

# Effect of Freeze-Thaw Cycles on Bacterial Communities of Arctic Tundra Soil

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**Abstract** The effect of freeze-thaw (FT) cycles on Arctic tundra soil bacterial community was studied in laboratory microcosms. FT-induced changes to the bacterial community were followed over a 60-day period by terminal restriction fragment length polymorphism (T-RFLP) profiles of amplified 16S rRNA genes and reverse transcribed 16S rRNA. The main phylotypes of the active, RNA-derived bacterial community were identified using clone analysis. Non-metric multidimensional scaling ordination of the T-RFLP profiles indicated some shifts in the bacterial communities after three to five FT cycles at  $-2$ ,  $-5$ , and  $-10^{\circ}\text{C}$  as analyzed both from the DNA and rRNA. The dominating T-RFLP peaks remained the same, however, and only slight variation was generally detected in the relative abundance of the main T-RF sizes of either DNA or rRNA. T-RFLP analysis coupled to clone analysis of reverse transcribed 16S rRNA indicated that the initial soil was dominated by members of *Bacteroidetes*, *Acidobacteria*, *Alpha-*, *Beta-*, and *Gammaproteobacteria*. The most notable change in the rRNA-derived bacterial community was a decrease in the relative abundance of a

*Betaproteobacteria*-related phylotype after the FT cycles. This phylotype decreased, however, also in the control soil incubated at constant  $+5^{\circ}\text{C}$  suggesting that the decrease was not directly related to FT sensitivity. The results indicate that FT caused only minor changes in the bacterial community structure.

## Introduction

Long and cold winters, relatively warm summers and consequently wide seasonal temperature variation characterize northern tundra soil ecosystems of Finland. Tundra environments are found in the northernmost parts of Lapland where summer temperatures rise above  $20^{\circ}\text{C}$ , while during the coldest winter months temperatures drop below  $-30^{\circ}\text{C}$ . Soil temperatures during the cold season are highly dependent on the thickness of insulating snow cover. Below thick snow cover, soil temperature is little affected by air temperature and remains close to  $0^{\circ}\text{C}$ , while in the wind-exposed ridges soil temperature may fall below  $-25^{\circ}\text{C}$ . Freeze-thaw (FT) episodes are common during autumn before snow accumulation as well as in spring. Global warming is expected to impact the Arctic ecosystems most severely as a temperature increase of  $3$ – $7^{\circ}\text{C}$  is predicted by the end of the twenty-first century [12]. Global climate change is predicted not only to increase summer temperatures but to increase temperature fluctuations and freeze-thaw cycles during the fall and spring [12].

Freeze-thaw events have been linked to a decline in microbial abundance during early spring and up to 50% die-off of microbes has been reported after a single freeze-thaw cycle [33]. FT cycles have been reported to decrease microbial biomass [17, 29] and FT cycles have been linked to shifts in soil microbial community metabolism [30]. Recently, FT

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cycles were shown to influence the enzymatic activities and relative densities of bacterial gene families involved in N cycling in Antarctic soil [38]. FT events have been reported to trigger a sudden, brief increase in microbial respiration which has been associated with a release of nutrients from the lysed cells [26, 29]. Freeze-thaws have thus been hypothesized to be a major driving force affecting microbial activity in cold soils. The effect of FT cycles on microbial community composition has been studied using phospholipid fatty acid profiles [5, 16] and DNA-based methods [38], but there is still little information on the different members of the microbial communities that survives or perishes after FT events, or which members of the microbiota remain active in carbon turnover. For instance, there is limited information on specific FT tolerant vs. sensitive species, although studies have suggested that multiple FT cycles select for FT tolerant strains [36]. Studies on Arctic [37] and alpine [20, 21] soils have indicated seasonal changes in the bacterial community composition. The communities were, however, even more controlled by vegetation type [21, 37] and consequently seasonal changes may be related to substrate availability rather than temperature per se.

In our previous studies we have used terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified 16S rRNA genes to distinguish differences in bacterial community structure of Arctic alpine tundra soil of northern Finland [24]. The bacterial communities appeared relatively stable and the main factor controlling bacterial species diversity was soil pH, which varies in the north-western part of Finland that contains both alkaline and acidic bedrock materials. DNA-based community analysis may, however, not detect rapid changes in microbial communities as the DNA pool contains both active and inactive microorganisms. In contrast, the ribosome per cell ratio is considered roughly proportional to the growth rate of bacteria and 16S rRNA an indicator of active bacteria [14, 15, 35]. In this study, we therefore compared the bacterial community composition derived from both DNA and RNA pools. Different freeze-thaw cycles and incubation below 0°C were used to evaluate whether the soil freezing leads to selective die-off of specific bacterial groups. Changes in the microbial community composition was estimated by T-RFLP analysis of amplified 16S rRNA genes and reverse transcribed 16S rRNA and compared to clone libraries constructed from soil 16S rRNA before and after freeze-thaw treatments.

## Materials and Methods

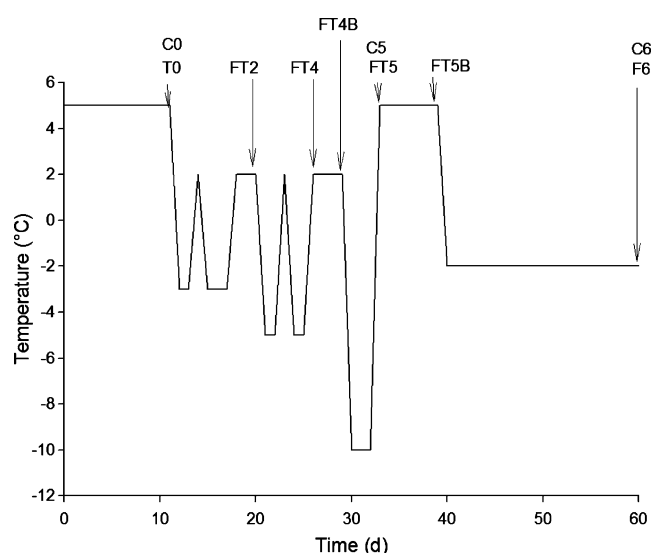
### Study Site

Tundra soil from an oligotrophic heath in Northern Finland (Malla nature reserve, Kilpisjärvi, 69°01' N, 20°50' E) was

used to elucidate the effect of seasonally fluctuating temperature on soil microbial community structure. In Kilpisjärvi, the mean annual air temperature is 2.2°C and growing season is approximately 100 days. The vegetation in the oligotrophic heath is patchy but dominated by dwarf shrubs, particularly *Empetrum nigrum*. Soil used for the experiment was sampled from the humus layer (top 5 cm) of a wind-exposed ridge at an elevation of 650 m in August 2005 and stored at 4°C for approximately 4 weeks prior to the experiment. The pH of the tundra soil was 4.9, the soil had a moisture content of approximately 70%, and an organic matter content of 80%. Soil was homogenized by hand and plant debris, large roots, and rocks were removed.

### Experimental Design

Fifty grams of homogenized soil was placed in capped 500-ml glass jars. Four replicate microcosms were subjected to various freeze-thaw cycles (Fig. 1) simulating fall/winter freeze-thaws observed in the field (data not shown). Two control microcosms were incubated at constant +5°C ( $\pm 1^\circ\text{C}$ ). In the beginning of the experiment (T0) and at various time points, two soil samples (0.3 g) were removed from each microcosm for DNA/RNA extraction. The first stage of the experiment consisted of FT cycles (two times at  $-2^\circ\text{C}$ , two times at  $-5^\circ\text{C}$ , and once at  $-10^\circ\text{C}$ ) after which incubation was continued at  $-2^\circ\text{C}$  for 4 weeks (Fig. 1). The incubations were done using a Lauda RUK 60 kryomat in which the cooling rate was adjusted to  $2^\circ\text{C h}^{-1}$ . Changes in soil bacterial communities were monitored after two, four, and five FT cycles at  $-3^\circ\text{C}$  (twice for 2 days),  $-5^\circ\text{C}$  (twice for 2 days), and  $-10^\circ\text{C}$  (once for 3 days). Soil was sampled either directly after the FT cycles (FT4 and FT5) or after 3-



**Figure 1** Schematic illustration of the temperature regime used in the experiment. Arrows indicate sampling points

day incubation at +2°C (FT2, FT4B) or 5 day incubation at +5°C (FT5B). After the FT cycles, incubation was continued at -2°C and soil was sampled after 21 days (F6, Fig. 1). T-RFLP analysis of amplified 16S rRNA genes and reverse transcribed 16S rRNA was used to estimate changes in the soil bacterial community. In addition, clone libraries were constructed from soil RNA before (sample T0) and after five freeze-thaw cycles (sample FT5) and used to identify dominant bacterial species active in the tundra soil before and after FT cycles and to link them to the T-RF sizes of the soil T-RFLP profiles.

#### Nucleic Acid Extraction

At each sampling time point duplicate samples of approximately 0.3 g were removed from each replicate soil microcosm and stored at -70°C until DNA and RNA extraction. Both DNA and RNA were extracted from the same sample using a modification of the method described by Griffiths et al. [8]. Soil samples were allowed to thaw briefly after which they were rapidly frozen in liquid nitrogen. After this, 0.5 g of glass beads (0.1 mm), 650 µl CTAB extraction buffer [8], and 650 µl phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0) were added and the samples were lysed by vortexing at full speed three times for 90 s. Separation of the phases and precipitation of the nucleic acids was performed as described by Griffiths et al. [8]. RNA and DNA were separated by DNAase (Promega, Madison, WI) and RNAase (Promega) treatment according to the manufacturer's instructions. All solutions used for RNA extraction were treated with 0.1% diethylpyrocarbonate (DEPC). RNA and DNA concentrations were measured using Qubit flurometer and Quant-iT RNA and dsDNA HS assay kits (Invitrogen, Carlsbad, CA), respectively.

#### cDNA Synthesis, PCR, and Terminal Restriction Fragment Polymorphism

Approximately 100 ng of RNA was reverse transcribed using M-MLV reverse transcriptase (Promega) and 907r reverse primer [11] according to the manufacturer's instructions. Partial bacterial 16S rRNA genes were amplified from DNA and reverse transcribed RNA using FAM-labelled 27f forward and unlabelled 907r reverse primers [11] as previously described [24]. PCR amplification from crude RNA was processed similarly as a negative control to check for the presence of DNA and all PCR amplification products were checked on 1% agarose gels. After this, PCR amplification products from the duplicate DNA preparations of FT-treated microcosms were pooled resulting in four replicate T-RFLP samples of each time point. PCR products were purified using the E.Z.N.A Cycle-pure kit (Omega Bio-Tek, Doraville, GA). Approx-

imately 15 ng of the purified PCR product was digested with restriction enzyme *MspI* (Fermentas, Burlington, ON) and the DNA was precipitated and frozen. T-RFLP profiles were analyzed by the fragment analysis service of University of Turku using an ABI PRISM 377 automatic sequencer and GeneScan-500 TAMRA (Applied Biosystems, Foster City, CA) DNA fragment length standard.

#### Cloning and Sequence Analysis of Clones

PCR products amplified from the reverse transcribed Malla soil RNA before (T0) and after five FT cycles (FT5) were inserted into pCR4-TOPO vectors and transformed to *Escherichia coli* TOP10 cells using the TOPO TA Cloning Kit for Sequencing (Invitrogen). Putative positive colonies were selected by blue/white screening and plasmid DNA was extracted using the Qiaprep spin miniprep kit (Qiagen). Approximately 100 clones from both soil samples were sequenced using plasmid primers T3 and T7, ABI Big Dye 3.1 kit, and an ABI3700 capillary sequencer. Sequences were combined and edited using the VectorNTI program (Invitrogen). Chimeric sequences were identified using the CHIMERA\_CHECK program of the Ribosomal Database Project and sequences that appeared chimeric were excluded from further analysis. Similarity searches were performed using BLAST [1]. Sequences were aligned using ClustalX software [34] and the phylogenetic tree of 424 unambiguously aligned nucleotide positions with 1,000 bootstrap replications was constructed using the Phylo\_Win package [7] and NJplot tree drawing program [27] by using the neighbor-joining method and Jukes-Cantor distances. Terminal restriction fragment (T-RF) sizes of the clones were analyzed from 1–2 ng of PCR products that were amplified and digested using the same protocol as described for the soil DNA and RNA samples. Sequences were clustered to operational taxonomic units (OTUs) at 95% sequence similarity level using the program CD-HIT (<http://www.bioinformatics.org/cd-hit>).

#### Data Analysis

T-RFLP data of the four replicate FT-treated soil and control microcosms were aligned and the relative abundance of each T-RF was used to estimate changes in the soil microcosms during the experiment. T-RFs of 60 to 500 bp in length and with heights of >50 fluorescence units were included in the initial analysis. T-RFLP peaks that differed by more than ±0.5 bases were considered unique. T-RFLP data was normalized to the total fluorescence of each sample, as described by Dunbar et al. [3] and normalized T-RFs with a peak area of <300 were excluded. This peak area corresponded, on average, to a peak height of 50 U. After normalization, the abundance of each T-RFLP peak

was calculated as the percentage of the peak area from the normalized total peak areas of each sample. To reduce background noise, and to focus on the dominant bacteria, T-RFLP peaks with abundance of less than 1% in all of the samples were omitted from further analysis.

T-RFLP patterns of the various sampling points were compared using non-metric multidimensional scaling (NMS) and cluster analysis using the program package PC-ORD [22]. NMS ordination was calculated using the Sorensen (Bray–Curtis) distance method and autopilot mode on slow and thorough speed. The graphical presentation of the NMS ordination was used to find the most (dis)similar sample points and to visualize differences in the T-RFLP profiles between different sampling points.

#### Accession Numbers

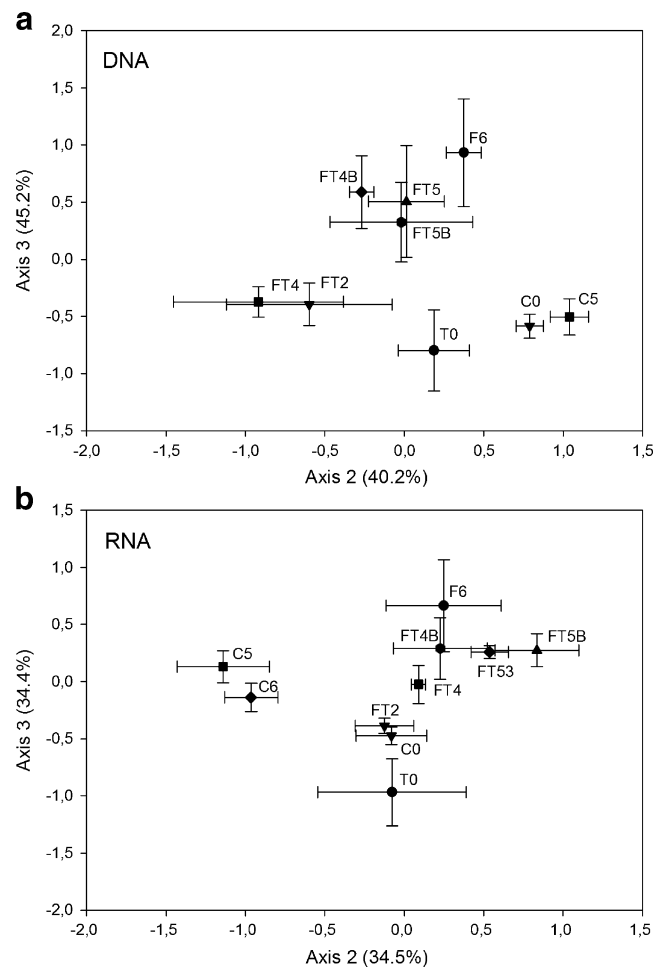
The partial 16S rRNA gene sequences are submitted to EMBL database under accession numbers AM945364 to AM945537.

## Results

### Effect of FT Cycles on Bacterial Community T-RFLP Profiles

Soil microcosms were subjected to FT cycles over a 60-day period as indicated in Fig. 1. T-RFLP analysis detected 58 and 51 distinct T-RFs from the amplified 16S rRNA genes and reverse transcribed 16S rRNA, respectively, that were present in the soil microcosms at a relative abundance of >1% in at least one of the samples. In addition, all of the peaks were present in multiple sample points and in at least two replicate microcosms. Non-metric multidimensional scaling of this data was used to visualize differences in the FT-treated soil compared to the initial soil and control soils incubated at 5°C. NMS ordination of the DNA-derived T-RFLP data distinguished four groups (Fig. 2): (1) the initial soil (T0), (2) control soils incubated at +5°C (C0, C6), (3) soil after two and four FT cycles (FT2, FT4), and (4) all other FT-treated soil samples (FT4B, FT5, FT5B, F6). Microcosms incubated at constant 5°C for 60 days (C6) grouped with the initial soil (C0) indicating that DNA-derived T-RFLP did not reveal shifts in the bacterial community at +5°C. The RNA-derived T-RFLP data at T0 and after the FT cycles clustered quite similarly as the DNA-derived data. Unlike in the DNA-derived T-RFLP, the microcosms incubated at +5 separated from the initial soil (C0) after 30- (C5) and 60-day (C6) incubation indicating shifts in the active bacterial community during the 30- to 60-day incubation at 5°C.

Further inspection of the results indicated that changes in the T-RFLP profiles were relatively moderate, as all main



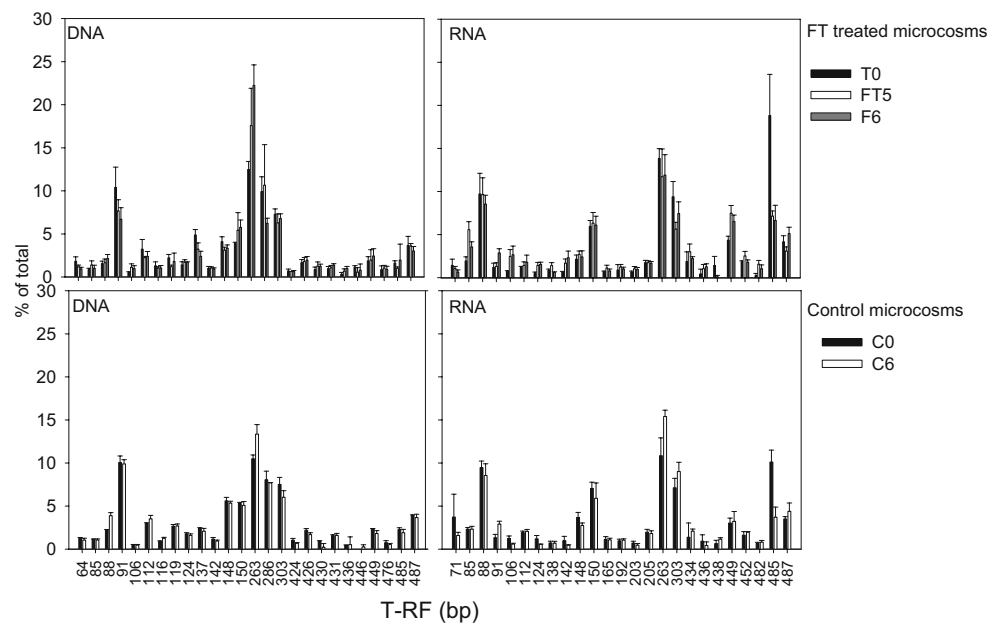
**Figure 2** NMS ordination of the T-RFLP (*MspI* digestion) profiles of DNA (a) and RNA (b) samples after incubation at the various temperatures. Points represent the average of T-RFLP profiles of four replicate FT-treated microcosms or two replicate control microcosms with the standard deviation indicated by error bars. See Fig. 1 for sample abbreviations

T-RFs (relative abundance >2%) were present both before and after the FT cycles. Figure 3 shows the T-RFLP profiles at T0, after five FT cycles and in the end of the experiment. In the DNA pool, the relative abundance of T-RF size 91 bp decreased while that of 263 bp increased after the five FT cycles. On the other hand, the relative abundance of T-RF 286 bp decreased after the final incubation at -2°C. The most notable change in the RNA derived profile was a decrease of T-RF 485 bp after five FT cycles. The relative abundance of this T-RF decreased throughout the experiment (data not shown) and also in the control microcosms.

### Identification of Dominant Bacterial Phylotypes before and after FT Cycles

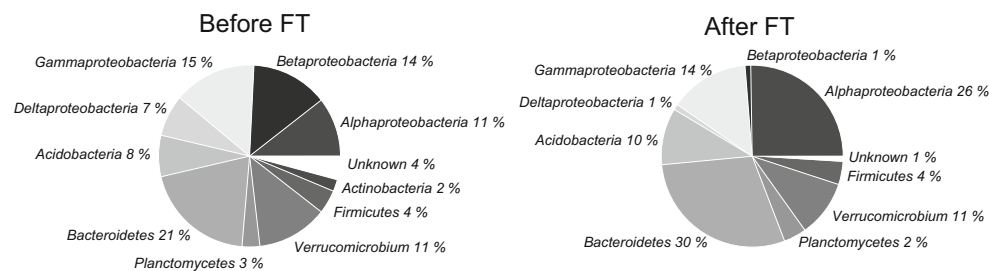
To identify bacteria active in the soil before and after the FT cycles, clone libraries were constructed from reverse

**Figure 3** Comparison of the soil DNA and RNA T-RFLP profiles of samples before (T0) and after five (FT5) FT cycles and after 3 week incubation at  $-2^{\circ}\text{C}$  (F6). Only T-RFLP peaks representing on average  $>1\%$  of relative abundance in at least one of the time points are shown

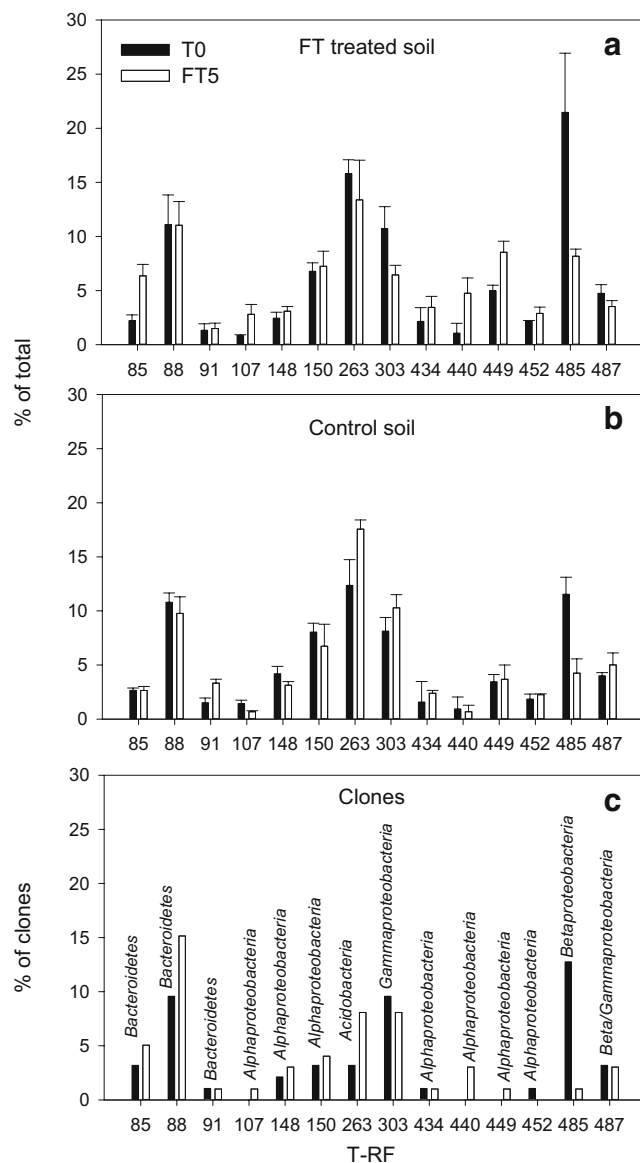


transcribed 16S rRNA of the initial soil (T0) and soil treated with five FT cycles (FT5). Sequencing of partial 16S rRNA genes indicated that before the FT treatment, soil was dominated by *Bacteroidetes*; *Gamma-*, *Beta-*, and *Alphaproteobacteria*; *Verrucomicrobia*; and *Acidobacteria*. After five FT cycles the relative share of *Bacteroidetes* and *Alphaproteobacteria* increased while that of *Betaproteobacteria* decreased (Fig. 4). The soil T-RFLP peaks were compared to T-RF sizes of clones from the same samples and 14 soil T-RFs present at a relative abundance of  $>3\%$  are shown in Fig. 5. For all of the 14 rRNA-derived soil T-RFLP peaks, there was a clone with the corresponding T-RF size (Fig. 5, Table 1). These T-RF sizes corresponded to sequences matching *Alphaproteobacteria* (T-RF sizes 107, 148, 150, 434, 440, 449, and 452 bp), *Bacteroidetes* (85 and 88 bp), *Acidobacteria* (263 and 148 bp), *Betaproteobacteria* (485 bp), and *Gammaproteobacteria* (303 and 487 bp). Moreover, the relative abundance of different T-RFs in the clone libraries matched well with the soil T-RFLP profiles (Fig. 5) indicating a good correlation between T-RFLP and clone analysis.

The correlation between T-RFLP and clone analysis was further verified by inspecting the diversity of clones matching the major soil T-RFLP peaks presented in Fig. 5. The clone sequences were grouped using cluster analysis and clones sharing at least 95% sequence similarities were placed in operational taxonomic units (OTUs). The number of different OTUs and Blast matches of representative clones matching the dominant soil T-RFs are presented in Table 1. In general, a particular T-RF size could be assigned to a single phylum, with the exception of T-RF sizes 148 and 485 that matched with clones representing two and three different phyla, respectively. The most abundant OTUs contained clones with T-RF sizes of either 304 or 485 bp. T-RF 485 that was initially the most abundant ribotype in the soil microcosms matched with clones that fell almost exclusively in a single OTU of *Herbaspirillum*-related *Betaproteobacteria*. The gammaproteobacterial T-RF 303 bp was represented by equally limited diversity, as 14 clones fell in to a single OTU. T-RF 263 bp matched exclusively with group 1 *Acidobacteria*. The two main acidobacterial OTUs contained seven and six



**Figure 4** Frequency of bacterial groups in clone libraries of soil 16S rRNA sampled in the beginning (T0) of the experiment and after five FT cycles (FT5)



**Figure 5** Comparison of the soil RNA T-RFLP profiles of samples before (T0) and after five (FT5) FT cycles (a), in control samples incubated at constant 5°C (b) and the frequency of different T-RF sizes of cloned sequences of soil before and after FT cycles (c)

sequences, respectively, that were most closely related to the recently described genera *Terriglobus* and *Edaphobacter*. The *Bacteroidetes* and *Alphaproteobacteria* matching soil T-RFs, as well as the clones matching these were, more diverse (Table 1, Fig. 6). A phylogenetic tree constructed from the Gram-negative phyla indicated that the alphaproteobacterial clones were related to acetic acid bacteria, the *Sphingomonadaceae*, *Caulobacteriaceae*, or *Rhizobiales*. The *Bacteroidetes* sequences were related to various *Sphingobacteriaceae* and *Flexibacteraceae* bacteria. The *Verrucomicrobia*- and *Planctomycetes*-related clones were even more diverse and could be assigned to

numerous T-RF sizes that were present at low abundance in the soil profiles.

#### Comparison of Soil Bacterial Community Structure Derived from RNA and DNA

T-RFLP analysis detected a total of 78 T-RF sizes that were present at a relative abundance of >1% in at least one of the sampling points. Of these, 27 T-RFs were present only in the DNA pool, 20 only in the RNA pool, and 31 in both DNA and RNA. The T-RF sizes that were present in either the DNA or RNA pool only were, however, all in relatively low abundance (<2%), while the dominating T-RF sizes (>3% in either pool) were detected in both pools. The most notable differences in the RNA vs. DNA-derived T-RFLP profiles were in the relative abundance of the T-RF sizes 85, 88 (*Bacteroidetes*), and 485 bp (*Betaproteobacteria*) that were substantially more abundant in the RNA pool and in the relative share of T-RF sizes 91, 137, and 286 that were substantially more abundant in the DNA pool throughout the experiment (Fig. 7).

#### Discussion

Studies on the effect of FT cycles on the soil microbial biomass have reported somewhat inconsistent results, as in some studies FT cycles have resulted in significant decrease in soil microbial abundance or activity [17, 29, 33], while others have reported insignificant effects [18, 19, 31, 32]. This discrepancy may result from methodological differences, such as in the number and amplitude of FT cycles [9], but is suggested also to reflect FT tolerance of communities that are naturally exposed to harsh conditions (e.g. [18]). This study attempted to identify bacterial species that are active in the Arctic alpine tundra before and after FT cycles and elucidate whether the FT cycles result in a decline of specific taxa. We used FT cycles that roughly represent FT events naturally occurring in the fall on the wind-exposed tundra heaths of Kilpisjärvi at a soil depth of ca. 3 cm (Männistö, unpublished data). Although large diurnal temperature fluctuations during fall and winter are typical in these areas, at 3–5 cm soil depth the temperature variation is highly muted relative to the surface temperatures and the number of FT cycles is generally less than five.

NMS ordination of the T-RFLP data suggested that multiple FT cycles resulted in shifts in the bacterial communities, as the samples after FT grouped together and separated from the T0 as well as the control samples (Fig. 2). However, when inspecting specific peaks in the T-RFLP profiles, there were generally no substantial changes in the main ribotypes and all predominant peaks were

**Table 1** Phylogenetic affiliation of the most abundant soil RNA T-RF sizes and corresponding clones

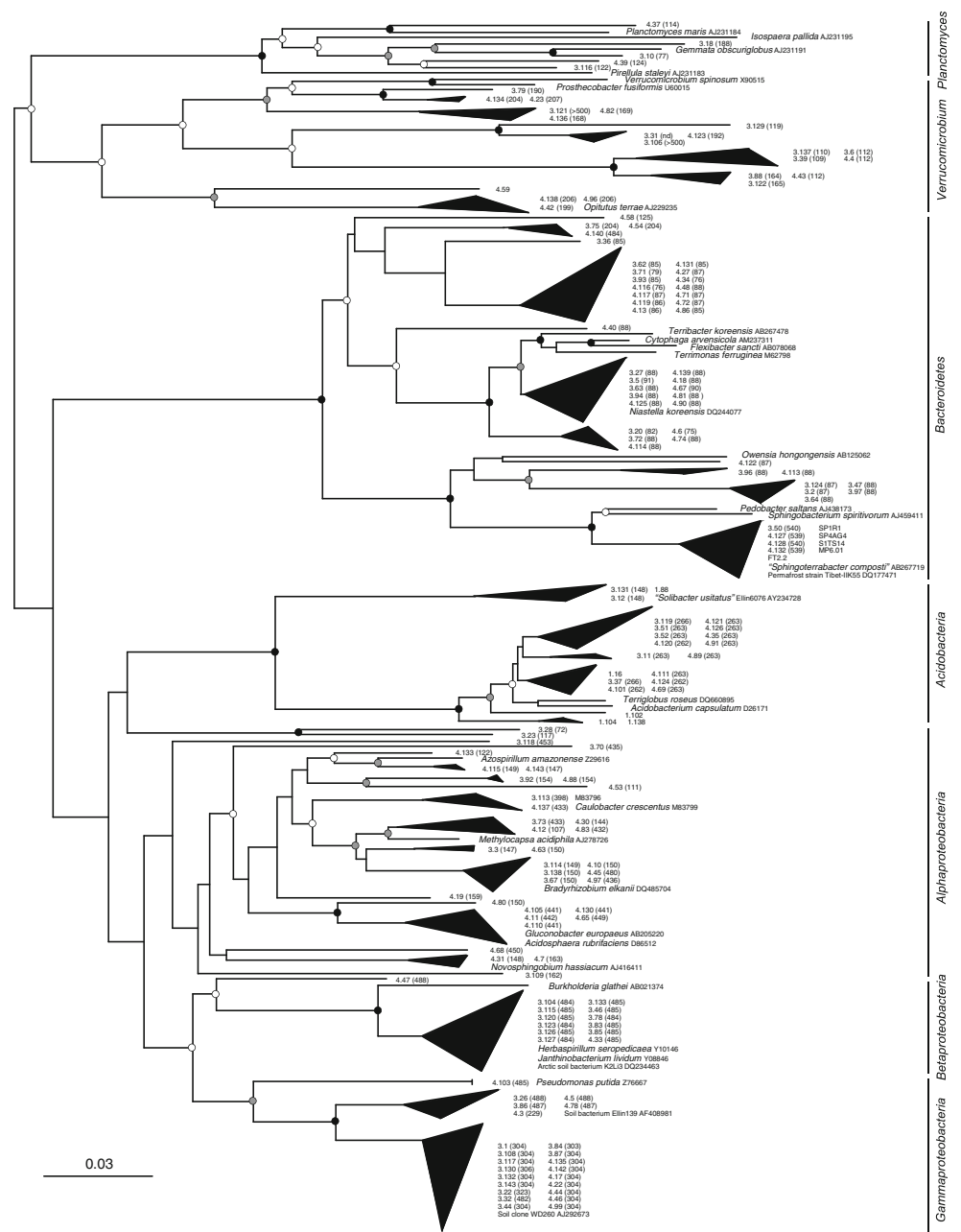
T-RF size (bp)	No. of clones	No. of OTUs <sup>a</sup>	Representative clone of main OTUs	No. of clones in the OTU	Closest match in GenBank <sup>c</sup> (accession number)	Similarity (%)	Phylogenetic group
85–86	8	4 <sup>b</sup>	4.13 (AM945481)	3	Uncultured lake water clone FSW11-13 (DQ501318)	94.7	<i>Bacteroidetes</i>
			4.117 (AM945458)	2	<i>Chitinophaga ginsengisoli</i> (AB245374)	89.8	<i>Bacteroidetes</i>
			3.93 (AM945443)	2	Uncultured soil clone GASP-MA4S3_F09 (EF664169)	97.6	<i>Bacteroidetes</i>
87–88	26	7 <sup>b</sup>	4.81 (AM945527)	4	<i>Chitinophaga ginsengisegetis</i> (AB264798)	92.8	<i>Bacteroidetes</i>
			4.71 (AM945521)	4	<i>Chitinophaga ginsengisegetis</i> (AB264798)	89.4	<i>Bacteroidetes</i>
			3.2 (AM945407)	3	Uncultured clone ADK-BTh02-48 (EF520590)	93.2	<i>Bacteroidetes</i>
			3.72 (AM945430)	3	<i>Terrimonas lutea</i> (AB192292)	89.9	<i>Bacteroidetes</i>
91	2	2	3.5 (AM945422)	1	<i>Niastella koreensis</i> (DQ244077)	92.2	<i>Bacteroidetes</i>
			4.67 (AM945517)	1	<i>Niastella jeongjuensis</i> (DQ244076)	91.8	<i>Bacteroidetes</i>
107	1	1	4.12 (AM945470)	1	<i>Methylocystis heyerii</i> (AM283543)	93.3	<i>Alphaproteobacteria</i>
148	4	3	3.12 (AM945388)	2	“ <i>Solibacter usitatus</i> ” Ellin6076 (AY234728)	93.9	<i>Acidobacteria</i>
			4.31 (AM945493)	1	<i>Novosphingobium pentaromativorans</i> (AF502400)	96.2	<i>Alphaproteobacteria</i>
			4.115 (AM945456)	1	<i>Azospirillum amazonense</i> (Z29616)	93.1	<i>Alphaproteobacteria</i>
150	6	3	4.10 (AM945451)	3	<i>Bradyrhizobium japonicum</i> (BJU69638)	99.0	<i>Alphaproteobacteria</i>
			4.80 (AM945526)	1	<i>Acidocella facilis</i> (ACD16SRNAG)	91.3	<i>Alphaproteobacteria</i>
			4.109 (AM945450)	1	Uncultured soil clone Elev_16S_429 (EF019250)	90.5	<i>Alphaproteobacteria</i>
			4.63 (AM945515)	1	<i>Beijerinckia indica</i> subsp. <i>indica</i> (CP001016)	94.5	<i>Alphaproteobacteria</i>
263	15	4 <sup>b</sup>	4.124 (AM945465)	7	<i>Edaphobacter modestus</i> (DQ528760)	95.7	<i>Acidobacteria</i>
			3.119 (AM945378)	6	<i>Terriglobus roseus</i> (DQ660892)	94.7	<i>Acidobacteria</i>
304	17	4 <sup>b</sup>	3.32 (AM945409)	14	<i>Steroidobacter denitrificans</i> (EF605262)	89.7	<i>Gammaproteobacteria</i>
434	2	2	3.73 (AM945431)	1	<i>Beijerinckia indica</i> subsp. <i>indica</i> (CP001016)	94.5	<i>Alphaproteobacteria</i>
			4.137 (AM945478)	1	<i>Caulobacter crescentus</i> (AE005673)	96.7	<i>Alphaproteobacteria</i>
441–442	5	3	4.105 (AM945449)	2	<i>Acidisphaera rubrifaciens</i> (D86512)	93.9	<i>Alphaproteobacteria</i>
			4.11 (AM945460)	2	<i>Acidisphaera rubrifaciens</i> (D86512)	93.9	<i>Alphaproteobacteria</i>
			3.91 (AM945441)	1	<i>Acidisphaera rubrifaciens</i> (D86512)	93.8	<i>Alphaproteobacteria</i>
449	1	1	4.65 (AM945516)	1	<i>Granulibacter bethesdensis</i> (CP000394)	93.2	<i>Alphaproteobacteria</i>
453	1	1	3.118 (AM945377)	1	Uncultured drinking water clone B3NR56D5 (AY957908)	90.7	<i>Alphaproteobacteria</i>
484–485	15	3	3.46 (AM945415)	13	<i>Herbaspirillum lusitanum</i> (AF543312)	95.4	<i>Betaproteobacteria</i>
			4.103 (AM945448)	1	<i>Pseudomonas putida</i> (AF094736)	99.3	<i>Gammaproteobacteria</i>
			4.140 (AM945482)	1	Uncultured forest clone GASP-MB2S2_C09 (EF665122)	96.8	<i>Bacteroidetes</i>
487–488	5	3	4.5 (AM945514)	3	Uncultured soil clone FAC29 (DQ451468)	97.8	<i>Gammaproteobacteria</i>
			4.78 (AM945524)	1	<i>Steroidobacter denitrificans</i> (EF605262)	89.1	<i>Gammaproteobacteria</i>
			3.86 (AM945438)	1	<i>Steroidobacter denitrificans</i> (EF605262)	89.0	<i>Gammaproteobacteria</i>

<sup>a</sup> Number of different OTUs that contained clones with the specific T-RF size. OTU was defined as clones sharing 95% sequence similarity

<sup>b</sup> Representative clones are shown only from the OTUs with >2 clones

<sup>c</sup> Closest match to the type strain of known species. In case the known species was <90% similar, the closest match to any sequence is shown

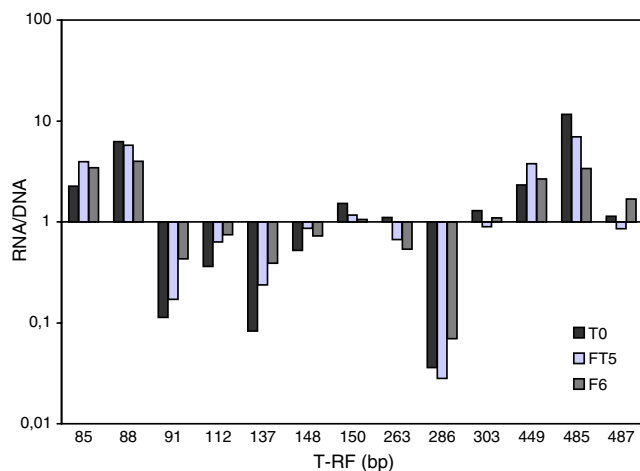
**Figure 6** Phylogenetic relationship of soil phylotypes and their closest relatives based on partial 16S rRNA sequences. Clones of reverse transcribed 16S rRNA with prefixes 3 and 4 refer to clones obtained from soil before (T0) and after five FT cycles (FT5), respectively. *Acidobacteria* sequences with prefix 1 from Kilpisjärvi tundra soil DNA [24] are included as reference. The T-RF sizes of the clones are shown in parentheses. The phylogenetic tree was constructed using the neighbor-joining method with Jukes–Cantor corrections of 424 unambiguously aligned nucleotide positions. The corresponding T-RF lengths of each clone are listed in parentheses. Bootstrap support is indicated as follows: closed circles over 90%, grey circles 70–90%, and open circles 50–70%. The scale bar indicates 0.03 changes per nucleotide



present throughout the experiment albeit some in slightly varying relative abundance. The most notable shift after the FT cycles was the decrease in the *Herbaspirillum*/*Janthinobacteria*-related phylotypes in the rRNA. As this T-RF size decreased also in the control microcosms incubated at +5°C, the decrease was not likely linked to FT sensitivity. This T-RF was initially tenfold more abundant in the RNA than DNA pool, indicating that it represented highly active organisms. We hypothesize that the initial high abundance of this T-RF correlated with high growth rate and cellular rRNA content of these organisms in the beginning of the experiment. The *Herbaspirillum*/*Janthinobacterium*-related phylotypes may have been growing on plant exudates or

nutrients released from soil aggregates after the soil cores were homogenized before the FT cycles were initiated. Soil samples for the experiment were obtained in late summer and stored at 4°C for several weeks prior to the experiment. Homogenization prior to the experiment presumably disrupted soil aggregates and released organic and inorganic nutrients. *Betaproteobacteria* have been suggested to represent copiotrophic bacteria that respond rapidly with high growth rates to available substrates [6]. Rapid growth, on the other hand, correlates with high ribosome content [4, 13, 15] which may decrease rapidly after exponential growth [13]. Rapid growth coupled to a peak in the cellular ribosome content would explain the high abundance of the





**Figure 7** Ratio of the relative abundance of the main T-RF sizes (present at >3% in either pool) in the RNA vs. DNA pools at T0, after five FTC and at the end of the experiment

phylotype in the RNA pool in the beginning of the experiment.

Clone analysis indicated an increase in sequences related to *Alphaproteobacteria* and *Bacteroidetes* after five FT cycles (Fig. 4). A slight increase of *Alphaproteobacteria*-related ribotypes 148, 107, 440, 449, and 452 bp and the *Bacteroidetes*-related ribotype 85 bp was detected also in the rRNA-derived T-RFLP after five FT cycles as compared to the T0 and soil incubated at 5°C (Fig. 5). Contrary to the *Betaproteobacteria* ribotype, which was affiliated to a single OTU, diverse *Alphaproteobacteria* and *Bacteroidetes* assigned ribotypes were detected in the RNA pool. Therefore FT-induced changes were not as obvious from the T-RFLP data as for *Betaproteobacteria* (Figs. 5 and 6, Table 1).

In the DNA pool, the most notable shift after FT cycles was the relative increase of the *Acidobacteria*-related T-RF size of 263 bp. This ribotype was equally abundant in both DNA and rRNA and matched exclusively *Acidobacteria* clones (Fig. 5, Table 1). *Acidobacteria* are among the most dominating members of Arctic as well as other acidic soils [10, 21, 24]. *Acidobacteria* have been suggested to represent oligotrophic bacteria with low growth rates [6] which would suggest low cellular rRNA content. This would agree with the somewhat lower abundance and diversity of *Acidobacteria* in the rRNA-derived clone library of this study compared to our earlier, DNA-based analysis of the Kilpisjärvi tundra soil [24]. All but two RNA-derived clones represented group 1 *Acidobacteria*. These sequences corresponded to the group 3 *Acidobacteria* (“*Solibacter*” group, Fig. 6). In our earlier study we have detected more abundant and diverse *Acidobacteria* from DNA of the Kilpisjärvi tundra soil [24], representing e.g. T-RF sizes 91, 148, 263, and 286 bp. In this study the T-RF sizes 91 and 286 were much less abundant in the

rRNA than DNA, suggesting that these organisms may not have been as active and readily detected from the rRNA.

The main differences in the T-RFLP profiles were between samples derived from soil DNA compared to soil RNA (Figs. 3 and 7). Our results are in agreement with previous studies that have compared 16S rRNA and 16S rRNA gene-based soil bacterial community structures [2, 23, 25] as only partial overlapping of the DNA- vs. RNA-derived microbial communities was observed. Nogales et al. [25] found that 29% of cloned 16S rRNA gene sequences were identical to 16S rRNA sequences of the same soil. Duineveld et al. [2] compared rhizosphere DGGE profiles and showed that profiles generated from RT-PCR products were less complex and represented a subset of bands visible in the DNA-based profiles. Ribosomal RNA content has been shown to correlate with the growth rate of bacteria [4, 28, 35] indicating that the RNA-based approach is more descriptive of active microbial communities. On the other hand, high abundance of the phylotype in the DNA pool may reflect high cellular abundance and/or multiple rRNA operons per cell [4]. However, in marine *Proteobacteria*, intracellular rRNA content was shown to be species specific since during early exponential growth phase there was up to 15-fold differences in the rRNA level of nine marine isolates [13]. Moreover, during non-steady state growth, 16S rRNA levels were not linearly related to growth rate and the 16S rRNA content/cell decay was very rapid. Interpretation of RNA-based community profiles is thus not necessarily straightforward.

The results of the T-RFLP analysis agreed well with clone libraries and the most abundant T-RF sizes detected in the soil samples were most frequently detected also in the cloned sequences (Fig. 5). T-RFLP analysis was most suitable in detecting variation in phyla with relatively low diversity, such as *Beta*- and *Gammaproteobacteria*, which were abundant but with low sequence and T-RF size diversity. *Acidobacteria* were nearly as low in T-RF size diversity and the 263 bp T-RF could be affiliated exclusively with group 1 *Acidobacteria* similar to our earlier studies [24]. The T-RF size and sequence diversity of other phyla was, however, higher. Consequently T-RFLP may not be as sensitive in detecting changes in these phyla.

In conclusion, this study indicates that although the relative abundance of some active members of the bacterial community varied, FT cycles did not drastically change the soil bacterial composition especially when inspected from the DNA. This is in agreement with studies of alpine and Antarctic soil where limited FT effects were reported for soil bacterial community [19, 38]. It appears that soils that are naturally exposed to harsh and changing environmental conditions may harbor extremely frost tolerant and resilient bacterial species. The tundra soil used in this study is

exposed to wide annual temperature fluctuation including very low (down to  $-15^{\circ}\text{C}$ ) winter temperatures (unpublished data). We hypothesize that these conditions have selected a stable bacterial community that is only little affected by temperature fluctuation and FT cycles.

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