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Complete List of Authors:	Zhao, Yeyi; Long-term Research Station of Alpine Forest Ecosystems, Institute of Ecology & Forest, Sichuan Agricultural University; Collaborative Innovation Center of Ecological Security in the Upper Reaches of Yangtze River, Wu, Fuzhong; Long-term Research Station of Alpine Forest Ecosystems, Institute of Ecology & Forest, Sichuan Agricultural University; Collaborative Innovation Center of Ecological Security in the Upper Reaches of Yangtze River, Yang, Wanqin; Long-term Research Station of Alpine Forest Ecosystems, Institute of Ecology & Forest, Sichuan Agricultural University; Collaborative Innovation Center of Ecological Security in the Upper Reaches of Yangtze River, Tan, Bo; Long-term Research Station of Alpine Forest Ecosystems, Institute of Ecology & Forest, Sichuan Agricultural University; Collaborative Innovation Center of Ecological Security in the Upper Reaches of Yangtze River, He, Wei; Long-term Research Station of Alpine Forest Ecosystems, Institute of Ecology & Forest, Sichuan Agricultural University; Collaborative Innovation Center of Ecological Security in the Upper Reaches of Yangtze River,
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Variations in bacterial communities during foliar litter decomposition in the winter and growing seasons in an alpine forest of the eastern Tibetan Plateau

Yeyi Zhao[1,2], Fuzhong Wu[1,2], Wanqin Yang[1,2]*, Bo Tan[1,2], Wei He[1,2]

[1]Long-term Research Station of Alpine Forest Ecosystems, Institute of Ecology & Forest, Sichuan Agricultural University, Chengdu 611130, China

[2]Collaborative Innovation Center of Ecological Security in the Upper Reaches of Yangtze River，Chengdu 611130, China

*Correspondence author: Wanqin Yang

E-mail: scyangwq@163.com

Tel: +86-28-86291112; Fax: +86-28-86290957

Yeyi Zhao, augustuszhao@gmail.com

Fuzhong Wu, wufzchina@163.com

Bo Tan, bobotan1984@163.com

Wei He, weihe19880408@gmail.com

Abstract Bacterial communities are the primary engineers during litter decomposition and related material cycling, and they can be strongly controlled by seasonal changes in temperature and other environmental factors. However, limited information is available on changes in the bacterial community from winter to the growing season as litter decomposition proceeds in cold climates. Here, we investigated the abundance and structure of bacterial communities using real-time quantitative PCR (q-PCR) and denaturing gradient gel electrophoresis (DGGE) during a two-year field study of the decomposition of four litter species in the winter and growing seasons of an alpine forest of the eastern Tibetan Plateau. Although the abundance of bacterial 16S rRNA gene during cypress and birch litter decomposition was relatively high in the first winter, the abundance of other litters was significantly lower relative to the growing season. A large number of bands were observed on the DGGE gels, and the intensities and number of the bands from the winter samples were lower compared with those from the growing season during the two-year decomposition experiment. Eighty-nine sequences from the bands of bacteria which had been cutted from DGGE gels were affiliated with 10 distinct classes of bacteria and an unknown group. A redundancy analysis indicated that the moisture, mass loss and elemental content (e.g., C, N, and P) of the litter significantly affected the bacterial communities. Collectively, the results suggest that uneven seasonal

changes in climate regulate bacterial communities and other decomposers, thus affecting their contribution to litter decomposition processes in the alpine forest.

Keywords alpine forest; bacterial community; DGGE; foliar litter decomposition; q-PCR

Introduction

Bacterial communities are the primary engineers during litter decomposition and are important in essential processes of biogeochemical cycles in terrestrial ecosystems (Jordan 1982; Aerts 1997; Didham 1998; Berg and McClaugherty 2008). However, bacteria are the most sensitive decomposer communities to changes in biotic and abiotic environments as microorganisms (Taylor et al. 2002; Young and Crawford 2004; Moscatelli et al. 2005; Monson et al. 2006). Minor changes in the environment often have strong effects on the structure and function of bacterial communities as well as on the decomposition of litter. Freezing temperatures in winter and warm temperatures in the growing season are the primary seasonal characteristics in cold climates; however, limited research has focused on seasonal changes in the bacterial community with litter decomposition.

The harsh environments in winter have long been considered to force microorganisms into dormancy or cause microorganism death (Uchida et al. 2005); thus, many studies have focused on bacterial communities in the growing season (Dilly et al. 2004; Korkama-Rajala et al. 2008; Thoms and Gleixner 2013). However, a growing number of cold-resistant bacterial groups were observed in winter beginning in the 1970s (Campbell et al. 2005; Zinger et al. 2009; Wang et al. 2010; Wilhelm et al. 2011). Therefore, it has been suggested that these active

bacterial populations might play an essential role in litter decomposition during winter.

Increasing evidence has shown that the loss of litter mass primarily occurs in the snow-covered season in cold biomes (Hobbie 1996; Aerts 1997, 2006; Konstantin 2010; Wu et al. 2013), which indicates that cold-adapted, cold-resistant and cryophilic microorganisms play important roles in litter decomposition in cold biomes. Recently, Zhu et al (2013) conducted a 2-year litter decomposition experiment along a subalpine forest gradient and demonstrated that the control of biological factors during litter decomposition in the growing season is significantly different from that in the non-growing season.

Cold biomes at high altitudes and latitudes are often characterized by obvious seasonal snow cover and freeze-thaw cycles. Baptist et al (2010) found that microorganisms and seasonal freeze-thaw cycles played significant roles in litter decomposition. Moreover, the biological activity of cold-adapted microorganisms has been detected in frozen soils (Clein and Schimel 1995), suggesting that litter decomposition in winter is primarily regulated by these microbes. During the growing season, favorable temperatures and humidity levels and adequate nutrient supply provide conditions for the proliferation and survival of most decomposers and lead to a diverse community structure that functions in the decomposition of litter. Compared with other microorganisms (such as

fungi, actinomycetes and protozoan), bacteria often displays a much stronger tolerance for cold temperatures and represent a major component of the microbial communities during litter decomposition in alpine regions (Neufeld et al. 2004; Mackelprang et al. 2011). Briefly, bacterial communities in foliar litter might regulate litter mass loss at different critical periods in the growing and non-growing seasons in cold biomes. However, limited research has focused on the relationships between litter mass loss and bacterial communities at different periods in cold biomes.

The alpine forests located along the upper reaches of the Yangtze River and eastern Tibetan Plateau play important roles in storing fresh water, conserving soil and water, nurturing biodiversity, regulating regional climate, sequestering carbon dioxide, and providing climate change indicators (Liu 2002; Yang et al. 2005, 2006, 2007). In previous studies, microbial diversity has been observed in completely frozen soils in winter through the use of denaturing gradient gel electrophoresis (DGGE) and PCR (Liu et al. 2010; Wang et al. 2010; 2012), and the mass loss of foliar litter in winter was 40 % to 65 % of the total loss for the year (Wu et al, 2013). Moreover, although significant changes have been observed in the diversity of bacteria along with climate dynamics and freeze-thaw patterns in soil (Wang et al. 2010; Wang 2012) and bacterial activity may directly contribute to the decomposition of litter, these relationships have received little attention. Based on previous studies, it is

hypothesized that the abundance, dominant groups and structure of bacterial communities in foliar litter change as litter decomposition proceeds, and these changes control the litter decomposition at different critical periods in the growing and non-growing seasons in alpine forest ecosystems.

To test these hypotheses, a field litterbag experiment was conducted in a Mingjiang fir (*Abies faxoniana*) primary forest. DGGE and real-time quantitative polymerase chain reaction (q-PCR) were used to evaluate the changes in bacterial abundance and community structure that occur during the decomposition of four different species of foliar litter throughout the winter and growing seasons over 2 years. The results of this study may clarify the mechanisms underlying bacterial decomposition of foliar litter and provide further insight into the process of foliar litter decomposition in alpine forests.

Materials and methods

Site description

The study was conducted in the Bipenggou Nature Reserve located in Lixian County in southwestern China (E 102º53′-102º57′, N 31º14′-31º19′; 2458-4619 m.a.s.l.), which is in the transitional area between the Sichuan Basin and Tibetan Plateau (Zhu et al. 2013). The mean annual temperature of the study site is 2-4 °C, the maximum

temperature is 23 °C, and the minimum temperature is -18.0 °C. The annual precipitation is approximately 850 mm. The soil is classified as Cambic Umbrisol (IUSS Working Group WRB 2007) and has a pH of 6.2 ± 0.3 (Gong et al. 2007). Snow cover remains for approximately 6 months in the winter season. The sample sites were located in Daxuetang (E 102°53′, N 31°15′; 3582 m.a.s.l.), which has an annual precipitation of approximately 801 mm and mean annual temperature of 2.9 °C. The canopy vegetation is dominated by fir (*Abies faxoniana*), birch (*Betula albo-sinensis*), larch (*Larix mastersiana*) and cypress (*Sabina saltuaria*). The shade density is approximately 0.7, and the average tree age is 130 years. The dominant understory plants are barberry (*Berberis sargentiana*), sedge (*Carex* spp.), fern (*Cystopteris montana*), sheep fescue (*Festuca ovina*), and azalea (*Rhododendron delavayi*) (Tan et al. 2014).

Foliar litter decomposition experiment

In September 2010, the fresh senescent foliar litter from larch, fir, cypress and birch was collected from the forest floor at the sample sites. The fresh foliar litter was air-dried for more than 2 weeks at room temperature, the air-dried litter was weighed to determine the moisture content, and the initial dry weight of the litter samples was determined by oven drying (65 °C, 48 h). Samples of air-dried foliar litter were placed inside nylon mesh bags (20 × 20 cm; 0.055-mm mesh sized nylon mesh

158 of bags' bottom side; 1.0-mm mesh size nylon mesh of bags' top side;
159 10.00 g per bag) (Keane 2008; Xia et al. 2011), and a total of 240
160 litterbags (3 sample plots × 4 species × 4 sample dates × 5 replicates)
161 were prepared. All of the litterbags with foliar litter were placed on the
162 forest floor under closed canopy in the sampled primary fir forests on
163 October 26, 2010, and intervals of at least 2-cm were placed between
164 each litterbag to avoid mutual disturbance upon collection (He et al. 2013;
165 Wu et al. 2013).

166 Based on previous studies, 3 sample plots (25 × 25 m) with similar
167 environmental characteristics were randomly selected within the sampled
168 primary fir forests, and the plots were separated by at least 100 m. To
169 determine the characteristics of bacterial abundance and community
170 structure in winter and the growing season during foliar litter
171 decomposition, the litterbags were collected on the following dates over
172 the 2-year experiment based on previous studies: March 3, 2011 (1st
173 winter stage, W1); August 19, 2011 (1st growing season, G1); March 7,
174 2012 (2nd winter, W2); and August 25, 2012 (2nd growing season, G2).
175 Five litterbags were randomly collected from each of the sample sites on
176 each sample date and then immediately transported to the laboratory in
177 cold storage. Additionally, Thermochron iButton DS1923-F5 loggers
178 (Maxim Integrated, San Jose, USA) were set up on October 26, 2010 to
179 record the litterbag and air temperature every 2 hours (Figure 1). The

positive accumulated temperature (PAT) and negative accumulated temperature (NAT) were calculated, and 0 °C was considered the normal threshold (Coakley et al. 1982) (Table 1).

Chemical analyses

Foreign materials such as roots and soil debris were carefully removed from the litterbags. The samples were divided into two parts, with one part used for the chemical analyses and mass loss measurements and the other part used for bacterial community analyses. The loss of foliar litter mass was calculated as follows: $M\ (g) = M_0 - M_t$, where M_0 is the dry litter mass when the bag was placed at the sample site (g) and M_t is the mass of the dry litter from the litterbag after it was removed from the site (g) (Zhu et al. 2012). The litterbag foliar litter was oven-dried at 65 °C for 48 h to a constant weight and then ground to pass through a 1-mm sieve to determine the total C, total N and total P as described by Lu (1999).

DNA extraction and PCR and DGGE analyses

DNA was extracted from approximately 0.3 g (dry weight) of litter using a DNA out kit (Tiandz, Beijing, China). The extracted DNA was purified through agarose electrophoresis using an E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Norcross, USA). The DNA purity was verified using electrophoresis on a 1 % agarose gel. For our community analysis, DNA was pooled in equimolar amounts from the five replicates at each

sample, species and sampling date combination to reduce the number of samples and the variation across replicates (Stone et al. 2015).

The primer pair 341f (5′-CCTACGGGAGGCAGCCAG-3′) and 534r (5′-ATTACCGCGGCTGCTGG-3′) was selected based on the conserved sequence of the V3 region of the 16S rRNA gene sequence to amplify the bacterial gene fragments. The purified genomic DNA was used as a template, and the 341f primer was modified with a GC-clamp: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC (Muyzer et al. 1993). To obtain the amplified target DNA, PCR was performed using the C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA) in 25 μL reactions that contained 12.5 μL Premix Ex Taq (TaKaRa, Dalian, China), 0.4 mg·mL^{-1} bovine serum albumin, 200 nmol·L^{-1} each primer, and 1.0 μL purified DNA (1-10 ng) as a template. The following conditions were used for the PCR: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 60 s, chain extension at 72 °C for 50 s, and a final extension step at 72 °C for 15 min. The GC-clamp product amplification was performed using a C.B.S. Denaturing Gradient Gel Electrophoresis System (C.B.S. Scientific Company, Inc.) according to the manufacturer's instructions. The electrophoresis was performed using 10 % acrylamide gels with a denaturing gradient of 35-65 %. The PCR products (25 μL) were used for each DGGE analysis. Acrylamide gels were run in 1× TAE buffer (40

224 mmol·L^{-1} Tris-HCl, 40 mmol·L^{-1} CH_3COOH, and 1 mmol·L^{-1} EDTA; pH
225 = 7.2) at 60 °C for 16 h at 100 V. The gels were stained with silver nitrate
226 and photographed with a GS-800^{TM} Calibrated Imaging Densitometer
227 (Bio-Rad), and the photographs were then digitized with Quantity One
228 4.0.1 (Bio-Rad) and the dominant DGGE bands were excised…..

229 **Phylogenetic analysis of bacterial genes**

230 Eighty-nine PCR products from the bands cut from the DGGE gels
231 were gel-purified and ligated into the pMD19-T vector (TaKaRa, Dalian,
232 China). The ligation products were cloned into *Escherichia coli* DH5α
233 competent cells according to the manufacturer's instructions. After the
234 recombinant clones were reamplified using the vector-specific primer pair
235 M13-47 and RV-M (TaKaRa, Dalian, China), the identity of the positive
236 clones was validated through sequencing and the National Center for
237 Biotechnology Information (NCBI) Basic Local Alignment Search Tool
238 (BLAST) program for each sample date. The 16S rRNA gene sequences
239 identified from the samples were compared with related 16S rRNA gene
240 sequences in GenBank using the NCBI BLAST program (Altschul et al.
241 1990). Based on the nucleotide sequences, the phylogenetic analysis was
242 performed using MEGA (molecular evolutionary genetics analysis)
243 version 4.0 (Tamura et al. 2007), and neighbor-joining phylogenetic trees
244 were constructed from dissimilar distance and pairwise comparisons with
245 the Kimura 2-parameter correction with 1000 replicates to produce the

bootstrap values.

Quantification of bacteria using q-PCR

The PCR-amplified products were gel-purified and cloned as described above. The ligation products were cloned into *Escherichia coli* DH5α competent cells according to the manufacturer's instructions. After the recombinant clones were reamplified using the vector-specific primer pair M13-47 and RV-M (TaKaRa, Dalian, China), the identity of the positive clones was validated through sequencing and the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program. The verified positive clones were selected for plasmid DNA extraction using an E.Z.N.A Plasmid Extraction Kit (Omega Bio-Tek), and these plasmids were used as the bacterial 16S rRNA gene standards. A ultraviolet-visible (UV-VIS) BioPhotometer (Eppendorf, Hamburg, Germany) was used to determine the concentration of plasmid DNA, and the number of bacterial 16S rRNA gene copies was directly calculated from the concentration of the extracted plasmid DNA (Okano et al. 2004). Tenfold serial dilutions of a known number of copies of the plasmid DNA that contained the sequenced bacterial 16S rRNA gene fragments were subjected to q-PCR in triplicate to generate an external standard curve.

Bacterial 16S rRNA gene from the purified foliar litter sample DNA was amplified by PCR using the primer pair Eub 338

(5′-ACTCCTACGGGAGGCAGCAG-3′) and Eub 518 (5′-ATTACCGCGGCTGCTGG-3′) (Lane 1991). The q-PCR was performed in 20 μL reactions that contained 12.5 μL SsoFast™ Eva Green Supermix (Bio-Rad), 0.4 $mg \cdot mL^{-1}$ bovine serum albumin, 200 $nmol \cdot L^{-1}$ each primer, and 1.0 μL purified foliar litter sample DNA (1-10 ng) as a template with the CFX96™ Real-Time system (Bio-Rad). Triplicate replicates were analyzed for each sample, and the optimized reaction conditions were as follows: pre-denaturation at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s. The plates were read at 72 °C after each cycle, and the product specificity was confirmed using a melting curve analysis (65-95 °C, 0.5 °C per read with a hold time of 5 s).

Statistical analyses

The digital images of the DGGE gels were obtained and analyzed using the Quantity One 4.0.1 software (Bio-Rad). Densitometric data obtained from the DGGE images were used to perform a principal component analysis (PCA) and multivariate redundancy analysis (RDA) with CANOCO 4.5 for Windows (Wageningen UR, Netherlands). Correlations between the bacterial abundance and foliar litter mass loss, litter elemental composition and environmental factors were analyzed using Pearson's correlation coefficients in SPSS statistical software (version 20.0, IBM, USA).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study were deposited in GenBank and were the following accession numbers: KJ 616619, KJ 616622-616624, KJ616640-KJ616652, KR 707618-KR707684.

Results

Bacterial abundance

Over the 2-year decomposition, the number of bacterial 16S rRNA gene copies was 1.34×10^6-1.26×10^9 g^{-1} dry litter for larch, 7.45×10^6-7.55×10^8 g^{-1} dry litter for fir, 2.99×10^7-6.49×10^9 g^{-1} dry litter for cypress and 1.92×10^8-1.47×10^{10} g^{-1} dry litter for birch (Figure 2). The abundance of bacterial 16S rRNA gene copies in the cypress and birch litter in winter was the highest at W1, whereas larch and fir litter had the highest abundance on G1, with this value subsequently decreasing to a minimum at W2 except for birch, which presented the lowest abundance at G2. The number of bacterial 16S rRNA gene copies was significantly correlated with the total P, C/N, C/P and N/P in all seasons, and temperature influenced the abundance of bacteria throughout the experiment (Table 2). In summary, the abundance of bacteria was lower in the 2^{nd} year compared with the 1^{st} year.

Diversity and dynamics of the bacterial community

Significant differences were observed in the structure of the bacterial

311 community in the four different types of foliar litter during the
312 decomposition process in winter and the growing seasons. The number
313 and intensity of the bands were lower in winter relative to the growing
314 season, and certain bands disappeared completely (Figure 3); however,
315 only the number of birch litter bands was greater in winter compared with
316 the other stages in the 2nd year (Table 3). In comparison between winter
317 and the growing seasons, the Shannon-Wiener indices were lower in
318 winter and inversely related to variations in the Simpson indices generally,
319 but there were inversely with cypress and birch in the 2nd years (Table 3).
320 To provide a more effective analysis of the bacterial community
321 composition at two different stages during foliar litter decomposition as
322 well as the DGGE band intensity, a PCA was performed to determine the
323 degree of similarity among samples in the winter and growing seasons.
324 The first two PCA factors accounted for 69.8 % of the variability (Figure
325 4), and variations were observed in the bacterial community between the
326 winter and growing seasons, although the communities were similar
327 during the foliar litter decomposition process. Additionally, the foliar
328 litter species acting as substrates affected the bacterial community.

329 **Phylogenetic analysis**

330 Using the sequences from the primary bands, all of the bacterial
331 sequences were grouped into ten distinct classes (Actinobacteria,
332 Alphaproteobacteria, Bacilli, Betaproteobacteria, Deltaproteobacteria,

Epsilonproteobacteria, Flavobacteria, Gammaproteobacteria, and Sphingobacteria) as well as an unclassified group based on neighbor-joining phylogenetic trees (Figure 5). The composition of these bacterial groups varied notably between the winter and growing seasons (Figure 6). Gammaproteobacteria dominated in winter largely in the larch and cypress litters, whereas Alphaproteobacteria and Sphingobacteria dominated in the 1st and 2nd growing seasons, respectively, in fir litter and were the dominant groups in the birch litter decomposition process, although their abundance was variable in the different stages. The bacterial community structure in winter was simpler than that in the growing season.

Redundancy analysis

The multivariate RDA showed that environmental factors and foliar litter elemental contents regulated the dynamics of the bacterial community structure (Figure 7), and the primary factor varied with the different stages of the litter decomposition process. In the 1st winter, the moisture was correlated with Alphaproteobacteria, mass loss was correlated with Betaproteobacteria, Gammaproteobacteria and Sphingobacteria, and the C/N ratio affected Flavobacteria. In the 2nd winter, moisture was the primary influencing factor on Betaproteobacteria, Flavobacteria and Sphingobacteria, and the mass loss and total C content were correlated with Alphaproteobacteria and Gammaproteobacteria,

355 respectively. In the 1st growing season, Actinobacteria, Bacilli,
356 Deltaproteobacteria and Epsilonproteobacteria were affected by the total
357 P content and C/P, Alphaproteobacteria and Gammaproteobacteria and
358 Flavobacteria were correlated with moisture, and Sphingobacteria were
359 affected by the total C content. Moreover, the Bacilli,
360 Epsilonproteobacteria and Flavobacteria were significantly affected by
361 the total N content, the moisture was correlated with the
362 Gammaproteobacteria and Sphingobacteria, C/P and the total C were
363 correlated with Actinobacteria and Alphaproteobacteria respectively.

364 **Discussion**

365 Two combined analysis methods were used here to explore the bacterial
366 abundance and community composition of four litter species throughout
367 2-year decomposition process in an alpine forest. Relatively abundant
368 groups of bacteria were detected during foliar litter decomposition in
369 winter, and bacterial abundance and community composition were
370 influenced by variations in the moisture and elemental contents in the
371 litter (C, N and P) in both the winter and growing seasons. These results
372 support our hypothesis that the abundance, dominant groups and structure
373 of bacterial communities in foliar litter change as litter decomposition
374 proceeds, and these changes control the litter decomposition at different
375 critical periods in the growing and non-growing seasons in alpine forest

ecosystems.

Bacteria survive in subzero temperatures and remain active at extreme cold temperatures (Carpenter et al, 2000; Junge et al, 2002). The bacterial community is an important part of the overall decomposer community and actively participates in the process of foliar litter decomposition. With continued foliar litter decomposition, seasonal variations in the number of bacterial 16S rRNA gene copies reflected the dynamics of bacterial abundance, and a statistical analysis of the q-PCR results indicated that variations in bacterial abundance were related to variability in environmental factors and elemental litter contents. The environment in winter, including freeze-thaw cycles, is particularly damaging to microorganisms (Walker et al, 2006) because of the formation of ice and reduction in soil solute concentrations, which result in protein denaturation, cell dehydration and low metabolic rates (Nedwell, 1999; Rodrigues and Tiedje, 2008). In general, low temperatures reduce microbial diversity and even kill certain bacteria species that have poor cold tolerance. Therefore, bacterial abundance should increase from winter to the growing season in cold regions (Deslippe et al. 2012), and higher abundances of bacteria should be observed during the growing season. Although the abundance of bacteria in larch and fir litter increased from winter to the growing season throughout the experiment, abundance was observed to decrease in the

cypress and birch litters from the 1st winter to the growing season, which may be explained by several possible underlying mechanisms. First, the lack of a snowpack during winter exposed the litter to extreme environmental conditions (e.g., large diurnal temperature changes); therefore, the region did not experience a relatively stable environment required to maintain bacterial activity in winter (Drotz et al. 2010; Bokhorst et al. 2010). Second, the foliar litter substrate on the forest floor, which lacked the protection of the snowpack, experienced increased mechanical disruption from the freeze events and freeze-thaw cycles in the winter, which resulted in an improved substrate environment for bacteria because of the greater availability of microbial substrates in the subsequent growing season (Berg 2000; Groffman et al. 2001; Herrmann and Witter 2002; Hentschel et al. 2008). Third, after the 1st year of decomposition, labile constituents were decomposed, which reduced the availability of nutrients required to sustain bacterial activities in the subsequent year (Dijkstra et al, 2011; Cotrufo et al, 2013). Fourth, the available nutrients decreased with the litter mass loss and might have been leached into the soil by rainfall (Wu et al, 2013); in addition, this loss may have been greater with the broad-leaved litter compared with the needle litter, which could explain the reduced abundance of bacteria in birch litter in the 2nd year of this study (Neff and Asner 2001; Park and Matzner 2003; Müller et al. 2009).

The diversity indices and bacterial group variations were direct indicators of bacterial community responses to the decomposition process of foliar litter. The Shannon-Wiener indices and Simpson indices were notably lower and higher, respectively, in winter compared with the growing season, and the values were consistent with the results for bacterial abundance. Although a large number of bacteria might have been killed and the Shannon-Wiener diversity index of the bacterial community was notably reduced in winter, certain bacterial species might increase their predominance in the community because of high tolerance for harsh environmental conditions (Walker et al. 2006; Wilson and Walker 2010). In addition, broad-leaved litter under freeze-thaw cycles can provide additional substrates for bacterial groups in winter, and improved environmental conditions, including sufficient moisture and mechanical disruptions of the litter caused by repeated freeze-thaw cycles, provide abundant substrates in the growing season and a good environment for bacterial communities (Lipson and Schmidt 2004); however, the low exchange of nutrients and water availability with longer periods of winter may present a greater stress to bacteria (Morozova and Wagner, 2007). Changes in the relative abundance of the bacterial groups during foliar litter decomposition (Figure 6) highlight the importance of understanding the relationships between changes in bacterial communities and environmental factors. The elemental contents, litter mass loss and

temperature during foliar litter decomposition might change the bacterial community structure. Based on a comparison of the results with the NCBI, the bacterial groups included Actinobacteria, Alphaproteobacteria, Bacilli, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Flavobacteria, Gammaproteobacteria, and Sphingobacteria. The bands from the winter DGGE gels were consistent with *Pseudomonas* (Gammaproteobacteria) in foliar litter, which was detected from alpine and glacial environments in other studies, indicating that these species can better tolerate the harsh environmental conditions during winter (Shivaji et al. 2004; Jiang et al. 2006; Zinger et al. 2009; Swan et al. 2010). However, Gammaproteobacteria reduced in the broad-leaf birch litter in this present study. Moreover, the bacterial communities in the subsequent growing season and winter were abundant, which suggests that the surviving and cold-active bacteria, which were masked by the more abundant species, adjusted their metabolic profiles (Nedwell, 1999; Schimel et al, 2007) in the subsequent growing season and winter. The multivariate RDA was used to illustrate the relationships between the bacterial community structure and environmental factors during foliar litter decomposition (Figure 7). The elemental contents, mass loss and moisture affected the bacterial community structure, although various primary factors affected the dominant groups of bacteria. In the 1st winter, the mass loss and elemental contents of the litter were the primary factors

limiting Gammaproteobacteria, which was the primary bacterial group. In the 2nd year, the moisture and elemental contents were the primary factors that affected the dominant groups. In the growing season, however, the dominant groups of bacteria were correlated with moisture (Figure 7).

In summary, the relatively rich abundance of bacteria detected in winter was notable and highlighted changes in the bacterial community structure between the winter and growing seasons during foliar litter decomposition. Changes in the loss of mass, moisture, and elemental contents in foliar litter are significantly related to the abundance and community composition of bacteria. Foliar litter substrates and environmental factors affected the bacterial community structure, which might have significance for understanding the process of foliar litter decomposition in the cold biomes. In the future, we should pay more attention to seasonal fluctuations of microorganisms and their related influencing factors as litter decomposition proceeds in the alpine forest. In addition to that, further more advanced methods should be used to study the microorganisms inorder to clearly understand their abundance, distribution and functions in litter decomposition.

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Figure captions

Figure 1. Dynamics of soil and air temperature during foliar litter decomposition from October 26, 2010 to October 25, 2012.

Figure 2. Changes in the number of copies g^{-1} dry matter litter of the bacterial 16S rRNA gene with foliar litter decomposition. Error bars indicate the standard error of the mean (SE) of triplicate q-PCR reactions, and different letters denote significant differences between different stages.

Figure 3. Denaturing gradient gel electrophoresis (DGGE) profiles of bacterial communities at different stages of foliar litter decomposition. M, marker.

Figure 4. Principal component analysis using DGGE band intensities. Points represent individual samples with different times, and the diameter of each point is proportional to the measured Shannon-Wiener index value. Abbreviations: L, Larch; F, Fir; C, Cypress; B, Birch.

Figure 5. Neighbor-joining phylogenetic tree based on bacterial 16S rRNA gene sequences derived from the DGGE fingerprint profiles. The numbers at branch points indicate bootstrap percentages of the Kimura 2-parameter.

Figure 6. Relative abundance of the bacterial groups as foliar litter decomposition proceeds.

Figure 7. RDA ordination diagrams for the first two dimensions of the relationship with the bacterial community structure at the class level.

Table 1. Chemical characteristics and temperature dynamics of foliar litter decomposition.

		Day	Moisture (%)	Mass Loss (g)	TC ($g \cdot kg^{-1}$)	TN ($g \cdot kg^{-1}$)	TP ($g \cdot kg^{-1}$)	C/N	C/P	N/P	PAT (℃)	NAT (℃)	Du-AT (℃)	Air (℃)
Larch	W1	1	31.48±8.69	1.26±0.19	512.64±7.03	8.60±0.07	2.18±0.11	59.62±0.58	235.10±9.35	3.94±0.19	0.00	-241.50	-3.47	-5.32
	G1	170	35.32±14.87	2.47±0.18	495.29±39.59	8.40±0.15	2.14±0.16	58.95±3.70	231.00±2.71	3.93±0.23	938.53	0.00	6.67	9.12
	W2	371	31.80±11.41	3.62±0.04	499.56±12.84	7.73±0.55	2.12±0.11	64.82±3.23	236.40±8.00	3.65±0.25	0.00	-230.23	-3.48	-5.74
	G2	542	20.83±4.18	3.93±0.07	464.84±1.58	8.18±0.11	2.11±0.03	56.81±0.89	220.41±3.30	3.88±0.03	1000.60	0.00	8.11	8.65
Fir	W1	1	26.29±8.14	0.84±0.14	486.46±5.19	10.59±0.15	1.35±0.03	45.96±1.14	359.61±11.21	7.83±0.19	0.00	-241.50	-3.47	-5.32
	G1	170	60.00±6.03	2.10±0.23	479.6±24.04	11.24±0.03	1.36±0.06	42.62±2.17	352.74±31.75	8.27±0.35	938.53	0.00	6.67	9.12
	W2	371	60.82±2.00	3.02±0.06	454.02±12.08	10.98±0.24	1.16±0.02	41.34±0.20	391.14±5.04	9.46±0.08	0.00	-230.23	-3.48	-5.74
	G2	542	65.52±0.67	3.28±0.06	429.05±0.84	10.9±0.09	1.15±0.02	39.36±0.38	373.77±8.66	9.50±0.14	1000.60	0.00	8.11	8.65
Cypress	W1	1	17.66±6.91	0.84±0.19	492.12±7.28	9.82±0.15	1.45±0.00	50.13±0.05	339.45±5.72	6.77±0.12	0.00	-241.50	-3.47	-5.32
	G1	170	48.58±18.79	2.27±0.18	501.53±0.91	9.61±0.03	1.40±0.05	52.20±0.56	357.68±14.75	6.85±0.21	938.53	0.00	6.67	9.12
	W2	371	40.79±9.13	3.41±0.30	476.71±20.23	9.44±0.23	1.42±0.07	50.51±0.94	335.40±3.92	6.64±0.19	0.00	-230.23	-3.48	-5.74
	G2	542	15.60±1.53	3.90±0.06	447.98±30.13	9.30±0.16	1.45±0.12	48.14±2.67	308.60±6.17	6.43±0.44	1000.60	0.00	8.11	8.65
Birch	W1	1	20.83±4.18	1.05±0.12	465.58±14.47	14.07±0.13	0.90±0.04	33.08±0.72	517.66±39.73	15.63±0.86	0.00	-241.50	-3.47	-5.32
	G1	170	65.52±0.67	2.79±0.19	439.72±1.68	14.43±0.37	0.97±0.01	30.49±0.88	452.53±6.71	14.85±0.48	938.53	0.00	6.67	9.12
	W2	371	15.60±1.53	4.10±0.09	426.67±21.67	14.52±1.20	0.97±0.07	29.44±1.06	441.36±22.06	15.02±1.17	0.00	-230.23	-3.48	-5.74
	G2	542	68.86±2.08	4.45±0.08	420.58±16.37	15.21±0.94	1.04±0.4	27.67±0.75	404.32±16.20	14.63±0.97	1000.60	0.00	8.11	8.65

Abbreviations: W1, 1st winter; G1, 1st growing season; W2, 2nd winter; G2, 2nd growing season; TC, total organic carbon; TN, total nitrogen; TP, total phosphorus; PAT, positive accumulated temperature; NAT, negative accumulated temperature; Du-AT, duration average temperature.

Table 2. Correlation analyses among the abundance of bacteria, C, N, P, degree-days, and average temperature.

	W1	G1	W2	G2	Whole Research
Log ratio of bacteria	0.801**	0.892**	0.794**	0.930**	0.627**
Moisture	-0.439*	-0.698**	-0.414	0.013	-0.361**
Mass Loss	-0.103	0.150	0.799**	-0.174	-0.479**
TC	-0.610**	0.383	-0.618*	0.413*	0.081
TN	0.740**	-0.398	0.870**	-0.484*	0.230*
TP	-0.636**	0.722**	-0.594*	0.566**	-0.240*
C/N	-0.693**	0.477*	-0.754**	0.545**	-0.182
C/P	0.726**	-0.643**	0.665**	-0.551**	0.409**
N/P	0.754**	-0.472*	0.825**	-0.507*	0.309**
PAT	-	-	-	-	-0.340**
NAT	-	-	-	-	-0.353**
Du-AT	-	-	-	-	-0.339**
Air	-	-	-	-	-0.331**

*, Correlation is significant at the 0.01 level (two-tailed). **, Correlation is significant at the 0.05 level (two-tailed).

Table 3. Bacterial diversity index of foliar litter decomposition.

		Bands number	Shannon-Wiener Index	Simpson Index
Larch	W1	11	2.387	0.093
	G1	39	3.584	0.029
	W2	11	2.348	0.099
	G2	18	2.865	0.058
Fir	W1	13	2.456	0.093
	G1	41	3.684	0.026
	W2	23	3.046	0.051
	G2	32	3.413	0.034
Cypress	W1	17	2.571	0.093
	G1	34	3.490	0.031
	W2	29	3.253	0.043
	G2	22	3.060	0.048
Birch	W1	17	2.786	0.064
	G1	29	3.318	0.037
	W2	41	3.645	0.028
	G2	29	3.312	0.038

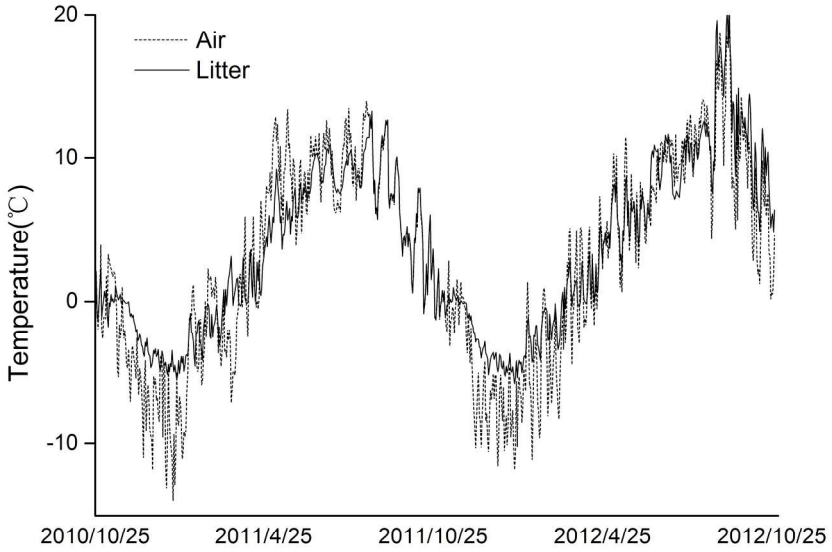

Figure 1. Dynamics of soil and air temperature during foliar litter decomposition from October 26, 2010 to October 25, 2012.
202x141mm (300 x 300 DPI)

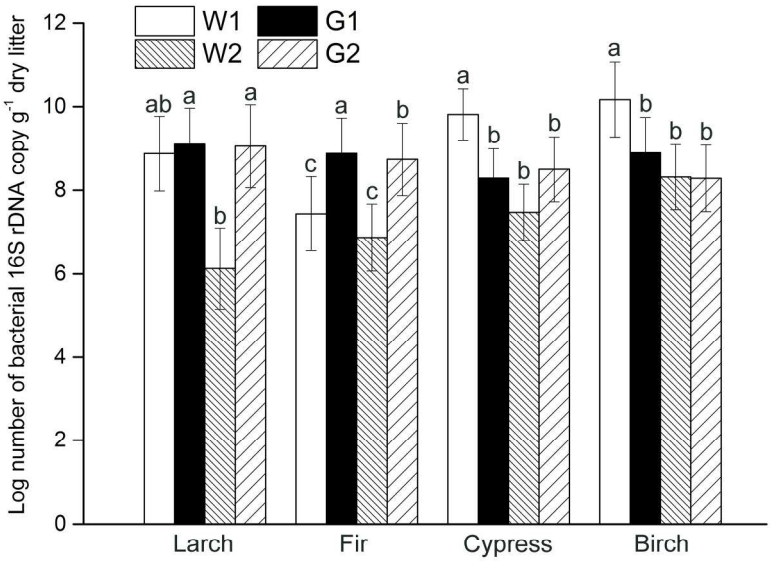

Figure 2. Changes in the number of copies g−1 dry matter litter of the bacterial 16S rRNA gene with foliar litter decomposition. Error bars indicate the standard error of the mean (SE) of triplicate q-PCR reactions, and different litters denote significant differences between different stages.
202x141mm (300 x 300 DPI)

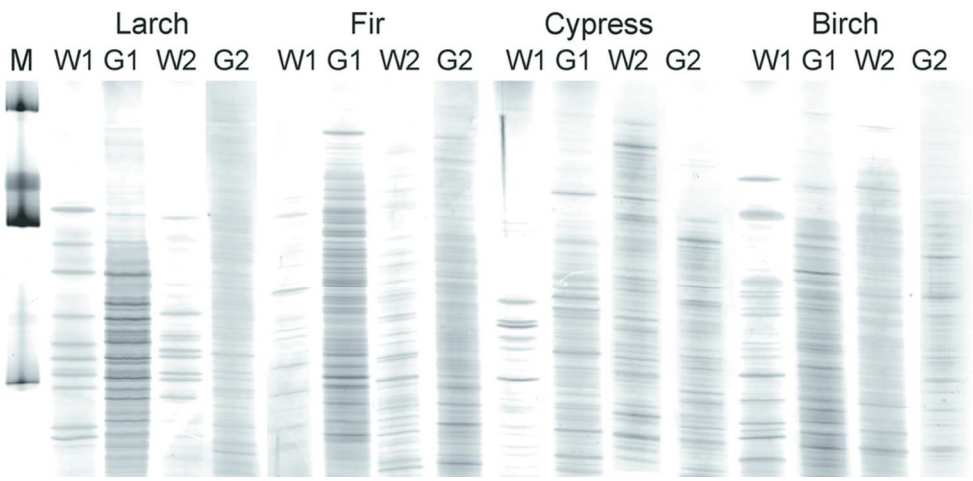

Figure 3. Denaturing gradient gel electrophoresis (DGGE) profiles of bacterial communities at different stages of foliar litter decomposition. M, marker.
89x42mm (300 x 300 DPI)

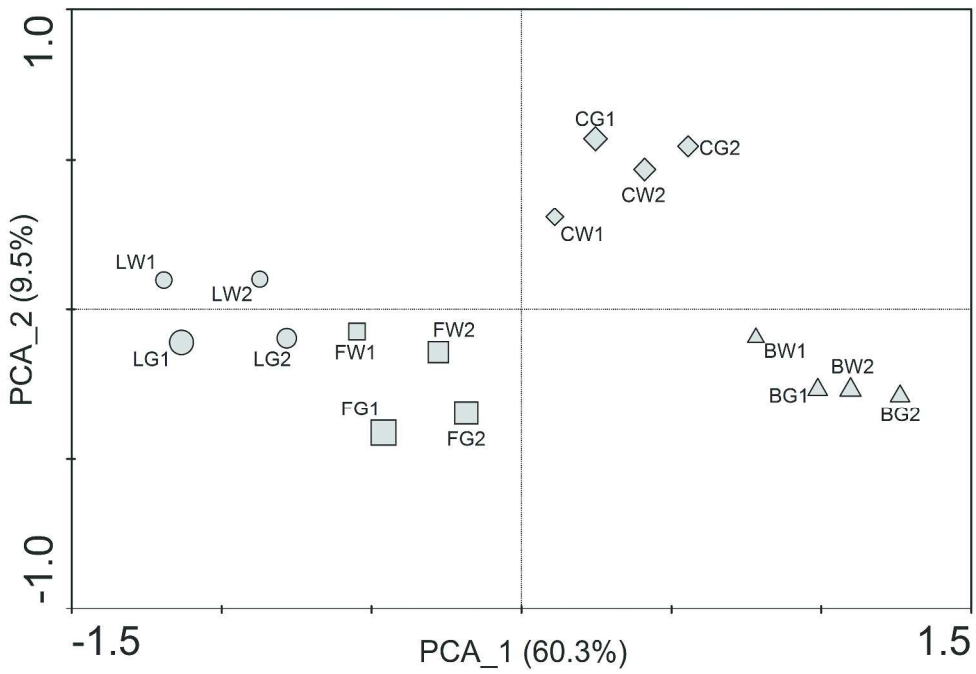

Figure 4. Principal component analysis using DGGE band intensities. Points represent individual samples with different times, and the diameter of each point is proportional to the measured Shannon-Wiener index value. Abbreviations: L, Larch; F, Fir; C, Cypress; B, Birch.
2414x1690mm (72 x 72 DPI)

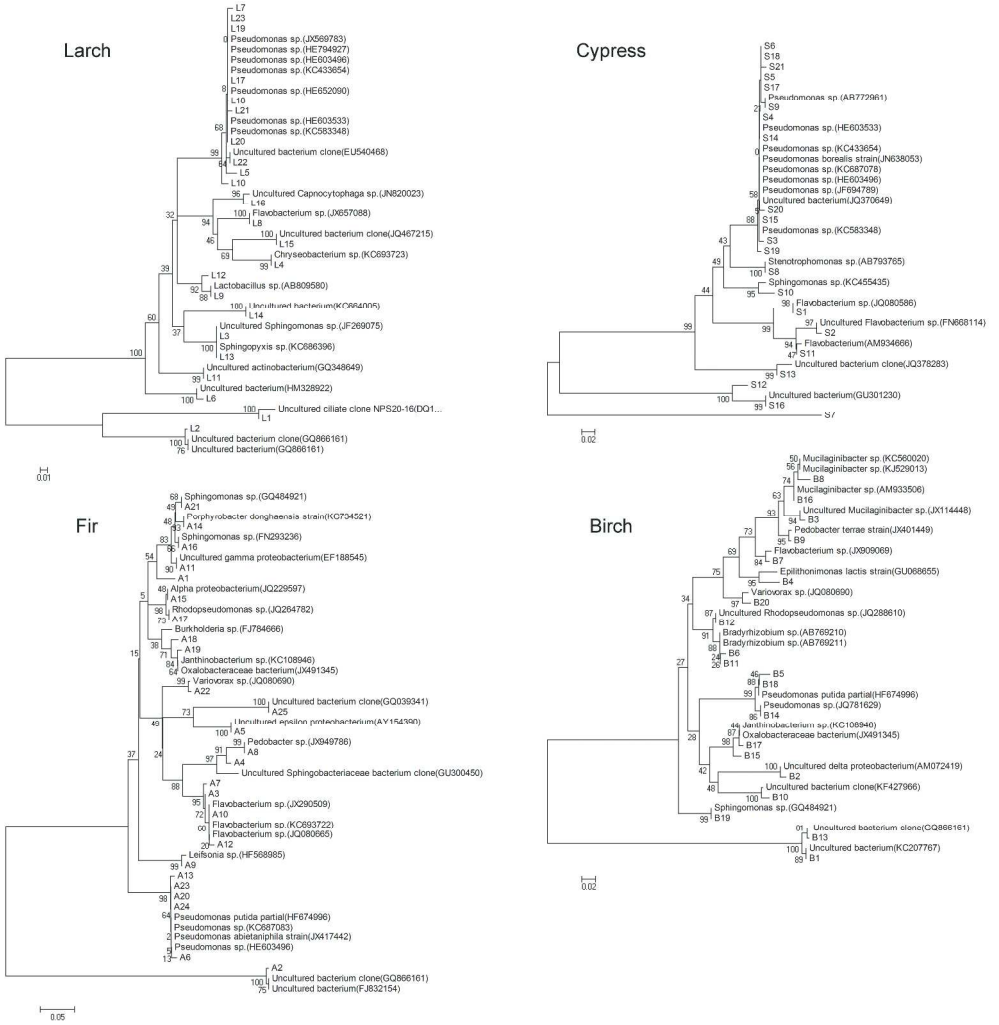

Figure 5. Neighbor-joining phylogenetic tree based on bacterial 16S rRNA gene sequences derived from the DGGE fingerprint profiles. The numbers at branch points indicate bootstrap percentages of the Kimura 2-parameter.
385x400mm (300 x 300 DPI)

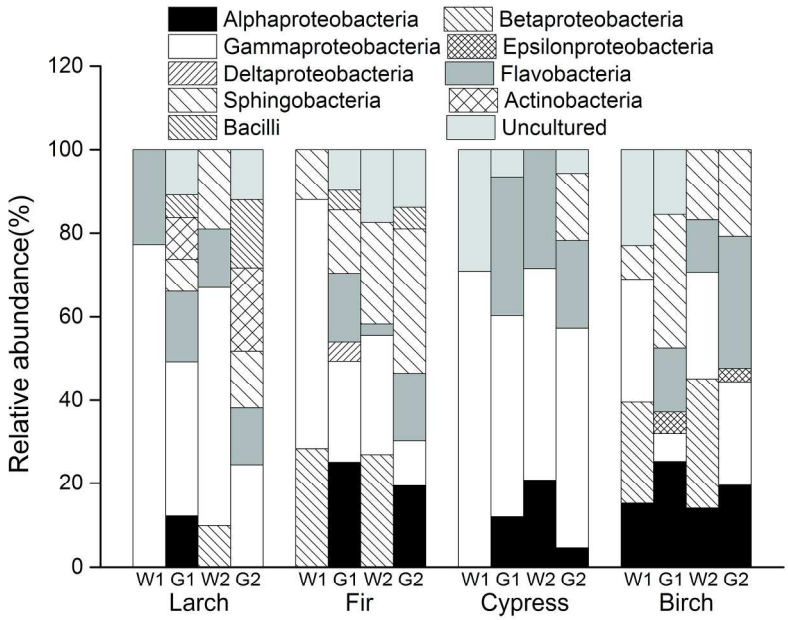

Figure 6. Relative abundance of the bacterial groups as foliar litter decomposition proceeds.
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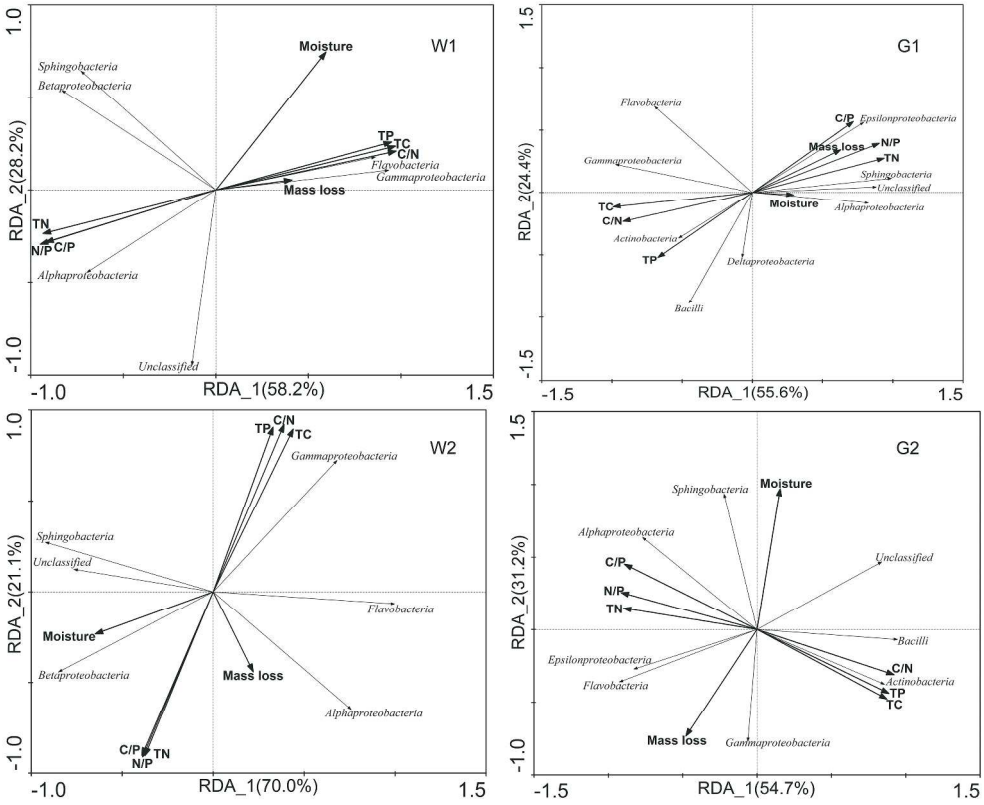

Figure 7. RDA ordination diagrams for the first two dimensions of the relationship with the bacterial community structure at the class level.
1164x948mm (72 x 72 DPI)