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Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic

Julie R. Deslippe [a], Keith N. Egger [a,*], Greg H.R. Henry [b]

[a] *Ecosystem Science & Management Program, University of Northern British Columbia, 3333 University Way, Prince George, BC, Canada V2N 4Z9*
[b] *Department of Geography, University of British Columbia, Vancouver, BC, Canada V6T 1Z2*

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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, which increased growing season temperatures by 1–3 °C, were randomly placed in a dwarf-shrub and cushion-plant dominated mesic tundra site in 1995. 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. However, moss cover was significantly positively associated with ARA rate. *NifH* gene variants were amplified from bulk soil DNA and analyzed by terminal restriction fragment length polymorphism analysis. Non-metric multidimensional scaling was used to ordinate treatment plots in *nifH* genotype space. *NifH* gene communities were more strongly structured by the warming treatment late in the growing season, suggesting that an annual succession in diazotroph community composition occurs.

Keywords: Nitrogen fixation; *nifH*; T-RFLP; Acetylene reduction assay; Arctic; Climate change; Moss

1. Introduction

Average annual temperature at arctic latitudes is predicted to increase by 2–5 °C over the next century as a result of climate change and will be greater than the global mean change [1]. 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 [2]. Arctic ecosystems are considered sensitive indicators of anticipated larger and slower global responses to climate change [3].

Low temperature and poor substrate quality often limit decomposition and N mineralization in many arctic ecosystems [4] and consequently plant production is often N limited [5–7]. In the future, higher rates of nutrient mineralization and an increased depth of thaw are likely to increase N available to plants [3,8] although plant acquisition of nutrients may be constrained by competition with soil microorganisms [4]. Longer-term changes in vascular plant species composition will also affect the rates of decomposition and mineralization through changes in litter chemistry [9]. 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 [10].

* Corresponding author. Tel.: +1 250 960 5860; fax: +1 250 960 5539.
E-mail address: egger@unbc.ca (K.N. Egger).

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It has been predicted that warmer temperatures will increase arctic nitrogen fixation rates by a factor of 1.5–2 [11]. Increased temperature is expected to induce the strongest direct change in N fixation rates by increasing metabolic processes in soil microorganisms, although increased moisture may be important for certain key photoautotrophic diazotrophs. Increases in the productivity of photosynthetic nitrogen fixers due to higher atmospheric CO_2 concentrations will also be important in securing this increase [11]. 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 negative feedback on 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 of terrestrial ecosystem responses to climate warming (see [7,12–14]). 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 vigour and abundance of plants and ultimately plant community composition [3,12,14,15]. 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 in a changing environment.

Nitrogenase is the enzyme responsible for nitrogen fixation. *NifH* is the gene that encodes for the iron protein subunit of nitrogenase and is highly conserved among all diazotrophic groups, making it an ideal molecular marker for these organisms. The purpose of this study was to investigate the structure and activity of diazotroph communities under conditions of simulated climate change. We assessed *nifH* community structure under experimental treatments as a measure of diazotroph community change. We used acetylene reduction assays (ARA) to estimate diazotroph activity. We expected that warming would result in increased rates of nitrogen fixation and change the composition of diazotroph communities.

2. Methods

2.1. Site, soils, experimental design

The study site was located in a glacial lowland adjacent to Alexandra Fiord, Ellesmere Island, Canada (78°53′ N, 75°55′ W). The vegetation of the experimental site is characterized as dwarf-shrub cushion-plant [16] and common vascular plants at the site are perennial woody species notably: *Salix arctica*, *Cassiope tetragona* and *Dryas integrifolia*. Herbaceous species include *Eriophorum angustifolium*, *Carex stans*, and *Carex membranacea*. Mosses typically account for 19% of the total vegetation cover in this plant community [16] and dominant species include *Hylocomium splendens* ssp. *alaskanum*, *Aulacomnium turgidum*, and *Tomenthypnum nitens* [17]. Small hummocks occur through much of the site and the active layer depth is approximately 35 cm. 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. Additional information on the ecology of the Alexandra Fiord site can be found in Svoboda and Freedman [18].

In 1995, sixteen transparent open top chambers (OTCs) [19] approximately 1 m in diameter, and sixteen 1 m^2 control plots were placed at random locations at the tundra site. In 2000, eight controls and eight OTCs were randomly selected for fertilization treatments. Fertilization treatments consisted of a single 5 $g\,m^{-2}$ addition of $20NH_4NO_3{:}20P_2O_5{:}20K_2O$ water-soluble fertilizer applied in mid June 2000. This was repeated in 2001. Unfertilized plots were treated with a volume of water equal to that used to dissolve the fertilizer. Soil samples were collected from eight OTC and eight control plots in 2001, and the remaining plots were sampled in 2002. This experiment will be referred to as the ''OTC experiment''.

In 2002, a second experiment was set up to investigate the short-term effects of fertilization on *nifH* community structure. Adjacent to the OTC site, three 1 m^2 fertilization plots and three control plots were established. The fertilization plots were treated with a single 5 $g\,m^{-2}$ addition of $20NH_4NO_3{:}20P_2O_5{:}20K_2O$ water-soluble fertilizer applied on the 28th of June, 2002. This will be referred to as the ''Short-term Fertilization experiment'' (STF). This experiment was sampled twice during the summer of 2002, in conjunction with the OTC experiment.

Out of concern for 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, the first two treatments were left undisturbed, three 25 cm^2 soil cores were removed from the third treatment, and nine 25 cm^2 soil cores were removed from treatment 4 (all soil cores were discarded). Two weeks later on the 31st of July, three 25 cm^2 soil samples were removed from treatments 2, 3, and 4, sealed in air tight bags, and frozen for subsequent DNA analysis. Thus, after this first sampling, only treatment 1 was undisturbed, 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 25 cm^2 soil samples were removed from all disturbance plots and stored as above. This experiment will be referred to as the ''Disturbance experiment''.

2.2. Acetylene reduction assays

Acetylene reduction assays were used to estimate nitrogen fixation rates in treatment and control plots of the OTC and STF experiments during the summer of 2002. In order to minimize the impacts of repeated samplings, three soil samples were randomly selected for collection in early summer (June 28–July 5) and six soil samples were harvested from treatment plots in peak summer (July 23–August 3). Using a grid and a random numbers table, soil samples with an area of 25 cm^2 and a depth of 5 cm (final volume = 225 cm^3) were removed from the plot using a sharp knife. In order to insure that desiccation would not limit ARA during the incubation, each sample was weighed on a top pan balance on collection, and then sprayed with approximately 10 ml of water from a spray bottle. The inside surfaces of the glass incubation cuvettes were also misted with water. After incubation, samples were re-weighed as a measure of water loss. The mean loss of water due to evaporation inside the cuvettes was 3.4 ± 1.2 ml and in no instance did the sample lose more water than was added by misting. Thus, the ARA values reported herein reflect potential ARA values when moisture limitation is removed. Moistened samples were placed on glass plates and covered with glass cuvettes fitted with rubber septa and a Vacugrease™ seal (for a detailed description of the cuvettes used see [20]). Acetylene gas was generated on-site from CaC_2 and water and injected into cuvettes to comprise 10% of the total headspace by volume. The ambient air temperature was recorded at a climate station at Alexandra Fiord using a Campbell Scientific 201 sensor fitted with a Fenwall UUT51J1 thermistor [21]. The mean air temperature during all incubation periods was 5.8 °C. On occasions when air temperature differed from this mean, the number of moles of acetylene injected into incubators was corrected for temperature. During the incubations, headspace gas was sampled twice, after approximately 45 and 60 h, by puncturing the rubber septa with a two-way needle and removal to a 2 ml Vacutainer™ (Becton–Dickinson, Franklin Lakes, NJ, USA). Twenty-four soil samples in glass incubators could be assayed simultaneously. 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. Soil temperature was monitored during each incubation period within a control incubator (sealed cuvette with a soil sample but no acetylene) with a hand-held digital thermometer fitted with copper–constantan thermocouple. Mean soil temperature was 8.8 ± 3.4 °C and ranged from a minimum of 3 °C to a maximum 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, Ottawa, ON, Canada) fitted with a Porapak column (Alltech Canada, Guelph, ON, Canada) and a flame ionization detector. Hydrogen was used as the carrier gas and was held at a constant pressure of 25 psi. After incubations the percent moss and lichen cover was estimated and the presence or absence of *Nostoc* sheets and/or vascular plants was determined for each sample. All soil samples were then placed in sealed plastic bags and frozen for future use. Acetylene Reduction Assay 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, percent cover of moss) variables to be analyzed simultaneously. All ANOVAs were performed using STATISTICA version 6.0 [22].

2.3. DNA extraction and PCR amplification

A 1 g sub-sample was removed from each soil sample collected for ARA analysis. In an attempt to obtain a sub-sample that included both photosynthetic and subsurface dwelling diazotrophs, one corner of the intact soil core (approximately 1 cm^2 by 5 cm in depth) was removed. Approximately one third of the soil samples were too coarse-textured to remain intact and could not be sub-sampled in this way. These samples were homogenized by shaking in an attempt to randomize the sub-sample. DNA was extracted using an UltraClean Soil DNA isolation kit according to the manufacturer's instructions (MoBio, Jefferson City, MO, USA). Extracted DNA was stored frozen at −20 °C. A half-nested polymerase chain reaction (PCR) protocol was used to amplify a 383 bp fragment of the *nifH* gene from a diluted extract. The primary amplification employed the primers Nh21F (5′-GCIWTYTAYGGNAARGG) and WidNhR (5′-GCRTAIABNGCCATCATYTC [23]). Both primers were synthesized by Invitrogen (Burlington, ON, Canada). The half-nested secondary amplifications employed dye-labeled primers (Integrated DNA Technologies, Coralville, IA, USA). 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. [23] 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, 10× PCR Buffer, 2 mM $MgCl_2$, and 0.72 U of Platinum *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada) 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 min and 10 s 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 min at 72 °C completed the program. A PTC-100 Programmable Thermal Controller (MJ Research Inc., Reno, NV, USA) was used for all amplifications.

2.4. T-RFLP analysis

Endonuclease digests were performed on 8 μl aliquots of dye-labeled PCR product with the enzyme *Hha*I (Invitrogen, Burlington, ON, Canada). Reactions were incubated at 37 °C overnight. Restriction products were kept frozen at −20 °C until analyzed. Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to generate unique *nifH* gene community profiles for each soil sample. *NifH* restriction products were denatured at 80 °C in formamide and run on vertical polyacrylamide gels for 45 min on Open Gene DNA sequencers (Bayer HealthCare LLC, Berkeley, CA, USA). Dye-labeled oligonucleotide markers of 101, 200, and 351 bp were used as internal standards. All resulting T-RFLP profiles were analyzed manually using GeneObjects 3.1 software (Bayer HealthCare LLC, Berkeley, CA, USA). Electropherogrammes of *NifH* T-RFs were characterized by large overlapping primer peaks in the first 8 min (below 70 bp). Between 8 and 35 min (80 and 400 bp) baseline fluorescence was stable (at or very near zero) between T-RF peaks. Internal marker T-RFs of 101, 200, and 351 occurred at approximately 11, 17, and 28 min, respectively. After 37 min the baseline fluorescence increased and large overlapping peaks, thought to be the result of contaminating fluorescence of the Cy 5.0 dye-labeled forward fragments, often occurred. Samples were rerun if total fluorescence from peaks between 8 and 35 min was less than 10,000 fluorescence units. Table 1 lists characteristics of electropherogrammes for samples from the OTC and STF experiments; in the OTC experiment fertilization treatments are included in their respective OTC or control plots.

T-RF peaks greater than 100 fluorescence units were selected for analysis from each electropherogramme. T-RFs from all electropherogrammes in each experiment were then entered in a spreadsheet program and sorted by fragment size. Like fragments were grouped into categories by selecting the midpoint whole values for fragment lengths. Sample numbers were concealed during the binning process to avoid bias. Contingency tables for the presence and absence of genotypes in each sampling plot were then constructed. Replicate T-RFLP profiles from each sampling plot were pooled so that the maximum number of times that a single genotype could occur in a given plot was equal to the number of replicates (six times). Genotypes that occurred greater than two times were included in the ordination analyses.

Non-metric multidimensional scaling (NMS) was chosen to visualize treatment plots in genotype-space. NMS [24] 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 [25]. All ordinations were run using PC-ORD version 4.0 in the "auto-pilot" mode which uses random starting configurations and assesses dimensionality by minimizing stress. Sørensen distance was selected as the distance measure for each initial matrix [25].

2.5. Elemental analysis of soils

Soil samples used for ARA and DNA extraction were oven dried at 90 °C for 24 h, mechanically ground in a soil grinder and analyzed for carbon and nitrogen concentration by elemental analysis using an AC 1500 Fisions NC autoanalyzer (Milan, Italy). Differences in N and C concentrations among treatments were analyzed with one-way ANOVA performed using STATISTICA version 6.0 [22].

3. Results and discussion

3.1. Treatment effects on moss and soils

The results of elemental analysis of total carbon and nitrogen for soils from the OTC experiment and the STF experiment are shown in Table 1. Soils had a mean

Table 1
Characteristics of T-RFLP profiles from soil cores collected during the second sampling period (July 23–August 3) 2002 from the OTC and the STF experiments

T-RFs per sample	OTC experiment (n = 48)			STF experiment (n = 18)	
	All	OTC	Controls	Fertilization	Controls
Mean	10	10	10	11	8
Median	8	10	7	10	8
Mode	6	6	6	7	3
Max	28	22	28	23	18
Min	2	2	2	1	1

nitrogen concentration of 14.0 ± 0.1 mg kg^{-1} and a C:N ratio of 18.4 ± 0.3 (S.E.). Changes in total soil N due to our fertilization treatments were not within detectable limits. The surface 5 cm of soil in the 1 m^2 control plots from which our soil samples were taken had a soil nitrogen content of 178.3 ± 15.4 g. The amount of N added with the fertilization treatment was approximately 1.7 g m^{-2} $year^{-1}$ (3.4 g in 2 years) an order of magnitude less than the margin of error.

Warming caused no significant change in the nitrogen concentration of soils but resulted in an increase in the carbon 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 STF plots (Table 2). Warming has been associated with elevated C:N ratios in arctic dwarf-shrubs when nutrients constrain growth [9]. The high C:N ratios observed in the OTC treatments may be due to increased carbohydrate in plant litter in those plots. This may also explain the higher C:N ratios observed in the OTC and fertilization plots since the amount of NPK fertilizer added was likely insufficient to offset the demands of plant growth.

In the OTC experiment warming was associated with a significant increase in the percent cover of mosses in soil samples ($p = 0.01$, Table 2). In fertilization treatments the opposite trend was observed, fertilization was associated with lower moss cover in both the control and warmed plots ($p = 0.06$). In combined warming and fertilization treatments the percent moss cover was not significantly different from controls (Table 2). Lower moss biomass as a result of shading due to enhanced growth of herbaceous plants and deciduous shrubs has been reported in several studies where fertilizers were applied to arctic tundra vegetation [12,7]. This mechanism may have been important in causing a decline in the moss cover in plots that received fertilizer in our study.

The positive response of moss to warming was unexpected. It is widely held that warming will lead to a decline in non-vascular plants as enhanced nutrient availability (an indirect consequence of warming) promotes over-story growth and canopy closure [12,26,7]. However, experimental warming treatments have often resulted in non-significant and even positive changes in moss biomass [[7] and references therein]. At a sub-Arctic site *Hylocomium splendens* showed a reduced growth in response to warming with Open Top Chambers but showed a positive response to temperature across its natural environmental gradient [27]. Species specific responses to warming are also possible and have been demonstrated in the case of nutrient addition [27]. As mosses were not identified to species in the present study, we can not rule out the possibility that the increased moss cover reflects the response of only a portion of the moss flora. However, the increased moss cover in the plots from the OTC treatments suggests that if cover by upper canopy plants remains nutrient constrained, increased temperature may enhance moss abundance.

3.2. Acetylene reduction assays

Warming and longer-term fertilizations in the OTC experiment did not significantly affect acetylene reduction activity. Moss, however, was a significant predictor of ARA rates in all samples ($p = 0.02$; $p = 0.04$ for OTC experiment samples alone). Mean ARA rate in the OTC experiment was found to be $2.6 \times 10^{-2} \pm 5.2 \times 10^{-4}$ μmol m^{-2} h^{-1} over the summer. ARA values were lower than those reported for a moist meadow site on Svalbard [28], despite similar sampling strategies and latitudes in the two studies. This difference likely reflects the greater abundance of moss at the Svalbard site, where mean percent cover of moss was 76% [28] as opposed to only 21% in the present study. However, higher ARA rates were also reported in a nearby wet sedge meadow at Alexandra Fiord [29], where moss typically covers 23% of the surface [16], but cyanobacterial mats are significantly more abundant (7% as opposed to 0% at our study site [16]) and were important contributors to acetylene reduction activity measured in this plant community [29].

In the STF experiment a different trend was observed. STF had no immediate effect on fixation rates but caused a significant increase in fixation ($p < 0001$) by

Table 2
Soil and moss cover characteristics and ARA rates of OTC and STF experiments from the second sampling period (July 23–August 3) 2002

Experiment	Treatment	Soil N (mg kg^{-1})	Soil C (mg kg^{-1})	C:N ratio	Moss % cover	ARA (μmol m^{-2} h^{-1})
OTC	OTC and fertilization (2000, 2001)	10.6^{a}	202.3^{a}	18.8^{a}	16.5^{a}	0.0089^{a}
OTC	OTC	$18.1^{b,c}$	360.3^{b}	18.8^{a}	32.3^{b}	0.039^{a}
OTC	Fertilization (2000, 2001)	11.7^{a}	208.5^{a}	18.7^{a}	9.1^{a}	0.045^{a}
OTC and STF	Control	$16.1^{a,c}$	266.8^{a}	16.7^{b}	24.6^{a}	0.03^{a}
STF	Fertilization (2002 only)	$18.9^{b,c}$	246.4^{a}	15.6^{b}	22.7^{a}	0.11^{b}

Different letters in each column denote a significant difference among treatments at $\alpha = 0.05$, as determined with Tukey's post hoc test.

the second sampling period (19–35 days post-fertilization). Ethylene production increased to $0.7 \times 10^{-2} \pm 6.5 \times 10^{-3}$ μmol m^{-2} h^{-1}, with the addition of NPK fertilizer in 2002. This suggests that fertilization treatments relieved some nutrient limitation to ARA activity in these plots. One possibility is that the nutrient amendments relieved P limitation of the diazotroph community. Phosphorus is thought to be a limiting nutrient for nitrogen fixation [30,31] and phosphorus fertilization has been shown to cause significant increases in ARA rates in the field [32,33]. A second possibility is that ARA rates increased in response to increased carbon exudation from plant roots. When salt-marsh communities were fertilized with NH_4NO_3, ARA 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 [34]. The much greater ARA rate observed in the STF experiment may also have come about through a combination of these two mechanisms.

3.3. Treatment effects on NifH communities

We examined *nifH* community structure under different levels of plot disturbance in the Disturbance experiment. We were unable to detect any change in *nifH* genotypes from "disturbed" plots immediately, 2 or 3 weeks post-disturbance even in plots that experienced a rate of sample removal four times that used in our study. Thus we suggest that changes we observed in *nifH* community structure were elicited by our intended treatments and not by a sampling artifact.

A NMS plot of sampling units in genotype-space for the OTC experiment revealed that *nifH* gene communities were strongly structured by warming late in the 2002 growing season. Fig. 1 shows that OTC plots grouped mostly in the top, right-hand quadrant of the plot while control plots mostly grouped in the bottom, left-hand-quadrant. Sixty-five iterations produced a 3D solution with a final stress of 9.40 and a final instability of 1×10^{-4}. Axis 1 and 2 (Fig. 1) account for 18 and 42% of the total variance in the dataset (cumulative $r^2 = 0.60$), while the third axis (not shown) accounted for 24% (total $r^2 = 0.84$). An overlay of ARA rates on this ordination (Fig. 1) shows that higher ARA rates were not associated with *nifH* communities from any particular treatment. This suggests a poor relationship between *nifH* community structure and nitrogenase activity at this site; factors that control the distribution of genotypes in soils may not be directly related to the expression of *nifH* genes. Similarly, 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 *nifH* community DGGE profiles were observed [34]. In this study, ARA rates were spatially variable and strongly controlled by the

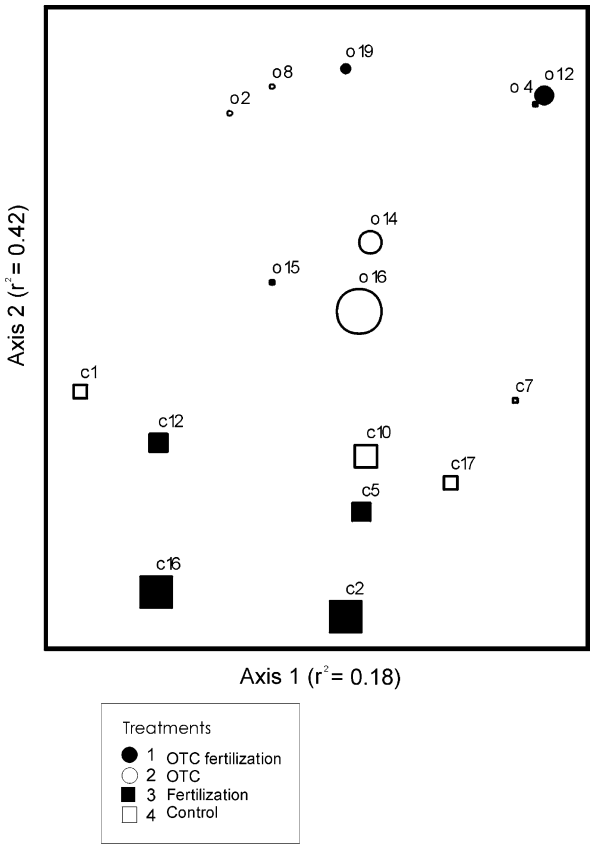

Fig. 1. Overlay of ARA rates (μmol m^{-2} h^{-1}) on an NMS plot of OTC experiment treatments in genotype space, the size of each symbol is proportional to the rate of nitrogen fixation in the treatment plot. OTC treatments are represented by circles, control treatment are represented by squares. Fertilization treatments are filled. Data were collected during the second sampling period (July 23–August 3) 2002.

presence of moss. This finding suggests that moss-associated diazotrophs, the cyanobacteria, were responsible for the majority of ARA activity at this site. The cyanobacteria are, however, a relatively homogenous group with respect to their *nifH* gene diversity when compared to all possible sub-surface dwelling diazotrophs. While warming elicited change in the structure of the diazotroph community as a whole (both sub-surface and surface dwelling organisms) it is possible that the portion of the *nifH* community responsible for higher ARA rates changed little or that any change in the community composition of cyanobacteria did not translate to the presence of different *nifH* genotypes.

Moss cover was positively correlated to ARA activity in soil samples, and the percent moss cover was significantly higher in samples from the OTC treatment, but not in plots that received OTC and fertilization (possibly a result of shading by over-story plants). This finding suggests that plots that received warming only should have higher rates of acetylene reduction than the control plots. Mean ARA activity was higher in the warmed plots ($p = 0.057$), but also highly spatially variable. This

suggests that factors other than percent moss cover must be important in determining ARA rates in our samples. As all soil samples were saturated prior to incubation, soil moisture, thought to be the primary limitation to nitrogen fixation in the field [11], could not account for this. The spatial heterogeneity of other vegetation may have influenced ARA rates. Macro-lichens were present in four samples from OTC plots and seven samples from control plots, and although these were not identified to species in this study, many of the common lichens at Alexandra Fiord such as *Nephroma* sp., *Stereocaulon* sp., and *Peltigera* sp. have cyanobacterial symbionts [11] and may have been actively producing ethylene during incubations. Soil crusts were not identified in this study, but these have also been shown to reduce ethylene at low rates [35]. No free living *Nostoc* sp. sheets were found in samples from the OTC experiment. Another consideration is microsite differences in ARA activity from moss associated diazotrophs. In a study of nitrogen fixation associated with the mosses *Calliergon richsonii* and *Sanionia uncinata* at Kongsfjorden, Svalbard ARA activity was found to be variable with growing location of the plant [34]. Factors such as moss health and photosynthetic rate may also have contributed to variability in ARA rates.

In general, our results agree with the prediction [11] that rates of nitrogen fixation in the High Arctic should increase with a future warmer climate. However, they also indicate that a complex relationship between diazotrophs, mosses and vascular plants, regulates nitrogen fixation rates in the field. As temperature increases, direct temperature effects on enzyme activity may increase N-fixation in the short term, but will also lead to increased decomposition and element turn-over on longer time scales. The combination of these "new" N-sources will result in vascular plant growth, shading and eventual decline of mosses, and a down-regulation of nitrogen fixation. Thus, any estimate of nitrogen inputs from nitrogen fixation in future arctic ecosystems should take into account the close relationship between moss cover and nitrogen fixation rate.

We observed seasonal variation in community structure. Earlier in the season (Fig. 2(a) – 2002, (b) – 2001), there was less differentiation between warmed and control plots. A NMS plot of sampling units in genotype-space from the first sampling period (June 28–July 5) (Fig. 2(a)) revealed no clear separation of *nifH* communities from warmed or control treatments. At the time of sampling, the study site had been snow-free for less than 2 weeks. Axis 1, which accounts for 45% of the variation in this 2D solution, produced after 82 iterations, indicates a weak separation of warmed plots on the left side of the axis, while control plots appear toward the right. No discernable trend exists along axis 2, which accounts for 37% of the variance in the dataset (cumulative $r^2 = 0.82$). These results must be interpreted with caution as the final stress of the ordination was somewhat high (13.8). Mid-season in 2001, an intermediate differentiation among warming treatments was observed. A 2D NMS ordination, resulting from 46 iterations, with a final stress of 11.5, and a final instability of 6×10^{-5}, is shown in Fig. 2(b). Axis 1 describes 8.9% of the variance in the data while axis 2 accounts for 78.2 (cumulative $r^2 = 0.87$). Fig. 2(b) shows warmed plots forming a

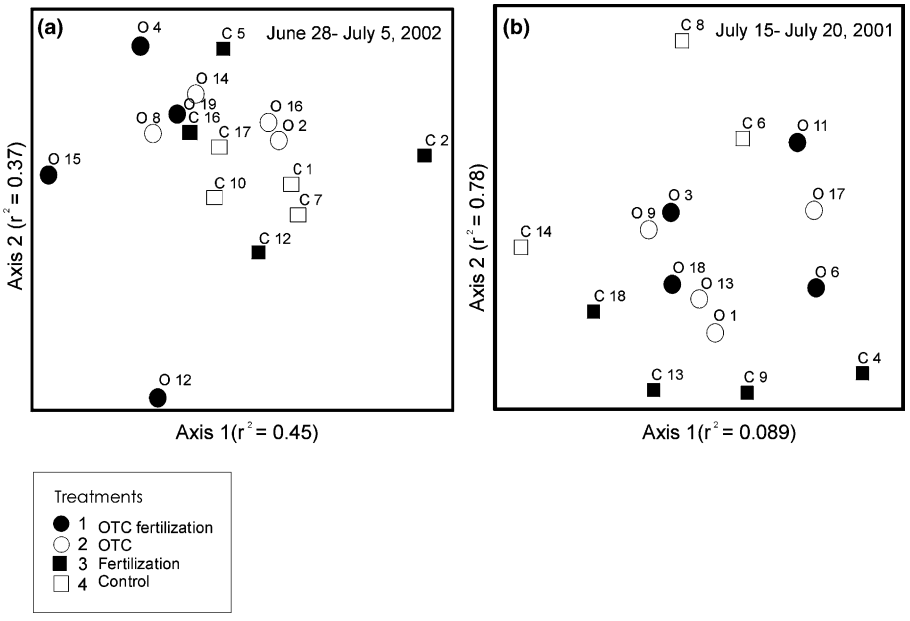

Fig. 2. NMS ordination of treatment and control plots from the OTC experiment in *nifH* genotype space. (a) Samples collected from June 28 to July 5, 2002. (b) Samples collected from July 15 to July 20, 2001. OTC treatments are represented by circles and control treatments by squares; fertilization treatments are filled.

loose group in the middle of the plot. Fertilized and unfertilized control plots appear to be differentiating on axis 2. In order to reach a stable NMS solution it was necessary to omit one unfertilized control plot that acted as an outlier.

The accumulation of thermal energy by soils as the growing season progressed may have contributed to the seasonal succession in *nifH* communities. Likewise, compositional changes in microbial communities in soils from a northern hardwood forest have been documented after incubation at elevated temperatures [36]. Increased soil temperature may alter diazotroph community structure in several ways. Firstly, temperature may have a direct impact on *nifH* 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 [37]. This process is metabolically expensive, and may place sufficient stress on certain members of the diazotroph community in warmed soils to effect a change in community composition. A second mechanism by which temperature may alter the structure of diazotroph communities is through increased mortality from grazing by soil fauna. In a study of a heath and a fell field site in Swedish Lapland, warming caused an increase in the density of bacterial and fungal-feeding nematodes [38]. 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 [38]. Changes in rates of nutrient mineralization with soil warming have been observed in many studies [12,26,38,39].

Despite the significantly higher rates of ARA in the STF plots in the second sampling period of 2002, we were unable to detect changes in diazotroph communities from fertilization treatments. NMS ordination of *nifH* genotypes from the STF experiment showed no separation of diazotroph communities due to treatment (data not shown). This finding is in contrast with rice rhizosphere diazotroph communities where fertilization was found to change *nifH* communities within 15 days of nitrogen application [40]. However our results agree with those from *Spartina alterniflora* rhizoplane diazotrophs where short-term nutrient addition (N and P) caused no detectable change in *nifH* DGGE profiles, while longer-term fertilizations (treatments applied every 2 weeks for 8 weeks) did elicit community change [34].

3.4. Characteristic changes in nifH community structure with warming

We identified 78 unique *nifH* genotypes in the OTC and STF experiments. Although the most abundant genotypes in the OTC experiment changed with warming treatment, the most common genotypes in both OTC and control plots belonged to members of the Alphaproteobacteria (Table 3). A member of the Betaproteobacteria occurred more frequently in the control plots, while genotype 108, which is common to many groups including the *Nostocales* occurred significantly more frequently in the OTC plots (Table 3). This is an interesting finding because moss cover also responded positively to OTC treatment. Since many members of the *Nostocales* live in association with mosses, it is consistent that the presence of cyanobacterial genotypes should respond to the same gradient as mosses.

Table 3
Characteristic differences in genotypes from T-RFLP profiles in OTC and control plots from the second sampling period (July 23–August 3) 2002

	OTC	Controls	Possible taxa
Most abundant T-RF	G324		Alphaproteobacteria; Rhodospirillales (*Azosprillum*)
		G158	Undetermined
		G184	Alphaproteobacteria (Rhizobiales – *Rhodopseudomonas*)
T-RFs that occur significantly more frequently[a]	G108**		Members of Actinobacteria (Actinomycetales); Alphaproteobacteria (Rhodospirillales, Rhodobacterales, Rhizobiales – *Rhodobacter*); Betaproteobacteria (Rhodocyclales); Cyanobacteria (Nostocales); Firmicutes (Bacillales)
		G158**	Undetermined
		G327*	Betaproteobacteria (Rhodocyclales – *Azoarcus*)
		G332**	Undetermined
Unique genotypes[b]	G213		Undetermined
		G160	Undetermined
		G165	Undetermined
		G190	Alphaproteobacteria (Rhizobiales – *Rhodopseudomonas*)
		G288	Undetermined

[a] Significance determined by χ^2 test with Yates correction for continuity (df = 1; $^{**}p < 0.05$; $^{*}p < 0.1$).
[b] Only unique genotypes that occurred two or more times in *nifH* community profiles were included.

Although the majority of genotypes recorded occurred in both OTC and control plots, one genotype was uniquely associated with warming treatments while four genotypes occurred only in control plots (Table 3).

Our results indicate that nitrogenase activity and *nifH* gene community structure are controlled by different factors at Alexandra Fiord. While ARA activity was spatially variable and strongly controlled by the presence of moss, *nifH* gene community structure was temporally variable in response to warming. Seasonal transitions in the structure of below ground diazotroph communities may be related to changes in plant allocation patterns throughout the growing season. Warming has been shown to increase C:N ratios in above ground tissues of vascular plants at Alexandra Fiord, when nutrients constrain growth [9]. Higher photosynthetic rates in plants from OTC plots may also lead to allocation of excess carbohydrate to roots and rhizosphere organisms. Increased exudation of labile carbon compounds by plant roots may select for certain *nifH* genotypes, and this mechanism may have been important in causing structural shifts in the below ground diazotroph community as the season progressed.

As a consequence of their greater *nifH* diversity, sub-surface diazotrophs accounted for the greatest change in *nifH* community structure with warming. Although photosynthetic diazotrophs may have been more common in warmed plots, and important contributors to ARA activity, changes in their species composition were difficult to detect using our method of ordinations based on the presence and absence of *nifH* genotypes. The poor relationship between *nifH* diversity and ARA activity found in this study suggests that considerable redundancy exists in the *nifH* community at Alexandra Fiord.

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References

[1] Intergovernmental Panel on Climate Change, 2001. Climate change 2001: impacts, adaptation, and vulnerability. IPCC Third Assessment Report, Cambridge University Press, Cambridge, 2001.

[2] Warrick, R.A., Shugart, H.H., Antonovsky, M., Tarrant, J.R., Tucker, C.J. The effects of increased CO_2 and climatic change on terrestrial ecosystems. In: Bolin, B. (Ed.), The Greenhouse Effect, Climatic Change and Ecosystems, Chichester, 1986, pp. 363–392.

[3] Shaver, G.R., Billings, W.D., Chapin, F.S., Giblin, A.E., Nadelhoffer, K.J., Oechel, W.C. and Rastetter, E.B. (1992) Global change and the carbon balance of arctic ecosystems. Bioscience 42, 433–441.

[4] Jonasson, S., Havstrom, M., Jensen, M. and Callaghan, T.V. (1993) In situ mineralization of nitrogen and phosphorus of arctic soils after perturbations simulating climate-change. Oecologia 95, 179–186.

[5] Chapin, F.S., Shaver, G.R. and Kedrowski, R.A. (1986) Environmental controls over carbon, nitrogen and phosphorus fractions in *Eriophorum vaginatum* in Alaskan tussock tundra. J. Ecol. 74, 167–195.

[6] Shaver, G.R. and Chapin, F.S. (1986) Effect of fertilizer on production and biomass of tussock tundra, Alaska, USA. Arct. Antarct. Alp. Res. 18, 261–268.

[7] Van Wijk, M.T., Clemmensen, K.E., Shaver, G.R., Williams, M., Callaghan, T.V., Chapin, F.S., Cornelissen, J.H.C., Gough, L., Hobbie, S.E., Jonasson, S., Lee, J.A., Michelsen, A., Press, M.C., Richardson, S.J. and Rueth, H. (2004) Long-term ecosystem level experiments at Toolik Lake, Alaska, and at Abisko, Northern Sweden: generalizations and differences in ecosystem and plant type responses to global change. Global Change Biol. 10, 105–123.

[8] Nadelhoffer, K.J., Giblin, A.E., Shaver, G.R. and Laundre, J.A. (1991) Effects of temperature and substrate quality on element mineralization in six arctic soils. Ecology 72, 242–253.

[9] Tolvanen, A. and Henry, G.H.R. (2001) Responses of carbon and nitrogen concentrations in high arctic plants to experimental warming. Can. J. Bot. 79, 711–718.

[10] Chapin, F.S., Jefferies, R.L., Reynolds, J.F., Shaver, G.R. and Svoboda, J. (1992) Arctic plant physiological ecology: a challenge for the future In: Arctic Ecosystems in a Changing Climate: An Ecophysiological Perspective (Chapin, F.S., Jefferies, R.L., Reynolds, J.F. and Shaver, G.R., Eds.), pp. 3–8. Academic Press, San Diego.

[11] Chapin, D.M. and Bledsoe, C.S. (1992) Nitrogen fixation in arctic plant communities In: Arctic Ecosystems in a Changing Climate: An Ecophysiological Perspective (Chapin, F.S., Jefferies, R.L., Reynolds, J.F. and Shaver, G.R., Eds.), pp. 301–319. Academic Press, San Diego.

[12] Chapin, F.S., Shaver, G.R., Giblin, A.E., Nadelhoffer, K.J. and Laundre, J.A. (1995) Responses of arctic tundra to experimental and observed changes in climate. Ecology 76, 694–711.

[13] Arft, A.M., Walker, M.D., Gurevitch, J., Alatalo, J.M., Bret-Harte, M.S., Dale, M., Diemer, M., Gugerli, F., Henry, G.H.R., Jones, M.H., Hollister, R.D., Jonsdottir, I.S., Laine, K., Levesque, E., Marion, G.M., Molau, U., Molgaard, P., Nordenhall, U., Raszhivin, V., Robinson, C.H., Starr, G., Stenstrom, A., Stenstrom, M., Totland, O., Turner, P.L., Walker, L.J., Webber, P.J., Welker, J.M. and Wookey, P.A. (1999) Responses of tundra plants to experimental warming: meta-analysis of the International Tundra Experiment. Ecol. Monogr. 69, 491–511.

[14] Henry, G.H.R. (2002) Effects of long-term experimental warming on High Arctic tundra ecosystems. Integr. Comp. Biol. 42, 1243.

[15] Shaver, G.R. and Chapin, F.S. (1995) Long-term responses to factorial, NPK fertilizer treatment by Alaskan wet and moist tundra sedge species. Ecography 18, 259–275.

[16] Muc, M., Freedman, B. and Svoboda, J. (1989) Vascular plant communities of a polar oasis at Alexandra Fiord (79°N), Ellesmere Island. Can. J. Bot. 67, 1126–1136.

[17] Bliss, L.C. and Matveyeva, N.V. (1992) Circumpolar arctic vegetation In: Arctic Ecosystems in a Changing Climate: An Ecophysiological Perspective (Chapin, F.S., Jefferies, R.L., Reynolds, J.F. and Shaver, G.R., Eds.), pp. 301–319. Academic Press, San Diego.

[18] Svoboda, J. and Freedman, B., Eds., (1994). Ecology of a Polar Oasis, Alexandra Fiord, Ellesmere Island, Canada. Captus University Publications, Toronto.
[19] Marion, G.M., Henry, G.H.R., Freckman, D.W., Johnstone, J., Jones, G., Jones, M.H., Levesque, E., Molau, U., Molgaard, P., Parson, A.N., Svoboda, J. and Virginia, R. (1997) Open top designs for manipulating field temperature in high-latitude ecosystems. Global Change Biol. 3 (Suppl. 1), 20–32.
[20] Larson, D.W. and Kershaw, K.A. (1975) Measurement of CO_2 exchange in lichens: a new method. Can. J. Bot. 53, 1535–1541.
[21] Labine, C. (1994) Meteorology and climatology of the Alexandra Fiord lowland In: Ecology of a Polar Oasis, Alexandra Fiord, Ellesmere Island, Canada (Svoboda, J. and Freedman, B., Eds.), pp. 23–39. Captus University Publications, Toronto.
[22] STATISTICA (data analysis software system), StatSoft, Inc., Tulsa, OK, USA, 2002.
[23] Widmer, F., Shaffer, B.T., Porteous, L.A. and Seidler, R.J. (1999) Analysis of *nifH* gene pool complexity in soil and litter at a Douglas Fir forest site in the Oregon Cascade mountain range. Appl. Environ. Microbiol. 65, 374–380.
[24] Kruskal, J.B. (1964) Nonmetric multidimensional scaling: a numerical method. Psychometrika 29, 115–129.
[25] McCune, B. and Grace, J.B. (2002) Analysis of Ecological Communities. MjM Software, Gleneden Beach.
[26] Hartley, A.E., Neill, C., Melillo, J.M., Crabtree, R. and Bowles, F.P. (1999) Plant performance and soil nitrogen mineralization in response to simulated climate change in subarctic dwarf shrub heath. Oikos 86, 331–343.
[27] Callaghan, T.V., Press, M.C., Lee, J.A., Robinson, D.L. and Anderson, C.W. (1999) Spatial and temporal variability in the responses of arctic terrestrial ecosystems to environmental change. Polar Res. 18, 191–197.
[28] Zielke, M., Stine Ekker, A., Olsen, R.A., Spjelkavik, S. and Solheim, B. (2002) The influence of abiotic factors on biological nitrogen fixation in different types of vegetation in the High Arctic, Svalbard. Arct. Antarct. Alp. Res. 34, 293–299.
[29] Henry, G.H.R. and Svoboda, J. (1986) Dinitrogen fixation (Acetylene Reduction) in high arctic sedge meadows. Arct. Alp. Res. 18, 181–187.
[30] Vitousek, P.M., Cassman, K., Cleveland, C., Crews, T., Field, C.B., Grimm, N.B., Howarth, R.W., Marino, R., Martinelli, L., Rastetter, E.B. and Sprent, J.I. (2002) Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57, 1–45.
[31] Vitousek, P.M. and Field, C.B. (1999) Ecosystem constraints to symbiotic nitrogen fixers: a simple model and its implications. Biogeochemistry 46, 179–202.
[32] Chapin, D.M., Bliss, L.C. and Bledsoe, L.J. (1991) Environmental-regulation of nitrogen-fixation in a high arctic lowland ecosystem. Can. J. Bot. 69, 2744–2755.
[33] Liengen, T. (1999) Environmental factors influencing the nitrogen fixation activity of free-living terrestrial cyanobacteria from a high arctic area, Spitsbergen. Can. J. Microbiol. 45, 573–581.
[34] Piceno, Y.M. and Lovell, C.R. (2000) Stability in natural bacterial communities. I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. Microb. Ecol. 39, 32–40.
[35] Solheim, B., Endal, A. and Vigstad, H. (1996) Nitrogen fixation in arctic vegetation and soil from Svalbard, Norway. Polar Biol. 16, 35–40.
[36] Zogg, G.P., Zak, D.R., Ringelberg, D.B., Macdonald, N.W., Pregitzer, K.S. and White, D.C. (1997) Compositional and functional shifts in microbial communities due to soil warming. Soil Sci. Soc. Am. J. 61, 475–481.
[37] Paul, E.A. and Clark, F.E. (1996) Soil Microbiology and Biochemistry. Academic Press, San Diego, pp. 27–30.
[38] Ruess, L., Michelsen, A., Schmidt, I.K. and Jonasson, S. (1999) Simulated climate change affecting microorganisms, nematode density and biodiversity in subarctic soils. Plant Soil 212, 63–73.
[39] Schmidt, I.K., Jonasson, S., Shaver, G.R., Michelsen, A. and Nordin, A. (2002) Mineralization and distribution of nutrients in plants and microbes in four arctic ecosystems: responses to warming. Plant Soil 242, 93–106.
[40] Tan, X.Y., Hurek, T. and Reinhold-Hurek, B. (2003) Effect of N-fertilization, plant genotype and environmental conditions on *nifH* gene pools in roots of rice. Environ. Microbiol. 5, 1009–1015.