WILL CLIMATE CHANGE ALTER ARCTIC NITROGEN BUDGETS? IMPACTS OF WARMING AND FERTILIZATION ON NITROGEN FIXING MICROBIAL COMMUNITIES AT ALEXANDRA FIORD, ELLESMERE ISLAND, NUNAVUT

by

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ABSTRACT

The impacts of simulated climate change (warming and fertilization treatments) on diazotroph community structure and activity were investigated at Alexandra Fiord, Ellesmere Island, Canada. Open Top Chambers were randomly placed in a dwarf-shrub, cushion-plant dominated mesic tundra site in1995. In 2000 and 2001 20N: $20P_2O_5$: $20K_2O$ fertilizer was applied at a rate of 5 g $m^{-2}year^{-1}$. Estimates of nitrogen fixation rates were made in the field by Acetylene Reduction Assays (ARA). Higher rates of N-fixation were observed 19-35 days post-fertilization but were otherwise unaffected by treatments and we hypothesize that microsite variation was a greater determinant of N-fixation rate than were the treatments applied. *Nif*H genes were amplified from bulk soil DNA and analyzed by Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis. Nonmetric Multidimensional Scaling (NMS) was used to ordinate treatment plots in *nif*H genotype space. *Nif*H gene communities were more strongly structured by warming treatment late in the growing season, suggesting that an annual succession in diazotroph community composition occurs. $\delta^{15}N$ analysis of plant and soil material from each treatment plot suggests that evergreen dwarf shrubs will depend more heavily on organic-N derived from mycorrhizae in warmer climates and that relative importance of symbiotic nitrogen fixation to the N-nutrition of *D. integrifolia* will decline at this site.

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LITERATURE REVIEW

1.1. Soil Microbial Ecology; a historical perspective

Although the term Soil Microbial Ecology is relatively recent designation, the study has deep roots in the history of science. By the mid-nineteenth century, the founders of modern science were conducting controlled experiments and making detailed observations of microorganisms in their environment, and the discipline originates from these early efforts. The rejection of spontaneous generation and the elucidation of anaerobic metabolism were achieved by the monumental works of Louis Pasteur (1830-1900). Charles Darwin is credited with pioneering quantitative studies on the transportive effect of earthworms on surface soil layers (1837, 1881). The widely credited founder of soil microbiology, Sergei Winogradsky, initiated the first studies of nitrification and sulfur-oxidation which led to the concept of microbial autotrophy. As the studies of microbiology, chemistry and biochemistry matured into the twentieth century, it became apparent that these disciplines were intricately connected in soils and the central role of soil organisms in nutrient cycling was recognized (Paul and Clarke 1996). The elucidation of the nutrient cycles provided a basic framework from which to interpret the processes that determine the distribution of organisms in the environment. Agronomy served as the subject of, and practical application for, most of the knowledge gained in the field. Consequently many of the basic paradigms of the field were uniquely suited to

agricultural systems (for a more extensive review of the history of soil biology see Coleman et al. 1983, Paul and Clarke 1996).

2.1. Ecology and nutrient cycling in natural systems

Much of the groundwork of soil microbial ecology took place in agricultural systems that are highly fertile when compared to natural ecosystems (Chapin 1980, Coleman et al. 1983). In nature, terrestrial biota is often limited by the availability of four key elements C, N, P, and S. The biogeochemical cycles of each of these essential elements are characterized by two pools in soils, a relatively large pool that is bound in organic forms (living and non-living), and a small pool that is present in a highly available (often inorganic) state. The flux between pools is mediated by two main biological processes; mineralization and immobilization. The balance between these processes often determines the potential to accumulate biomass for the organisms in the ecosystem. Biological activity that determines the rate of nutrient mineralization and immobilization exists in an abiotic environment controlled by three basic parameters, soil texture, soil age and regional climate. These abiotic factors strongly influence the interactions of organisms and characterize the nutrient cycling in a system.

Nitrogen is the primary limiting nutrient in many natural terrestrial ecosystems (Dugdale et al. 1967; Paerl et al. 1987; Dawson 1992). With few exceptions, primary producers are not carbon-limited by virtue of their metabolisms. Nitrogen is required in all of the basic

building blocks of life notably; protein, RNA and DNA, and is required in relatively large quantities compared to the other limiting nutrients. Atmospheric N_2 is a huge reservoir of N that is largely unavailable to organisms. The two soil pools interact with the atmospheric reservoir by three biologically mediated processes, nitrification, denitrification, and nitrogen fixation. Nitrification is the microbially-mediated process by which ammonium is oxidized to nitrate for energy directly, or simultaneously with CO_2 reduction. Denitrification is the opposite process, whereby nitrate is reduced during anaerobic respiration (oxidation of CH_2O) to nitrous oxide (N_2O) and dinitrogen, returning N to the atmospheric pool. Nitrogen fixation, the only source of new nitrogen in terrestrial systems, is the process by which atmospheric N_2 is reduced to ammonium.

2.2. Arctic soils are nitrogen limited

Soil ecosystems at high latitudes are characterized by cold and saturated conditions for much of the growing season (Chapin and Bledsoe 1992b). These conditions limit decomposition and thus, the internal recycling of nitrogen by mineralization and nitrification as well as the recruitment of new N by fixation. Depending on the outcome of competitive interactions, available N can be rapidly assimilated by plants, retained in plant tissue, and returned to the soil as litter fall. Conversely, available N will be assimilated into microbial biomass and retained until mineralized once more. Nitrogen leaves soil organic matter by leaching, transport by soil water, denitrification and ammonia volatilization (Shaver et al. 1992). Most of the nitrogen in tundra ecosystems is

held in soils and supply to plants (via mineralization) is a major bottleneck to plant growth (Chapin et al. 1980). Consequently, the productivity of many terrestrial arctic plant communities is strongly nitrogen limited (Ulrich and Gesper 1978; Shaver and Chapin 1980, 1986).

2.3. Global warming will affect arctic nitrogen budgets

Temperature increase as a result of climate change in the arctic is predicted to be 2-5°C over the next century (Houghton et al. 1995, 1996). This is far greater than the global mean increase of 1-3.5°C (Boer et al. 1990). Changes in temperature are predicted to induce widespread change in all of the earth's ecosystems. The responses of arctic ecosystems to climate change are of particular interest because arctic communities have responded disproportionately to past climate transitions, suggesting that future climate change will induce widespread alterations of these systems (Warrick et al. 1986).

Warmer temperatures, a result of an amplified 'greenhouse effect' due to increasing atmospheric CO_2 concentrations, may affect arctic nitrogen cycling by a variety of mechanisms. Directly, warmer temperatures will increase rates of all enzyme-mediated reactions in soils. Secondary effects of warming include the potential alteration of hydrological regimes at regional and local scales. In the arctic, warmer temperatures may be associated with increased rainfall due to decreased albedo as sea ice melts and an increased evaporative load. At local scales, increased evaporation may make dry sites

drier while increased melting of glacial ice may make low lying areas wetter. Increased temperature should also result in an increased depth of thaw of permafrost releasing large stores of organic nitrogen that will be mineralized to ammonium (Shaver et al. 1992). Likewise, increased microbial activity and element turnover, including increased nitrification, is expected (Nadlehoffer et al. 1992). As long as sufficient water is available, many of these affects may feedback positively, resulting in a more rapid turn-over of the large soil N-pool, and consequently, a relief of the 'bottleneck effect' of N-mineralization on plant N-nutrition in the short term.

However, since nitrogen fixation is the primary source of new nitrogen in arctic plant communities, variation in its input may be a major regulator of ecosystem productivity in the long term (Chapin and Blesoe 1992a). Climate warming is expected to increase nitrogen fixation rates by a factor of 1.5-2 times current values in the arctic. Increased temperature is expected to have the strongest direct effect on all nitrogen fixing organisms, while increased moisture is expected to be important for certain key photoautotrophic groups (particularly cyanobacteria). Direct effects of increased CO_2 concentrations on the metabolism of photosynthetic nitrogen fixers will also be important in securing this increase (Chapin and Blesoe 1992a). If these predictions prove true, the warmer arctic climates of the future should be less nitrogen limited than they are today.

3.1. Diazotrophs

Organisms that fix atmospheric nitrogen are collectively called diazotrophs. It is believed that nitrogen fixation has been present since the evolution of eukaryotes (Postgate and Eady 1988), and all organisms depend either directly or indirectly on diazotrophs as a source of nitrogen. Diazotrophic organisms are a comprised of a diverse array of prokaryotic phyla from two domains; the eubacteria and the archeabacteria. Members of these two groups employ nearly every life history strategy; there are free living and colonial photoautotrophs, and free living heterotrophs and chemolithoautotrophs (Paerl 1998). Many symbioses exist between diazotrophs and other organisms. Diverse groups of bacteria, such as the actinomycetes and the proteobacteria (eg. *Rhizobium*), form symbiotic associations with plants, while other diazotrophs are endosymbiotic with animals, and may live in the guts of wood-eating termites and pelagic copepods (Zehr et al. 1998).

3.2. Diazotrophs in Arctic Soils

It is widely held that the most important diazotrophs in arctic terrestrial ecosystems are cyanobacteria. Cyanobacteria are photosynthetic surface dwellers with heterocysts that are likely the primary source of newly fixed nitrogen in these systems (Chapin and Bledsoe 1992a). Principle genera include *Nostoc, Anabaena, Scytonema, Stigonema, Hapalosiphon, Tolypothrix*, and *Fischerella* (Alexander 1974; Granhall and Lid-Torsvik

1975). The importance of cyanobacteria that lack heterocysts and fix nitrogen primary in the dark (Liengen 1999) is still unclear as the presence of heterocysts is the only morphological attribute that indicates that an organism has the ability to fix nitrogen.

Another group whose contribution to nitrogen fixation in arctic soils is unclear is the free-living anaerobic bacteria. This group is often abundant in arctic soils (Stutz 1977) and may be locally important at some sites (Granhall and LidTorsik 1975) but low soil temperatures and low availability of carbon substrates is thought to limit their contribution to the overall nitrogen budget of arctic soils (Jordan et al. 1978).

Dryas integrifolia is common at many high arctic lowland sites and is known to be colonized by actinorhizal bacteria (Henry and Svoboda 1986). Symbiotic nitrogen fixation in plants is, however, believed to be rare in the high arctic (Stutz 1977). Where actinorhizae do exist they likely contribute significantly to the local nitrogen budget. For example, at Sarcpa Lake, NWT the highest rates of N-fixation were observed in habitats densely colonized by legumes and it was concluded that rhizobial symbioses contributed significantly to the N-budget at that site (Karagatzides et al. 1985).

3.3. Limitations to nitrogen fixation in arctic soils

The temperature optimum for nitrogen fixation by arctic diazotrophs has been determined by several authors to be near 20°C (Davey 1983, Chapin et al. 1991, Lennihan et al. 1994,

Liengen and Olsen 1997a, Liengen 1999). Consequently nitrogen fixation is considered to be temperature limited in cold arctic soils. Soil moisture has also been implicated as a strong control on N-fixation (Alexander et al. 1974, Alexander et al. 1978, Chapin et al. 1991). Soil moisture may limit N-fixation at dry sites and buffer soil temperature in wet sites preventing maximum rates. Phosphorus is the primary nutrient limiting N-fixation in most natural systems (Vitousek 1999) and phosphorus limitation of N-fixation in arctic soils is also well documented (Fritz-Sheridan 1988, Chapin et al. 1991, Liengen 1999). Positive correlations between magnesium and calcium concentrations and nitrogen fixation rates of high arctic cyanobacteria have been reported (Liengen and Olsen 1997a, 1997b); however direct limitation of nitrogen fixation by either element has not yet been established. Depending on the physiology of the diazotroph in question, light limitation or carbon limitation may be a factor. Photoautotrophs, particularly cyanobacteria are important in arctic sites and limitation of N-fixation by shading has been reported by some authors (Henry and Svoboda 1986, Liengen 1999). Similarly, chemoheterotrophs may be carbon limited, and increased concentrations of labile-C compounds, either from root exudation, or sucrose amendments have been associated with increased rates of N-fixation in the field (Li et al. 1995, Piceno and Lovell 2000a, 2000b).

3.4. Free-living diazotrophs at home: the mycorhizosphere

The concept of the rhizosphere, the portion of the soil that is influenced by the presence of a root or its exudates, can be expanded to include those areas inhabited by the plant's

mycorrhizae. The mycorrhizosphere is inhabited by diverse and dynamic microbial populations (Linderman 1988). Benefits that bacteria may derive from fungi include habitat. For example, the extra-matrical hyphae of arbuscular fungi exude substances that cause mineral and organic fractions of soils to aggregate (Sutton and Sheppard 1976). Within these soil aggregates microorganisms flourish (Forster and Nicholson 1981). Certain bacteria appear to be favored by fungal exudates (Gilbert and Linderman 1971) implying fungal control the development of the bacterial communities in the mycorrhizosphere to some extent.

Diazotrophs are present in the mycorrhizophere. Mycorrhizae may provide the high levels of phosphorus required by diazotrophs (Bowen 1987, Miller 1987). A nitrogen fixing, spore-forming bacteria of the genus *Bacillus* was found to be active in ectomycorrhizal tubercles on Douglas fir (Li et al. 1995). Similarly, some ectomycorrhizae are known to secrete mannitol, a carbohydrate utilized by nitrogen-fixing organisms (Hassouma and Wareing 1964). These findings suggest that an active component of the diazotrophic community may be in association with mycorrhizal fungi and that the fungi may be important in the carbohydrate nutrition of mycorrhizosphere diazotrophs.

4.1. *Nif*H can be used to detect diazotrophic communities in nature

Traditional studies of bacteria from soils and the rhizospheres of plants involved culturing colonies on selective media (often nitrogen deficient) followed by cell counts,

DNA extraction, and sequencing techniques (Oyalzu-Masuchi and Komagata 1988). Culturing techniques are considered to be limited in value when studying natural communities of diazotrophs as only a small percentage of prokaryotes in nature can be cultured (Wayne et al. 1987). Further, the act of culturing likely alters community attributes such as species abundance and community structure from natural levels by altering selective conditions (Dunbar et al. 1997). A culture independent approach was clearly desirable for the study of natural diazotrophic communities.

All diazotrophs possess the multimeric enzyme complex nitrogenase. Nitrogenase is a tetramer composed of two identical Fe_4S_4 cluster, and FeMo cluster subunits (Dean and Jacobson 1992) that are highly conserved among all diazotrophic groups (Bothe 1982). The genes that encode the protein subunits are also well conserved; a character that makes them ideal molecular markers (Postgate and Eady 1988).

In diazotrophs, nitrogen is fixed through the action of the enzyme nitrogenase. There are twenty genes that encode for the proteins that compose nitrogenase in the diazotroph *Klebsiella pneumoniae* (Dean and Jacobson 1992). The twenty genes (*nif* genes) are arranged into eight transcriptional units, some of which appear to overlap (Beynon et al. 1988). All *nif* genes are well conserved among diazotrophs (Postgate and Eady 1988), a quality that makes them useful molecular tools for the construction of degenerate oligonucleotide primers. The first degenerate oligonucleotide primers were developed for *nif*H gene sequences of the marine cyanobacterium *Trichodesmium thiebautii* (Zehr and McReynolds 1989). *Nif*H is the gene that encodes for the iron protein subunit of

nitrogenase. Its product forms the homodimer, the basic structure of the enzyme. In conjunction with the polymerase chain reaction, these primers proved to be useful tools for examining diazotrophs from natural communities. Further support for the application of *nif*H as a molecular tool came from the confirmation that *nif*H sequence phylogenies are largely consistent with the widely accepted 16S rRNA phylogeny for diazotrophic microbes (Young 1992).

4.2. Measurements of diazotroph diversity

Genetic diversity in diazotrophs has been assessed by a variety of techniques based on PCR amplification with degenerate *nif*H primers from natural samples. Many studies involve the amplification of *nif*H sequences followed by direct sequencing of the genes (Kirshtein et al. 1991; Ueda et al. 1995; Borneman and Triplett 1997; Jeong and Myrold 2000). However, because of the high cost and time associated with DNA sequencing this method appears to be best suited to the analysis of single or relatively small populations (Dunbar et al. 2000).

When analyzing large populations or when comparing multiple populations of diazotrophs, several techniques have been employed. Probably the simplest technique is RFLP analysis of PCR products. In this method PCR products are digested with restriction enzymes and the resulting fragments are electrophoresed on agarose or polyacrylamide gels. A community profile is represented as a banding pattern on the gel

and these can be compared among samples. Gene richness (the number of bands) and gene evenness (the relative brightness of the bands) have been successfully estimated in this way (Widmer et al. 1999; Shaffer et al. 2000; Poly et al. 2001).

RFLP analysis is not without criticism despite its wide use in the analysis of diazotrophic communities. The most common criticism of the technique is summarized well by Tiedje et al. (1999) in their review of the techniques used in microbial ecology. The authors state that because single base pair substitution can alter the restriction site of a DNA fragment, resulting in the production of two bands but no functional difference in the gene (because of codon degeneracy), RFLP analysis tends to overestimate the genetic diversity of a population. Further, populations of only a few organisms can produce banding patterns that are so complex that they are not interpretable (Liu et al. 1997). These criticisms led Tiedje et al. (1999) to conclude that RFLP analysis is of limited value when used on highly diverse soils composed of non-dominant populations of microbes.

Denaturing Gradient Gel Electrophoresis (DGGE) has been successfully used to measure the diversity of *nif*H fragments in *Paenibacillus azotofixans* strains from soil and rhizosphere samples (Rosado et al. 1998). The technique involves the electrophoresis of double stranded DNA on gels with increasing concentrations of formamide and urea, and is sensitive to single-nucleotide differences. DGGE is considered to be a rapid way to assess intraspecific genetic diversity from environmental samples (Rosado et al. 1998).

4.3. T-RFLPs in the assessment of microbial diversity

Terminal Restriction Length Polymorphism (T-RFLP) is a technique related to RFLP, in that restriction enzymes are used. However, T-RFLP differs from RFLP by the addition of a dye label to the 5' end of the oligonucleotide primer. The dye label allows an automated DNA fragment analyzer to detect the position of the dye-labelled terminal fragment in a polyacrylamide gel. Since only the terminal fragment is visualized, each genotype corresponds to one PCR product. In T-RFLP the DNA fragments are represented quantitatively as peaks on a computer generated graph. The peak area is then integrated to determine the number of terminal DNA fragments it represents. These functions allow for estimates of gene diversity through characterization of sequence evenness and richness. This process is considered a more sensitive quantitative measurement than RFLP (Tiedje et al. 1999).

T-RFLPs have been used in the assessment of *nif*H gene diversity in the guts of termites (Ohkuma et al. 1996, 1999) and the technique holds considerable promise for use in natural soil samples. T-RFLP analysis of microbial diversity by 16S rRNA has been used extensively in soils and aquatic samples (Liu et al. 1997; Clement et al. 1998; Moesender et al. 1999). Further, it was shown that T-RFLP and DGGE (which has been used on soil diazotrophs) identified similar relationships among marine cyanobacteria for the 16S of rRNA (Moesender et al. 1999). Dunbar et al. (2000) calibrated the T-RFLP method by comparing community composition, richness, and evenness of four soil microbial communities that had been previously analyzed by 16S rDNA cloning. They

demonstrated that T-RFLP is also an excellent method for rapidly comparing microbial communities from environmental samples.

5.1. Measurements of nitrogen fixation

Analysis of diazotroph community structure under conditions of simulated climate change is helpful for predicting how these communities will respond to environmental perturbation. If we wish to understand how altered diazotroph communities will function it is necessary to measure diazotroph activity under different treatments. Changes in the composition or structure of diazotroph communities have the potential to alter nitrogen fixation rates and ultimately N input to plants. These changes may take place on multiple timescales; miniscule changes in the activity of diazotrophs may or may not be measurable over several hours or days, but if these changes are sustained over the life of a long-lived woody plant, their cumulative effect may be dramatic. Thus, field measurements of nitrogenase activity are needed at two timescales; one measurement should indicate the potential of a given community to fix nitrogen at any point in time, while the second should indicate the longer-term trends in the nitrogen fixation rate at a site.

5.2. ARA

Perhaps the most commonly used technique to measure nitrogenase activity in the field is the Acetylene Reduction Assay (ARA) (Paerl 1998). The technique is possible because nitrogenase will reduce the triple bond in acetylene (producing ethylene) preferentially over dinitrogen. Acetylene and ethylene are easily separated by gas chromatography, and because it has been determined that acetylene will be preferentially reduced over nitrogen at a set ratio (4:1) (Crawford et al. 2000), estimates of the rate of nitrogen fixation can be made (Stewart et al. 1967; Burris 1974; Bergerson 1980). The ARA is considered a rapid, inexpensive and extremely sensitive technique (Shearer and Kohl 1986).

5.3. Natural ^{15}N isotopes

^{14}N is the dominant form of nitrogen found in nature. The addition of a single neutron to the nucleus of the nitrogen atom produces the ^{15}N isotope. The ^{15}N isotope is less favored by kinetics resulting in only 0.37% of total nitrogen found in this form, while ^{14}N represents 99.63% of the total nitrogen pool (Mook and de Vries 1999). The extremely stable ratio of ^{15}N:^{14}N in the large atmospheric reservoir of N_2 lends itself well as a reference value. As such the atmospheric value has been designated as 0‰ (note that the ratio of ^{15}N: ^{14}N is expressed as a thousandth). As nitrogen cycles through soil, vegetation, and microbial biomass, slight fractionations of the isotopes occur. With each biological transformation, discrimination against the heavier isotope causes ^{15}N to be less

abundant in the new pool. This leads to a pattern of ^{15}N and ^{14}N abundance in terrestrial ecosystems, where vegetation is depleted in ^{15}N and soil and litter are enriched in ^{15}N compared to the atmospheric signature (Nadelhoffer and Fry 1994). Typical values for ^{15}N from different nitrogen pools are shown in Figure 1. The symbol $\delta^{15}N$ represents a change in ratio of ^{15}N:^{14}N above or below the atmospheric value.

Figure 1 depicts variation in the ^{15}N values for each nitrogen pool. As nitrogen becomes more limiting to plant growth plants will quantitatively extract all nitrogen from the soil, resulting in little or no discrimination against the heavier nitrogen isotope (Nadelhoffer and Fry 1994). However, if N-competition among soil organisms is great (as is often the case in natural systems), considerable partitioning of the N-pool may occur. Patterns in the ^{15}N content of vegetation can provide insights to these interactions. For instance, $\delta^{15}N$ content of arctic plants is thought to be indicative of plant-mycorrhizal interactions (Hobbie et al. 2000) and provides some evidence that different mycorrhizal-types access different sources of soil nitrogen (Michelsen et al. 1996). Despite large variation in the N concentrations of new, mature and senescent foliage, seasonal fluctuations in the $\delta^{15}N$ value were found to be small for most species (one exception was Aspen growing at nutrient rich sites) (Kielland et al. 1998). This finding provides some confidence in the value of ^{15}N as an integrator of plant-nitrogen relations (Kielland et al. 1998), at least in N-limited high arctic sites, suggesting that $\delta^{15}N$ values may be a useful indicator the long-term trends in nitrogen contributions from diazotrophs as well.

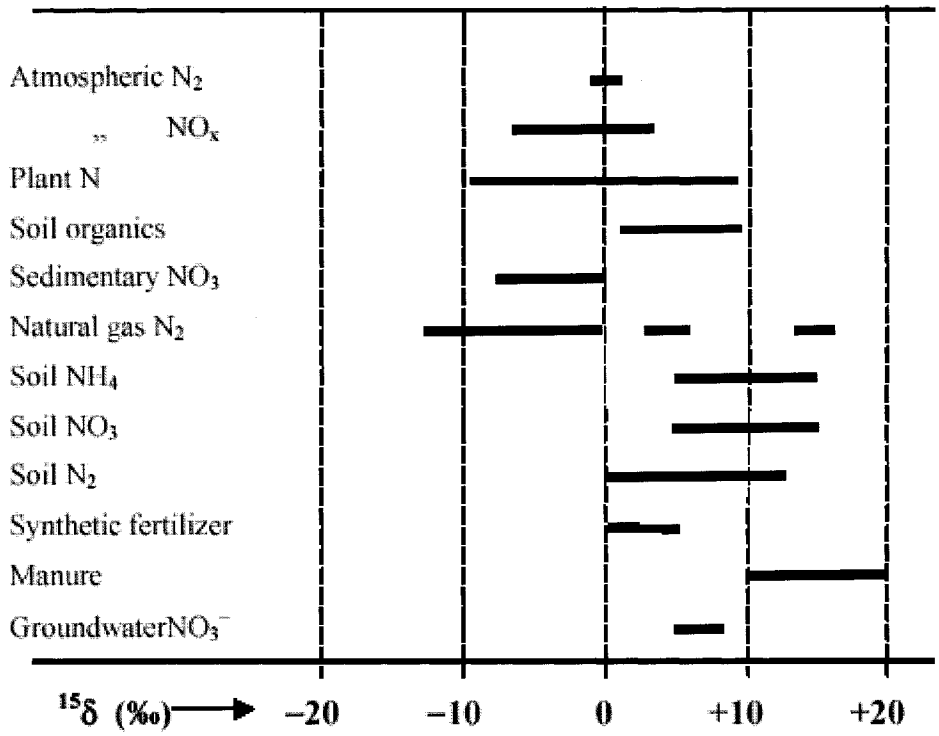

Figure i. A survey of the $\delta^{15}N$ values of nitrogen containing compounds in nature. The $\delta^{15}N$ values are given relative to the isotopic composition of atmospheric N_2 (0‰). (from: Mook and de Vries 1999).

6.1. Studies of community response to simulated climate change

In the future, arctic ecosystems are predicted to be less nitrogen limited than they are at present (Shaver et al. 1992, Chapin and Bledsoe 1992b). A combination of elevated CO_2, increased air and soil temperatures, and increased depth of thaw in the permafrost are thought to be the major factors that will increase nitrogen supply, through faster recycling of the soil N pool (Chapin and Bledsoe 1992b). To assess the impact of climate change on terrestrial arctic ecosystems researchers have employed a variety of tools to simulate

the physical and chemical effects of climate change. Two such tools are the warming of air and soil (plant microclimate) and the addition of soluble nutrients.

Open Top Chambers (OTCs) are commonly used to simulate warming of soils and air (Marion 1997; Arft et al. 1999). OTCs are hexagonal structures with walls of transparent polycarbonate or fiberglass that passively warm the enclosed microenvironment (Marion et al. 1997). Detailed study of the structures in the field has shown that the mean daily near-surface air temperature and soil temperatures increased by 1.2°C to 1.8°C while unwanted side-effects such as altered light, moisture, and gas exchange are minimized (Marion et al. 1997). Water-soluble commercial fertilizer has been used to increase inorganic nutrient supply to tundra communities (Haag 1974; Chapin et al. 1975, 1986; Henry et al. 1986). In all of these studies plant growth and/or vigor was the response variable of interest.

Some key findings from experiments where tundra plant communities were treated with warming include the observation that air warming alone had no effect on plant biomass. This result was interpreted to mean that nutrient limitation is a stronger constraint on tundra plant biomass than is temperature (Shaver et al. 1992). Subsequently, when soil temperature was increased, nutrient availability increased in unfertilized plots and plant nutrient uptake and growth followed this trend (Shaver et al. 1992). The authors hypothesize that the release of plants from nitrogen limitation, due to increased microbial mineralization of nitrogen with warmer soils, accounted for the increase in plant biomass. Evidence for this hypothesis was supplied by the application of greenhouses to four

Swedish tundra soils (Schmidt et al. 2002). Here, warming was found to increase nutrient mineralization rates but, the increased nutrient supply was only immobilized into microbial biomass when competition with plant roots was excluded (Schmidt et al. 2002).

Increased mineralization rates with warming have been reported by many authors (Chapin and Bloom 1976, Chapin et al. 1995, Hartley et al. 1999, Ruess et al. 1999, Schmidt et al. 2002). In a study of a heath and a fellfield site in Swedish Lapland, warming caused increased densities of bacterial and fungal- feeding nematodes and an associated increase in microbial activity, and nutrient mineralization (Ruess et al. 1999). Increased rate of mineralization may be a result of increased activity of soil mesofauna. Alternatively, another mechanism suggested for this change is that the fraction of the microbial population that is favored by higher temperatures may have the ability to metabolize a range of substrates unavailable to microbes at lower temperatures (Zogg et al. 1997).

Nutrient (NPK) amendments have been shown to change plant species composition in arctic tundra communities. Henry et al. (1986) showed that a single addition of 20:20:20 fertilizer at the beginning of three growing seasons resulted in an increased dominance of forbs and graminoids over woody species in a three-year period. Similarly, when N-fertilizer was applied at a much higher rate to tussock tundra vegetation it was found that acquisition of added N was specific to plant functional groups. N-accumulation was greatest in mosses and least pronounced in evergreen shrubs (Chapin et al. 1995). In both studies the authors concluded that sustained increases in nutrient availability would

change the plant-species composition of the tundra communities they studied (Henry et al. 1986, Chapin et al. 1995).

In each of the studies examined, the increased nutrient content of soils, whether directly applied or achieved by microclimate warming, influenced the vigor and abundance or the activity of the organisms in question. These results indicate that climate change may have significant effects on the structure and activity of future arctic ecosystems. Simulated climate change may have similar impacts on communities of nitrogen fixing organisms, and changes in activity or community structure may be observed under experimental conditions.

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2. WILL CLIMATE CHANGE ALTER ARCTIC NITROGEN BUDGETS? IMPACTS OF WARMING AND FERTILIZATION ON NITROGEN FIXING MICROBIAL COMMUNITIES AT ALEXANDRA FIORD, ELLESMERE ISLAND, NUNAVUT

INTRODUCTION

Temperature increase at arctic latitudes as a result of climate change is predicted to be 2-5°C over the next century (Houghton et al. 1995, 1996) and will be far greater than the global mean change (Boer et al. 1990). In the past, climate transitions have led to a disproportionate response by arctic communities, suggesting that present day ecosystems are especially vulnerable to future climate change (Warrick et al. 1986). Arctic ecosystems are considered sensitive indicators of anticipated larger and slower global responses to climate change (Shaver et al. 1992).

Low temperature limits decomposition and subsequent N-mineralization in many arctic ecosystems and consequently plant production is often N-limited (Ulrich and Gesper 1978; Shaver and Chapin 1980, 1986, Chapin et al. 1986). In the future, higher rates of nutrient mineralization and an increased depth of thaw are likely to relieve N-limitation of plants in the short-term (Naddlehoffer et al. 1992, Shaver et al. 1992). Ultimately though, arctic plant production is dependent on the input of new N. Nitrogen fixation is the primary source of new N to terrestrial arctic ecosystems and variation in its input may be a major regulator of ecosystem productivity in the long term (Chapin and Bledsoe 1992b).

It has been predicted that warmer temperatures will increase arctic nitrogen fixation rates by a factor of 1.5-2 (Chapin and Bledsoe 1992a). Increased temperature is expected to induce the strongest direct change in N-fixation rates by increasing all metabolic processes in soil microorganisms, although increased moisture may be important for certain key photoautotrophic diazotrophs (particularly cyanobacteria). Increases in the productivity of photosynthetic nitrogen fixers due to higher atmospheric CO_2 concentrations will also be important in securing this increase (Chapin and Bledsoe 1992a). If these predictions prove true, future arctic plant communities may enjoy a greater N-supply allowing for greater sequestration of atmospheric CO_2 in plant biomass and a down-regulation of the CO_2 induced greenhouse effect. Alternatively, if climate warming does not result in higher rates of N-fixation and an increased supply of N, plant productivity will be tightly constrained by the mineralization of organic N in soils.

Extensive research effort throughout the circumpolar arctic has been devoted to the study terrestrial ecosystems response to climate warming (see Chapin et al. 1995, Arft et al. 1999). A principal finding of these studies has been that increased nutrient content of soils, whether achieved by direct application of nutrients or by microclimate warming, influenced the vigor and abundance of plants and ultimately plant community composition (Henry et al. 1986, Shaver et al. 1992, Chapin et al. 1995). However, the response variable of interest in these studies has often been plant growth and reproduction and relatively little is known about the subsurface ecosystem. Specifically, it is unknown if, or how, the nitrogen-fixing (diazotroph) community changed in these studies. Given the importance of diazotrophs to the long-term productivity of these sites,

this knowledge would greatly improve our ability to predict the fate of N-limited arctic plant communities.

The purpose of this study was to investigate the structure and activity of diazotroph communities under conditions of simulated climate change. Our null hypothesis was that warming or fertilization would not alter diazotroph communities or N fixation rates. We expected that warming would relieve the N-limitation of arctic plants partly through increases in nitrogen fixation rates and/or changing the composition of diazotroph communities. We also assessed the relative dependence of arctic plants on nitrogenase derived N as a function of warming.

METHODS

Site, soils, experimental design

The study site selected lies in a glacial lowland adjacent to Alexandra Fiord, Ellesmere Island, Canada (78° 53′ N, 75° 55′ W). The dominant landform is an outwash plain and the vegetation type is characterized as dwarf-shrub cushion-plant, while the most common soil type is characterized as an Orthic Static Cryosol (Muc et al. 1994). Small hummocks occur through much of the site and the active layer is approximately 35 cm in depth. A fluctuating water table produces mottled mineral soils under a layer of organic matter 5-10 cm thick, organic matter is often mixed into mineral soil. A second soil type,

a Gleysolic Static Cryosol (Muc et al. 1994), occurs locally along the south western margin of the site boardering a drainage channel. Here larger diameter organic hummocks occur which are surrounded by water channels, eroded to the mineral substratum. Table 1 provides physical and chemical data for these two soil types. All data in Table 1 is from Muc et al. (1994). Dominant plants at the site consist of perennial woody species notably: *Salix arctica*, *Cassiope tetragona* and *Dryas integrifolia*. Herbaceous species include *Eriophorum angustifolium*, *Carex stans*, and *Carex membranacea*.

Table 1: Physical and chemical data for the two dominant soil forms at the study site. All data is complied from Muc et al. (1994)

Soil	Horizon (depth) (cm)	pH	Organic matter (%)	Total N (g/kg)	Available P (ppm)	Soil texture		
						Sand (%)	Silt (%)	Clay (%)
Orthic Static Cryosol	LF (6-0)	5.1	30	0.018	1	71	17	12
	Bm (0-8)	5.3	1.5	0.006	12	84	1	15
	C (8-35)	5.4	1.7	0.007	2	68	16	16
Gleysolic Static Cryosol	Om (8-10)	4.9	29	0.011	1	67	18	15
	Cg (0-50)	5.1	4.0	0.0011	7	67	17	16
	Cz (50+)	No data	No data	No data	No data	No data	No data	No data

* Total N was determined by micro-Kjeldahl method and extractable P was determined by weak acid extraction (see Muc et al. 1994).

In 1995, 16 transparent fibreglass Open Top Chambers (OTCs) approximately 1 m in diameter, and 16, 1 m^2 control plots were placed at random locations at the tundra site. In 2000, 8 controls and 8 OTCs were randomly selected for fertilization treatments.

Fertilization treatments consisted of a single 5 g m^{-2} addition of 20N: $20P_2O_5$: $20K_2O$ water-soluble fertilizer applied in early June 2000. This was repeated in 2001. Nitrogen was present as ammonium nitrate (NH_4NO_3). Unfertilized plots were treated with a volume of water equal to that used to dissolve the fertilizer. Soil samples were collected from 8 OTC and 8 control plots in 2001, remaining plots were sampled in 2002. This experiment will be referred to as the "OTC experiment".

In 2002, a second experiment was established to investigate the temporal effects of fertilization on nitrogen fixation and *nif*H gene community structure. Three 1 m^2 fertilization plots and three control plots were established adjacent to the OTC site. The fertilization plots were treated with a single 5 g m^{-2} addition of 20N: $20P_2O_5$: $20K_2O$ water-soluble fertilizer applied on the 28th of June, 2002. This will be referred to as the "Temporal fertilization experiment". The temporal fertilization experiment was sampled twice during the summer of 2002, in conjunction with the OTC experiment.

To address potential shifts in diazotroph communities due to repeated sampling, a disturbance experiment was also established in 2002. Three replicate plots were established for each of four levels of disturbance. On July 15th, 2002 the first 2 treatments were left in a pristine state, three 225 cm^3 soil plugs were removed from the third treatment, while nine 225 cm^3 soil plugs were removed from treatment 4 (all soil plugs were discarded). Two weeks post-disturbance on the 31st of July, 2002, three 225 cm^3 soil samples were removed from treatments 2 (pristine), 3 (3 samples removed), and 4 (9 samples removed), sealed in air tight bags, and frozen for subsequent DNA analysis.

Thus, after the first sampling treatment 1 was pristine, treatment 2 had 3 samples removed, treatment 3 had 6 samples removed and treatment 4 had 12 samples removed. Finally on August 7th, 23 days after the initial disturbance and 7 days after the second disturbance (first sampling date), three 225cm^3 soil samples were removed from all disturbance plots and stored as above. This experiment will be referred to as the "Disturbance experiment".

Acetylene Reduction Assays

Acetylene reduction assays (ARA) were used to estimate nitrogen fixation rates in treatment and control plots of the OTC and Temporal Fertilization experiments during the summer of 2002. In order to minimize the impacts of repeated samplings, 3 soil samples were randomly selected for collection in early summer (June 28 to July 5) and 6 soil samples were harvested from treatment plots in peak summer (July 23 to August 3). Two hundred and twenty-five cubic centimeter soil samples were weighed and placed on glass plates and covered with glass cuvettes fitted with rubber septa and a Vacugrease™ seal. Before sealing the cuvette, soil samples were moistened with creek water from a spray bottle to prevent desiccation during incubations. Acetylene gas was generated on-site from CaC_2 and water and injected into cuvettes to comprise 10% of the total headspace by volume. Prior to the first sampling period, the length of incubation time required to detect ethylene in samples was found to be about 30 hours, and ethylene peaks of repeatable size were obtained after 35 hours. During the incubations, headspace gas was

sampled twice, after approximately 45 and 60 hours, by puncturing the rubber septa with a two-way needle and removal to a 2 ml Vacutainer™. Twenty-four soil samples in glass incubators were assayed per sampling period. Incubators were kept on a wooden table top painted white, and surrounded with ice and snow in sealed plastic bags, to minimize the thermal energy gained by the incubators. On sunny days the incubators were also covered in white shade cloth for the duration of the incubation. Mean incubator temperature was 8.3°C and ranged from 5°C to 14°C through out the sampling season.

The ratio of acetylene to ethylene in gas samples was measured in the field with a portable gas chromatograph (SRI 8610A, Wennick Scientific Corporation) fitted with a Porapak column and a flame ionization detector. Hydrogen was used as the carrier gas and held at a constant pressure of 25 psi. During each incubation period, point measurements of temperature were taken within a control incubator (sealed cuvette with soil sample but no acetylene) with a hand-held digital thermometer fitter with copper-constantan thermocouples. These were used to correct the volume of acetylene for incubator temperature.

Due to a technical problem, no ambient air temperature data were available for Alexandra Fiord during the entire sampling period. In order to correct ARA data for temperature differences among sampling periods, hourly mean temperatures at the Environment Canada weather station at Eureka were used. Eureka is also a sea level site located approximately 100 km east of Alexandra Fiord. Excellent correlation (p=00000) was found between hourly mean temperatures at Alexandra Fiord and Eureka weather station

in July and August 2001. ARA data in 2002 was corrected for the mean temperature difference among sampling periods using Eureka temperature data. The mean ambient air temperature at Eureka during all incubation periods was 5.3°C. ARA rates that were determined for incubation periods that deviated from the mean ambient air temperature were corrected to this temperature using a $Q_{10} = 5.6$ (Stutz and Bliss 1975, Henry and Svoboda 1986). The conversion factor for acetylene reduction to nitrogen fixation used was 4 (Jensen and Cox 1983, Liengen 1999, Crawford et al. 2000). After incubation, all soil samples were placed in sealed plastic bags and frozen for further use. Nitrogen fixation data were analyzed with a General Linear Model ANOVA that allowed for the effects of categorical (OTC and nutrient amendments) and continuous (moisture content, soil %N and %C) variables to be analyzed simultaneously. All ANOVAs were performed using STATISTICA version 6.0 (Statsoft Inc. 2002).

DNA extraction and PCR amplification

A 1 g sub-sample was removed from each soil sample collected for ARA analysis and allowed to thaw at room temperature in the lab. DNA was extracted and purified from these soils using a commercial kit according to the manufacturer's directions (MoBio UltraClean Soil DNA isolation kit). Bulk DNA was kept frozen at –20°C. A half-nested polymerase chain reaction (PCR) protocol was used to amplify a 365 bp fragment of the *nif*H gene from a diluted extract. The primary amplification employed the primers Nh21F (5'GCIWTYTAYGGNAARGG) and WidNhR (5' GCRTAIABNGCCATCATYTC, (see

Widmer et al.1999)). Both primers were synthesized by Invitrogen. The half-nested secondary amplifications employed dye-labeled primers (IDT Technologies). The forward primer, Cy5Nh21F, had the same nucleotide sequence as the Nh21F primer above, while the reverse primer, Cy55Nh428R (5′ Cy5.5-CCRCCRCANACMACGTC) was similar in sequence to one developed by Widmer et al. (1999) with a few substitutions to optimize amplification efficiency. PCR cocktails consisted of genomic DNA (approximately 100 ng), 0.2 mM dNTPs, 0.4 μM primers, 10X PCR Buffer (Life Technologies), 2 mM $MgCl_2$, and 0.72 U of Platinum Taq DNA polymerase (Life Technologies) in a final volume of 30 μl. PCRs were performed with a single thermocycler program consisting of an initial denaturing temperature of 94°C for 2 minutes and 10 seconds followed by 35 cycles of: denaturing at 94°C for 45 s, annealing at 53°C for 45 s, and extension at 72°C for 45 s. A final extension period of 3 minutes at 72°C completed the program. A PTC-100 Programmable Thermal Controller (MJ Research Inc.) was used for all amplifications.

T-RFLP analysis

Endonuclease digests were performed on 8μl aliquots of PCR product with the enzymes *Taq*I and *Hha*I (Invitogen). *In silico* assays were performed on *nif*H genes using all of the restriction endonucleases available from the manufacturer Invitrogen. The enzymes *Taq*I and *Hha*I were complimentary; *Taq*I has a GC-rich recognition sequence ($G_{\uparrow}CG^{\downarrow}C$), while *Hha*I has an AT-rich recognition sequence ($T^{\downarrow}CG_{\uparrow}A$) and both were high frequency

cutters. Reactions were incubated overnight at temperatures optimal for enzyme function (65°C and 37°C respectively). Restriction products were kept frozen at -20°C until analyzed. Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was used to generate unique *nif*H gene community profiles for each soil sample. *Nif*H restriction products were denatured at 80°C in formamide and run on vertical polyacrylamide gels for 45 minutes on OpenGene DNA sequencers (Bayer/Visible Genetics). Dye-labeled-oligonucleotide markers of 101, 200, and 351 base pairs were used as internal standards. All resulting T-RFLP profiles were analyzed manually using GeneObjects 3.1 software (Visible Genetics). *Nif*H genotypes were manually binned by fragment size and the frequency of each genotype in soil samples from replicate treatment plots was determined.

Nonmetric Multidimensional Scaling (NMS) was chosen to visualize treatment plots in genotype-space. NMS (Mather 1976; Kruskal 1964) is an ordination technique that uses an iterative approach to position n entities on k dimensions that minimizes the stress of the k-dimensional configuration (McCune and Grace 2002). All ordinations were run using PC-ORD version 4.0 in the 'auto-pilot' mode which used random starting configurations and assessed dimensionality by minimizing stress. Sorensen distance was selected as the distance measure for each initial matrix (McCune and Mefford 1999). Where necessary, Beals smoothing was applied to *nif*H frequency matrices to reduce noise and enhance the strongest patterns in the dataset (Beals 1984, McCune 1994).

Elemental analysis and moisture content

Soil samples used for ARA and DNA extraction were oven dried at 90°C for 24 hours and reweighed as a measure of moisture content. A small amount of each sample was reserved for elemental analysis. These samples were mechanically ground in a soil grinder and analyzed for carbon and nitrogen concentration by elemental analysis using an AC 1500 Fisions NC autoanalyzer. Differences in N and C concentrations among treatments were analyzed with one way ANOVA performed using STATISTICA version 6.0 (Statsoft Inc. 2002).

^{15}N analysis of plants and soils

In August of 2002, leaves and stems of *Salix arctica*, and *Dryas integrifolia* were sampled from each of the 16 treatment and control plots from the OTC experiment. These species were selected because while are both long-lived, ectomycorrhizal, woody, shrubs that were present in every treatment plot, they differ in that *Dryas integrifolia* is also actinorhizal and may access N derived from the atmosphere. Plant materials were sealed in air-tight plastic bags and frozen for transport to the laboratory. Plant samples were transferred to paper bags and dried at 90°C for 24 hours, then ground with a mortar and pestle. Small amounts of oven-dried and ground soil samples were also allocated for isotope analysis. Plant and soil samples were analyzed for ^{15}N with a Finnigan MAT 252 mass spectrometer. The significance of OTC, nutrient amendment and species on the

$\delta^{15}N$ value of plant material was tested by ANOVA using STATISTICA version 6.0 (Statsoft Inc. 2002).

RESULTS

Elemental analysis of plants and soils

Elemental analysis of total carbon and nitrogen revealed that the 5 g m^{-2} additions of 20N: $20P_2O_5$: $20K_2O$ fertilizer to the OTC experiment in 2000 and 2001 had no significant effect on total soil N or C by mid-summer in 2001 or in 2002. Soils had a mean nitrogen concentration of 0.014 ± 0.001 g kg^{-1} and a C: N of 18.4 ± 0.3 (S.E.). In 2002, the fertilization treatments from the temporal fertilization experiment had the highest N-concentration, however these were still not significantly different from those of the control plots. Table 2 depicts mean soil N and C concentrations and C: N ratios for the OTC and temporal fertilization experiments from the second sampling period. Different letters denote significant differences at alpha = 0.05, using a Tukey's post hoc test.

Table 2: Mean soil N and C concentrations and C: N ratios for OTC and Temporal fertilization experiments from the second sampling period (July 23-August 3) 2002

Experiment	Treatment	N g kg^{-1}	C g kg^{-1}	C:N ratio
OTC	OTC and fertilization (2000, 2001)	0.01065^{a}	0.2023^{a}	18.83^{a}
OTC	OTC	$0.01808^{b,c}$	0.3603^{b}	18.83^{a}
OTC	Fertilization (2000, 2001)	0.01167^{a}	0.2085^{a}	18.69^{a}
OTC and Temporal fertilization	Control	$0.01612^{a,c}$	0.2668^{a}	16.68^{b}
Temporal fertilization	Fertilization (2002 only)	$0.1889^{b,c}$	0.2464^{a}	15.61^{b}

* Different letters denote significant differences at alpha = 0.05, as determined with Tukey's Post hoc test

Warming caused no significant change in the N-concentration of soils but resulted in an increase the C-concentration of soils treated with OTCs only (Table 2). However, this increased C did not correspond to higher C: N ratios in the soil of OTC plots when compared to OTC and fertilization plots or fertilization plots (Table 2). The C: N ratios of all treatment plots from the OTC experiment (OTC fertilization, OTC, and fertilization treatments) were significantly higher than those of the control plots or the temporal fertilization plots (Table 2).

Elemental analysis of plant materials in 2002 indicates a strong species bias for nitrogen concentration. The non-actinorhizal species (*Salix arctica*), had significantly higher ($p<0.0001$) nitrogen concentration than did the actinorhizal species (*Dryas integrifolia*) in all treatments. Warming caused a significant decrease ($p=0.029$) in the nitrogen concentration of *D. integrifolia* (Figure 1), but had no effect on the non-actinorhizal species.

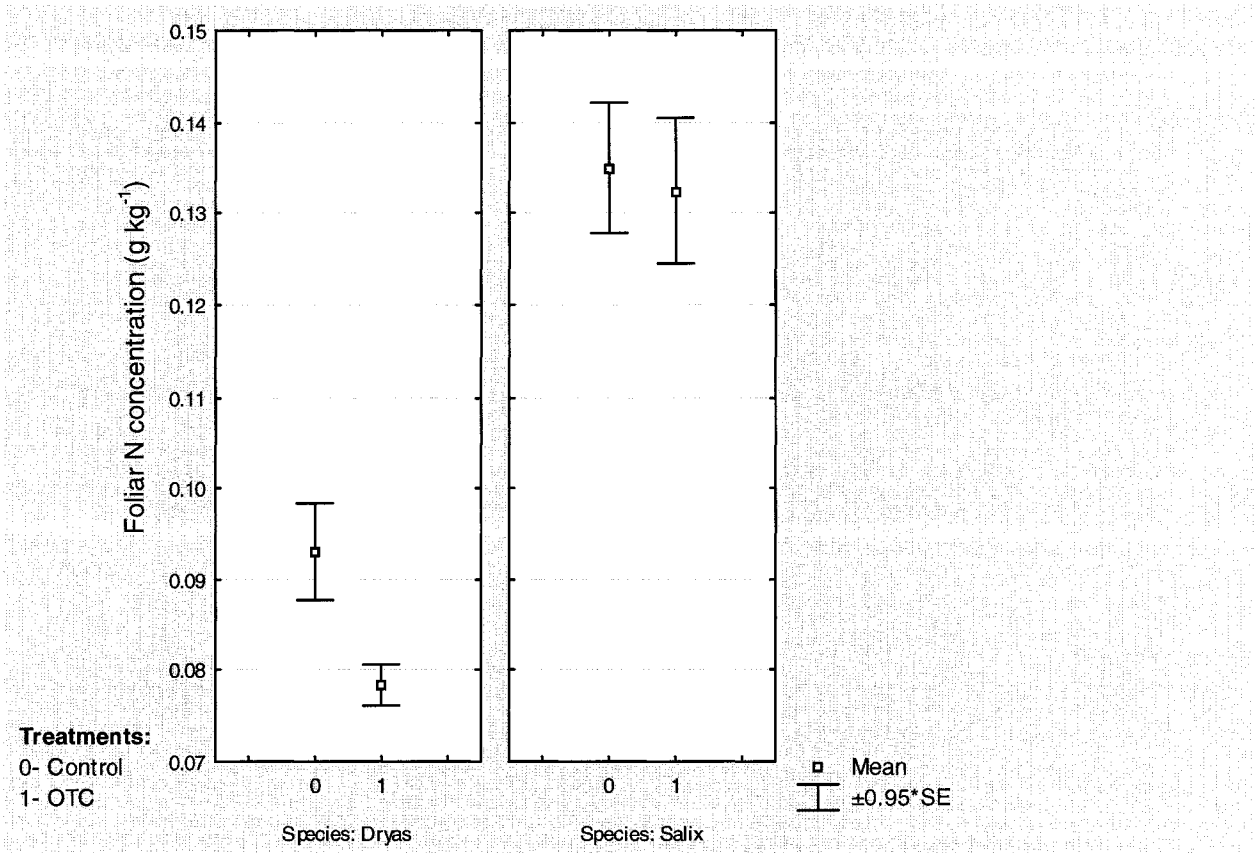

Figure 1: Foliar N content of *Salix arctica* and *Dryas integrifolia* from warmed and control plots

S. arctica showed a weak trend (non-significant at alpha=0.05) toward higher plant nitrogen concentrations with fertilization (Figure 2), while the N-concentration of *D. integrifolia* was unchanged with fertilization.

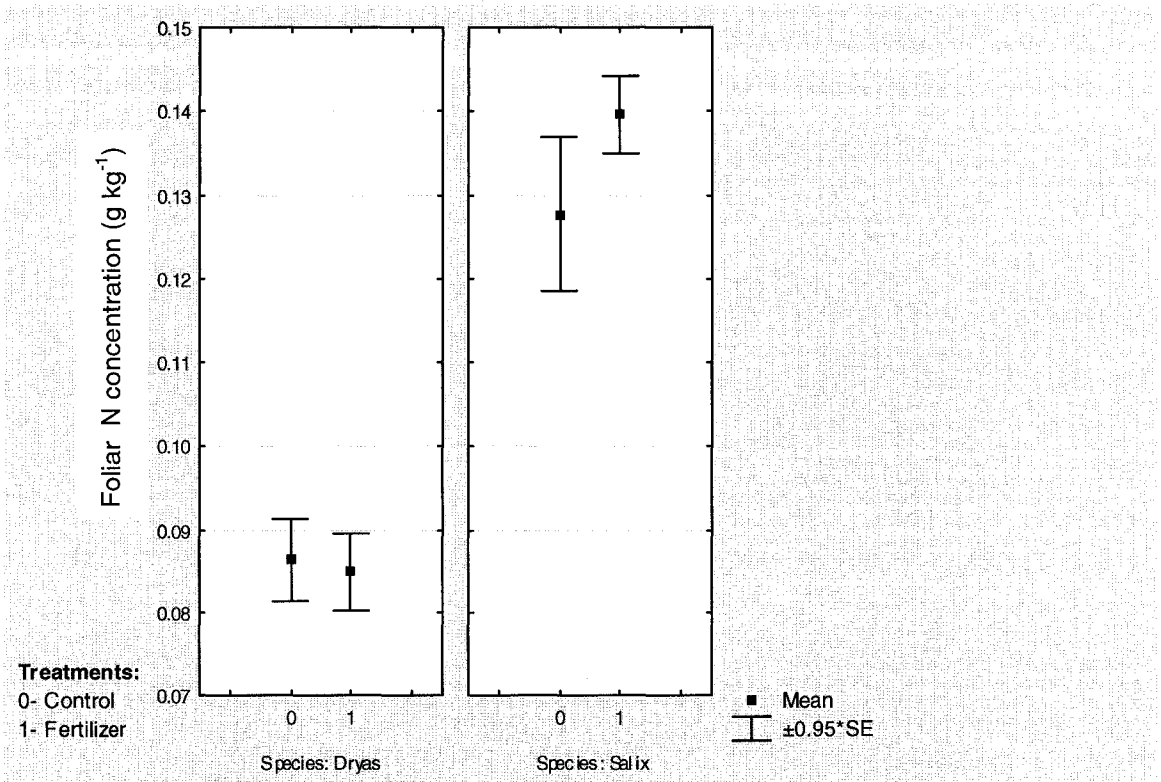

Figure 2: Foliar N content of *Salix arctica* and *Dryas integrifolia* with and without fertilizer amendments

Acetylene Reduction Assays

No differences in fixation rates were found due to warming or to longer-term fertilizations in the OTC experiment. Fixation rates were spatially variable, with replicates from some sampling plots showing no activity after 60 hours of incubation. The mean rate of N-fixation was found to be $3.5 \times 10^{-2} \pm 5.2 \times 10^{-3}$ mg $N \cdot m^{-2} \cdot hr^{-1}$ over the summer. Short-term fertilization had no immediate effect on fixation rates but caused a significant increase in fixation ($p=0.00000$) by the second sampling period (19-35 days post-fertilization). Mean rates increased to $0.136 \pm 3.4 \times 10^{-2}$ $N \cdot m^{-2} \cdot hr^{-1}$, with the addition of 20N: $20P_2O_5$: $20K_2O$ fertilizer in 2002 (Figure 3).

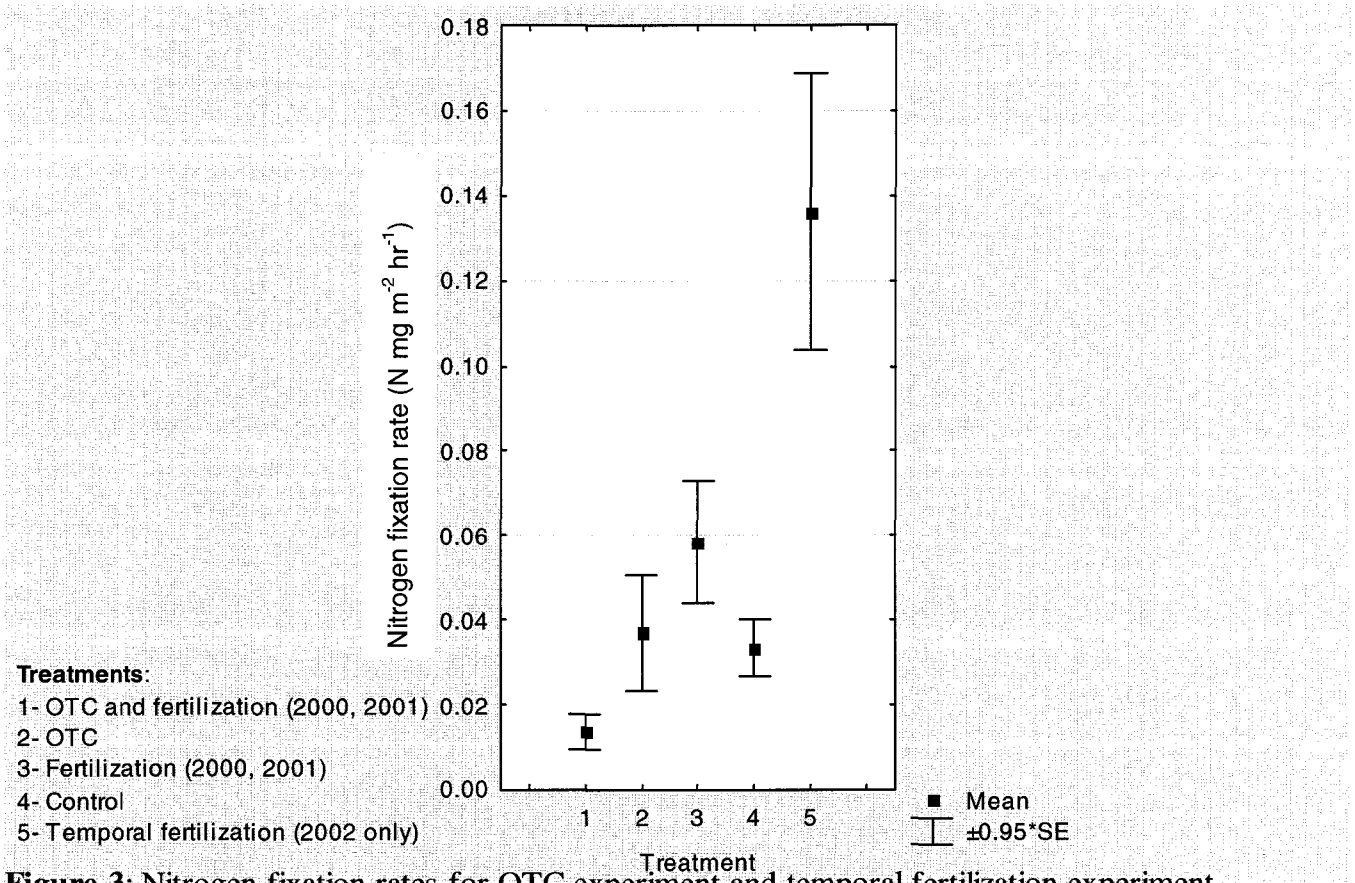

Figure 3: Nitrogen fixation rates for OTC experiment and temporal fertilization experiment for the second sampling period (July 23- August 3), 2002

T-RFLP analysis

NMS plots of sampling units in genotype-space revealed that *nif*H-gene communities were most strongly structured by warming late in the 2002 growing season. Figure 4 shows soils that received the OTC-treatments grouped in the top, right-hand corner of the plot while fertilized and control soils formed a looser group on the bottom, left-hand-side. Sixty-five iterations produced a 3-dimensional solution with a final stress of 9.43 and a final instability of 0.00009. Axis 1 and 2 shown here, account for 6 and 49% of the total

variance in the dataset (cumulative r^2 = .54), while the third axis accounted for 18 % (total r^2 = .730). An overlay of nitrogen fixation rates on the same NMS ordination (Figure 5) shows that higher rates of N-fixation were not associated with *nif*H communities from any particular treatment.

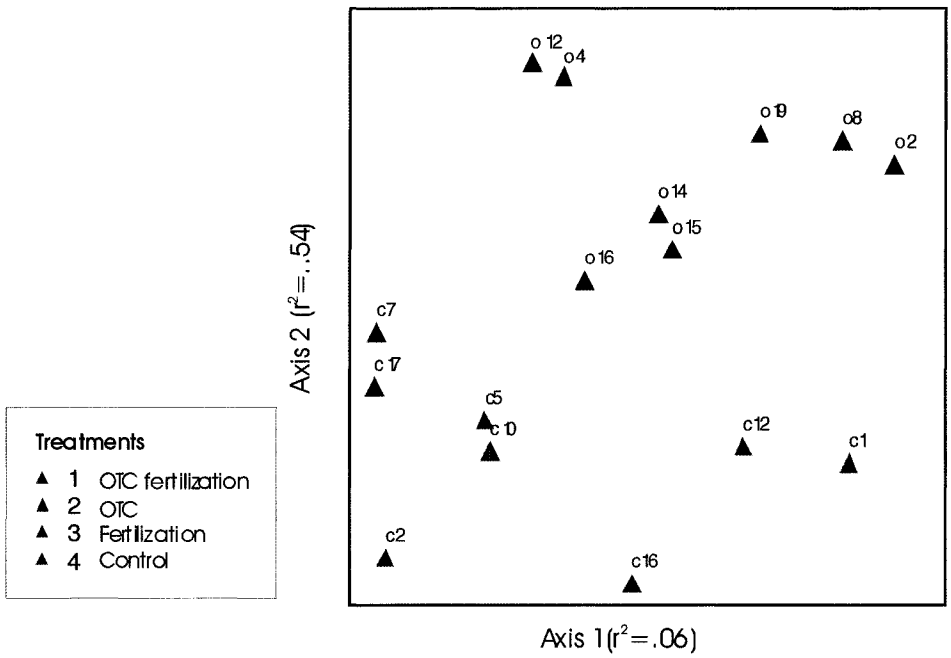

Figure 4: NMS plot of treatment and control plots in *nifH* genotype space. T-RFLP data collected during the second sampling period (July 23-August 3) 2002.

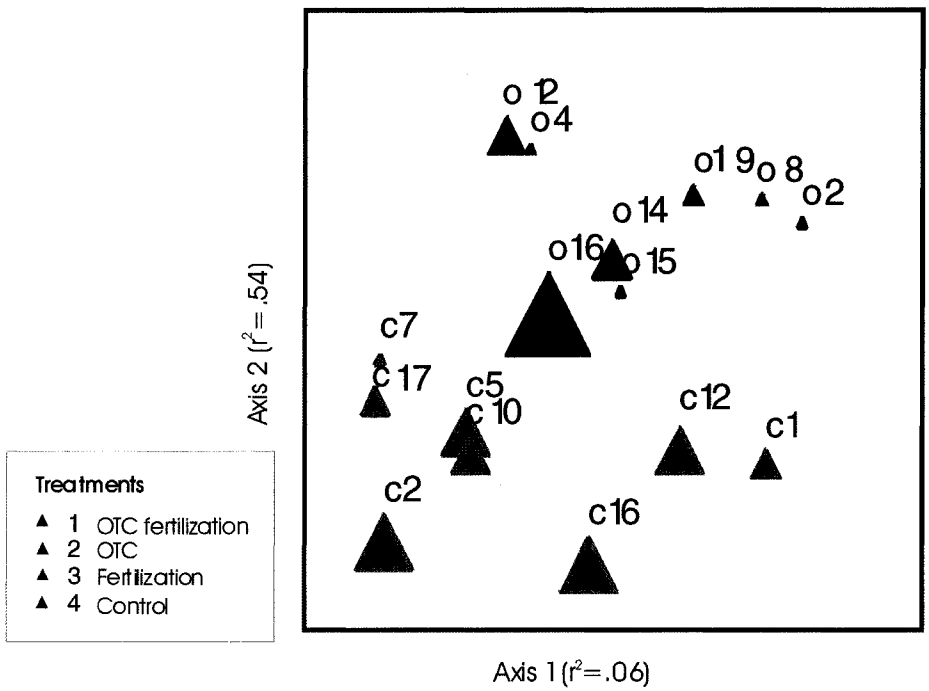

Figure 5: Overlay of nitrogen fixation rates (N mg m^{-2} hr^{-1}) on an NMS plot of treatments in genotype space, data for second sampling period (July 23-August 3) 2002.

Earlier in 2002, no strong relationship among diazotroph communities was found to be due to warming. A NMS plot of sampling units in genotype-space (Figure 6) from the first sampling period (June 28- July 5) revealed no clear separation of *nif*H communities from warmed or control treatments. Axis 1, which accounts for 45% of the variation in this 2-dimensional solution, produced after 82 iterations, indicates a weak trend of warmed sampling units on the lower half of the axis, while un-warmed plots appear toward the upper half. No trend exists along axis 2, which accounts for 37% of the variance in the dataset (cumulative r^2 = .82). These results must be interpreted with caution as the final stress and instability of this ordination were both rather high (13.79 and 0.0001, respectively).

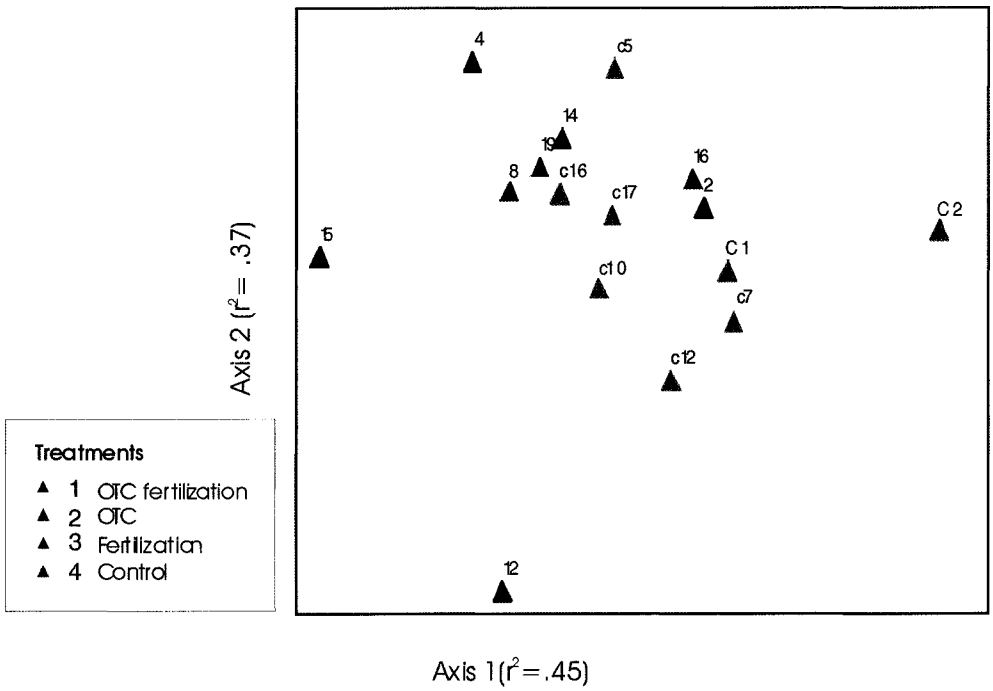

Figure 6: NMS plot of treatment and control plots in *nif*H genotype space. T-RFLP data collected during the first sampling period (June 28-July 5) 2002.

Fertilization treatments from the temporal fertilization experiment were not associated with detectable changes in diazotroph communities in 2002. Short term fertilization treatments had *nif*H gene profiles similar to those from control soils. Figure 7 shows the second and third axis (40% and 33% of the variation, respectively) of a 3-dimensional solution (cumulative r^2 = .805) produced after 37 iterations, with a final stress of 8.69 and a final instability of 0.00008. Longer-term fertilization plots had diverse *nif*H communities. Two of four long-term-fertilization plots (c16 and c5) were similar to control soils while two plots (c2 and c12) ranked very low on axis 2 (Figure 7). These two sampling plots grouped more closely with soils that received OTC-treatment than with control soils when all sampling units were combined (data not shown).

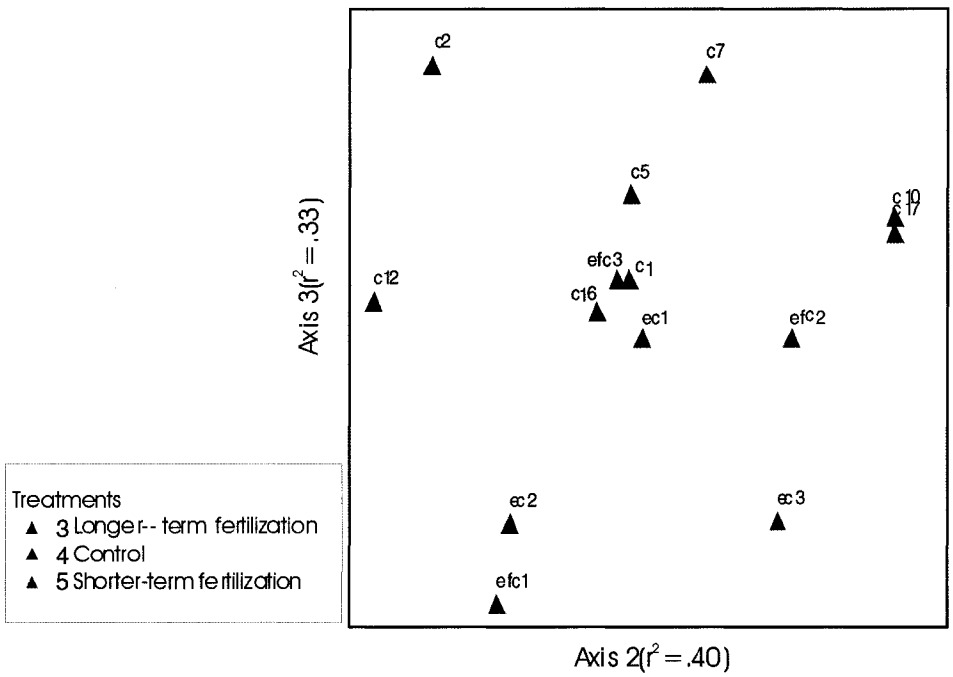

Figure 7: NMS ordination of fertilized and control plots in *nifH* genotype space. T-RFLP data collected during the second sampling period (July 16- August 3) 2002.

In 2001, warming and fertilization treatments were associated with different patterns of *nif*H-gene community composition. Figure 8 is a plot of a 2-dimensional NMS ordination, resulting from 46 iterations. The solution has a final stress of 11.5, and a final instability of 0.00006. Axis 1 describes only 8.9% of the variance in the data while axis 2 accounts for 78.2 (cumulative r^2= .871). In order to reach a stable NMS solution it was necessary to omit one control plot that acted as a strong outlier. Figure 8 shows warmed plots forming only a loose group in the mid-range of axis 2 and the middle and upper range on axis 1. Fertilized plots without warming occur very low on axis 2, but throughout axis 1. Control plots tend toward the upper portion of axis 2 but are diverse and do not form a coherent group. In contrast to the *nif*H profiles from 2002, the 2001 control plots are distinct from the soils that received fertilization without warming.

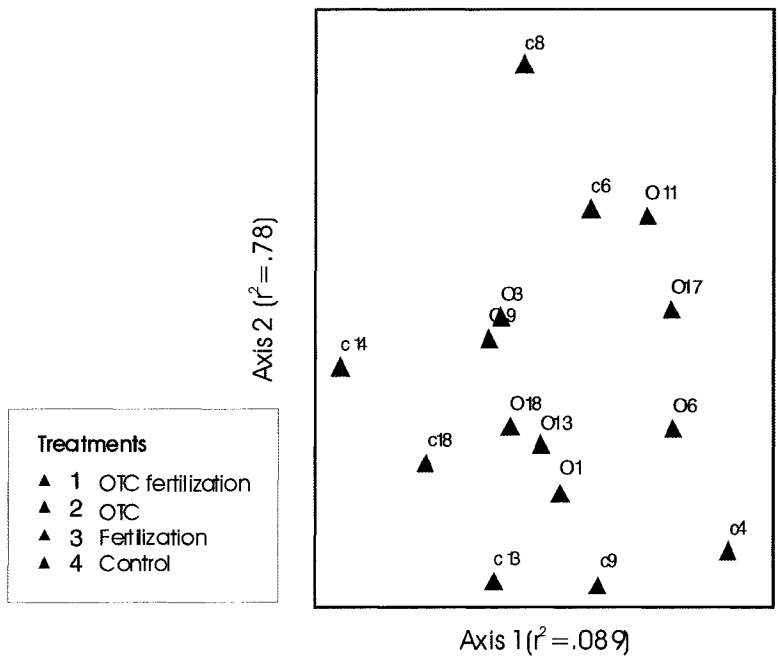

Figure 8: NMS plot of treatment and control plots in *nifH* genotype space. T-RFLP data collected in 2001.

No acceptable NMS solution was achieved for the ordination *nif*H genotypes from disturbed plots with those from OTC and fertilization plots making it difficult to assess the similarity of diazotroph communities from these treatments. However, Correspondence Analysis (CA) of the disturbance treatments alone revealed no clear grouping of *nif*H gene communities according to the level of disturbance they received (Figure 9). Furthermore CA ordination of *nif*H genotypes from disturbed plots with those from OTC and fertilization plots revealed no detectable patterns at all (data not shown).

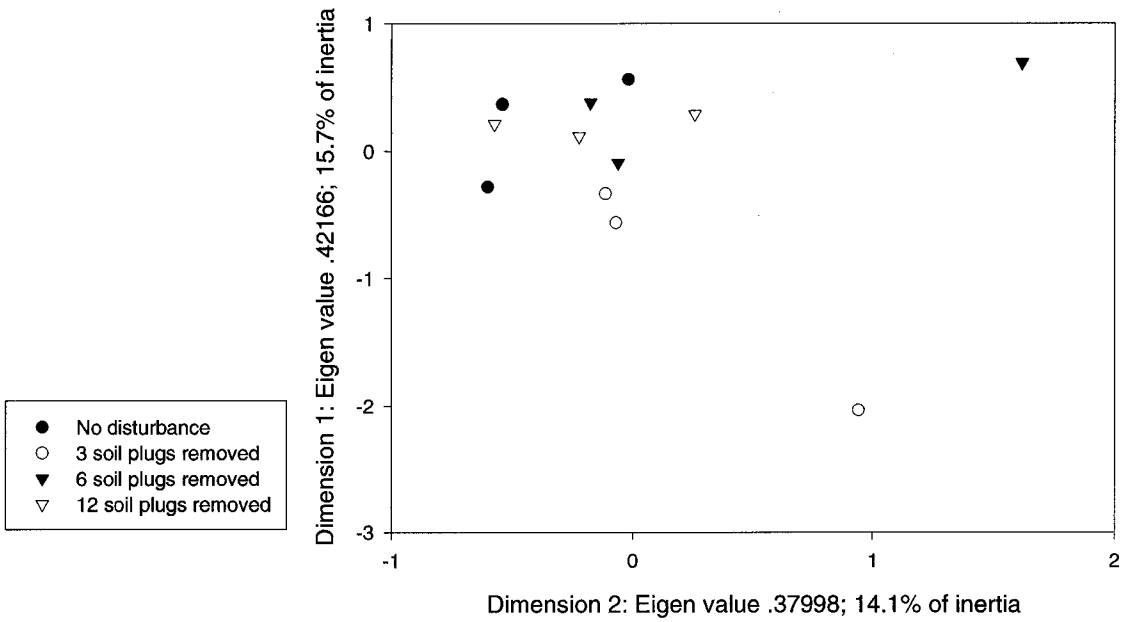

Figure 9: Correspondence Analysis generated plot for disturbance experiment

$\delta^{15}N$ Analysis

$\delta^{15}N$ analysis of foliage from *Salix* and *Dryas* plants in 2002 revealed a significant decline in delta ^{15}N values with warming treatments ($p=0.01$), while fertilization treatments did not significantly alter the $\delta^{15}N$ values. Additionally, *Dryas integrifolia* was found to be more depleted in the heavier isotope than *Salix arctica*. Figure 10 shows $\delta^{15}N$ values for *S. arctica* and *D. integrifolia* from warmed and control plots. Good correlation was found between foliar %N and the $\delta^{15}N$ values of these species (adjusted $r^2=.60$) (Figure 11).

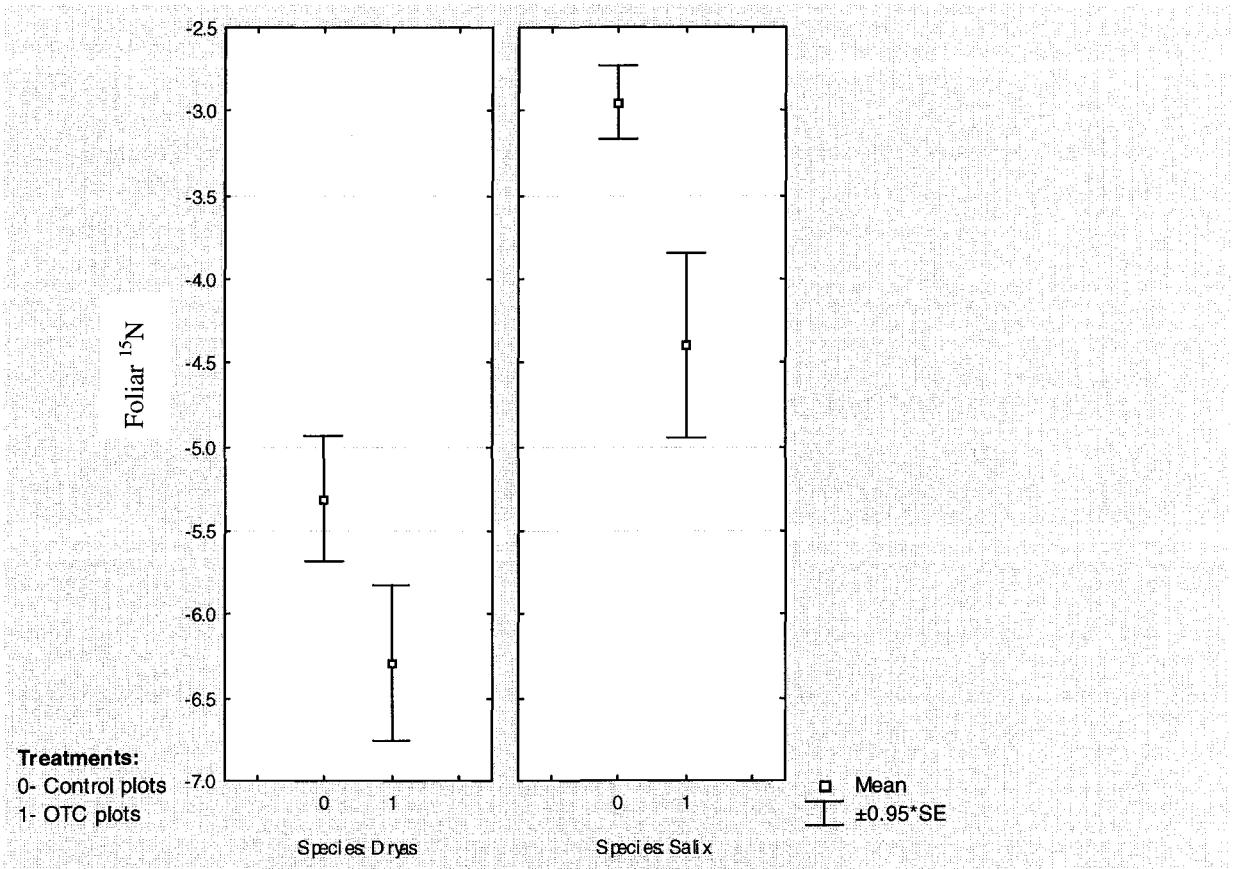

Figure 10: Foliar delta ^{15}N values for *Dryas integrifolia* and *Salix arctica* with OTC treatment

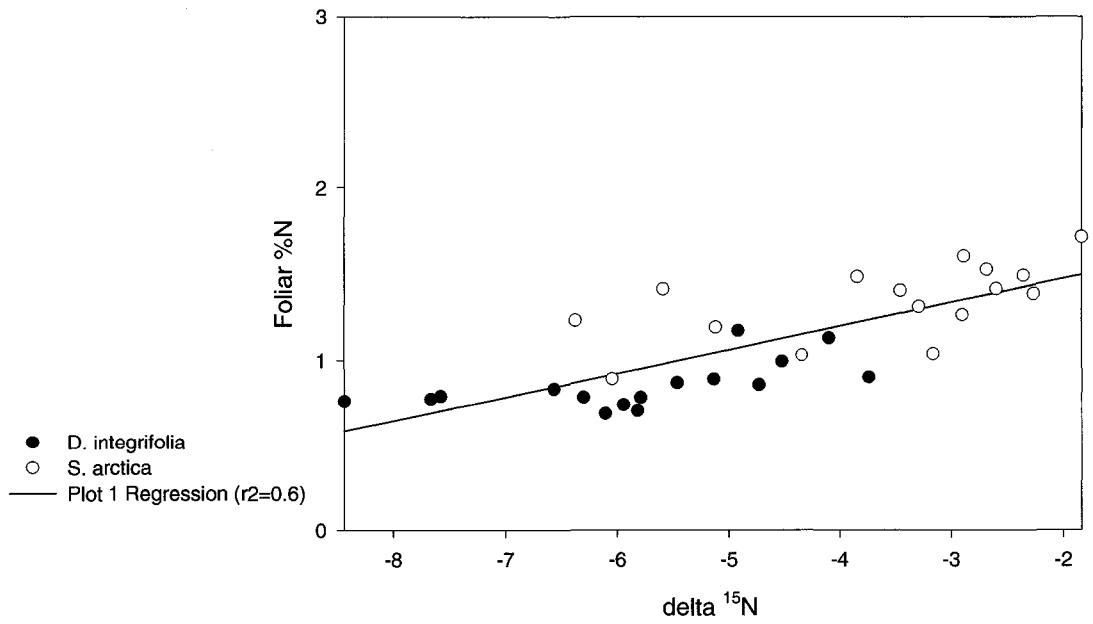

Figure 11: Linear relationship for foliar %N and delta ^{15}N values in *S. arctica* and *D. integrifolia*

No differences were found among soil $\delta^{15}N$ values from all treatments. The mean $\delta^{15}N$ value for soils (+0.15) was close to the atmospheric value, and similar to that of the fertilizer applied (+0.40).

DISCUSSION

Methodological considerations

Elemental analysis of total nitrogen in control plots revealed no change in N concentrations of soils due to the fertilization treatments. Control plots had a nitrogen concentration of 178.3 ±15.4 g m^{-2}. The amount of N added with the fertilization treatment was approximately 1.7 g m^{-2} $year^{-1}$ (3.4 g m^{-2} in two years) an order of magnitude less than the margin of error. Thus, we lacked the ability to detect changes in total soil N that were due to our fertilization treatments.

We were unable to detect changes in fixation rates due to treatments in the OTC experiment. In this experiment two treatments were applied; warming and fertilization. The lack of response of N-fixation to fertilization treatments may be explained by the lack of detectable change in soil N with nutrient amendment. Higher rates of fertilizer application may have been required to cause changes in N-fixation. Other studies have found that high rates of fertilization (particularly ammonium addition) suppresses N-

fixation in the field (Krupka 1984, Okoronkwo and Van Hove 1987, Liengen 1999, Piceno and Lovell 2000), while lower rates of nutrient amendment tend to increase N-fixation in some systems (Bagwell and Lovell 2000, Piceno and Lovell 2000).

The lack of response to warming is of particular interest. Warming is generally expected to increase the rate N-fixation in the field due to the direct effect of temperature on microbial metabolism (Chapin et al. 1992a). However, some evidence exists to suggest that N-fixation by arctic diazotrophs may be subject to acclimation. For example, after soil cores from a *Salix*-moss-hummock community were incubated at 15°C for two weeks it was found that optimal rates of N-fixation occurred at this temperature (Chapin et al. 1991). Further, it was found that the temperature optimum for N-fixation in field-cores from Truelove Lowland approximated maximum surface temperatures measured in the study (Chapin et al. 1991). If a complete picture of diazotroph activity in response to warming is to be gained from field studies of N-fixation it may be necessary to consider the possibility of diazotroph acclimation.

In the present study, incubators were kept at a relatively constant temperature. In contrast, temperature data collected inside and outside Open Top Chambers for a two-week period prior to the 2002 sampling season revealed one maximum daily temperature of 20.5°C within an OTC while the corresponding control plot had a maximum temp of only 14.5°C (both measured 15cm above the surface). If it is true that arctic diazotrophs acclimate to the maximum daily temperature they are exposed to, we would expect diazotrophs from OTC treatments to have higher temperature optima for N-fixation than those from control

plots. Figure 11 is an idealized temperature response curve (based on data from Liengen 1999) for N-fixation by diazotrophs from warmed and control soils. From Figure 11 it becomes clear that the experimental conditions provided temperatures closer to the physiological optima of diazotrophs from control plots and we would expect higher fixation rates from those samples.

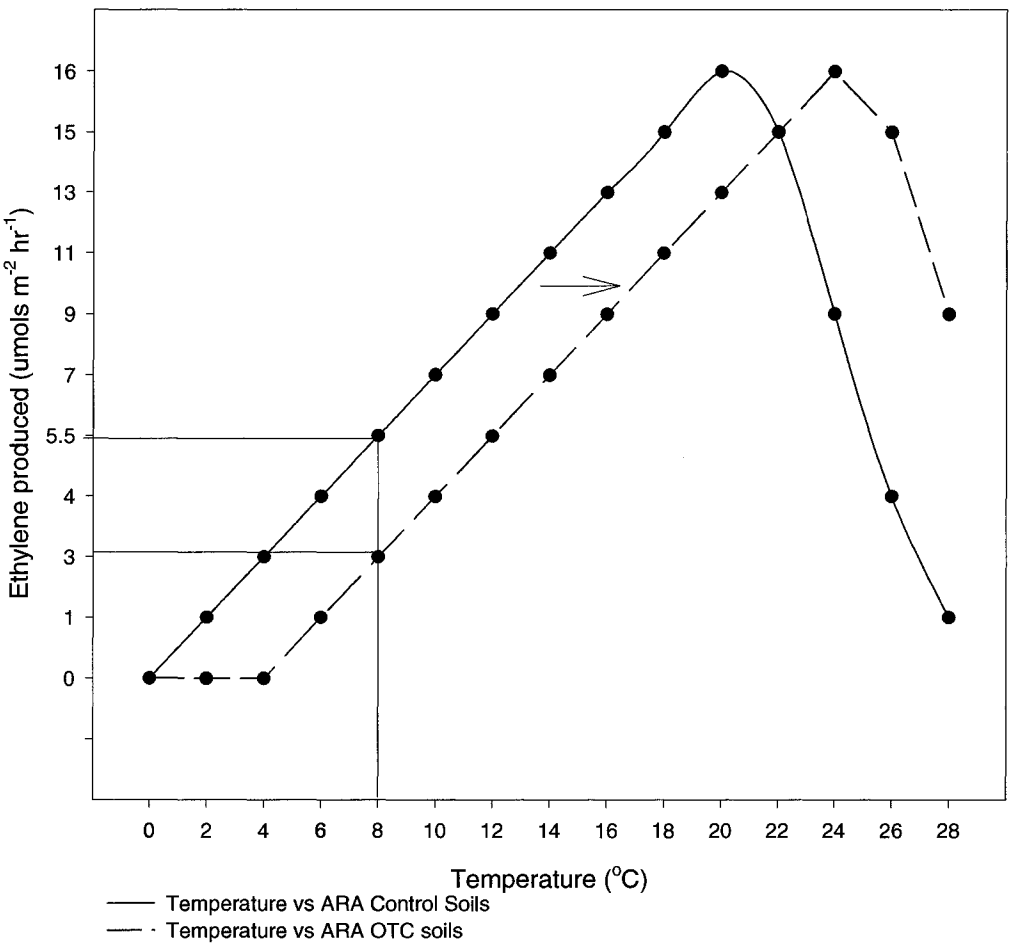

Figure 12: Idealized temperature response curves for arctic soil diazotrophs acclimated to maximum daily temperatures of 20°C and 24°C

Acetylene Reduction Assays

As described above, warming had the potential to increase fixation rates (through direct effects on diazotroph metabolism) or decrease fixation rates (through acclimation of nitrogenase activity to temperature). However, we were unable to detect changes in fixation rates due to any treatment in the OTC experiment. Fixation rates measured in this study and at many other arctic sites (Table 2) are low when compared to nitrogen fixation rates in temperate regions (Lennihan et al. 1994). Moreover, N-fixation rates were spatially variable. The spatial variability of arctic N-fixation has been noted by many authors (Alexander and Schell 1973, Karagatzides et al. 1985, Henry and Svoboda 1986, Chapin et al. 1991), and it has been suggested that N-fixation responds most strongly to factors that can vary on a microsite scale (Chapin et al. 1991, Liengen et al. 1999). It is possible that the range of microsites that occurred in control plots were inherently as variable as the changes that accrued due to the treatments applied.

Microsite differences that have influenced N- fixation in other studies and may apply to the present study include (1) microtopography, (2) moisture status, and (3) plant community composition. Henry and Svoboda (1988) reported higher rates of N-fixation in hollows than in hummocks, where cyanobacteria were more plentiful. They attributed this difference to shading of cyanobacteria by dead plant material on hummocks. In another study, nitrogen fixation was found to be greater in the depressed centers of polygons as compared to the raised rims (Alexander and Schell 1973). Here, the difference was attributed to a moisture limitation of fixation on drier polygon rims.

Additionally, moisture differences between raised or depressed microsites may control maximum daily temperatures locally. This may result in diazotrophs from drier-microsites that are acclimated to warmer temperatures than water-insulated, depression-dwelling diazotrophs. In a study of N-fixation across site types at Sarcpa Lake, highest rates were associated with plant communities with high densities of legumes (Karagatzides et al. 1985). Although no legumes are found at Alexandra Fiord , spatial heterogeneity in the distribution of lichens, *D. integrifolia* and *Nostoc* mats in the present study may have obscured any differences due to treatments.

Rates of acetylene reduction observed in the OTC experiment were similar to values reported for other non-brackish lowlands sites in the Canadian high-arctic (Table 3).

Table 3: Acetylene reduction activity reported for non-brackish, low-land sites in the Canadian high-arctic

Location	**Site type**	**Acetylene Reduced ($\mu mol\ m^{-2}\ hr^{-1}$)**	**Year of study**	**Reference**
Alexandra Fiord	Wet sedge meadow	2.65 (1.3)*	1988	Chapin et al. 1991
Alexandra Fiord	Wet sedge meadow	6.82 (1.2)	1983	Henry and Svoboda 1986
Sarcpa Lake	Rocky tundra	7.37 (2.2)	1982	Karagatzides et al. 1985
Alexandra Fiord	Mesic dwarf-shrub cushion-plant tundra	9.28 (1.4)*	2002	Present study
Truelove Lowland	*Salix*-moss hummocks	14.10 (1.8)*	1988	Chapin et al. 1991
Truelove Lowland	Herb-moss hummock	17.39 (6.8)*	1988	Chapin et al. 1991

* These value have been corrected to 9.6°C (Q_{10}=5.6) to facilitate direct comparison with other studies

In the temporal fertilization experiment a very different trend was observed. During the first sampling period (1-7 days post-fertilization) fertilized plots had N-fixation rates near those of controls. By the second sampling period (19-35 days post-fertilization) the fixation rates in fertilized plots had more than tripled. This indicates that the fertilization treatments (1.7 g m^{-2} N) did not provide sufficient nitrogen to suppress fixation at this site. In a *Spartina* salt marsh 16.3 g m^{-2} additions of N (also as NH_4NO_3) were insufficient to suppress N-fixation across all treatment plots (Piceno and Lovell 2000). Although the amount of N required to suppress fixation may be specific to a site or even to a diazotroph community, it is likely that much greater additions of nitrogen were required to suppress fixation at Alexandra Fiord.

The significantly greater rates of N-fixation observed during the second sampling period suggests that fertilization treatments relieved some limitation to N-fixation in these plots. One possibility is that the nutrient amendments relieved P limitation of the diazotroph community. Phosphate has a diffusivity in soils that is an order of magnitude lower than ammonium and two orders of magnitude lower than nitrate (Paul and Clark 1996). Consequently, phosphorus is more strongly retained in soils than nitrogen (Chapin et al. 1995, Black 1968), and may have been available to soil microorganisms after the added N was assimilated into plant biomass. Phosphorus is thought to be a limiting nutrient for nitrogen fixation (Gorham et al. 1979) and phosphorus fertilization has been shown to cause significant increases in N-fixation in the field (Chapin et al. 1991, Liengen 1999). The retention of P by soils after the added N was removed may explain the higher rates of N-fixation in fertilized plots several weeks post-fertilization. A second possibility is that

nitrogen fixation rates increased in response to increased C-exudation from plant roots. When salt-marsh communities were fertilized with NH_4NO_3 N-fixation rates increased significantly 2 and 8 weeks post-fertilization; this change was attributed to an increase in plant productivity and root exudation in response to fertilization (Piceno and Lovell 2000). It is also possible that the much greater rate of N-fixation observed in the temporal fertilization treatment plots came about through a combination of these two mechanisms.

T-RFLP analysis

Our inability to detect strong patterns in the *nif*H gene frequency data from disturbance plots assured us that *nif*H community structure was not altered in a predictable fashion by sampling during our study. Although altered carbon availability may (in theory) have the potential to induce structural change in the diazotroph community, and although plants damaged during sampling may have exuded labile carbon into surrounding soil, these do not appear to have been major factors that influenced *nif*H community structure. We suggest that changes in the structure of diazotroph populations were induced by the intended treatments and not by a sampling artifact.

The NMS ordinations of *nif*H genotype frequencies from treatment and control plots suggest that warming was an important determinant of diazotroph community structure (Figure 4). Similarly, compositional changes in microbial communities from a northern hardwood forest were reported after incubation at elevated temperatures (Zogg et al.

1997). In 2002, the study site had been snow-free for less than 2 weeks prior to the first sampling period. The similarity of *nif*H communities from warmed and control plots in the first sampling period (June 28-July 2) (Figure 6) suggest that an accumulation of thermal energy (estimated as degree days) leads to succession in the *nif*H community, resulting in detectable changes only later in the season. The intermediate influence of warming in 2001 (Figure 8) is consistent with 2002 results as 2001 samples were taken in mid-July between the first and second sampling periods in 2002.

Increased soil temperature may alter diazotroph community structure in several ways. Firstly, temperature may have a direct impact on *nif*H community structure by selecting for organisms with higher physiological temperature optima. Change in the lipid composition of cellular membranes is believed to be a major strategy for acclimation of soil microbes to different temperatures (Paul and Clarke 1996). For example, in cyanobateria in pure culture an increase of less than 4°C has been associated with a shift in membrane lipid composition from saturated and monounsaturated fatty acids to polyunsaturated fatty acids (Russell and Fukunaga 1990). The synthesis of cell membrane components is metabolically expensive, and synthesis of new membrane lipids may place sufficient stress on certain members of the diazotroph community in warmed soils to affect a change in community composition. A second mechanism by which temperature may alter the structure diazotroph communities is through increased mortality from grazing by soil fauna. In a study of a heath and a fellfield site in Swedish Lapland, warming caused an increase in the density of bacterial and fungal- feeding nematodes (Ruess et al. 1999). Increased grazing by soil fauna at higher temperatures may be

associated with a decline in free-living soil diazotrophs. Another repercussion of increased grazing is an accelerated rate of nutrient mineralization. In the same study, increased rates of nutrient mineralization were a noted effect of the high rates of grazing by nematodes (Ruess et al. 1999). Changes in rates of nutrient mineralization with soil warming have been observed in many studies (Chapin and Bloom 1976, Chapin et al. 1995, Hartley et al. 1999, Ruess et al. 1999, Schmidt et al. 2002). A second mechanism suggested for this change was that the fraction of the microbial population that is favored by higher temperatures may have the ability to metabolize a range of substrates unavailable to microbes at lower temperatures (Zogg et al. 1997).

Despite the significantly higher rates of N-fixation in the short-term fertilization plots in the second sampling period of 2002, fertilized *nif*H communities were not different from those from control plots (Figure 7). This finding is consistent with a fertilization study of salt marsh diazotrophs where the authors report detection of every genotype by reverse-sample genome probing in each sample, regardless of treatment (Bagwell and Lovell 2000). Similarly, short-term nutrient addition (N and P) caused no detectable change in *nif*H DGGE profiles of *Spartina alterniflora* rhizoplane diazotrophs, causing the authors to conclude that the diazotroph assemblage showed substantial short-term stability to environmental change (Piceno and Lovell 2000). However, longer-term fertilizations (treatments applied every 2 weeks for 8 weeks) did result in detectable differences in DGGE profiles in the same study (Piceno and Lovell 2000).

It has been hypothesized that the stability of the inorganic nitrogen concentration of soils is the primary determinant of *nif*H gene pool structure (Poly et al. 2001). Although diazotroph communities appear to be stable to short term changes in nutrient status (Bagwell and Lovell 2000, Piceno and Lovell 2000), longer-term nutrient increases appear to bring about compositional change. In the present study, the 5g m^2 $year^{-1}$additions of 20N: $20P_2O_5$: $20K_2O$ fertilizer in 2000 and 2001 generally appear to have been within the range of nutrient levels for which diazotroph communities were adapted (exceptions will be discussed below). In contrast, OTC treatments resulted in notable community change. It is possible that while fertilization treatments were insufficient to induce compositional shifts in the diazotroph community, warming with OTCs resulted in prolonged alteration of the nutrient regime. This change appears to have been sufficient to lead to typical community structures in warmed soils at least late in the growing season (late July to early August).

Two details from the NMS ordinations of *nif*H gene fragments from plots that received various fertilization treatments deserve special consideration. Data from 2001 (Figure 8) shows that fertilized soils grouped low on Axis 2, closer to plots that had received OTC treatment than to controls. These plots also had significantly higher %N ($p=0.006$) and %C ($p=0.016$) than the fertilization treatment plots sampled in 2002. Similarly when the long and short-term fertilization treatment were compared (Figure 7), two of four long-term-fertilization plots (c16 and c5) were similar to control soils while two plots (c2 and c12) ranked very low on axis 2. These two sampling plots grouped more closely with soils that received OTC-treatment than with control soils when all sampling units were

combined (data not shown). These findings suggest that the fertilization treatments used were heterogeneous in their effects on diazotroph community structure and that a limited response to the fertilization treatments may have occurred in some plots. However, our data from 2002 suggest that if fertilization treatments did alter diazotroph community structure within the same year, this alteration was not sustained.

Higher rates of N-fixation were not associated with specific *nif*H community structures in this study (Figure 5). This suggests that the relationship between *nif*H genotype frequency and nitrogenase activity is not simple at this site. Community structure (as defined in this study) is a function of both the richness and abundance of *nif*H genotypes. The number of genotypes present in a given system (richness) is generally thought to be a function of environmental selection, however, in a recent cross-system comparison it was noted that the diversity of nitrogenase genes was not directly related to the degree of N-limitation of the system (Zehr et al. 2003). This finding prompted the authors to suggest that physical and chemical factors, including the transport of cells between and among environments, are also important determinants of gene distributions in natural assemblages (Zehr et al. 2003). Once present in a soil, the abundance of a given genotype should reflect its ability to compete for limited resources in the ecosystem. However, because several diazotrophs are known to carry multiple copies of the *nif*H gene (Young 1992) it would perilous to suggest that the fitness of a given diazotroph in an environment could be directly implied by the presence or absence of a specific *nif*H genotype. If environmental conditions cause selection against a microorganism, we would expect that all of its *nif*H gene variants would decline at the same rate, but

problems can arise if diazotrophs with different physiological optima share some but not all copies of *nif*H genes. Nonetheless, the distribution of N-fixing organisms is non-random and can be predicted on the basis of habitat characteristics (Poly et al. 2001, Zehr et al. 2003).

Factors that control the presence of genotypes in soils (natural selection, movement of cells or DNA) may not be directly related to the expression of *nif*H genes where they occur. In this study, nitrogen fixation rates were spatially variable and may have been more strongly controlled by abiotic factors (moisture, temperature, microsite differences in C and N availability) than by the potential of the diazotroph community to express *nif*H genes. In a Spartina salt-marsh, acetylene reduction rates increased in N and N & P amended plots 2 weeks post fertilization although no corresponding change in *nif*H community DGGE profiles were observed (Piceno and Lovell 2000). It appears from this study and others (Chapin et al. 1991, Liengen et al. 1999), that factors that structure diazotroph communities may be different than the factors that control N-fixation. Further, it appears that these operate on different timescales, allowing any given diazotrophic community to acclimate to short term environmental perturbation within a limited range.

Elemental analysis of plants and soils

Although we did not have the power necessary to detect changes in soil N due to the fertilization treatments applied in this study it is quite possible that none occurred. There

is increasing evidence to suggest that arctic vegetation competes well with soil microbes for added nutrients (Chapin et al. 1995, Schmidt et al. 2002). In this study, some evidence suggests that the nitrogen added in nutrient amendment plots was assimilated in plant biomass. *Salix arctica* from fertilized plots had slightly higher nitrogen concentration than did plants from unfertilized plots. In another study (Chapin et al. 1995), tussock tundra vegetation was found to readily accumulate added nitrogen. After 3 years of fertilization, at a rate six-times that used in this study, the total vegetation N-pool doubled, acquiring 62% of the added N. Moreover, the response of vegetation was specific to functional groups. N-accumulation was greatest in mosses and least pronounced in evergreen shrubs. Similarly, Henry et al. (1986) found that fertilization increased forb and graminoid growth much more than dwarf shrub growth across moisture regimes at Alexandra Fiord. Woody species such as *S. arctica* are known to have relatively slow rates of nutrient uptake (Chapin and Tyron 1982) when compared with mosses, forbs, and graminoids. It is possible that much of the 3.4 g m^{-2} additions of N from fertilizer added over a two year period was stored in tissues of plants that were not sampled in this study.

The significantly higher C: N ratios of the treatment plots in the OTC experiment suggest that OTC and fertilization treatments caused a decline in soil organic matter quality. The mechanism for this decline is uncertain, but it likely reflects changes to one or more soil processes. In the warmed treatments, increased decomposition and subsequent N-mineralization may have led to increased N-cycling. This should result in the acquisition of more soil N by plants as the number of competitive interactions among plants and soil

microorganisms increases (Kaye and Hart 1997). Consequently, the remaining soil organic matter (alive and dead) would have higher C: N ratios. This hypothesis is consistent with the findings of other studies where tundra warming has led to increased N acquisition by plants (Chapin et al. 1995, Hartley et al. 1999). In the fertilization treatment without warming the explanation for the higher C: N ratios requires an additional step. Assimilation of fertilizer into plant biomass and a subsequent increase in root exudation of labile C may stimulate a fraction of the microbial community unable to mobilize C bound in plant complex organic matter. These microbes (termed copiotrophs by Semonov et al. 1999) may be prone to rapid decline as root exudation slows (Semonov et al. 1999), and N-mineralized from their biomass may be acquired by plants. This could result in the lower soil C: N ratios observed in fertilization treatments. The finding that soil C: N ratios decline with fertilization treatment is consistent with results from Norwegian spruce forests. In one study, authors report that NH_4NO_3 fertilization caused a decline in microbial biomass N and lower microbial respiration rates (Smolander et al. 1994). Similarly in a second study, N-fertilization decreased the immobilization of N by microbes and increased N-mineralization rates (Priha and Smolander 1995).

The species bias for N-concentration of the two dwarf shrubs may reflect differing life history strategies. These two species may co-exist in this N-limited system in part because *D. integrifolia* can tolerate higher C: N ratios and access different N-pools (actinorhizal or mycorrhizal symbioses) to meet its N-requirements, while *S. arctica* is the better competitor for inorganic soil N. This is consistent with the observation that *S.*

arctica tended to have increased vegetative N-pools with fertilization while *D. integrifolia* did not.

The species specific response to warming also points to differing life-history strategies, but results contrast with expectations. Warming was expected to augment N-fixation and thus the N-concentration of *D. integrifolia*. The significant decrease in N-concentration with warming suggests a more complex response. One possibility is that warming accelerated N-mineralization in soils sufficiently to suppress N-fixation. However, this scenario is unlikely given propensity of arctic plants to accumulate available inorganic N. The lower foliar N-concentration of *D. integrifolia* with warming will be addressed in more detail below.

$\delta^{15}N$

The $\delta^{15}N$ value of soils measured in this study were similar to those found at other arctic sites (Nadelhoffer et al. 1996, Michelsen et al. 1998) and were unaffected by warming or fertilization. Given the similarity of the isotopic signatures of the fertilizer (+0.40) and the control soils (+0.15) it is not surprising that fertilization had no effect on the isotopic signature of the soils. The unchanged $\delta^{15}N$ signatures of warmed soils suggest that either processes that discriminate against the heavy isotope of N were unaffected by temperature, or that each altered process was changed by the same amount. Soil $\delta^{15}N$ values were higher than plants sampled in every treatment plot, and the range of soil $\delta^{15}N$

values was smaller than the range of values observed in plants. These findings agree with those from other arctic sites (Nadelhoffer et al. 1996, Michelsen et al. 1998, Hobbie et al. 2000).

Plant $\delta^{15}N$ values from Alexandra Fiord were in good agreement with those reported for other arctic sites. *Salix arctica* from control plots was -3.0 ‰ which is the same value reported for this genus growing at Toolik Lake, Alaska (Nadelhoffer et al. 1996) and for *S. myrsinites* from heath tundra in northern Sweden (Michelsen et al. 1998), though slightly higher than the value for *S. arctica* from Greenland (-4 ‰) reported in the same study. *Dryas integrifolia* from control plots had a mean $\delta^{15}N$ value of -5.3 ‰ which is comparable to the -5 ‰ value reported by Michelsen et al. (1998) for the heath tundra in northern Sweden. It is noteworthy that in many study sites where *Dryas* sp. and *Salix* sp. occur together *Dryas* is more depleted in $\delta^{15}N$ (the present study, heath tundra in Greenland [Michelsen et al. 1998], heath tundra in north Sweden [Michelsen et al. 1996]).

If $\delta^{15}N$ values of plants and soils are to provide insight to the nitrogen nutrition of plants at Alexandra Fiord, they must be taken in context with all other evidence. Important findings about the nitrogen nutrition of *Salix arctica* and *Dryas integrifolia* gathered in this study include; (1) in fertilization trials *S. arctica* accumulates foliar N, while *D. integrifolia* does not; (2) in OTC treatments *S. arctica* accumulates foliar N, while *D. integrifolia* does not; (3) *Salix arctica* has greater foliar N-concentration than *Dryas integrifolia* in all treatment and control plots; (4) *Salix arctica* has less negative foliar

$\delta^{15}N$ values than *Dryas integrifolia* in all treatment and control plots; (5) a linear relationship between foliar N concentration and $\delta^{15}N$ values exists for both species. What insight can a synthesis of these observations provide? We will review key findings about $\delta^{15}N$ values and N status of arctic plants and soils and then put forth a hypothesis to explain these findings.

Processes in arctic plants and soils that can lead to discrimination against the heavier isotope of N include microbially-mediated N-transformations and partitioning of N pools among plants (Nadelhoffer et al. 1996). Fractionation during plant N acquisition is generally considered negligible in N-limited arctic systems (Nadelhoffer and Fry 1994). Soil N-transformations may lead to differences in the $\delta^{15}N$ status of different N-pools. A strong discrimination during the reduction of nitrate to N_2 occurs, with products being -14‰ to -23‰ depleted over substrate NO_3^- (Blackmer and Bremner 1977). Similarly, the fractionation that occurs during the oxidation of ammonium to nitrate (nitrification) can result in product $\delta^{15}N$ values being -8‰ depleted over reactants in field settings (Feigin et al. 1974). If anaerobic conditions result in high rates of denitrification in soils, NO_3^- pools may be highly enriched in ^{15}N. Conversely, if aerobic conditions dominate and nitrification occurs then nitrate pools will be depleted in ^{15}N, while ammonium pools will be enriched. As soils are spatially heterogeneous environments, it is possible that all of these conditions are met at any point in time. Although the $\delta^{15}N$ values of ammonia and nitrate pools were not measured in this study, others have found that the two pools can have very different ^{15}N signatures (Yoneyama 1996, Nadelhoffer et al. 1996).

It has been suggested that differences among plant species in $\delta^{15}N$ values reflect differences in the forms of N used (Nadelhoffer et al. 1996, Hogberg 1997). Further, forms of N used by plants may be a result of rooting-depth, or mycorrhizal type (Nadelhoffer et al. 1996, Michelsen et al. 1998). In arctic soils, $\delta^{15}N$ values generally increase with depth, and this has been related to N-pool partitioning among plant functional groups (Nadelhoffer et al. 1996). In the present study, both species have similar rooting depths and thus rooting depth cannot explain the significant difference in ^{15}N concentrations of the two species.

In studies of $\delta^{15}N$ values of vascular plants from arctic sites ^{15}N abundance was found to be closely associated with the presence and type of mycorrhizae (Michelsen et al. 1998, 1996, Hobbie et al. 2000). A general pattern emerges in the $\delta^{15}N$ data whereby values are greatest (positive or near zero) in non-mycorrhizal and arbuscular mycorrhizal plants, lower (negative) in ectomycorrhizal plants and lowest (most negative) in plants with ericoid mycorrhizae (Michelsen et al. 1998, 1996, Hobbie et al. 2000). A strong discrimination against the heavier N isotope is believed to occur on transfer of N from the fungus to the host plant leaving the plant depleted in ^{15}N while the fungus is enriched. Field evidence in support of this concept comes from a heath tundra in Greenland where common sporocarps of fungi known to form mycorrhizal associations were collected (Michelsen et al. 1998). All sporocarps sampled had positive $\delta^{15}N$ values, while mycorrhizal plants in the study site where depleted in $\delta^{15}N$. The most common fungi *Russula* sp., *Cortinarius* sp. and *Lactarius* sp. had $\delta^{15}N$ values that ranged from +2‰ to +4‰ (Michelsen et al. 1998).

Another trend that has been made apparent through investigations of $\delta^{15}N$ values of plants across multiple study sites is that foliar N-concentration is closely correlated to the $\delta^{15}N$ value. The mechanism that has been put forth is that as soil N availability declines, foliar N declines, and dependence on mycorrhizae increases. This causes a filtering of N through the fungus which results in fungal ^{15}N enrichment and foliar ^{15}N depletion (Hobbie et al. 2000). Thus, foliar %N and $\delta^{15}N$ values may be predictive of the mycorrhizal status of plants across landscapes. Further, increased plant dependence on ectomycorrhizae or ericoid mycorrhizae in N-limited systems is supported by the observation that strongly N-limited systems have mycorrhizal plants with much lower $\delta^{15}N$ values (Michelsen et al. 1998, Hobbie et al. 2000).

Now that we have reviewed key findings from isotope studies of the N-nutrition of arctic plants, what insight can be gained to the N-dynamics of the two plant species studied under conditions of simulated climate change? As we shall see, three main concepts may explain all observations about N-nutrition in of *Salix arctica* and *Dryas integrifolia* made in this study, these are: (1) *S. arctica* is a better competitor for inorganic N than is *D. integrifolia*; (2) an external limitation to N-fixation triggers a transition from dependence on actinorhizae to ectomycorrhizae derived N in *D. integrifolia*; (3) warming results in increased N-limitation, and increased dependence on mycorrhizae in the woody shrubs studied, this may result in changing cost-benefit ratios for some mycorrhizal symbioses. Let us examine each of these in turn.

The five observations described above of the N-nutrition of the two plant species studied suggest that *S. arctica* is a better competitor for inorganic N than is *D. integrifolia.* In the fertilization trials *S. arctica* showed a trend (although not significant at alpha = 0.05) toward higher foliar N concentrations with fertilization, while *D. integrifolia* showed no such trend. Given the relatively small additions of N in the fertilization treatments and given the propensity of woody shrubs to be out competed for inorganic N by herbaceous plants, it is remarkable that such a trend could be detected at all. In a fertilization study of a similar ecosystem it was reported that deciduous shrubs (such as *Salix* sp.) were better competitors for applied nutrients than were evergreen shrubs (such as *Dryas integrifolia*) (Chapin et al. 1995). Warming is associated with increased rates of decomposition and subsequent nutrient mineralization. In OTC treatments *S. arctica* accumulated foliar N ($p=0.02$) while *D. integrifolia* did not, as suggested by the T-RFLP data for *nif*H communities from OTC treated soils, warming may result in more sustained alterations to N-cycling than do small and infrequent additions of fertilizer. As with the high rates of fertilizer applied in the study by Chapin et al. (1995) *S. arctica* may acquire more of the newly mineralized N than does *D. integrifolia.* In support of these suggestions are the observations that *D. integrifolia* appears to be more N-limited in this system than is *S. arctica. Salix arctica* had greater foliar N-concentration, and less negative foliar $\delta^{15}N$ values than *Dryas integrifolia* in all treatment and control plots, while both species displayed a linear relationship between foliar N concentration and $\delta^{15}N$ values. According to the hypothesis put forth by Hobbie et al. (2000) lower foliar N-concentration and lower $\delta^{15}N$ values suggest *D. integrifolia* depends more heavily on N derived from its mycorrhizal symbionts than does *S. arctica.* Another interesting

observation comes from a survey of the mycorrhizal status of plants from the Alexandra Fiord site (Kohn and Stasovski 1990). In this study, both species were classified as ectomycorrhizal however, *S. arctica* was less so; with 84.6% (252 of 298) of root tips sampled showing fungal colonization, while 96.6% (311 of 322) of root tips sampled were colonized in *D. integrifolia*. The greater proportion of uncolonized roots in *S. arctica* suggests that this species may acquire some N through direct root uptake. The acquisition of soil N, which has a ^{15}N:^{14}N ratio near zero, may explain the more positive $\delta^{15}N$ values and greater foliar N concentration observed in this species. Conversely, the lower $\delta^{15}N$ values and lower foliar N concentration of *D. integrifolia* may reflect its almost complete dependence on fungal-derived N, suggesting that it is a relatively poor competitor for soil N acquired through direct root uptake.

The finding that *D. integrifolia* had lower $\delta^{15}N$ values than *S. arctica* was somewhat surprising in light of the knowledge that *Dryas* forms actinorhizal associations with the diazotroph *Frankia* and that we observed root nodules on *D. integrifolia* in the field. Despite reports that isotope discrimination during N_2 fixation (via nitrogenase) can be up to -6‰ (Robinson 2001), most values reported for actinorhizal plants in field settings are much closer to the atmospheric value (0‰). For example, *Frankia* infected *Alnus glutinosa*, *A. incana*, and *A. cripa* have $\delta^{15}N$ values of -1.9 ‰, -1.8 ‰, and -1.5 ‰ respectively (Domenarch et al. 1989, Nadelhoffer et al. 1996) while *Shepherdia* had a $\delta^{15}N$ value of -0.3‰ (Hobbie et al. 2000). It follows that if *D. integrifolia* at this study site were heavily dependent on nitrogenase derived N, it would have $\delta^{15}N$ values closer to the atmospheric value. In a study of a successional sequence of a glacial retreat at Glacier

Bay, Alaska, *D. integrifolia* occurred in one site which represented the earliest seral stage (Hobbie et al. 2000, 1998). At this oligotrophic site, *D. integrifolia* had a mean $\delta^{15}N$ value of -1.14‰, and a foliar N concentration of 1.88%. Both of these values are much higher than those reported in this and other studies (Michelsen et al. 1998, 1996), and were explained as dependence on nitrogenase-fixed-N. It is possible that these discrepancies reflect a changing life-history strategy for this long-lived species. In early seral-stages *Dryas* may perform a role similar to that of other pioneer plants with N-fixing endosymbionts, depending heavily on actinorhizae and enriching the system with N. As the ecosystem ages three factors may contribute to a transition to dependence on fungal derived N. First, soils may become increasing inoculated with mycorrhizal fungi leading to increased opportunity for colonization. Second, as arctic systems age they tend to accumulate organic N, access to this new N-pool is favored by symbiosis with mycorrhizal fungi. In later seral stages, *Dryas integrifolia* may compete with other woody dicots for access to a potentially large organic-N pool. Third, the accumulation of a thick organic layer as arctic soils age, may limit access of shallow-rooted woody shrubs to phosphorus pools (derived from the parent material) held in the mineral soils below. Phosphorus has been shown to be a major limiting nutrient to N-fixation (Chapin et al. 1991, Vitousek 1999, Liengen 1999) and P-limitation is hypothesized to limit N-fixation more severely in later seral stages (Walker and Syers 1976, Gorham et al. 1979, McGill and Cole 1981). It is possible that a declining productivity of actinorhizae as arctic soils age precipitates *Dryas*'s transition from dependence on atmospheric N (N-fixation) to dependence on mycorrhizal N. It is also possible that a nutrient deficiency other than phosphorus limits N-fixation as arctic sites age. However, since carbon and nitrogen

cannot be limiting in actinorhizal symbioses by virtue of the physiologies of the partners, some external mechanism must act to limit N-fixation in later successional-stage tundra sites.

Warming caused a significant decline in the $\delta^{15}N$ content of both species studied. This change was associated with lower foliar-N concentration in *D. integrifolia*, and slightly (but not significantly) lower foliar N concentration in *S. arctica*. This evidence may suggest increased N-limitation of woody-dicots, and increased dependence on mycorrhizal-N, under conditions of warming. Several mechanisms may explain this observation, these include; (1) changes in mineralization rates, (2) changes in competitive interaction among plant species, (3) changes in the cost-benefit ratio for mycorrhizal symbioses.

It is well documented that warming arctic tundra soils results in increased rates of nutrient mineralization (Chapin and Bloom 1976, Chapin et al. 1995, Hartley et al. 1999, Ruess et al. 1999, Schmidt et al. 2002). Higher temperature is associated with increased microbial activity and with higher densities of fungal-feeding nematodes in arctic soils (Ruess et al. 1999). The fate of this newly mineralized N is of particular interest. In a 9-year study at Toolik Lake, Alaska, elevated temperature treatments resulted in an increase in exchangeable ammonium in soils, attributed to increased mineralization. Interestingly, the authors found no change in total nutrient pools of vegetation after 3 or 9 years. The increase in available nutrients was however, associated with species-specific changes in plant biomass that 'cancelled-out' at the ecosystem level resulting in no net change in

total plant biomass (Chapin et al. 1995). These findings imply that (1) increased N availability was allocated to growth (in some species) rather than altering foliar nutrient concentrations; (2) increased nutrient availability altered the competitive interactions among plant functional groups. Indeed, these findings prompted the authors to predict that increased nutrient availability resulting indirectly from warming should increase the abundance of deciduous shrubs relative to evergreen shrubs and non-vascular plants (Chapin et al. 1995).

The lower $\delta^{15}N$ values of *S. arctica* and *D. integrifolia* from warmed plots are consistent with our knowledge that woody shrubs are inferior competitors for newly mineralized N. Although N availability increases with temperature, increased plant growth outstrips N supply and woody shrubs are out-competed for soil N by more productive species such as herbaceous perennials. Warmer temperatures, however, place the same physiological demands (higher respiration rates, increased cell elongation, increased apical growth) on woody shrubs as other species. Thus, ectomycorrhizal shrubs may be required to meet their increased demand for N by becoming increasingly dependent on their mycobionts, which results in the more negative $\delta^{15}N$ values observed in *Dryas* and *Salix* in this study.

Mycorrhizae are symbiotic associations between fungi and plants that arise because of different physiological limitations of the partners. In many natural systems fungi are carbon-limited while plants are nutrient-limited. Mycorrhizal symbioses can range from mutualistic (the fitness of both partners is increased) to antagonistic (the fitness of one partner increases while the other decreases) (Egger and Hibbett in press) and most are

thought to exist in a continuum between these two endpoints (Bronstein 1994, Egger and Hibbett in press). Environmental perturbation may alter the balance in plant-fungi symbioses shifting the physiological optimum in favor of one partner at the expense of the other. If warmer conditions alter the cost-benefit ratio for plant-fugal symbiosis very little or not at all we would expect to find no difference in the foliar N concentration of plants from warmed and control plots, as is the case of *S. arctica*. If however, warming shifts the ecological optimum closer to the physiological optimum of the fungal symbionts, we might expect the fungi to be able to acquire the same amount of carbon from their host while providing less nitrogen to the plant. Thus, the combination of lower $\delta^{15}N$ and lower foliar N concentration in *D. integrifolia* with warming may suggest changes in the cost-benefit ratio for its mycorrhizal symbioses.

CONCLUSIONS

Climate warming will likely result in higher mineralization rates in mesic-tundra sites at Alexandra Fiord. These warmer soils, which will be relatively depleted in organic N, will support different diazotrophic communities than those present today. The question of how these communities will differ is still unclear. Standard diversity indices (Simpson, Shannon, Shannon-evenness) for *nif*H genes were generally unaffected by treatments with one exception, *nif*H gene richness was found to be more variable with OTC treatment. Diazotroph communities may acclimate to warmer temperatures resulting in no net change in nitrogen fixation rates due to warming, but the factors that control N-

fixation rates are likely to remain variable at the microsite scale. Possible secondary effects of warming such as increased moisture content of soils and increased labile-C exudation from plants may result in local increases of N-fixation in certain microsites. The *nif*H community structure data and the ARA data suggest that diazotroph communities are adaptable to a range of environmental perturbations and that field sites may already be inherently more variable than the changes due to the treatments applied. Given the enormous variation in the environments inhabited by diazotrophs today, it follows that the capacity to adapt to warmer climates is retained by these organisms.

An interesting finding, which requires further investigation, is that seasonal transitions in diazotroph community composition may be more pronounced in a warmer climate. The reasons for this are unknown, but may include a limitation of P mineralization by low soil organic matter quality earlier in the season (see Nadelhoffer et al. 1991) or earlier limitation by some other annually-finite nutrient supply. A second possibility, especially for rhizosphere diazotrophs, is that C-limitation accounts for this change. OTC treatments cause vascular plants to flower earlier in the growing season (Arft et al. 1999), and flowering places high demands on plant C-stores. There is evidence to suggest that plants reduce root C-exudation to the rhizosphere during times of the greatest plant need. For example, 42 day old wheat allocates 37% of its photosynthate below ground while 98 day old wheat, in the midst of producing grain, allocates only 9% (Whipps 1990). If anthesis corresponds annually to the onset of C-limitation for rhizosphere diazotrophs, a shift in community composition from copiotrophic to oligotrophic bacteria may ensue. It is

possible that the *nif*H communities typical of OTC treatments were composed of organisms well adapted to nutrient or carbon limitation.

Contrary to our expectations, symbiotic N-fixation did not relieve N-limitation in the actinorhizal species studied, and it appears that at later seral stage sites, this will not be the case as long as organic N stores are mineralized. *Dryas integrifolia* may utilize two distinct life history strategies depending on the organic matter content of the soil. In oligotrophic sites *D. integrifolia* is heavily dependent on symbiotic N-fixation, causing the accumulation of nitrogen at the site, in more eutrophic sites *D. integrifolia* reduces its dependence on its N-fixing endosymbionts and relies heavily on the cycled organic N-pool. Warmer temperatures will likely amplify competitive interaction among plant functional groups, and less successful species will derive a higher proportion of their N-requirements from mycorrhizae if possible. This may be associated with changing cost-benefit ratios for some mycorrhizal symbioses. It also appears that the relative importance of diazotrophs to the N-nutrition of evergreen dwarf shrubs declines with warming. The underlying mechanism for this requires further study but it must be due to an external limitation on N-fixation in arctic actinorhizal systems.

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