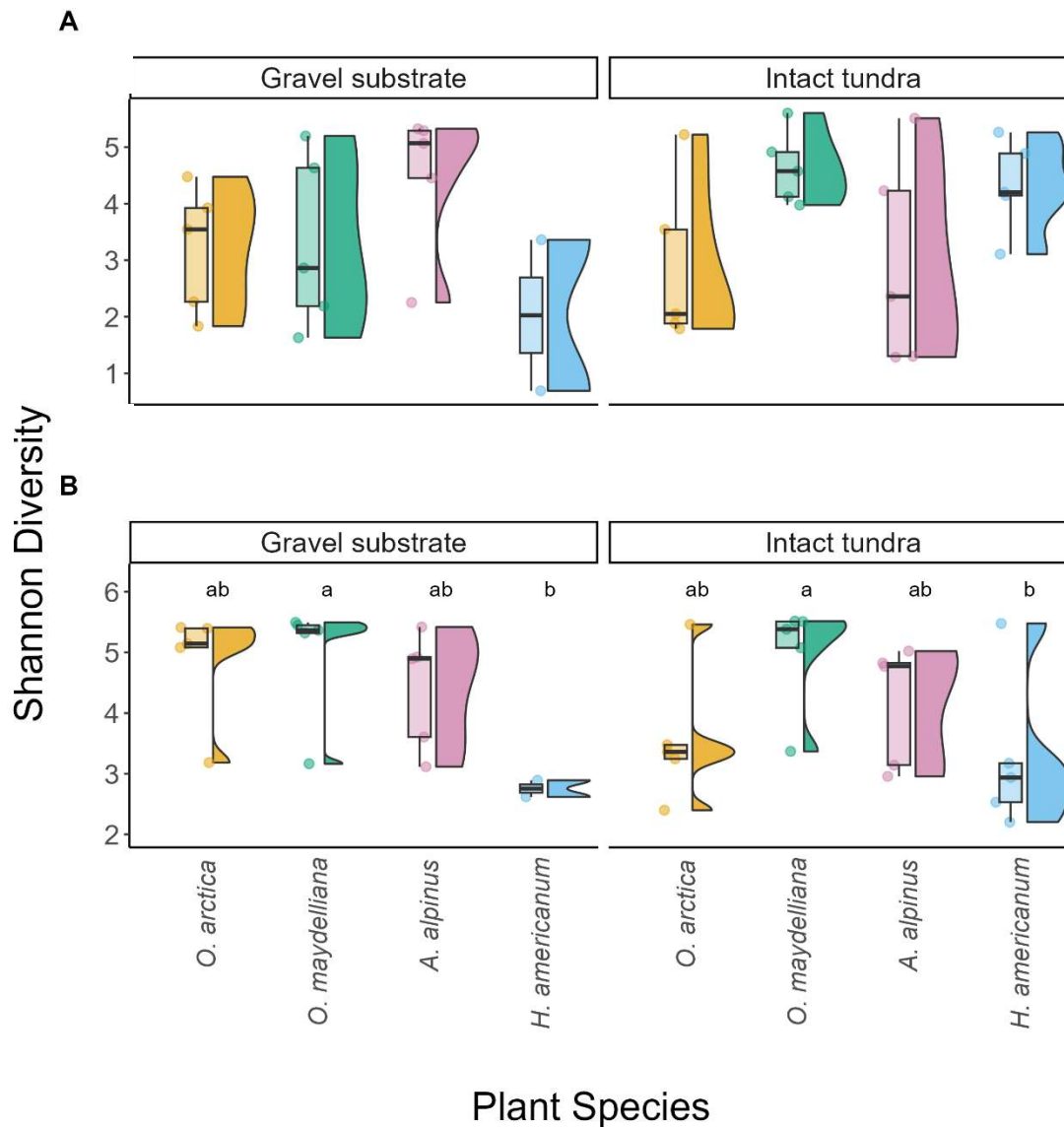


Figure 3.3. Boxplots and violin plots of A) 16S and B) *nifH* Shannon diversity in nodule communities of four Arctic legume species – *O. arctica*, *O. maydelliana*, *A. alpinus*, *H. americanum* from gravel substrate and intact tundra areas. Boxes encompass 25%- 75% percentiles of the data. The median is indicated by the horizontal black line, with each nodule sample represented by dots.

3.4.3. Contribution of 16S ASVs with high loadings to nodule community structure

Of the 310 16S rRNA ASVs, the ten with the most positive species scores on PC1 axis classified to Alphaproteobacteria (6), Gammaproteobacteria (2) and Bacteroidia (2). The ten with the most negative species scores on PC1 classified to Alphaproteobacteria (4), Bacteroidia (2), and Acidimicrobiia, Saccharimonadia, Phycisphaerae, and Gammaproteobacteria at 1 each (Table A2). Six of the above ASVs were found in more than ten samples. These ASVs were classified as bacterial genera *Mesorhizium* (2), *Pseudomonas* (2), *Asticcacaulis* (1), and *Caulobacter* (1). There were five other ASVs that did not contribute greatly to the differentiation of communities along the ordination axes but were found in more than 10 samples. Differential abundance analysis using ALDEx of these 11 ASVs showed that although the two substrates had nodule communities that overlapped in composition, the selected ASVs were differentially abundant between the two substrates (**Error! Reference source not found.**). In contrast to the prevailing trend, we saw no difference in abundance of the six ASVs with high loadings compared to the ASVs with smaller scores (Table 3.2). A two-way analysis of variance also did not show differences in abundance of the six ASVs with higher species scores between the four legume species. However, one *Pseudomonas* ASV showed a significant difference in abundance between gravel and tundra substrates. Additionally, a significant interaction term was found for one *Mesorhizobium* ASV, with its abundance appearing to vary between substrates for certain legume species. (**Error! Reference source not found.**; Table 3.2).

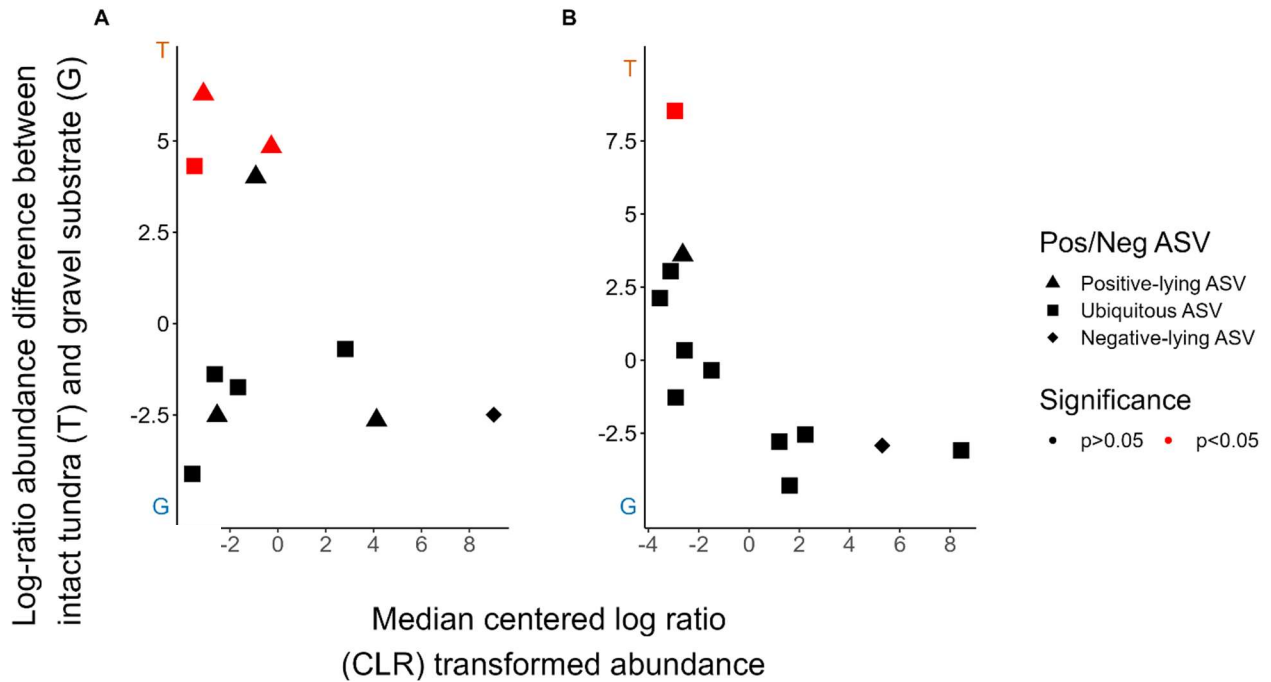


Figure 3.4. ANOVA-like differential expression (ALDEx) plot of the most abundant A) 16S and B) *nifH* ASVs. X axis values are median centred-log ratio transformed abundances and y-axis values are per feature median centered-log ratio transformed abundance differences. Differences significantly different from zero are in red while those not significant are in black. Triangles are ASVs negatively loaded on PC1 in the ordinations in Figure A2, and diamonds are positively loaded ASVs. Squares are ASVs that did not load strongly on PC1. Positive y-axis values indicate higher abundance in intact tundra and negative higher abundance in gravel substrates.

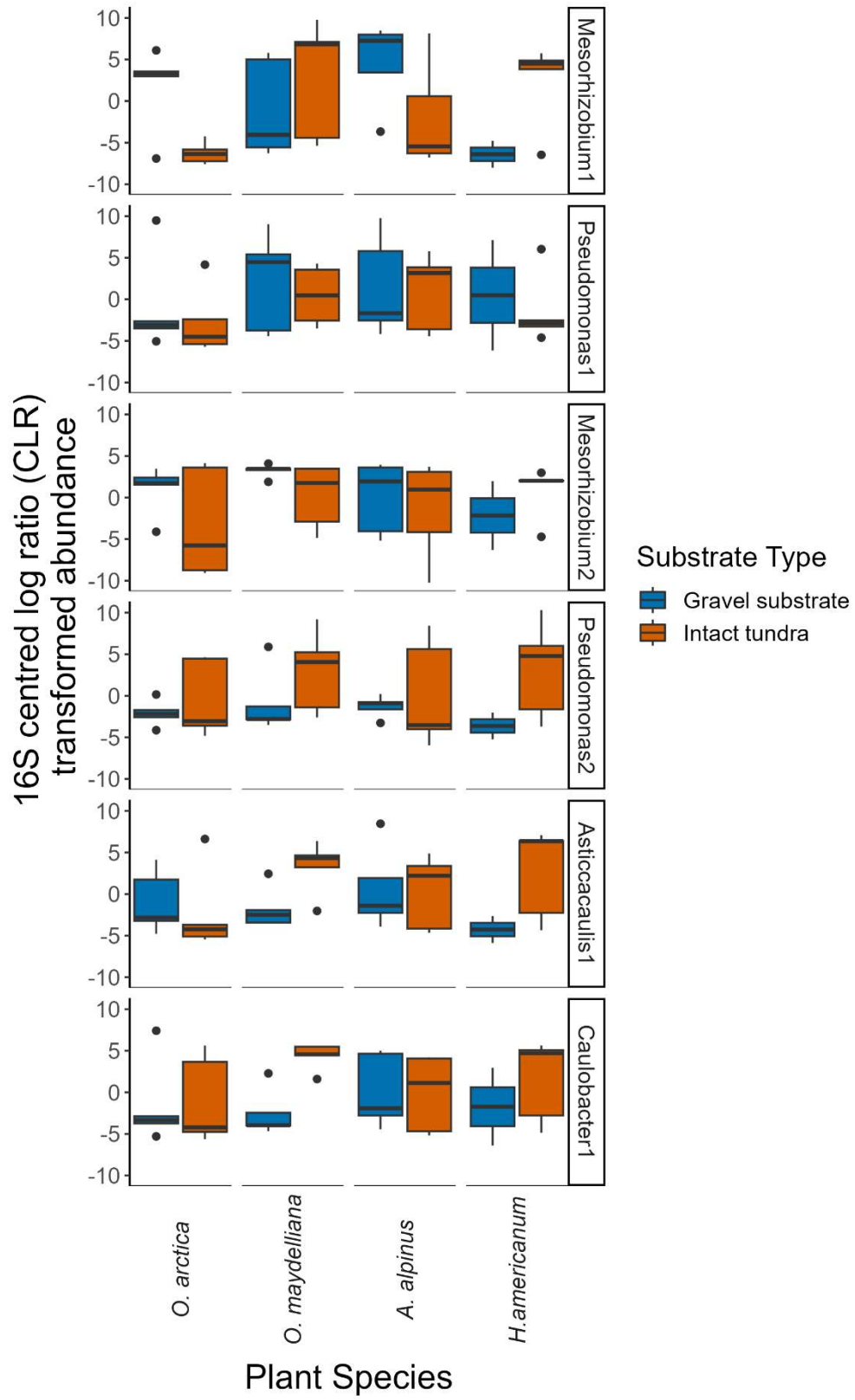


Figure 3.5. Boxplots of the six 16S ASVs with highest species scores on PC1 in **Error!**
Reference source not found. A2 with the centered-log ratio transformed abundance on the y-axis and the four legume species on the x-axis. ASVs that were present in more than ten samples and were also within the top ten negative and top ten positive loadings in PC1 were chosen for analysis. Boxplots are coloured by substrate type and separated by ASVs codes. Boxes represent 25% to 75% percentiles of the data and points represent samples that lie beyond 1.5* interquartile range (IQR). There is no statistical difference in the centered-log ratio transformed abundances across plant species nor substrate type.

Table 3.2. Results from two-way ANOVA analyses on the centered-log ratio transformed abundances of the six 16S ASVs and the two *nifH* ASVs that contributed greatly to their respective PC1 axis (Figure 3.2; Figure A2). ASVs that were present in more than ten samples and were also within the top ten negative or top ten positive loadings on PC1 were chosen for analysis.

ASV	Species	Substrate	Species x Substrate
<i>16S Asticcacaulis1</i> (<i>ae41a7e7ad08cd926075f8ab35eaa0eb</i>)	F _{3,29} = 0.725; p=0.545	F _{1,29} = 2.137; p=0.155	F _{3,29} = 1.833; p=0.163
<i>16S Caulobacter1</i> (<i>6d1de170c8d9c378c1bacf72d93283c2</i>)	F _{3,29} = 0.479; p=0.700	F _{1,29} = 2.976; p=0.095	F _{3,29} = 1.325; p=0.285
<i>16S Mesorhizobium1</i> (<i>e010f6ec05db89e11d623899272ecde1</i>)	F _{3,29} = 0.890; p=0.458	F _{1,29} = 0.838; p=0.368	F_{3,29} = 4.677; p=0.009
<i>16S Mesorhizobium2</i> (<i>7d850db295ca96fe18f859c33034a116</i>)	F _{3,29} = 0.786; p=0.512	F _{1,29} = 1.723; p=0.200	F _{3,29} = 0.894; p=0.456
<i>16S Pseudomonas1</i> (<i>3aa080fdc0ba7fe7aa9153d11c5fb3fa</i>)	F _{3,29} = 0.877; p=0.465	F _{1,29} = 0.668; p=0.420	F _{3,29} = 0.039; p=0.990
<i>16S Pseudomonas2</i> (<i>0f6640911d66a8b028b2b091f1cbf0c0</i>)	F _{3,29} = 0.689; p=0.566	F_{1,29} = 4.137; p=0.051	F _{3,29} = 0.575; p=0.636
<i>nifH Mesorhizobium-1</i> (<i>88ad6d4de9880fdeb52ea15e8f11c0e0</i>)	F_{3,29} = 3.779; p=0.021	F _{1,29} = 1.842; p=0.185	F _{3,29} = 0.863; p=0.471
<i>nifH Mesorhizobium-2</i> (<i>439a5c6fc811c01591732533d407d094</i>)	F _{3,29} = 2.118; p=0.120	F _{1,29} = 0.033; p=0.857	F _{3,29} = 0.558; p=0.647

3.4.4. *nifH* composition and diversity of nodule bacterial communities

nifH gene sequences showed a similar pattern to the 16S rRNA sequences with no significant differences in composition between the nitrogen-fixing nodule bacterial communities of either substrate type (Figure 3.2; Table 3.1). There was a significant difference between the *nifH* nitrogen-fixing bacterial community composition of *O. maydelliana* and *H. americanum* (Table 3.1; Table 3.4) and *nifH* Shannon diversity was different between these two species (Figure 3.3; Table 3.5; Table 3.1).

Table 3.3. Results of pairwise comparisons among *nifH* nodule samples of four legume species, both on gravel substrates and intact tundra substrates, assessed based on the Euclidean distance matrix of centred-log ratio transformed abundance. O-arc = *Oxytropis arctica*, O-may = *Oxytropis maydelliana*, A-alp = *Astragalus alpinus*, H-ame = *Hedysarum americanum*. *O. maydelliana* and *H. americanum* centred-log ratio transformed abundances are significantly different with $p= 0.011$.

<i>Pairs</i>	Df	SumsOfSqs	F.Model	R2	p.value
<i>O-arc vs O-may</i>	1	2422.694	1.429	0.074	0.18
<i>O-arc vs A-alp</i>	1	1385.736	0.748	0.04	0.53
<i>O-arc vs H-ame</i>	1	2984.663	1.579	0.095	0.149
<i>O-may vs A-alp</i>	1	3169.086	2.198	0.109	0.079
<i>O-may vs H-ame</i>	1	6430.021	4.604	0.235	0.011
<i>A-alp vs H-ame</i>	1	2333.143	1.472	0.089	0.153

Table 3.4. Summary of Tukey’s HSD on *nifH* gene Shannon diversities of nodule samples from four legume species – O-arc = *Oxytropis arctica*, O-may = *Oxytropis maydelliana*, A-alp = *Astragalus alpinus*, H-ame = *Hedysarum americanum*. Shannon diversity was significantly different between *O. maydelliana* and *H. americanum* at p = 0.005.

	diff	p adj
<i>O-arc vs O-may</i>	0.748	0.379
<i>O-arc vs A-alp</i>	0.052	0.999
<i>O-arc vs H-ame</i>	-1.097	0.156
<i>O-may vs A-alp</i>	-0.696	0.441
<i>O-may vs H-ame</i>	-1.846	0.005
<i>A-alp vs H-ame</i>	-1.149	0.129

3.4.5. Contribution of *nifH* ASVs with high loadings to nodule community structure

Of the 306 *nifH* ASVs, the ten with the highest species scores on PC1 axis classified to *Mesorhizobium* (7) and *Rhizobium* (2) with one ASV only able to be classified as bacteria. The ten with the most negative species scores on PC1 classified to *Mesorhizobium* (1) and *Rhizobium* (9) (Table A3). Two of the above ASVs were found in more than ten samples. Both the ASVs were classified as bacterial genus *Mesorhizobium*. There were ten other ASVs that did not contribute to the differentiation of communities along the ordination axes but were found in more than 10 samples. Differential abundance analysis using *ALDEx* of these 12 ASVs showed no significant difference in the relative abundance of ASVs between the two substrate types (Figure 3.4; Table 3.2), similar to our results of the *nifH* nodule community composition analysis. When comparing the two ASVs that were found in more than ten samples, there was a significant difference in the relative abundance of these ASVs between *O. maydelliana* and *H. americanum* and between *A. alpinus* and *H. americanum* (Figure 3.6; Table 3.2, Table 3.4).

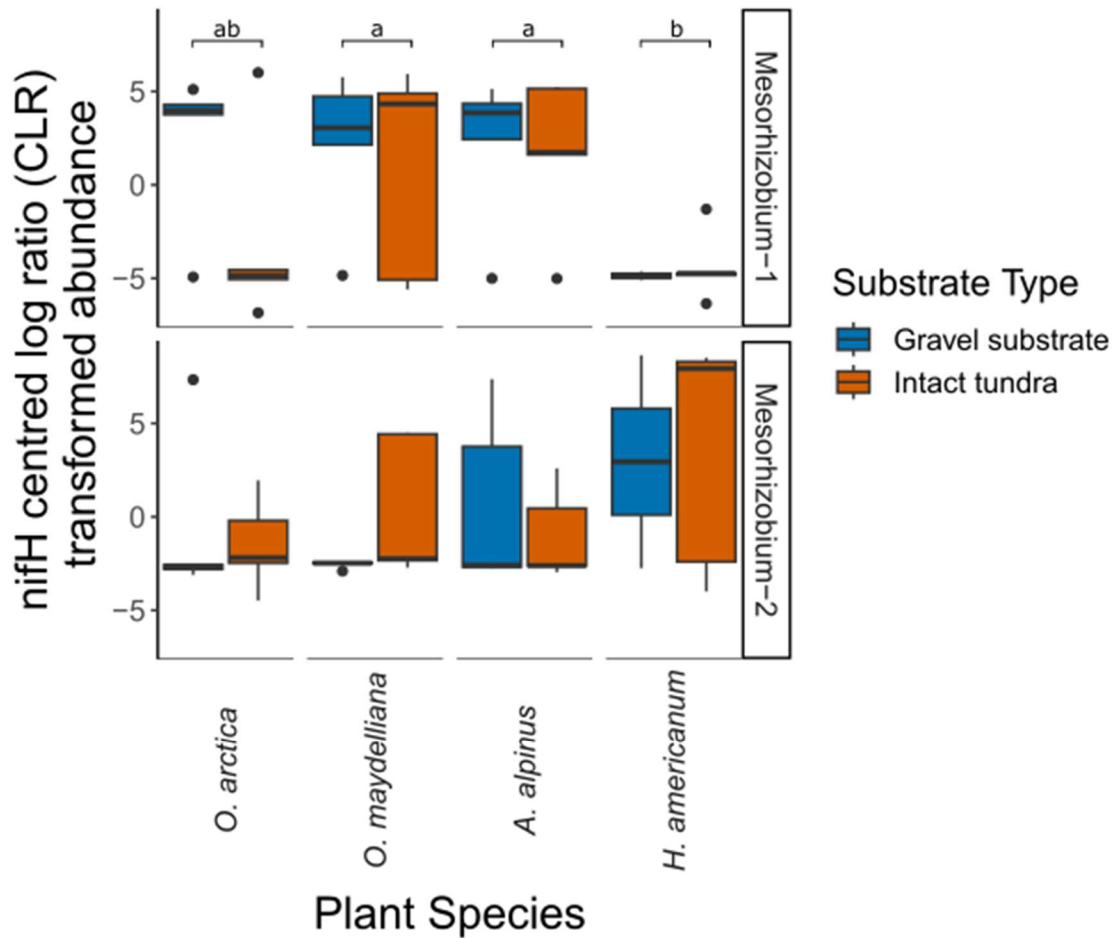


Figure 3.6. Boxplots of the two *nifH* ASVs with the highest species scores on PC1 in Figure A2 with the centered-log ratio transformed abundances on the y-axis and the four legume species on the x-axis. ASVs that were present in more than ten samples and were also within the top ten negative and top ten positive loadings in PC1 were chosen for analysis. Boxplots are coloured by substrate type and separated by ASVs codes. Boxes represent 25% to 75% percentiles of the data and points represent samples that lie beyond 1.5* inter-quartile range (IQR). For Mesorhizobium-1, *O. maydelliana* – *H. americanum* are significantly different with $p = 0.032$ and *A. alpinus* – *H. americanum* are significantly different with $p = 0.02$. Abundances were not significantly different between legume species for Mesorhizobium-2.

Table 3.5. Summary of Tukey’s HSD on Mesorhizobium-1 *nifH* ASV (88ad6d4de9880fdeb52ea15e8f11c0e0) centered-log ratio transformed abundances from four legume species – O-arc = *Oxytropis arctica*, O-may = *Oxytropis maydelliana*, A-alp = *Astragalus alpinus*, H-ame = *Hedysarum americanum*. Centered-log ratio transformed abundances of Mesorhizobium-1 were significantly different between *O. maydelliana* and *H. americanum* at $p = 0.032$, and between *A. alpinus* and *H. americanum* at $p = 0.02$.

	diff	p adj
<i>O-arc vs O-may</i>	1.842	0.766
<i>O-arc vs A-alp</i>	2.256	0.636
<i>O-arc vs H-ame</i>	-4.217	0.201
<i>O-may vs A-alp</i>	0.415	0.996
<i>O-may vs H-ame</i>	-6.059	0.032
<i>A-alp vs H-ame</i>	-6.473	0.02

3.5. Discussion

Despite the near absence of vascular plants and substantially lower organic carbon in the gravel substrates compared to nearby tundra (Hnatowich et al., 2023a), there were no significant differences in the composition or diversity of either the overall bacterial community or the potentially nitrogen fixing bacterial community among plant nodules collected from these distinct substrates (**Error! Reference source not found.**). This result is unexpected because highly disturbed gravel substrates with limited soil development or vegetation have lower abundance of soil microflora and distinct microbial communities (Filcheva et al., 2000; Hamidović et al., 2020) and studies in similar northern areas have suggested that nitrogen cycling is affected by topography (Stewart et al., 2014). Furthermore, while the high degree of disturbance might be expected to reduce populations of potential rhizobial inocula, there is evidence of successful nodulation in some highly disturbed environments. For example, both *Lotus corniculatus* and *Trifolium arvense* nodulated in early sandy overburden soils from a reclaimed mine, suggesting that the inocula necessary for nodulation can be found even in highly disturbed substrates (Boldt-Burisch et al., 2015). Rhizobia can persist in substrates without host plants for up to five years (Denison & Kiers, 2011; Hirsch, 1996), so it is not unreasonable that small amounts of residual inoculum are responsible for the successful nodulation observed here. There is evidence that vertical transmission of nitrogen-fixing bacteria by seed is possible (Ai et al., 2023; Jooste et al., 2019; Rout et al., 2013), but has not yet been documented in legumes (Frank et al., 2017).

We found relatively few differences in the nodule communities between the four legume species. There were no differences in nodule communities when analysed using 16S sequencing data. Some diversity differences were identified between *H. americanum* and *O. maydelliana* nodules when analysed using *nifH* sequencing data. This finding is interesting considering that we explored a diverse group of four species spread across three genera. *Astragalus* and *Oxytropis* are closely-related sister taxa within the Fabaceae, diverging an estimated 14.8 million years ago, and the clades with the descendants of the most recent common ancestors of *Hedysarum*, *Astragalus*, and *Oxytropis* diverged 33 million years ago. There is a reasonable expectation that species-driven differences in nodule communities should be present between two genera that diverged 14.8 million years ago (Lavin et al., 2005).

However, some studies have shown that small genetic differences can be overridden by environment to alter the nodule microbiome. For example, there was no genotype effect of two varieties of *Vigna unguiculata* (cowpea) but a strong soil type effect on the composition of the nodule microbiome (Leite et al., 2017). The similarity in the composition of inocula across our sampling sites could potentially override the genetic differences among our legume species, leading to the observed nodule community composition. While plant genetics do play a role in shaping nodule microbiome assembly, this is only one aspect of a broader set of biotic and abiotic factors influencing the environment. Research conducted in similar environments, such as alpine regions, suggests that the interactions between bacterial species and host plants may be less dependent on biotic factors with environmental filters playing a more dominant role (King et al., 2012; Pang et al., 2021). Recent studies have shown that soil pH plays a significant role in shaping bacterial communities in polar regions, directly impacting the colonization of the rhizosphere by rhizobia (Amores-Arrocha et al., 2023; Bordeleau & Prévost, 1994; Chu et al., 2010; Ji et al., 2022; Malard et al., 2019, 2021). Further studies may be required to compare the resident soil microorganisms at each collection site and correlate soil abiotic factors.

Our expectation was that *nifH* sequences would be conserved in nitrogen-fixers (Rösch et al., 2002). We largely observed this; however, our ability to detect differences may have been hindered by limitations in our database. Of the 306 total *nifH* sequences, 41 remained classified as “unknown bacteria” or simply proteobacteria, and of the 255 sequences that were classified to genera, all but two were identified as *Rhizobium* or *Mesorhizobium*. Given the limited study of microbial genomes in the Arctic (Edwards et al., 2020), it is likely that these unclassified sequences include new taxa. Whole-genome sequencing approaches may be required in future studies to target taxa that may not respond to culturing and amplicon-based methods.

Overall, our study demonstrated that early colonizing native Arctic legumes on gravel quarries were able to acquire nitrogen fixing nodule communities that were no different than those of legumes collected from nearby intact tundra. This is an important result for restoration practitioners as it suggests that seeding or transplanting native legumes in disturbed Arctic sites can lead to associations with locally adapted nodule communities without artificial inoculum treatments. Restoration efforts can be more cost-effective and environmentally friendly, reducing reliance on commercial inoculum treatments, which may not be well-suited to Arctic conditions.

By capitalizing on the natural ability of early colonizing native legumes to acquire nitrogen-fixing endophytes and stimulate ecosystem processes through nitrogen-rich plant litter deposition, restoration practitioners can enhance the success and sustainability of their restoration projects in Arctic ecosystems.

4. Use of Positron emission tomography to study the root systems of Arctic legumes

4.1. Abstract

Native Arctic legumes show promise for restoring soil functionality due to their nitrogen-fixing bacterial associations, yet their belowground interactions are not well understood. In this study, samples of native legume species were collected from intact tundra near Rankin Inlet, Nunavut, Canada. The samples were used to optimize [^{11}C]CO₂ dosing and imaging procedures adapted from recommended procedures on agricultural crops. *Oxytropis maydelliana* was successfully exposed to [^{11}C]CO₂ tracer and root structures imaged using the GNEXT PET/CT imaging system. Non-invasive imaging technologies like positron emission tomography (PET) offers insights into intact plant processes associated with belowground ecology, aiding in recommending better reclamation plans for Arctic sites.

4.2. Introduction

Revegetating tundra ecosystems after major disturbances, such as resource extraction, proves challenging due to factors like short growing seasons and low plant productivity. Native Arctic plant seed is generally not commercially available, and logistical challenges in transporting plant material to remote sites make using established vegetation impractical for many restoration projects. Therefore, research that centers on the re-establishment of functioning soils to facilitate natural colonization from the surrounding tundra areas may be better suited for restoration initiatives. Nitrogen-fixing legumes are often used in restoration plantings due to their contributions to improved soil conditions from nitrogen fixation. Legumes host rhizobia within their nodules which fix atmospheric nitrogen, and as a result can produce nutrient rich plant exudates and litter that may influence microbial activity and contribute to soil formation through deposition of plant litter (Dakora & Phillips, 2002; S. E. Hobbie, 1996; Reynolds et al., 2003). Non-invasive imaging technologies such as positron emission tomography (PET) may allow us to understand intact plant processes associated with the belowground ecology of these plants and recommend better reclamation plans for Arctic sites.

The use of positron emitting tracers, a technique initially applied to barley plants in 1939, has gained traction in monitoring plant processes such as water uptake, sugar transport, and the accumulation of nano-particles in plant tissues (Davis et al., 2017; Fatangare & Svatoš, 2016;

Hubeau & Steppe, 2015; Schmidt et al., 2020). Common isotopes used in plant studies include ^{11}C , ^{13}N , ^{15}O , and ^{18}F , with short half-lives requiring specialized facilities for prompt manufacturing and delivery, aside for ^{18}F . While most research using positron emitting tracers has focused on hydroponic systems and uniform substrates with easily cultivated crop plants, exploring ecological samples represents a novel field offering insights into in situ interactions (Hubeau & Steppe, 2015; Jahnke et al., 2009; T. Jones & Townsend, 2017).

This study represents one of the first attempts to administer [^{11}C]CO₂ to Arctic legume plants with intact belowground structures and substrates. The aim was to observe the real-time movement of photosynthates from sources to sinks in the roots and rhizosphere of Arctic legumes. Existing protocols were tested and modified to assess the feasibility of further experiments aimed at understanding source-sink relationships in early colonizing plants on disturbed tundra mine sites. Our objectives were to identify translocation pattern differences among four native Arctic legume species in two distinct habitats. The techniques established in this study may serve as a valuable guide for future PET studies on ecological samples.

4.3. Protocol Development Methods

4.3.1. Sample collection

Four native legume species were collected from locations near the Agnico Eagle Mines Ltd. Meliadine mine, approximately 25 km northwest of Rankin Inlet, Nunavut, Canada (63°01'22.9"N 92°11'41.1"W). The local vegetation is mostly comprised of heath tundra, heath-lichen, and lichen-rock vegetation, with common vascular plant species including dwarf shrubs such as *Cassiope tetragona* (Arctic Mountain Heather), *Dryas integrifolia* (White Mountain-avens), *Salix reticulata* (Net Veined Willow), and *Vaccinium uliginosum* (bog blueberry). Forbs included *Astragalus alpinus* (Alpine Milkvetch), *Cardamine digitata* (Richardson's Bittercress), *Pedicularis flammea* (Red-tipped Lousewort), and *Stellaria longipes* (long-stalked starwort) (Hnatowich et al., 2023a, 2023b).

Three abandoned gravel pits and adjacent undisturbed upland heath tundra located within 100 m of gravel pits were selected to provide a habitat comparison. The substrate texture of these old disturbance areas consisted primarily of gravel and sand particle sizes with limited soil organic matter (Hnatowich et al., 2023a). The four native legume species were collected from both disturbed substrate and intact tundra were *Astragalus alpinus*, *Hedysarum americanum*,

Oxytropis arctica, and *Oxytropis maydelliana* (Porsild & Cody, 1980). All species were abundant in the adjacent intact tundra; *A. alpinus* and *O. arctica* were more commonly found establishing on the disturbed substrates compared to the other two.

Pots used for plant collection were 30 cm tall and 10 cm in diameter plastic tubes with a removable base that had drainage holes drilled into it. Plants with rosettes around 5 cm in diameter were selected to be able to fit inside the plastic tube without severing roots and were collected from July 14 to July 18, 2021, inclusive. To extract the plant with its root system and associated soils intact, we centered the plastic tube over the plant and the substrate was carefully dug around the outside of the tube while simultaneously pressing down to surround the plant until the full depth of the pot was reached. We then slid a spade under whole plant to lift the plant out of the ground. Organic material (primarily landscaping jute) was used to stabilize the substrate in the pot before placing the plastic base at the bottom. The plants were placed in coolers with ice packs and kept in a shaded area. Ice packs were changed out daily until the coolers were sealed and transported by air to the University of Saskatchewan. Once there, plants were placed in a growth chamber set to 15 °C/5 °C (day/night), 65% relative humidity, 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$, and 18 h days. Plants were watered three times a week. Due to pandemic-related delays, protocol development was completed, and the plants were dosed in March 2022.

4.3.2. Gas dosing equipment

[¹¹C]CO₂ is produced at the Sylvia Fedoruk Centre for Nuclear Innovation at the University of Saskatchewan through bombardment of nitrogen gas with protons accelerated by a cyclotron. This process produces a mixture of gas impurities that can be removed using a specifically designed purification apparatus that traps the [¹¹C]CO₂ on a molecular sieve and diverts the other gaseous molecular impurities toward waste gas. The purified [¹¹C]CO₂ is then delivered to the plant dosing chamber using helium gas; following dosing, plants are scanned in the SOPHIE instrument. Extensive method refinement work detailed in Subedei (2024) determined the plant dosing and scanning procedures outlined below.

A dosing chamber previously used in plant dosing experiments proved incompatible with the short stature of Arctic legumes as the chamber had been manufactured to seal taller agricultural crops such as wheat and lentil (Subedei, 2024). Our plants stood no higher than 2 cm from the substrate surface and we were unable to seal around the main stem without damaging the plant

leaves. We modified the existing design of the chamber and designed an acrylic chamber that sealed around the plant pot. The substrate surface was covered with heavy weight paper and the edges sealed with acrylic latex and silicone caulking to prevent the [^{11}C]CO₂ from interacting with soil moisture and causing noise and distortion during imaging. The acrylic dosing chamber was fit with an O-ring that was lubricated with vacuum grease to create an airtight seal around the plant pot. Two gas valves were installed at the top of the chamber for gas input and output. Additionally, a grow light was positioned approximately 10 cm above the dosing chamber to allow for photosynthesis during the dosing process. Since the dosing period was relatively short and the plants were transferred directly from the growth chamber beforehand and returned immediately afterward, we did not follow highly specific procedures for light regulation during dosing.

4.3.3. Plant dosing and visualization

The plants used for our dosing experiment were the ones collected in July 2021 as well as some preliminary test plants that had been collected several years earlier and kept in the phytotron (Hnatowich et al., 2023b). Three days before dosing with [^{11}C]CO₂, the plants were moved from the primary growth chamber into a radiation shielded growth chamber at the Sylvia Fedoruk Centre for Nuclear Innovation set at identical growth conditions. Before dosing, the plant was removed from the growth chamber and placed under the grow light for at least 5 minutes. The base of each pot was also removed to facilitate scanning in later steps. Each plant was prepared for dosing as per Section 4.3.2 and was dosed with 2 GBq of [^{11}C]CO₂ at 5 – 7 PSI helium flow rate. The trapped [^{11}C]CO₂ was unloaded off the molecular sieve until radiation was detected on a downstream ascarite trap, ensuring that sufficient [^{11}C]CO₂ had been unloaded into the dosing chamber. The helium flow was then turned off to allow passive diffusion of the isotope within the system and the plant was allowed to uptake the [^{11}C]CO₂ for 30 minutes under the grow light before the gas was purged from the system for 5 minutes into a waste bag. We were then able to safely disconnect the plant from the dosing system and transfer it to the shielded growth chamber to allow for translocation of photosynthates from the photosynthetic tissues into the roots.

The GNEXT PET/CT imaging system (SOFIE, Virginia, USA) was employed to capture images of plants. Specifically designed for animal studies, the GNEXT PET/CT imager features an axial and trans-axial field of view of 10.4 cm and 12 cm, respectively. Consequently, the plants were

positioned horizontally. Each PET scan lasted for 10 minutes, with the field of view centered at 5 cm from the base of the plant. The GNEX PET/CT generates DICOM files from the PET scans, which can be analyzed using image post-processing programs. VivoQuant™ (Invicron, LLC) was used to remove the scanning bed from images and subsequently visualize the 3D reconstructions.

Following recommendations outlined by Subedei (2024), the plants underwent imaging every 30 minutes for a duration of 2.5 hours, starting 30 minutes after their transfer into the shielded growth cabinet. Between scans, the plants were returned to the growth chamber. A timelapse of images was created, with optimal root visualization achieved after the plants had spent 120 minutes in the shielded growth chamber.

4.4. Plant Dosing Proof of Concept

We were able to dose seven of the plants that had been collected from the gravel substrate areas in 2021 with varying rates of success. Unfortunately, due to pandemic delays, by the time we completed protocol development, the plants had been living in artificial environments for too long and did not respond well to the disturbances required to prepare for radioactive gas exposure. We were not as successful in maintaining plants that had been collected from intact tundra areas despite our efforts in maintaining sufficient relative humidity levels and soil moisture levels. Additional challenges included equipment availability at critical timepoints during experimentation. Of the plants we were able to dose, we were able to obtain a timelapse series of 3D images from the SOFIE scanner, illustrating successful dosing of [¹¹C]CO₂ and subsequent translocation of the radioisotope into the belowground tissues. From these images, we recommend for *O. maydelliana* that the optimal time for assimilation is between 120 minutes and 150 minutes after the plant has been dosed with [¹¹C]CO₂ (Figure 4.1).

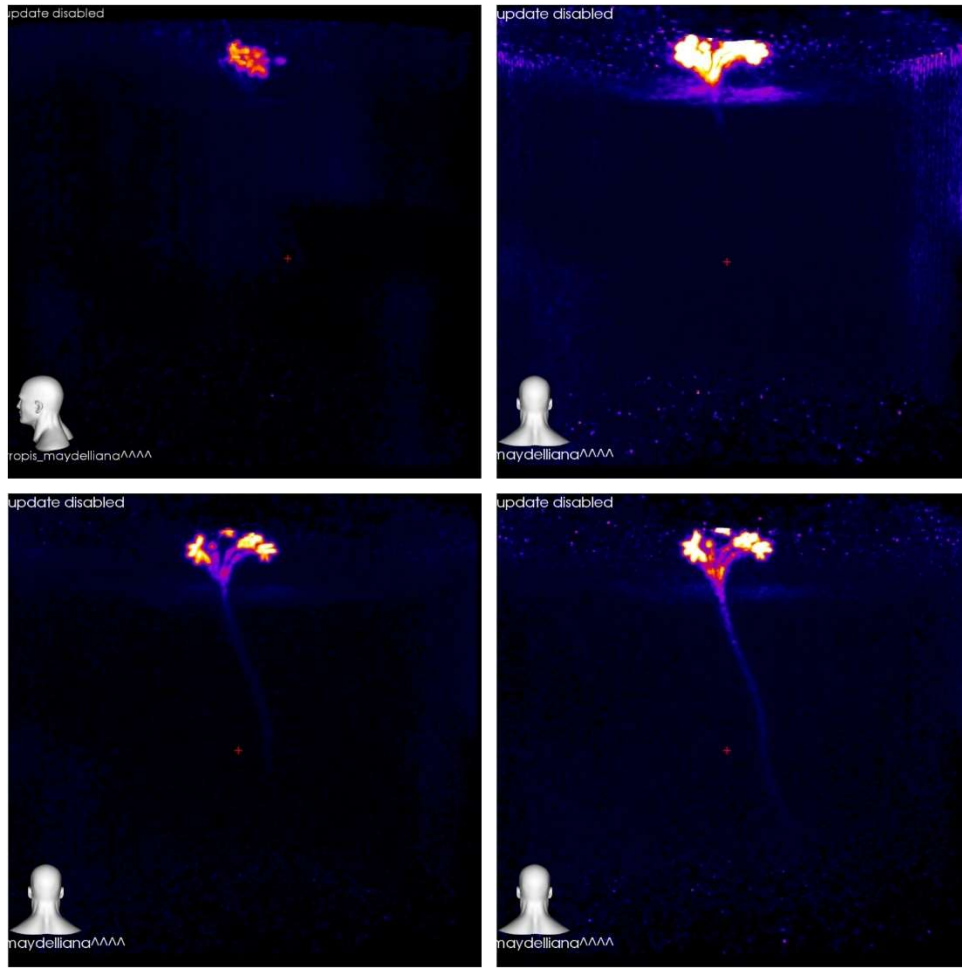


Figure 4.1. Positron emission tomography collage of *O. maydelliana* collected from gravel substrate after 30 minutes [^{11}C] CO_2 exposure and A) 30 min of assimilation time, B) 90 min of assimilation time, C) 120 min of assimilation time, and D) 150 min of assimilation time.

4.5. Discussion

In our study, we successfully optimized dosing and imaging procedures for the native Arctic legume *O. maydelliana*. Following protocol optimization methods recommended by Subedei (2024), our experiments focused on dosing rate, dosing time, and chamber design. The appropriate amount of radioactive tracer to be applied depends on the plant species. For instance, previous studies found that 5 GBq was required to successfully dose radish roots, while only 200 MBq was needed for maize roots (Garbout et al., 2012; Hidaka et al., 2019). Given that our experiment used the same sized pots and studied legumes as well, we decided to adopt the 2 GBq dosing rate and a 30-minute initial exposure time as recommended by Subedei (2024). Chamber

design was particularly crucial, as previous studies on other plant species were able to dose just one leaf and observe the entire root system (Kiser et al., 2008). However, due to the low-lying stature of Arctic legumes, it was nearly impossible to isolate and seal off only one leaf nor implement the clam-shell method of sealing for airtightness as used in Subedei (2024). Additionally, considering the slow growth rate of many Arctic species, translocation of photosynthates may be limited if only a portion of the plant is dosed.

One common concern when working with $[^{11}\text{C}]\text{CO}_2$ is that it readily attaches to water molecules, causing significant noise in the final image and rendering identification of plant structures much more difficult. This challenge is particularly acute for *O. maydelliana* given the prostrate growth form that precludes sealing around the stem as done by Subedei (2024). Our isolation technique using silicone-latex caulking to seal off the soil surface from exposure to $[^{11}\text{C}]\text{CO}_2$ proved successful in preventing CO_2 from attaching to the soil surface. After 30 minutes of assimilation time, the largest amount of radioactivity was concentrated at the leaflets of the legume, indicating CO_2 uptake at these sites, with no radioactivity observed along the substrate surface (Figure 4.1A). As the plant undergoes translocation over time, the radioactivity begins to move downward through the plant vasculature, allowing for identification of petioles, stems, and eventually the primary root (Figure 4.1B to Figure 4.1D). However, at 150 minutes, while roots are discernible, they appear less bright compared to the leaf tips and are relatively challenging to trace.

Despite the valuable insights provided by translocation studies, several challenges persist in optimizing experimental protocols, as highlighted by previous research (Subedei 2024). The short half-life of $[^{11}\text{C}]\text{CO}_2$ requires precise timing by researchers and equipment readiness, as delays can potentially disrupt the experimental workflow. In addition to the challenges presented while in the field with locating and extracting intact plants into containers that are small enough to fit into the imaging container, sandy and gravelly substrates do not hold their structure very well when the samples are being transported and handled. Another ongoing challenge for studies aiming to understand plant roots under field conditions is obtaining combined structural and radioactive tomography images. For instance, X-Ray Computed Tomography (X-ray CT) systems struggle to differentiate root material from soil organic matter, and in rocky substrates, the density difference between rocks and air-filled pores poses a challenge for the system to

distinguish organic materials (Schmidt et al., 2020; Taina et al., 2008). Without information about root structure, images obtained by the PET approach can only provide limited insight (Jahnke et al., 2009).

5. Synthesis and Conclusions

The studies conducted here aimed to answer questions about the ecology of native Arctic legumes and to use that knowledge to make recommendations for enhancing the use of these legumes in specific targeted restoration strategies. I compared the bacterial and nitrogen-fixing communities within nodules isolated from four common legume species found at the study site in two differing habitats: gravel substrate areas and adjacently located, intact tundra areas. These legume species (*Astragalus alpinus*, *Hedysarum americanum*, *Oxytropis arctica*, and *Oxytropis maydelliana*) appeared to be some of the first colonizers on disturbed areas that had little or no organic matter. The substrates that I collected the nodules from had different particle size profiles and substantial soil chemistry differences (Hnatowich et al., 2023a), yet I found relatively few differences in nodule communities between environments. Between species there were no differences in 16S communities and *nifH* diversity differences only between *H. americanum* and *O. maydelliana* nodules. The lack of differences in nodule occupants between environments and species demonstrate that these legume species are cosmopolitan hosts and sources of inoculant are readily found on disturbed substrates.

I extended the positron emission tomography (PET) techniques developed by Subedei (2024) to administer [¹¹C]CO₂ to Arctic legume plants to observe the real-time movement of photosynthates from sources to sinks in the roots and rhizosphere of the same four common Arctic legumes species from the previous study. My aim was to identify translocation pattern differences among four native Arctic legume species in two distinct habitats to understand the source-sink relationship, but technical challenges prevented replicated comparisons. The techniques that were established may serve as a valuable guide for future PET studies on ecological samples.

5.1. Future Research Directions

The findings from the nodule bacterial community analysis are interesting given that we were studying four different species spread across three genera. We expected the communities to be more different as *Astragalus* and *Oxytropis* diverged an estimated 14.8 million years ago, and the clades with *Astragalus* and *Oxytropis* diverged from clades containing *Hedysarum* 33 million years ago (Lavin et al., 2005). The legume species studied here are clearly cosmopolitan hosts that allow nodulation by several rhizobia species rather than engaging in more specific

interactions. Other genera in the Fabaceae including *Cicer*, *Trifolium*, and *Vicia* can be more restrictive hosts (Broughton & Perret, 1999; Peix et al., 2015; Zurdo-Piñero et al., 2009). Those species are found in the same Fabaceae crown node, however, the clade containing those genera diverged from the clade containing our species 33 to 39 million years ago (Lavin et al., 2005). Whether the more cosmopolitan rhizobial communities in our species are representative of the nodule communities of other *Oxytropis*, *Astragalus*, and *Hedysarum* species remains unstudied.

As the overall bacterial communities were not significantly different aside from a couple of sequence variants and knowing that nodule occupants are recruited through plant chemical signalling, we may infer that our study species may all have similar chemical signalling. Perhaps the four native Arctic legume species may have comparable exudate profiles as other known cosmopolitan hosts. Recent studies suggest that root traits may be key in explaining plant ecosystem roles (T. W. N. Walker et al., 2022), and some root traits have been shown to be linked to root exudation (Rathore et al., 2023). A future study conducted to examine the link between the root architecture of our species and any differences in root exudate pattern in different habitat type could be interesting. We could continue extending the PET techniques developed by Subedei (2024) and refined here to obtain 3D morphological data and subsequently extract the metabolites from the rhizospheric soil to more closely link morphology and physiology. It would be particularly interesting to examine differences in exudate profiles of plants collected from different habitats as plant metabolome differences may only be apparent under abiotic or biotic stress (Montesinos-Navarro et al., 2020).

The studies here demonstrated that it was not nodule occupants that were influencing the observed distribution of species at our study site. In addition to examining exudate differences between plant species, we could have conducted some rRNA studies to observe potential differences in actively transcribed genes or performed whole genome sequencing comparisons since study of microbial genomes in the Arctic is limited (Edwards et al., 2020).

5.2. Implications for Arctic restoration

Plant species in the Arctic exhibit remarkably slow growth rates and are adapted to the brief growth seasons. While producing seedlings commercially for restoration purposes may not be cost-effective due to their slow growth, there may still be potential for seed collection. Our study saw plants naturally colonize disturbed tundra environments and thrive independently. By

gathering seeds from native tundra plants and integrating them with the turf transplant approaches described in (Hnatowich et al., 2023a), we may be able to enhance restoration efforts. Combining seeding and transplantation may be important given the challenges in scaling up turf transplantation for larger areas.

We suggest restoration strategies that prioritize legumes given that those species play a crucial role in nitrogen fixation within nutrient-poor environments (Hnatowich et al., 2023a). Field observations indicate that *O. maydelliana* and *H. americanum* tended to favour lower-lying hollows in the tundra, while *A. alpinus* and *O. arctica* were more prevalent in exposed areas. When implementing hardscaping techniques during initial restoration efforts, it will be essential to replicate the hummock-hollow tundra landscape for optimal results.

Restoration efforts often rely on commercial rhizobial inoculum to kickstart ecological recovery in areas lacking nitrogen, a vital nutrient for restoring ecological function (A. Bradshaw, 1997; Chapin et al., 1994; Kidd et al., 2006). However, the use of commercial inoculum raises important considerations for ecosystem health and stability. While inocula can provide immediate benefits by efficiently fixing nitrogen, there is also a risk of the inoculant species becoming invasive as has been identified as a risk for mycorrhizal inoculants (Hart et al., 2018; Thomsen & Hart, 2018). Research has shown that rhizobial populations can persist at high levels for up to five years in substrates without host plants (Denison & Kiers, 2011) and commercial inoculants released into soils has been documented to endure in environments with minimal competition from native rhizobia (Hirsch, 1996). Our findings suggest that inoculation may not always be necessary, and a more cautious approach to enhancing nutrient conditions in disturbed tundra is feasible. Decisions regarding using commercial inoculant should be tailored to each site, with practitioners aiming to minimize the risk of introducing invasive species. This can be achieved through conducting test plantings of local legumes and monitoring nodulation as an indicator of whether inoculation is warranted. By embracing such approaches, we can better assess and mitigate the potential risks associated with inoculation practices, thereby safeguarding the integrity of the tundra ecosystem.

6. References

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7. Appendix A



Figure A1. Location of the three gravel quarries near the Agnico Eagle Mines Ltd. Meliadine mine. Sampling area is approximately 25 km NW of Rankin Inlet, Nunavut, Canada

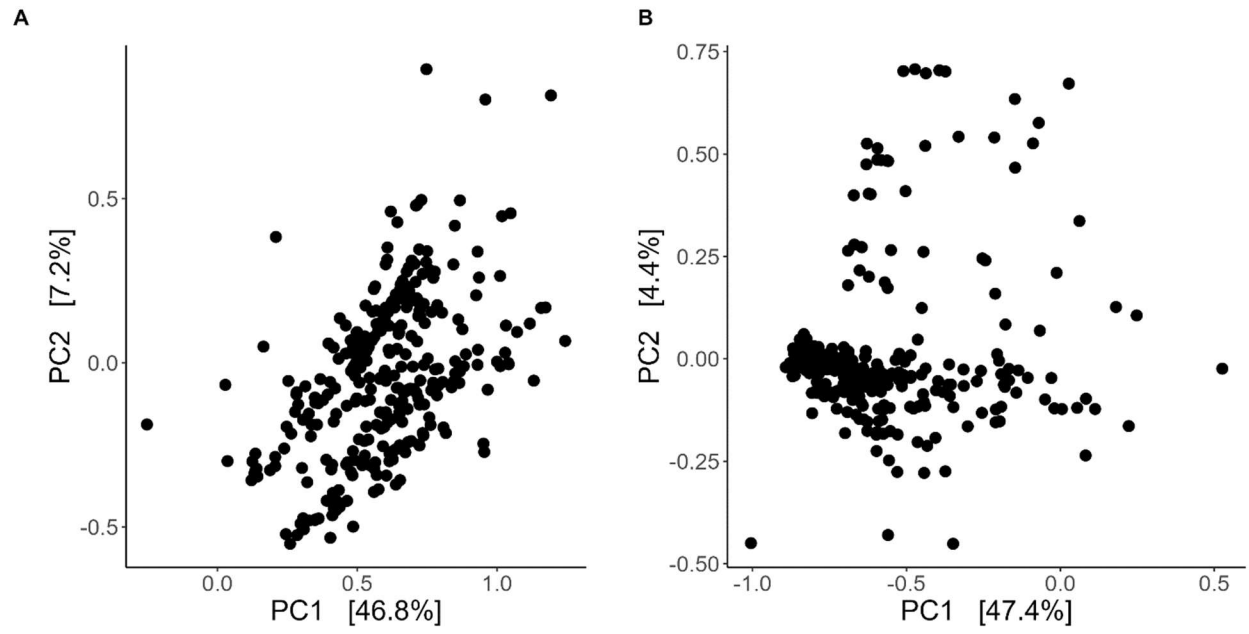


Figure A2. A) Species scores for the 310 16S ASVs in the PCA ordination. B) Species scores for the 306 *nifH* ASVs in the PCA ordination.

Table A1. Nodule collection sites and number of samples collected per legume species and site. Samples were collected by pooling roots of individuals within a 30 m diameter. Each site was separated by at least 300 m. Gravel and tundra substrate areas were within 100 m of each other for each site.

Site	Quarry	Substrate Type	<i>A. alpinus</i>	<i>O. arctica</i>	<i>O. maydelliana</i>	<i>H. americanum</i>
1	Q2	Gravel	1	1	1	0
1	Q2	Tundra	1	1	1	1
2	Q2	Gravel	1	1	1	0
2	Q2	Tundra	1	1	1	1
3	Q1	Gravel	1	1	1	1
3	Q1	Tundra	1	1	1	1
4	Q1	Gravel	1	1	1	0
4	Q1	Tundra	1	1	1	1
5	Q3	Gravel	1	1	1	1
5	Q3	Tundra	1	1	1	1
Total			10	10	10	7

Table A2. 16S ASV PCA scores

ASV NCBI code	PC1	PC2	PC3	PC4	PC5	PC6
7d850db295ca96fe18f859c33034a116	-0.2514	-0.2013	0.3069	-0.5967	-0.0119	0.1132
ec8a98fa1531f920c8df629f4fc7f568	0.0283	-0.0718	0.1032	-0.0334	0.0973	-0.0271
2b6531317d2a0446eead1f9b59d2c67b	0.0364	-0.3047	0.1229	0.0021	-0.0166	-0.2651
434bb8893ebe80ef5089a8361f8a6233	0.1222	-0.3643	0.1715	-0.0104	-0.1183	-0.3094
b2cff293bd9c9a72bdb443df3b9498b8	0.1256	-0.3059	0.1445	0.0090	-0.0925	-0.2583
61b464895cda438b2ce4bb2573b7446b	0.1325	-0.3437	0.1657	-0.0037	-0.1313	-0.2998
235b7d4b0cb8a336597a89b1e9dd9467	0.1365	-0.2835	0.1535	-0.1072	-0.0571	-0.0661
c6fed87b3b730590dc2f7dd39c4db911	0.1370	-0.3331	0.1697	0.0031	-0.0393	-0.2790
f501bf9a881c6c991bbe6a2113cbbf06	0.1406	-0.3290	0.1687	0.0063	-0.0440	-0.2800
a98d7c979b509f23955ea9a54cc51a00	0.1437	-0.3537	0.1877	-0.0249	0.0132	-0.3816
d177a13c79ae88def2b8cfb1cc0cac6b	0.1647	0.0439	0.1302	0.0093	0.1559	-0.0681
flf02851a65a8c21d2e87633f79ac377	0.1885	-0.3329	0.1668	0.0179	-0.1676	-0.3004
556b203c628ce54afda850974b151394	0.2071	-0.3210	0.1810	0.0057	-0.1825	-0.3013
a47081da0df3f368178caaec919cc545	0.2072	-0.2940	0.1893	0.0133	0.0084	-0.3864
d755f095a776385647793573fbd6ec8d	0.2090	0.3782	0.0976	-0.2064	0.2693	0.1430
75ca39b738ac0b680b3c31812723c883	0.2391	-0.2668	0.1631	0.0627	-0.1001	-0.2872
90b4679f66c4d4cfded222b62d4954a5	0.2452	-0.5238	0.0557	0.0742	-0.1024	-0.1938
528bc64d6c7248e07604d88ca561e707	0.2478	-0.2013	0.1659	-0.0603	-0.0887	-0.0690
6620dc4582d2e9084972edcbeb7c6bc9	0.2540	-0.0664	0.2635	-0.2488	0.2299	0.2022
5045bdc432b0a48e7ae18e9c3fa9dee0	0.2604	-0.5523	0.0381	0.0743	-0.1017	-0.1595
ca07074ee4baa7ef3688764c274149d4	0.2642	-0.2255	0.2608	-0.3572	-0.1620	-0.0166
510c633c17b1302bc26338db10e88991	0.2780	-0.1568	0.1837	-0.1713	-0.0051	-0.0509
352d79b9aeedce91430c114d695ce8bc	0.2831	-0.1046	0.2026	0.0506	-0.0424	0.0530
883b281458566f19bfc3728a93b09a55	0.2836	-0.0986	0.2240	0.0306	-0.0401	0.0279
2c099201f920cf32c20fc69cdeb68292	0.2856	-0.5258	0.0398	0.0961	-0.1193	-0.1824
1ef749ddaf52c9f940841a455648b956	0.2892	-0.1355	0.2084	-0.0578	-0.0150	-0.0893
b619cba42098457bebcd63a6c4020a93	0.2974	-0.4905	0.0367	0.1137	-0.1348	-0.1899
fc9d7042a0d7cc08b44930ed31a9eeea	0.3033	-0.3327	0.2603	0.0572	0.2590	-0.0790
2f83c47cedd1dba8ee1eb2d0888dbd6e	0.3065	-0.4756	0.0837	0.0642	-0.2363	-0.2197
e5bec664eefc8a1379875cc5e231bde6	0.3067	-0.1798	0.1461	-0.0378	0.0711	-0.1503
44fcfe131d85fb11ba9b3c4cefef8e2c	0.3083	-0.5032	0.0296	0.1109	-0.1307	-0.1673
ed93b2670371be3dedf9ff46f90c22c2	0.3099	-0.5138	0.1426	0.1033	0.1506	-0.0883
758a9388851ec1b7fb542372bded784d	0.3146	-0.0663	-0.1742	-0.1472	0.0520	-0.0366
0755d889c789944a48f178d827c5d986	0.3214	-0.3661	0.0663	-0.0114	-0.1789	0.0194
e829756dd8a1f562b92b125649e7567d	0.3272	-0.1620	0.1806	-0.0580	-0.1251	-0.0551
84e5f5abcb6223c2444509c946dcc579	0.3283	-0.4806	0.0369	0.1267	-0.1520	-0.1833
1d9b54a3dd948f4c1dd872bbdba4a71c	0.3351	-0.2361	0.2601	-0.1505	0.3357	0.0906
ec553972e5e8642b724cc7045faf4532	0.3369	-0.1960	0.2002	-0.0526	-0.0435	-0.1908
594c00aef6096809d226c172b3cb71b4	0.3488	-0.4792	0.0322	0.1306	-0.1571	-0.1700
bfed5f001d9569443a6f31809f0aaf9b	0.3488	-0.1152	0.2603	-0.3427	-0.1251	0.0403

582d72b2be6315959976556abd74e0ee	0.3501	-0.1293	0.1974	-0.0407	0.1576	-0.2030
25edd76ce21740f5a1ae73f23e0b2201	0.3513	-0.4771	0.0223	0.1170	-0.1427	-0.1257
3ae0226e9d894fc251c28cff8c8b121	0.3537	-0.2173	0.3021	0.0339	0.2501	0.1406
6e180169382afac5a86c5bef501c9280	0.3570	-0.2016	0.2686	0.0105	0.3107	0.0898
e72b86f2ab497eef25453316c6194fe4	0.3612	-0.1331	0.2735	-0.4372	-0.0695	0.0368
f66488c9dc5a4635ce68bdf42369d9df	0.3622	-0.4744	0.0321	0.1348	-0.1649	-0.1541
7e79ea955fd00780cbfcf356bb1ee2b1	0.3632	-0.1112	-0.1962	-0.0203	-0.0269	-0.2732
f39ab2b519204339394f517786186ed7	0.3681	-0.0431	-0.1890	-0.1387	-0.0073	-0.0753
b47bbe0bfff9972cc435d343389efaa2d	0.3780	-0.1238	0.2774	-0.4391	-0.0817	0.0423
d6731a10b95b189aa8e16937f0ae0dd8	0.3897	-0.2986	0.0882	0.1212	-0.1071	0.1260
e43b9b8ac191e25d79b4828e13e8c988	0.3911	-0.4211	0.0267	0.0444	-0.0677	0.0402
4234852fc5b10cea6fd8253097f1e057	0.3947	-0.1051	0.2068	0.0157	-0.1018	0.0531
6e9aca2bad612d15479bc95c08aa9fa7	0.3972	0.0492	0.2627	0.1361	-0.1303	0.0772
cd29c6b7cb119046c0a1503a56a13c0c	0.4034	-0.0648	0.1708	-0.0260	-0.1430	-0.0773
0af29748b4ed62cb624dd045389d795a	0.4046	-0.5387	0.1224	0.1417	0.1245	-0.0430
8bae333b07e4e4b400a0dd98b7972efe	0.4072	-0.3291	0.1113	-0.1086	0.0447	-0.0793
249d4344e0a9a20efbbecf23e95e798b	0.4104	-0.3141	0.0927	0.0252	0.0021	-0.1068
0bcc54af6809356946dc7372e568d5d7	0.4119	-0.4706	0.1049	0.0466	0.1933	0.1479
f8fc8f2c96d434feafc0d26b54185ae3	0.4129	-0.3975	0.0254	0.0563	-0.0791	0.0473
e39bf075239916707cf6285bc03bb8f9	0.4158	0.0367	0.2521	0.1644	-0.1575	0.1081
67438bb8aceda7b27cf0d08ca1f092a9	0.4164	-0.4431	0.0484	0.1451	-0.1892	-0.1981
df7bc96b746cae85fda54aba5d7e8783	0.4167	-0.1925	0.2703	-0.0177	0.3173	0.1462
7c53a33d60c8aa473cb3dac5cc87e1f6	0.4213	-0.0869	0.2611	-0.3216	-0.1542	0.0612
f24feeb5d5c45a091a6fa292231c67a7	0.4255	-0.1693	0.2858	0.0412	0.2951	0.0972
0c686ba404284890bb0ad8b4d33ff64b	0.4256	-0.4547	0.1131	0.0504	0.2098	0.1609
492a1fd7fdacccb51aeb4d4d123a3df	0.4257	-0.4272	0.1224	0.0521	0.2342	0.1728
a237d09946474c03abfd42f1b62222f3	0.4343	-0.3891	0.0456	0.0434	-0.1811	0.0336
dc6c7dc6ca6e0e92f67808af8cb24872	0.4344	-0.0062	0.3559	0.2187	0.2171	0.2700
b8e26d1256bd85c5dfd0aca3d8a38236	0.4355	-0.4437	0.0804	0.0629	0.1373	0.1471
0bd8c37170486daa644e0088fba6c826	0.4380	0.1268	0.2038	0.0756	0.2078	0.1448
8afc4c07d0ddc356d793ac56a6c4c3f8	0.4382	-0.4438	0.0950	0.0628	0.1840	0.1667
75605a2f75c28ccc7a5c819530ecb414	0.4588	0.0352	-0.2074	-0.2223	0.1122	-0.0410
f83617c0f27efd0a9bcfac112e9d86c6	0.4593	0.1047	0.2391	0.1492	-0.1251	0.0432
8915f5bddb0587aed3d6b9d92e440024	0.4609	-0.3115	0.0608	0.1406	-0.1368	0.1427
a0aae1717cc4c1a609f7737b84984811	0.4627	-0.1386	0.2020	-0.0578	-0.1074	-0.0930
d027f83dd2821931ad0520fc7943bdbe	0.4644	-0.4266	0.1141	0.0685	0.2102	0.1862
06c155adfeffd384e1d46418be22b9968	0.4661	-0.3060	0.0905	0.0435	-0.0146	-0.0998
5896c07d0fc34d317c078f1e9eca303b	0.4766	0.0768	-0.2403	-0.1044	0.0569	-0.0309
f1743987bd5f6251bab20ae28a2ba289	0.4774	-0.3340	0.0580	0.0433	-0.2030	0.0194
37533e8c42deec0047e07e0e1d2983a7	0.4781	-0.0347	0.2769	-0.3183	-0.1670	0.0577
3dc47ea8a4df1118e76ce016f7dbb3ce	0.4824	0.0338	-0.0988	-0.4056	-0.0494	0.0287
85160436f7580b6b9b3c112ca39a04d0	0.4825	-0.0292	0.2773	-0.3190	-0.1685	0.0581
05a00038f4a0213405868614c1b41606	0.4830	-0.3431	0.0237	0.0821	-0.1087	0.0730

d57b9445efce9a0b3e31ad560a843075	0.4835	0.0508	-0.2065	-0.2163	0.1016	-0.0344
3dff02d387ab60dad6cafeb9e2ff7b	0.4848	-0.2711	0.0571	0.0578	-0.0230	-0.0468
709392b1218d5f3e7fd869c7339d156d	0.4854	-0.4978	0.0249	0.1464	-0.2195	-0.3136
da064cbaef476ee57fb1aacc398f1457	0.4874	-0.0212	0.2799	-0.3154	-0.1726	0.0638
b253f16c81886ed7445c036863450913	0.4897	-0.2987	0.0637	0.0742	-0.0242	0.0169
ffde3e4e4e2683d7069c5378a0426e52	0.4921	-0.0500	0.2965	-0.4300	-0.1187	0.0531
f2f93ddd392aa5c2d53358e83b6a47a9	0.4926	-0.0150	0.2800	-0.3170	-0.1740	0.0634
1c20590d3f92ba830cf461ad106fb381	0.4933	-0.1308	0.2705	0.0089	0.3912	-0.0230
479ea7d7633e34c70149b9c318a572a1	0.4972	-0.0075	0.2533	-0.1494	0.0955	-0.0792
8e57d6f13cf6cf4d3b1cf19a3a212238	0.4976	0.0856	0.1893	0.0238	-0.0523	-0.0369
d525d878ec5902aec3dfbff1ac893ad3	0.4989	-0.1228	0.3211	-0.0126	0.3358	0.0513
5eb0a0182458892eb7ea436b0b4a32ce	0.4993	0.0994	-0.1761	-0.1096	0.1475	-0.0980
7db11a4136e4f675feca5a73e12a3b4e	0.5004	0.0708	-0.1511	-0.3612	-0.0339	0.0276
9cc470906e3b73a5fc1f7f90e3e94c66	0.5063	-0.2204	-0.3352	-0.0562	0.0141	0.0333
562d52b933c294a85a6ad53f9e425504	0.5082	-0.0734	0.2746	0.0256	0.3596	0.1481
4d5adfcce4899e32fcb1bf603d86f8f	0.5102	-0.0245	0.3029	-0.4384	-0.1215	0.0547
af19b529d914fef361fafeec9fe334	0.5105	0.0776	-0.2458	-0.0932	0.0444	-0.0183
dd3fff10e2c7bfb62702b820f1c5adc	0.5113	0.0100	0.2818	-0.3225	-0.1791	0.0606
33697e448f62b063a797dfdee11d9763	0.5121	-0.0243	0.3032	-0.4440	-0.1170	0.0535
9a9a74b85543de7188b2ac2880a4f658	0.5180	-0.3141	0.0637	0.0265	-0.0274	-0.0775
950136af9b4f4845f83b28908abff69d	0.5184	0.0111	0.4167	-0.1315	0.0983	0.1756
cbb267d7950f2777fb4d906f57947f73	0.5247	0.0428	-0.1282	-0.2203	0.0843	-0.0185
e24d974fe8f9602e466c1d000c1b3013	0.5255	-0.2260	-0.3245	-0.0366	0.0013	0.0321
b0546ef2f7ccf2c60ff4de3c012738de	0.5260	0.0866	-0.1491	-0.3512	-0.0455	0.0342
8afa0cc3c20bd3653096da7e1df52635	0.5273	-0.2860	0.0973	0.0836	-0.0778	-0.0482
db0aea107f42ffcf3132622b303e4de	0.5294	-0.1311	0.3048	0.0061	0.4243	-0.0040
9326dd55f55ce77eb818042fb2ae1b79	0.5300	0.1651	0.2485	0.1058	-0.0282	-0.0774
5efc0d9d15bd5788eac6781d92128e84	0.5323	-0.1816	-0.3357	-0.0466	0.0091	0.0271
c3799799242747e59c6390324085b286	0.5334	0.0264	0.2831	-0.3283	-0.1849	0.0570
faf15dac5bdf02e8d30336428a2b8d30	0.5336	-0.0022	0.2235	-0.1683	0.0993	-0.1810
5d858d9a46632a9c5477a71bab7dbaf6	0.5396	-0.2225	-0.2946	-0.0059	-0.0245	0.0537
35863a64c91014976aa42d3c40101afd	0.5408	0.0380	0.2815	-0.3313	-0.1870	0.0591
887724b5d6939ec32a920c82b11027ce	0.5442	-0.0594	0.2684	0.0397	0.4041	0.1706
2e3f5cdc94db0abfea4fe5544cbefaff	0.5454	-0.1235	0.2481	0.0040	0.3657	-0.1478
0030dd7cec9d1c942e4344a710e78aaf	0.5477	-0.3136	0.0323	0.0850	-0.1293	0.0780
2d771253641a6fa7ea6b39b9d5e376d9	0.5497	-0.0981	0.3416	0.0785	0.3367	0.2153
22a19f99f0c3e7745af026b996e802c5	0.5524	0.0587	0.2787	-0.3356	-0.1907	0.0624
6354b3803957976854da4ae4bfa72095	0.5545	0.1448	0.3037	0.1845	-0.2077	0.1392
15fef17f1766dd1516each686560ec4b	0.5550	0.0625	0.2782	-0.3368	-0.1914	0.0631
16d0f6d83e8beaede758f986f020d42a	0.5593	0.2156	0.2451	0.1899	-0.1386	0.0744
e74c73fd18649c477ec394d5bab1c8c5	0.5597	-0.3904	-0.0272	0.2140	-0.2170	-0.1492
aec1085687998137cbc1e1c8e0e92e03	0.5609	-0.3259	0.1295	-0.1848	-0.0860	-0.0755
7c1e46993526898a2ff9157b4909ac1b	0.5615	0.0703	0.2805	-0.3373	-0.1874	0.0607

4a824a87a80dd7c1c23eeabf16beb6d2	0.5617	0.0707	0.2804	-0.3375	-0.1875	0.0608
20695e225ad82857043ea5b6a6d93305	0.5639	0.2241	0.2404	0.1932	-0.1400	0.0761
be9744ff0513ea3ad629314972d509f8	0.5643	-0.3035	0.0265	0.0941	-0.1333	0.0800
21dda6f26274577c6be9227020898591	0.5675	0.1509	0.2338	0.0340	0.0367	-0.0937
1ed4b99c8fc5b28bcc01be229fdb95d	0.5687	-0.1926	0.1098	0.2011	-0.1975	0.1648
f39a6fb7b4a07834d7f99f2c2ec37f46	0.5688	0.1266	-0.2250	-0.0984	0.0286	-0.0199
6d58215d58780c740eb458605738e6fd	0.5704	-0.3499	0.1655	0.0410	0.2008	0.1291
8b203d385b5afd5699943fe2ad6d86f6	0.5746	0.0900	0.2748	-0.3451	-0.1963	0.0684
9b959e9dbbb93e6f6573277503c482cb	0.5766	-0.3908	0.1070	0.0900	0.1582	0.2011
a958fd62f75304231af430884f22e1c6	0.5799	0.0979	0.2725	-0.3482	-0.1998	0.0715
074741e0f088ff6f633737aa9ce99668	0.5814	-0.1402	-0.2612	0.0222	-0.0650	0.1549
e85d5ee5b47e98193b7ab853b3439e09	0.5820	-0.0632	0.2839	0.0393	0.4890	0.0509
f8236bb54b5367ee917652c79b2bfc57	0.5825	-0.0337	-0.3107	-0.0477	0.0019	0.0283
7fe830307ffdd6cfb52ea962500797a9	0.5843	0.1011	-0.0675	-0.3786	-0.0977	0.0624
07fd5a8d533c0fcea2f726cf24977496	0.5859	-0.1880	-0.3058	0.0016	-0.0386	0.0635
15da43626d34c564dcfd6fb4392dbf6e	0.5871	0.1118	0.2399	0.1345	-0.0618	0.0869
4f333539ecb7124a121f1f0c6ed3d44a	0.5908	0.1586	-0.2259	-0.0838	0.0239	-0.0169
0908193cf70e5d90d24280812fe62ff6	0.5914	0.1153	0.2674	-0.3550	-0.2076	0.0783
d820d79a6d5cd0c31d50e7174fa37ec6	0.5916	-0.1413	-0.3386	-0.0176	-0.0221	0.0541
81c417d4d6834619ea67930c2056e67c	0.5924	-0.2578	0.1155	0.0629	0.0166	-0.1044
c1b3b46d596b62e1615ade5e76102944	0.5933	0.1181	0.2665	-0.3561	-0.2089	0.0794
fbfcada4d938a89bdb4e74bab9eb8119	0.5934	0.1620	-0.2264	-0.0829	0.0239	-0.0176
c44137f70947c3fbf7cef89728c308b7	0.5967	-0.1834	-0.3036	0.0043	-0.0458	0.0700
8be39ac6071e900e863b29724e7b3750	0.5985	-0.0197	-0.0783	-0.3887	-0.0838	-0.2062
c0f8d7461154f8c2b23b686a08d715b0	0.5989	0.1265	0.2641	-0.3593	-0.2127	0.0827
5ea0b5724c159cf2cf6eae49a7620740	0.6013	0.1764	-0.2262	-0.0794	0.0236	-0.0218
b6f135f89579414e084cf2d988851f92	0.6016	0.2930	0.1977	0.2170	-0.1489	0.0878
6dea10a45a94fed7d1662c8371c0bed0	0.6029	-0.1375	-0.3360	-0.0124	-0.0311	0.0663
3f48c2ab1c356d3aa59c03d00966d1e1	0.6032	-0.0691	0.3090	0.0478	0.2953	0.0092
76e9b1a55985f97e88238e728b5a9cf8	0.6047	-0.3495	0.1274	0.1571	0.1336	0.2670
a78385f237715a64d899990353444c94	0.6069	0.3066	0.2058	0.2317	-0.1572	0.0973
2243a553ecdd8ae1e85fb7b43d281162	0.6078	0.3425	0.2455	0.3152	-0.2032	0.1962
910a569763c548382bbc5d0cbbb47964	0.6081	-0.1706	-0.2614	-0.2416	-0.0408	0.0175
2bef9586e73e24579676bdc7bbaa6d29	0.6100	0.1462	0.2647	-0.3564	-0.2227	0.0926
9e272043620dfc4da5c846c52d594e17	0.6123	0.0405	-0.0868	-0.0677	0.3851	0.1378
71012596916991ad48dafca99052d01b	0.6126	0.1175	-0.1213	-0.0435	0.0709	-0.1408
70b926817f3fd292736acab4720a1feb	0.6152	-0.3004	0.0381	0.0918	-0.1390	0.0644
fa1a049eb785d66621a0496ca3de5234	0.6153	-0.2760	0.0122	0.1172	-0.1441	0.0817
40de59ada6b55a7e3ba9bebb710c4fe8	0.6160	0.0204	0.2883	-0.3083	-0.1784	0.0132
10d7fb71e786537a3f52b51ac3d06c64	0.6200	-0.2729	0.0113	0.1186	-0.1462	0.0808
e4a2683f7e1a3ea1307e1deeb25aa4ba	0.6203	0.4447	0.3720	0.4330	-0.1659	0.2747
9821a68da094d8b64937c85842c18578	0.6205	-0.1002	-0.3374	-0.0198	-0.0242	0.0547
3d0eb5dbb2cdc3ce47e2add43a613df2	0.6212	-0.2709	-0.1995	0.0191	0.1966	0.1801

98ab2470e690c55393c53c56bb07b647	0.6214	-0.1594	-0.3204	0.0024	-0.0429	0.0691
e87d8cd72dde75ec958e0d894ac83a60	0.6230	0.1924	-0.2288	-0.0760	0.0226	-0.0270
1b2d58aabb9aa1f7ad0f98c7f7c1a1ba	0.6250	0.1958	-0.2297	-0.0750	0.0226	-0.0270
aafe6d6d0afcd29e9ecb3373fb676003	0.6276	-0.2684	0.0055	0.1234	-0.1474	0.0830
ef251cb9079cf95b031bdd2e319ee3ac	0.6277	-0.3023	0.1531	0.1031	0.2966	0.2338
c2d74a56a295b7e45b78b3b2bb07721d	0.6318	-0.0271	-0.3095	-0.0484	-0.0203	0.0451
237d27e623a0f8c8f762861410de8d54	0.6332	0.1626	0.1936	0.2696	-0.1014	-0.5248
d54326137b3290b399dcb6f9e456b4a0	0.6368	-0.2629	-0.0016	0.1293	-0.1489	0.0856
a48a9855570be3f740a4f63be454dc84	0.6378	0.2171	-0.2353	-0.0689	0.0217	-0.0281
b384c24783e7caa39146ec2e7cabfb88	0.6381	-0.3655	-0.0796	0.2218	-0.1372	-0.2186
1c4a8119160f366f7919b1fcfb21373d	0.6418	-0.0601	-0.4869	0.1000	0.4557	0.4066
b5f3a6ad486b06f7b38a8278ad58e470	0.6426	0.0313	0.3240	-0.3489	-0.0817	-0.0767
fd0c4c24fe2103c12055909d856fd762	0.6433	0.4201	0.2342	0.3159	-0.1986	0.0928
c5cda2bc51fd9bcddab8544320cc5138	0.6447	0.1120	-0.1909	-0.1065	0.0193	-0.0538
4fde78fdfd326601fba16a2e72685668	0.6473	-0.0769	0.0486	0.2404	-0.1858	0.0257
fffbe9d2786df3f04a5c6d042a79ebdd	0.6482	0.0762	0.3532	-0.4949	0.0021	-0.1240
84698f4607adec306623517ccb356f0a	0.6490	0.1905	0.1666	0.0702	0.1137	-0.1940
2f4be782ad31585c793c47fade4f4a68	0.6499	-0.1173	-0.3406	-0.0109	0.0136	-0.0034
0e86e38c7dfd53c98f8d0a0cdfce3f79	0.6502	0.0841	0.2296	-0.0695	0.0192	-0.1576
97524f39624ce2bd32829f25d6949df0	0.6503	-0.1397	-0.3161	-0.0127	-0.0547	0.0797
017159bddf3d24a82770911d674f33d6	0.6507	-0.1448	-0.3117	-0.0096	-0.0570	0.0807
10db31583f27d458ce195dec4676c4d9	0.6510	-0.1120	-0.3412	-0.0123	0.0130	-0.0025
f996e6d781cdd8c35c05d908950bb369	0.6517	-0.2499	-0.0071	0.1371	-0.1457	0.0852
5a70819b20eceed4c48de0fbcc63063f	0.6523	-0.1298	0.1400	0.1784	-0.1234	0.1447
7d2e3db5257617d6c73dafc590977ce1	0.6527	0.2143	0.2576	0.0847	-0.2373	0.0629
a874bae2bb3177d5ae46803b6068c12b	0.6537	-0.3622	0.0927	0.1256	0.1866	0.2249
a15eb2f5a10de50f2ff39734e155e56e	0.6549	0.2473	-0.2417	-0.0599	0.0265	-0.0298
50de0d7bc2bce0ecee78696d84a4f287	0.6576	-0.0002	-0.3082	-0.0376	-0.0214	0.0352
878f23589782505ac0362d771af5ef88	0.6577	0.1065	0.2245	0.0179	0.0117	-0.1196
6658933c85ed4ecc45be7f8c83a5c9ed	0.6610	0.2021	0.2230	0.1027	-0.0415	0.0093
b09162c247969d643ca7bef232c354bb	0.6611	-0.1879	0.1447	0.1280	0.3576	0.2576
e18b328248308aab81e093a20d878184	0.6614	0.2592	-0.2484	-0.0553	0.0247	-0.0273
e37a1683cc86d9fef066d9a658bb1960	0.6615	-0.1042	-0.3303	-0.0183	-0.0468	0.0716
98464fec49b22ceccaba269d3c52b5af	0.6643	0.2276	0.2262	0.1589	-0.0833	-0.1534
a7d55b5b0f4bc9bc8a11630424be91e9	0.6661	-0.0960	0.1714	-0.2741	-0.2532	0.1359
ab1669c7c6c33b62197b7ea819d47500	0.6714	0.2404	-0.1251	-0.0572	0.0997	-0.0852
aea99196d81cd58231c618fd6efaf537	0.6719	-0.2727	0.0258	0.1074	-0.1483	0.0554
3c9ec0b3dbaa328cbfd44154fa09ee72	0.6728	0.2233	-0.1716	-0.2649	-0.0550	0.0215
f575a95ccad9af28de8f7c9fe4593ef4	0.6731	0.0274	-0.0974	-0.1843	0.4750	0.0412
b03ce1f96470279e92dbefe63ef87304	0.6762	0.1690	0.0412	-0.5253	-0.0808	0.0553
afc9c413094a37859ba15ca04b93c5c5	0.6768	0.2877	-0.2643	-0.0444	0.0202	-0.0215
e7782fe39cc4510982f7fc950c8278ac	0.6773	-0.1013	0.3459	-0.0908	0.3875	0.0105
0e93b31b5035f68d31e1fa5e97da21d8	0.6776	-0.0778	-0.1926	0.0758	0.2014	0.1676

e22df544bb703aaf436e2cb605be81ab	0.6799	-0.1279	-0.3168	0.0019	-0.0593	0.0721
aae2af53c62eee58462bdcd8288b4008	0.6817	-0.2391	-0.0386	0.1585	-0.1585	0.1008
8d340abfea8f9efb57170c07d36d0cfd	0.6837	0.3005	-0.2714	-0.0394	0.0183	-0.0189
d4bdc931ae2709bcf15598eb54565709	0.6856	0.2211	-0.0263	-0.4006	-0.1353	0.0636
671f8921f0ab269feed99f955d46c21e	0.6887	0.1922	-0.1883	-0.0482	0.2808	0.0869
7da7eac82b1d8dd3a9f9c244da67d88d	0.6894	-0.0654	-0.3348	-0.0106	-0.0446	0.0597
d4085f4e28fcd349eebb1ed6e7ee9225	0.6927	-0.2351	-0.0502	0.1664	-0.1631	0.1065
a3db846395ef3ee54ea4d82ab2bbd173	0.6939	0.0742	0.2123	0.0364	-0.0506	-0.1520
11750af138312812b3d242e67dcd498b	0.6947	0.3221	-0.2783	-0.0280	0.0128	-0.0123
fb7e83c7c527aa0d987ed8bc0bc2b6c9	0.6996	-0.0241	0.2685	-0.0745	0.3380	0.0778
46ab46b942c0174d23ec7f9593bcaa31	0.7025	-0.0661	-0.3378	0.0021	-0.0520	0.0679
0ef25c5272f003dc7a2cbe533e4088d6	0.7053	0.2869	0.3334	0.3144	-0.2335	0.2721
ed076b76b84c8b579593fbf270247736	0.7083	0.2527	-0.1668	-0.0688	0.1456	-0.2012
d1a75003cbc69c762a2e05ef3928157b	0.7098	0.4721	0.2001	0.3361	-0.2378	0.1154
f47847f360ef20833d69e19f86c49a75	0.7113	-0.0794	-0.3379	0.0128	-0.0592	0.0711
4136c2cd503112b96fac25eae5d42daf	0.7129	0.1993	-0.0638	-0.0047	0.0153	-0.1469
a8e9971931fdac5e190f76109ea978fe	0.7141	0.4641	0.4327	0.4475	-0.2299	0.1579
87392072de2cd92b0abb6e982339bf00	0.7142	0.0720	-0.1943	0.1069	0.5258	-0.0602
9c45d9c71781c8cfd842c268616cfa85	0.7173	0.1821	0.1607	0.0879	-0.0379	-0.2151
4717c2d48012e3b8a057c4d41316eb39	0.7175	-0.0937	-0.3318	0.0221	-0.0668	0.0755
b1959ff9eefe517f1f5094b71cde6497	0.7181	0.1373	0.1417	0.0355	-0.0714	-0.0839
f266d7f5baf6e23d2883ebb6a3a1f2ab	0.7201	0.1883	-0.1396	-0.0619	0.0000	-0.0711
dac881080ad09ad0b86c8e6c404ba048	0.7206	-0.2479	0.0038	0.1284	-0.1505	0.0580
e71a75ba44b8cd6009da8ba3823fa379	0.7211	0.3583	-0.3376	-0.0356	0.0211	-0.0129
23672617680a03f942d3d721207f38be	0.7225	-0.2474	-0.0748	0.1722	-0.1193	0.0390
1c0c71e332a57b4d9d66cc458de8748a	0.7226	-0.1145	-0.3135	0.0372	-0.0779	0.0805
fff3221656ed281c1763e273e21daf2	0.7233	0.1389	0.4559	0.4106	0.1743	-0.0541
cc51b5118d29cc0a5c74591a02e3700a	0.7252	0.1369	0.1429	0.0345	-0.0720	-0.0868
873652884a0223a3337fa94a9d21a62d	0.7255	-0.0567	0.1081	0.1382	-0.0963	-0.0893
ae30bed6526b52f725fb51bdf74f3ba9	0.7294	0.4949	-0.0288	0.1960	-0.0135	0.1306
4c202fc2ad15750c064fdbcb4a9f8df	0.7340	-0.2063	-0.2253	0.0445	0.1719	0.1912
f32439dfdeb79efafc56cdeb3f214917	0.7341	0.2531	0.4171	0.3156	-0.0531	-0.3179
468bcb2c48bcfd0abe0b4cd4aa244aa7	0.7359	-0.2127	-0.0983	0.1950	-0.1794	0.1264
e74bbce3f26be82ebf9e76c557d4fdcc	0.7370	-0.2196	-0.0366	-0.1791	0.2739	-0.1807
02f6f3989328879aff35bc822b147340	0.7372	0.1816	-0.0150	-0.3890	-0.1829	-0.0769
20e61891951af607a1d11c8e5373e778	0.7382	0.0073	-0.3443	0.0045	-0.0462	0.0495
ea136dd566093c1a808fd8c15b0ad044	0.7389	-0.1139	-0.3185	0.0003	0.0398	-0.1107
9ceebb52461c587c5fb59564ec197f30	0.7415	0.1082	0.3715	-0.2494	-0.2144	-0.0598
76eed1edd5963498d5185ae077d6c88d	0.7461	0.3113	-0.0882	-0.4200	-0.1565	0.0906
3aa080fdc0ba7fe7aa9153d11c5fb3fa	0.7474	0.8827	0.2530	0.6752	-0.2724	0.4381
0c113731fff36168882d7ece52e79570	0.7503	0.3229	0.4129	0.2890	0.0876	-0.4353
77ce40782224b2520cff2ea1a3d26084	0.7520	0.0271	-0.3547	0.0131	-0.0466	0.0517
2b7861162fbd8a77c7eb101b0dab0bbd	0.7574	-0.1569	-0.2228	0.0840	-0.0661	0.0040

5085c17b907212fdf242168dd6f8f034	0.7604	0.2736	0.2187	0.6580	-0.3724	0.3480
e805b68e6071b0a75ebd159787aea0a5	0.7624	-0.1806	-0.2602	0.0199	0.2990	0.1238
3f95c500153b7af691b10dda9ad728df	0.7645	-0.0692	-0.3540	0.0379	-0.0739	0.0757
627e191307635e5e85405934bc9c5fe6	0.7662	0.1502	0.0331	0.0021	0.4523	0.0102
87d104355268e39f582534db982a9df9	0.7709	-0.0476	-0.3177	0.0700	-0.0810	0.0369
f451781f522a29f1e8aa99661d992a67	0.7717	0.2583	0.0572	-0.5375	-0.0358	-0.0564
94c64ff9d53bbeb57dbe1f43c501645d	0.7747	-0.0653	-0.3595	0.0473	-0.0776	0.0798
b8995aa46ead915a75ee85ae1ab7a716	0.7771	0.2817	-0.0957	0.0337	0.0037	-0.1507
2d0efff6b9587a471a1b281d211aeb5f	0.7786	-0.0295	0.2346	0.3121	-0.2049	0.1591
b0ca42591410d67519bd9ded946ea1af	0.7847	-0.0840	-0.3620	0.0366	-0.0127	-0.0182
1889a3b7ad714ca6d5c891fe9c7ee332	0.7864	0.1688	0.1629	-0.5296	0.3061	0.1646
0dccc6110a04d6f9df4b6e238a03b0e0	0.7898	-0.0538	-0.3676	0.0539	-0.0746	0.0801
9eb7cd236103fefeec81cd18b90176ae	0.7899	-0.0211	0.1102	0.1496	-0.0377	-0.2302
48c547822adba3bcea7228b61241fdf	0.8041	0.1286	0.5566	-0.2912	0.2366	0.3434
2c9bef6dce80e4951db8bc3e41cfd45a	0.8053	-0.0426	-0.3867	0.0625	-0.0791	0.0868
9d7828fa9d27484fd07f0ef74dc1ae5e	0.8081	-0.1837	-0.3061	0.0522	-0.1875	0.0327
7030bdb7506dd423e7144afd77012562	0.8176	-0.2264	0.2678	0.2167	0.2221	0.0527
0e7bdf8b98c89e7b419c7560f36fc0	0.8369	-0.0635	-0.2320	-0.2344	-0.2068	0.1625
9483af845458460943c7bdfd4e9d61ea	0.8444	0.2748	0.5494	0.4927	-0.0020	0.4316
2cb79d7b27e6026f2e81087de2b67e22	0.8459	-0.0385	-0.4119	0.1060	-0.1068	0.1162
e477379b146ea3f57418865e84d90732	0.8484	0.4224	-0.0732	-0.4628	-0.0843	0.0449
47b1f7991ebf4fae0074b435196b36dd	0.8499	-0.0147	-0.4297	0.0972	-0.0990	0.1118
80761af9fab37120981e4acb81197d99	0.8501	0.0145	-0.2341	0.0091	-0.0034	-0.0259
2ad8313f393a41026b06d9839d59c138	0.8614	0.1239	0.2296	-0.0916	0.0043	-0.2189
f3fb5ca602e0d27a5c0e9264c328b092	0.8644	-0.0435	-0.4326	0.1186	-0.1152	0.1233
62fbd976034f64c25f5fa1f7b640f336	0.8663	0.4893	0.2230	0.4659	0.0962	-1.0498
a5cf2fd490cd08c5f0d7ede266ebee58	0.8663	-0.0117	-0.3439	0.0949	-0.0386	0.0616
d4f42917efcaadc4816149f338e01a60	0.8757	0.1114	-0.2031	-0.0391	0.0769	0.0002
d986fbe27a5ea20376d666555ed4b899	0.8805	0.0063	-0.4043	-0.0632	-0.0267	0.0456
52bb2782971dec2ade343002bfc90f2f	0.8879	0.0418	-0.3776	0.0654	0.0593	-0.0670
56d0ec50b5195ab3cb5ff02ae4b72909	0.9255	0.1937	0.2965	0.6659	-0.4460	0.4409
576a7beee23c206f54692fe8f4961e56	0.9297	0.0077	-0.3292	0.1453	-0.0902	-0.0366
917fb0fd40d70a67b678770750c90fdf	0.9311	0.3120	0.6159	0.2784	0.4133	-0.2921
4b4eeeb1577758c7204e4c34285f0372	0.9312	0.0495	-0.2086	-0.0378	0.0619	-0.3364
2e4d26a70c8b9783694c6587f11ef6f8	0.9353	0.2478	0.3715	-0.3745	-0.1537	-0.1103
9634df012bd2cfbbfcc7ee503dd0a95a	0.9511	-0.2450	0.0209	0.1486	-0.1900	-0.2165
7fe5571370c2044de1205ea436b6dd90	0.9546	-0.2747	0.0673	0.2074	0.1405	0.0919
37b30ec33ee7cf8fe5c082f193bc1a42	0.9573	0.8150	-0.3028	0.2140	0.0342	-0.3565
d9b53728a07e2a883710b00f4cb060e2	0.9666	-0.0784	-0.1041	0.0926	0.2826	0.1514
0ea83172bf7ec6439830bdb7f4101435	0.9998	0.0058	-0.0878	-0.0495	0.5645	-0.0419
0a111b1952f1b3fbd682afb79c761c37	1.0100	0.2733	-0.1531	-0.3982	0.0244	-0.0542
4566a634f8d744212928063c71276064	1.0110	0.0051	-0.3628	0.1510	-0.0919	-0.0456
72f4f198589786db73e3303368bb1ac1	1.0182	0.4333	0.2459	0.3332	0.2675	0.2772

89853878d5d463e6e887e454c1cc61ec	1.0283	0.0421	-0.2318	-0.4378	-0.1735	0.1223
7888d5725b99c81b4883e44de26c597d	1.0312	0.1217	-0.1446	-0.3251	-0.2377	0.1990
b9325d7d717e26d4d63e00e25dcb8905	1.0395	0.0197	-0.4940	0.0598	-0.0101	-0.0727
295c8d3c651acb8c58fb6e4dba947b3b	1.0416	0.0011	-0.1457	0.1110	0.0592	-0.2530
ec63a7e08dfcdce05aa4fe15debd99ab	1.0481	0.4592	-0.0725	0.0848	-0.0762	-0.2629
71fff63e5dc662c38aa7f38d102265d6	1.0711	0.1017	-0.1856	0.3332	-0.2522	0.2464
fe870ca30c828f6d18387786618e0d20	1.1166	0.1303	-0.2374	-0.1359	0.1695	-0.2125
6493139d9b078eb565965264e4426ca8	1.1309	-0.0373	-0.3961	0.1148	0.0054	-0.3058
6d1de170c8d9c378c1bacf72d93283c2	1.1576	0.1626	0.1391	0.0948	0.2607	-0.1305
ae41a7e7ad08cd926075f8ab35eaa0eb	1.1733	0.1683	-0.0272	-0.0131	0.4500	0.0616
e010f6ec05db89e11d623899272ecde1	1.1918	0.8164	-0.0005	0.2583	0.1205	-0.9286
0f6640911d66a8b028b2b091f1cbf0c0	1.2435	0.0645	0.0666	-0.0878	0.4922	-0.3225

Table A3. *nifH* ASV PCA scores.

ASV NCBI code	PC1	PC2	PC3	PC4	PC5	PC6
88ad6d4de9880fdeb52ea15e8f11c0e0	-1.0053	-0.4499	-0.3126	-0.6664	-0.5427	-0.0710
1bf3bce129ff9de40a38848041a33aa4	-0.8922	-0.0203	-0.0192	-0.0509	0.0595	-0.0648
667eb78ef99fcd0d39d250c5e92e99c6	-0.8790	-0.0103	0.0195	-0.0023	0.0981	-0.0370
0416d767426d2e8042468485eed23d34	-0.8702	-0.0426	-0.0251	-0.0900	0.0737	-0.1182
7bda7ef63b865c97ce1092eb0c85e870	-0.8684	-0.0322	0.0114	-0.0367	0.0907	0.0528
e738c0937b1df0884c3b22ca2c38c8bb	-0.8682	0.0257	0.0871	0.0711	0.0179	0.0778
bffbbba5c5eedb95ed88c5eb0ba49da	-0.8672	-0.0116	0.0150	-0.0111	0.0188	-0.0175
be092b8cd5cfe1adf3615994cbdc6dba	-0.8653	0.0132	0.0927	0.0696	-0.0409	0.0622
8f5a253e98af21ebd0c24c618e5977dd	-0.8628	-0.0404	0.0261	-0.1063	-0.0736	0.0297
8cff7ae6f343aeb73b0341a9ae4d52b	-0.8585	-0.0234	0.0500	-0.0037	-0.0504	0.0015
76eb4f51465bed0383a30aba19983e67	-0.8576	-0.0253	0.0487	-0.0069	-0.0509	-0.0008
b692e29c54857a73102a4c4738c8cb6f	-0.8509	0.0238	0.0812	0.0595	0.0256	0.0751
28ff4b5a55ea59ad6e7a3a21f7d5ea6b	-0.8506	0.0100	0.0442	0.0156	0.0667	-0.0764
fa84673462d343cc15817ed92266937a	-0.8496	0.0107	0.0521	0.0174	-0.0764	0.0207
f9106c883533536561acd4142b2ea41a	-0.8489	-0.0069	0.0147	0.0010	0.0151	0.0638
a0cff32a69aa113c1c076abdb5033f78	-0.8468	0.0473	0.0175	0.0067	0.0027	0.0775
6863a4e2ba8782e1f892115443ada027	-0.8462	0.0117	0.0569	0.0411	-0.0027	-0.0027
58190588b6bbcb67b521e3f6c76998d5a	-0.8461	-0.0090	0.0354	0.0097	0.0513	0.0700
5b8fdfdb2469972fcd56c438eea22ce	-0.8438	0.0291	0.0564	-0.0157	-0.0317	0.1090
6c3981698b9099da87959cb6a7bedfec	-0.8397	-0.0148	0.0051	-0.0293	0.0051	0.0535
cf657bc172db4d2089b50110010665f6	-0.8365	0.0319	0.1243	0.0954	0.0197	0.0413
eb855455562675f1a0ed14cd61c32085	-0.8348	-0.0046	0.0158	-0.0050	-0.0077	0.0671
c3f7673194d37064b677da8a56b533e8	-0.8348	-0.0046	0.0158	-0.0050	-0.0077	0.0671
96e7e44947b77f986ea30c0ee807f833	-0.8348	-0.0046	0.0158	-0.0050	-0.0077	0.0671
eed0310036837effc8efba2a71c58fde	-0.8347	-0.0047	0.0165	-0.0030	-0.0090	0.0648
5f87b620fb2caee5601357f5f79b6217	-0.8344	-0.0091	0.0049	-0.0383	0.0759	-0.0819
9c8ef0173aac777e3d0000c2458c0ad1	-0.8343	-0.0083	0.0321	-0.0069	0.0249	0.0512
be262360e93192d14d6184c862708e79	-0.8335	0.0605	0.0209	0.0335	0.0107	0.0781
53b00ac49b89f7bc7e8c0d0ed2758264	-0.8327	-0.0087	0.0032	-0.0435	0.0856	-0.0775
2a1c0b2b7ab482ffe617f75b1675474c	-0.8323	-0.0016	0.0425	0.0268	0.0425	0.0537
17ad9f153c1e271a9ecf644575741e95	-0.8308	0.0232	0.0858	0.0738	0.0080	0.0440
36dea33c5bf4d4438077c860e875a0b3	-0.8298	-0.0197	0.0004	-0.0592	0.0580	-0.1329
a13f5c0480e338cb475aecfc71ad7119	-0.8267	-0.0278	0.0505	-0.0235	-0.0604	0.0388
74aedb0cdb294cf7c041e8dd773bbe90	-0.8248	0.0491	0.1095	0.0517	-0.0312	0.0832
a1b26d75cd6517ee0992aae18955f7b4	-0.8226	-0.0133	0.0064	-0.0266	0.0027	-0.0166
976372c2320e0c15f92daaf3feec4246	-0.8205	-0.0185	0.0551	-0.0154	-0.0282	0.0771
f69c9176306607f35c58d67b6e6d077d	-0.8192	-0.0101	0.0022	-0.0416	0.0757	-0.0956
9aa6ea46ac2fbb3d0678e1f037346b9f	-0.8162	0.0100	0.0881	0.0589	-0.0609	0.0336
580a768e7b41353442559a159e47c062	-0.8153	-0.0351	0.0314	-0.0345	-0.0563	-0.0279
f80ec914035eb5d3a6186a1c0410bb2b	-0.8151	-0.0100	0.0019	-0.0424	0.0737	-0.0947

fe73cf61d90103596f783fec5e1d747c	-0.8102	0.0094	0.0675	0.0415	-0.0286	0.0507
bb66c87d726493e5b2dab24c58c80439	-0.8080	-0.0080	0.0239	-0.0299	0.0339	0.0671
920998e6233d3c6019bbc02f21c49860	-0.8074	-0.0115	0.0679	0.0044	-0.0341	0.0590
553031c1ab6f030c8bf28dd8511643cd	-0.8074	-0.0833	-0.0368	-0.1168	-0.0767	-0.1588
36478cf23cac4448316ed86f5aa0b760	-0.8066	-0.0085	0.0079	-0.0171	-0.0057	0.0508
82c5a97eacde91bdbdc1c56849e6650d	-0.8065	-0.1326	-0.1137	-0.2219	-0.0132	-0.2417
c18d4b46c461dff82a1febfa01b2cae	-0.8059	0.0307	0.0216	-0.0442	-0.0393	0.1355
bf37a7ce8b067730b015f87cf85c38d4	-0.8052	0.0092	0.0668	0.0404	-0.0297	0.0488
42815bd456d891e2f223231aa56b0692	-0.8052	-0.0116	-0.0007	-0.0471	0.0797	-0.0985
151b7f3425dc06d3744e05530346fcd6	-0.8044	0.0166	0.0407	-0.0548	-0.0751	0.1320
f3b6b384f9525c1768883824de95aed5	-0.8027	-0.0119	-0.0011	-0.0478	0.0793	-0.0996
96508f48c7333b2b2b7f3f485029c225	-0.8022	-0.0010	0.0392	0.0117	-0.0554	0.0316
4c0edf29262ad172613f2dbf5eaca1fe	-0.8021	-0.0119	-0.0012	-0.0480	0.0792	-0.0999
d997ba6a5649f52386d4e6bb0a34b051	-0.8018	0.0307	0.0212	-0.0449	-0.0333	0.1324
8c0fb18b6568b61b7e6118a02fedcc7a	-0.8013	0.0313	0.0218	-0.0434	-0.0412	0.1312
f76dbe0c6372a87cf538393c0ac66949	-0.8008	-0.0121	0.0665	0.0024	-0.0345	0.0555
ad4306a991aaa5e4c209e50ab809c9e4	-0.7990	-0.0001	0.0444	-0.0553	-0.0216	0.0457
e0f1b3811ca5fb6634b8b50a4ebb0491	-0.7961	0.0022	0.0699	0.0372	-0.0133	0.0251
00e1cddc9d2a473c73759a248fb0261d	-0.7947	-0.0787	-0.0408	-0.0978	0.0528	-0.1530
af84a044ef581a933d62d558f6df02db	-0.7942	-0.0135	-0.0020	-0.0511	0.0751	-0.1060
81027fd4cc3cd8c5247182cbda435779	-0.7927	-0.0292	-0.0176	-0.0694	0.0515	0.0300
bf9491552b186a943c54de45f008190c	-0.7911	-0.0564	-0.0394	-0.1116	-0.0056	-0.0889
38e61b5011d0a9e47f85e49aec75dc73	-0.7892	-0.0833	-0.0853	-0.1641	-0.0761	-0.1623
c6ecbf7ec11e1887e7f0b26c40fe0114	-0.7860	0.0292	0.0183	-0.0498	-0.0362	0.1253
5e7321d2cd1393f01ec4146514310061	-0.7845	-0.0361	-0.0115	-0.0502	-0.0111	-0.0840
3fe475f15d0c9c4616e44de827a5a501	-0.7764	-0.0235	-0.0152	-0.0622	0.1107	0.0337
372176e41de4cd264a129bff92534756	-0.7712	0.0109	0.0881	0.0368	0.0233	0.0271
40ee8b212e0fa73c1a359554860272b6	-0.7694	0.0087	0.0444	0.0070	-0.0875	-0.0142
0ee8eae0ecd4d537db2c0864deafdd41	-0.7675	-0.0094	0.0232	-0.0340	0.0283	0.0737
43be9a613f9e1b310abb3a19935be51	-0.7670	-0.0914	-0.0968	-0.1750	0.0728	-0.2848
06385469ee89ee59b45a4e1de5e0e8fc	-0.7659	-0.0680	-0.0633	-0.1398	0.0799	-0.2161
e13c22d29521b16d54407b13985f6a80	-0.7631	-0.0613	-0.0147	-0.0737	0.0117	-0.1183
67475b3603b60641aebec8009541d089	-0.7623	0.0099	0.0858	0.0330	0.0108	0.0177
d26669227a58f01f0f55c5c5c28b206f	-0.7612	0.0067	0.0600	0.0302	-0.0399	0.0317
cce5e7cc4e0825aa4e731b68e45ab7a5	-0.7580	0.0082	0.0430	0.0046	-0.0882	-0.0184
6c1b69c9fd76171ba55c6b5b166cb313	-0.7580	-0.0257	-0.3153	0.4171	-0.0015	0.0626
1211e7ff9ee6c37f0a94820f82f096d3	-0.7569	-0.0242	-0.0187	-0.0534	0.0595	-0.1624
de777b0deb418b72246b1c6ec2f23e8c	-0.7533	-0.0171	0.0569	-0.0140	-0.0440	0.0279
2e5bcecff5de4054e9440f43358aa571	-0.7524	-0.0805	-0.0295	-0.1115	0.2027	0.2289
74970996faa65e31203d26361b41a4d7	-0.7425	-0.0146	0.0876	0.0441	0.1357	0.0628
15240339bbea1fd5f7ec82f6256932b3	-0.7420	0.0249	0.0095	-0.0642	-0.0471	0.1041
b64228672efbb69acd71b41e36c3e98c	-0.7380	-0.0933	-0.0809	-0.1455	0.0803	-0.1693
cdba8bad167417547ec5b593385b96ef	-0.7379	-0.0892	-0.0849	-0.1603	-0.0068	-0.1170

725b24d36cae4e6f48e8cda849101605	-0.7371	0.0072	0.0404	0.0003	-0.0895	-0.0258
86c0360ba99fab5c04bfb2dea1049c7e	-0.7328	-0.0290	-0.0336	-0.0762	0.0652	-0.2096
31d01e64767c0a57d6ea565e706f82c9	-0.7264	-0.1217	-0.0859	-0.2054	0.0394	-0.1656
7efcef74c682c791ce76658d1f300439	-0.7258	-0.0189	0.0019	-0.0248	0.0244	-0.0906
342f7b1d59501900812706f2606df85d	-0.7258	-0.0251	-0.3310	0.4408	0.0280	0.0364
758158b19aaf5b8ff3804a4c8448993f	-0.7252	-0.0785	-0.0108	-0.0949	0.2234	0.2904
28257afea45ae25d75ae3ca60becde17	-0.7240	-0.0791	-0.0483	-0.1207	0.0168	-0.1259
e7657ab9f053ac79991019d6c371aac1	-0.7127	0.0026	0.0522	0.0165	-0.0386	0.0077
cb1d569a67c5574516c219c1e75742e4	-0.7052	-0.0138	0.0884	0.0326	0.1242	0.0593
c1ddf6c4bf970fab282b979d3a81f027	-0.7046	-0.0625	0.0210	-0.0434	0.0952	0.0028
b00e1a9f8612c0fc417cdb008e2282d2	-0.7035	-0.0140	0.0889	0.0345	0.1225	0.0557
dd184ba122e175581c26646b9b195ac9	-0.7034	0.0284	-0.0171	-0.0227	-0.0050	-0.0805
969e46a4eda51e10fce9e1481b6882b4	-0.7031	-0.0439	0.0681	-0.0444	0.2756	0.2995
34ec9a8e9bcbec5af13486418d42edeb	-0.7029	-0.0379	-0.3708	0.4178	0.0288	0.0417
e6798a32620f6ca687472825c3ef1651	-0.7012	-0.0219	-0.0195	-0.0787	0.0700	-0.1444
5ab87b5e6abe9a0d2dfb566fdceb308d	-0.7011	-0.0812	-0.0252	-0.1184	0.1620	-0.0744
9770adf8f340f749b76f27f422330a7c	-0.7001	-0.0219	-0.0196	-0.0788	0.0702	-0.1445
bc5e28a57033478cb4555d8e23ff0a26	-0.6994	-0.1813	-0.1728	-0.3144	0.1161	-0.4286
c9fd6b7c6331ed8ea61509d9a646578b	-0.6956	-0.0505	0.0618	-0.0384	-0.2146	-0.0458
27c237141eb5cd9913b3f8964e6e3e85	-0.6919	-0.1299	0.0779	-0.0409	-0.1601	0.1379
405b490b17434ea0858eb4248fd7dc7e	-0.6903	-0.0048	0.1266	0.0521	0.1223	0.0388
64eb2220cb903290b347f98f9ca850ba	-0.6898	0.2639	0.1316	0.0452	0.1477	-0.1209
cf85a420192dce78ee3ffcdab0f46d40	-0.6897	0.1799	0.0366	-0.0696	0.1745	-0.2343
0380c4b717b5f7053486361b353f7de8	-0.6882	0.0177	-0.3821	0.4140	-0.0125	0.0682
63daea83b596c2564dd1e094d3d4b063	-0.6866	-0.0762	0.0133	-0.1192	0.1651	0.1683
a6bf2e099caf1c43cf2f73ddda337cd7	-0.6855	0.0030	0.0306	-0.0164	-0.0921	-0.0552
6f876bce4bb3b0bdd6c204261a9279b5	-0.6801	-0.0214	0.3380	0.2458	0.0746	-0.0853
98483193132d218b486c009c96cf2fc7	-0.6783	-0.0288	0.3222	0.2258	0.0843	-0.0754
b2ba818e0b24a60e72450ccaed2099c	-0.6780	-0.0188	-0.0242	-0.0827	0.0721	-0.1447
9b546486480668110cb10af662e68f27	-0.6757	-0.0038	0.0540	0.0082	-0.0213	-0.0235
212013288ac367183ec4c6314817c7fe	-0.6750	-0.1004	0.0864	-0.0698	-0.2219	0.0008
d240c9eb26f8fec0e795cf35b6714df9	-0.6733	-0.0566	0.0585	-0.0675	0.2259	0.2837
199fc0851ad4a166e178b94023b7788f	-0.6732	-0.0165	0.0828	0.0236	0.1281	0.0424
e7ff4aa70995b7ad23187e8cb8de994c	-0.6711	0.3993	-0.0362	-0.1159	-0.1493	0.1945
6027b159d1ed21b82435f194b4a71069	-0.6694	0.2790	0.1341	0.0163	0.1690	-0.1250
10887abb57072761e9d7f62edbeba2d7	-0.6677	-0.0309	0.3192	0.2202	0.0825	-0.0815
fcc75bf9bc931996dc89d205ca93f31f	-0.6658	-0.0311	0.3189	0.2197	0.0822	-0.0823
1c6c2e66e99c5ae643a3465c445b1d7d	-0.6658	-0.0311	0.3189	0.2197	0.0822	-0.0823
e9a4b30cfd737c63e219de800fef358	-0.6641	-0.1363	0.1438	0.0289	-0.2409	0.1692
d0edfa3f1aed635e4711b0153eabdf7d	-0.6614	-0.0768	0.0509	-0.1097	-0.2363	0.0504
16f74566589298c00afe2711f4791dd4	-0.6608	-0.0900	-0.0244	-0.1126	0.1743	0.2509
19a510fbd8c71f503b8c161e9e5090c6	-0.6602	-0.0388	-0.3865	0.4118	0.0062	0.0374
1b7c803de985d0dbafa7e33fb1a9ea84	-0.6580	-0.0389	-0.3867	0.4115	0.0063	0.0363

fedc794d0b88d4cefebd05850f13ad4f	-0.6577	-0.0950	-0.0318	-0.1121	0.1878	0.2267
6991ee8fb022b05b79cba406265c039d	-0.6571	-0.0381	-0.3843	0.4138	0.0141	0.0350
34a4fcf4222b9132981671c09f31b6d	-0.6568	-0.0308	0.3201	0.2195	0.0708	-0.0906
9225b6ab3b76226a979b9bfc7df670f75	-0.6530	-0.0913	-0.0281	-0.1170	0.1726	0.2469
27849aae3359a62cd01069133c9c45c3	-0.6528	-0.0500	0.0621	-0.0650	0.2451	0.2867
f35f55a5a76452180107ea020d432f8a	-0.6528	-0.1467	0.1480	0.0256	-0.2536	0.1784
bb27c3b35555490e31b462053cc324ca	-0.6522	0.2162	0.0433	-0.0745	0.1951	-0.2592
18c8f179dba66ad6d0aa7c273be734b8	-0.6510	-0.0243	-0.0097	-0.0430	0.0176	-0.1225
acec84446468cbe4f7b5d2e1ff4cb381	-0.6499	-0.0482	-0.3672	0.3475	0.0208	-0.0725
29bcd53fc2e41976e120aae1b7732d80	-0.6498	-0.0244	-0.0099	-0.0432	0.0176	-0.1228
e4e67fed9297b1f2191791a1ce0a7a4c	-0.6481	-0.0468	0.0047	-0.0771	-0.0288	-0.0800
b08f6c6d0a30ce0fb351b8106b8d3a78	-0.6464	-0.0392	-0.3866	0.4110	0.0122	0.0301
5889fa689d79983388a4f1770e28f651	-0.6464	0.2731	0.0999	-0.0077	0.1651	-0.1344
f27841e39c9ef6a27d57f3d8a17db67b	-0.6464	-0.0328	0.3155	0.2140	0.0698	-0.0953
ecd1ebb2d3ddb2c76e067e7403b606d1	-0.6462	-0.1086	0.0840	-0.0804	-0.2287	-0.0145
2cef883332338817031818453d563e10	-0.6458	-0.0392	-0.3867	0.4108	0.0121	0.0299
10d3351c7a0217f1cab765f3396f0e6c	-0.6449	0.2724	0.0991	-0.0089	0.1673	-0.1376
5e1dbfb8b202f223661ebe61bb69f8d1	-0.6418	-0.1143	0.0757	-0.0849	-0.2589	0.2386
2a47665eb1262948fb62311bf6b2baa6	-0.6411	-0.0215	0.0384	-0.0449	-0.0396	-0.0022
47f23d615878150693806418b8c569b1	-0.6389	-0.1477	0.1109	-0.1068	-0.3278	0.2133
ad5c07c652413f46741195c5ea3cef2e	-0.6368	-0.0541	0.0589	-0.0679	0.2741	0.2813
126a856bc558ed0d6b44379980acc334	-0.6350	0.0047	0.0271	-0.0222	-0.0985	-0.0547
d9f952b5c21ae7fe3ff2b12ba97a377e	-0.6343	-0.0604	0.0326	-0.0538	0.0972	-0.1031
544b78c2eeb3e76b9d529f4b0cfd47df	-0.6336	-0.0672	0.1444	0.0839	-0.0795	0.0344
f60e75d45e14ea3c2b52c0021c594dd5	-0.6335	0.0046	0.0270	-0.0223	-0.0984	-0.0550
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842b6bed9754c00372f569b120bf51b7	-0.6319	0.0193	-0.0066	-0.0895	-0.0400	0.0736
e74700f5e381d3a2a67976c83f31db3e	-0.6302	0.4749	-0.0463	-0.0630	-0.1299	0.1357
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aad54d4b5f4db7a0b1b44151d30ca337	-0.6272	-0.1758	-0.1325	-0.2805	0.0514	-0.3301
48c8c7d1a8cfb2d737eb37fbb1a20ced	-0.6270	-0.0422	-0.3928	0.4023	-0.0021	0.0150
28a4fc2655ddd881c0d6c3137b06a85f	-0.6269	-0.1537	0.1569	0.0036	-0.2416	0.1773
5bb11711f162621eef75df37743e0498	-0.6225	0.4033	-0.0475	-0.1347	-0.1611	0.1753
97ca89c2d5e3123eeb808d82ec3eb51f	-0.6220	0.2005	0.0804	-0.0463	0.1370	-0.3175
b7869efcea323c5085f987a722787eb8	-0.6214	-0.0425	-0.3936	0.4012	-0.0017	0.0134
4f1d045e9b5b2fa6d9b637f4b5b165df	-0.6190	-0.0427	-0.3940	0.4008	-0.0015	0.0127
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94dd0b54837f91687de380e83677fef0	-0.0496	-0.0991	-0.2114	-0.2234	0.2374	-0.4477
60885538781464af19a23c23b4dea2a1	-0.0291	-0.0467	-0.0058	-0.0339	0.1936	-0.0022
331f13ff1433695c2f56544ee9ae6633	-0.0193	-0.1208	-0.1063	-0.1749	0.2345	0.1738
e1d25842050f851e510d01b810296b9e	-0.0120	0.2101	-1.0895	-0.4106	-0.6375	-0.1244
5dc2744f1e8db692bc769f6af2e0ec2b	0.0053	-0.1225	-0.1757	-0.1975	0.1144	-0.1433
f7920ccd712e17fba4597dfcb15e9251	0.0276	0.6720	-0.0268	-0.1472	0.0725	-0.1385
82eb66297b137308501fd04f09b0b438	0.0548	-0.1195	0.0498	-0.0722	-0.1190	-0.0731

80b15f67ed3942a1af81b8f4116f9ef8	0.0622	0.3365	-0.0752	-0.2061	-0.1106	0.2906
c69e404b02b1af95b6cdee5dc102cb65	0.0822	-0.2358	-0.2042	0.1436	-0.2205	0.0832
5c85ff3e906a5e2980287666f09ca4d2	0.0835	-0.0973	0.0446	0.0423	0.0006	0.0182
b6549246867515ce1844d87a491b05d5	0.1130	-0.1224	-0.3373	0.2200	0.2303	-0.0835
2c8f655ca93e7e640d642efc9ccb99c2	0.1809	0.1266	0.3222	0.1820	0.2085	-0.2804
b351200bba2529fdaf47a770e96a506f	0.2225	-0.1641	-0.1290	-0.2604	0.4667	0.5072
0fec274e15ecedcb37fb6a8f07ed3f9e	0.2482	0.1061	0.0127	-0.1537	0.5105	-0.1303
439a5c6fc811c01591732533d407d094	0.5264	-0.0239	-0.1664	-0.1529	0.5704	-0.1311