Succession of litter-decomposing microbial organisms in deciduous birch and oak forests, northern Japan

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Abstract

Biological litter decomposition and the litter-associated microbial organisms were monitored for three years to characterize litter decomposition in early and late successional stages. Two forests were used for the investigation: pioneer a forest dominated by birch (*Betula platyphylla* var. *japonica*) and a climax forest by oak (*Quercus mongolica* var. *grosseserrata*) in the cool-temperate region of northern Japan. Three types of litter were used: birch, oak and mixed litter. The litter decomposition was effective during the first year but 50% of the original litter remained even after three years. Carbon-to-nitrogen ratios in the litter decreased largely in the first year and became stable thereafter. The litter decomposition rates were not different among the litter types and between the forests. The temporal changes in phospholipid fatty acids (PLFAs) showed that fungal biomass reached its peak in the first year and the bacterial biomass increased steadily until the end of the experiment. The concentrations of fungal PLFAs in the litter did not differ between the litter types but were lower in the oak forest. The litter decomposition was performed mostly by fungi, in particular in the early stages, while bacterial decomposition depended on the litter types and/or the forest types. Gram-negative bacteria reached their peak of PLFAs in the second year while gram-positive bacteria PLFAs increased gradually during the three years. Therefore, the succession of microorganisms in the litter occurred from fungi to bacteria and from gram-negative bacteria to gram-positive bacteria in the two forests. Unlike in the case of coniferous or monotonic forests, the effects of forests and litter types on litter decomposition for the first year were weak. The forest types on litter decomposition appeared only for the long-term litter decomposition. The successional changes of microorganisms occurred from fungi to bacteria for long-term litter decomposition processes with increasing N concentration in the litter.

Keywords: Microbial succession · Leaf litter decomposition · Bacterial and fungal ratio · Carbon to nitrogen ratio · Phospholipid fatty acids (PLFAs) · broad-leaved forest

Abbreviations: PLFAs, phospholipid fatty acids; PPFD, photosynthetic photon flux density; C/N ratio, carbon-to-nitrogen ratio; B/F ratio, bacteria to fungi ratio; GLM, generalized linear model; GLMM, generalized linear mixed-effects model; CCA, canonical correspondence analysis; HFA, home-field advantage

1. Introduction

Litter decomposition determines N and C dynamics in terrestrial ecosystems and is promoted by three processes, physical, chemical and biological degradation (Wardle et al., 2004; Hobbie, 1992). Of these processes, the biological decomposition, conducted mostly by bacteria and fungi, affects C/N flux between forest floor and trees (Lladó et al., 2017) and controls the fertility of forest ecosystems (Osono, 2007; Møller et al., 1999).

The litter decomposition by bacteria and fungi is regulated by the micro-climate, influenced by temperature, precipitation, vegetation and litter type (Parton et al., 2007). In addition, forest structures affect the composition of fungal and bacterial communities in the soils (Prescott and Grayston, 2013) and in the litter (Urbanová et al., 2015). Litter decomposers regulate C and N consumption in the early litter decomposition stages, while moisture and legacy in the soils play a major role in the later stages (García-Palacios et al., 2016). Since litter decomposition is slow in cool-temperate region, to investigate the microbial activities in the litter, long-term monitoring is required. However, few studies have been conducted for long term in this study area so far.

The composition of microorganisms involved in litter decomposition varies depending on the stage of decomposition. The fungi/bacteria ratio decreases greatly in the first eight months in the oak forests of Czech Republic (Voříšková et al., 2013). The diversities of bacteria and fungi change with succession (Tláskal et al., 2016; Purahong et al., 2016), although the relationships between bacteria and fungi are unclear. In the cool temperate regions of Japan, the dominant species along a successional sere change from birch to oak. This implies that litter mixed with birch and oak are often developed. During vegetation succession in temperate and cool-temperate regions, including Japan, birches (*Betula*) often develop pioneer forests and oaks (*Quercus*) form later and/or climax forests (Ishikawa and Ito, 1988). These two forest types are broadly distributed in Japan (Krestov et al., 2015). Therefore, the comparisons of these forests are invaluable to understand successional changes in litter decomposition and microbial composition. Furthermore, the conservation

of these forests provides diverse ecosystem services, such as forestry, recreation and education, including learning succession (Mölder et al., 2019; Yokoyama and Tsuyuzaki, 2015). When the succession of microorganisms is observed in relation to litter decomposition as well as vegetation succession, these changes are applied to the evaluation of ecosystem health.

The home-field advantage (HFA) hypothesis suggests that litter decomposition is encouraged by litter mixed from only a few species. This hypothesis is examined well by litter mixed with broad-and needle-leaved leaves (Gartner and Cardon, 2004; Asplund et al., 2018; Ayres et al., 2009). However, this hypothesis has not been examined well in broad-leaved forests where the litter should be made of various broad-leaved plants (Gao et al. 2016). Since the litter components are different between broad-leaved and mixed forests, the different HFA effects are expected in broad-leaved forests. Also, the succession of microorganisms in these forests has not been investigated well. Therefore, the decomposition of litter consisting of birch, oak and their mixture were monitored.

Litterbag experiments are often used to investigate litter decomposition and its related factors (Wieder and Lang, 1982; Kazakou et al., 2006). In addition, biological litter decomposition is commonly estimated by indicators of biomass and composition of microorganisms, represented by quality and quantity of phospholipid fatty acids (PLFAs) (Chapman and Newman, 2010; Šnajdr et al., 2011). The concentrations of carbon (C) and nitrogen (N) in litter show the litter quality and N/C fluxes (Berg and McClaugherty, 2008). The present study provides new insights into microbial succession by using these techniques with considering differences in litter quality in cool-temperate regions. We monitored the successional changes in the biomass of each microbial taxon and litter decomposition at two forest sites of mixed forest examining birch and oak litter to investigate how the litter decomposition and the microbial succession develops and changes over time. Using these surveys, two hypotheses were examined: (1) HFA effects were weak to decompose litter in broad-leaved forests, as compared with mixture of broad- and needle-leaved litter, because of the similarity of litter species. (2) Microbial succession

occurred during the litter decomposition for a few years with changes in nitrogen and carbon concentrations in the litter.

2. Materials and methods

2.1. Study sites

The study was conducted in a lowland at the foot of Mount Toishi (826 m a.s.l.) in the city of Sapporo, Hokkaido, northern Japan. The survey was conducted for three years (33 months) from December 2009 to August 2012. The mean annual temperature was 9.3-9.8°C during the surveyed period at the distance of 8 km from the study site (Japan Meteorological Agency, 2012). The maximum daily temperature was recorded as 34.1°C in August 2010 and the minimum was at -12.6°C in February 2010. The annual precipitation during the three years was between 1254 mm and 1325 mm, of which 21% was supplied by snow during November and April.

Birch and oak forests were selected for the study. The birch forest was dominated by *Betula platyphylla* Sukaczev var. *japonica* (Miq.) Hara that is characteristic to the early successional stages (Bradshaw et al., 2005). The oak forest was dominated by *Quercus mongolica* Fisch. ex Ledeb. var. *grosseserrata* Rehd. Wils. and represented late successional stages developed after the birch forest stage as the climax. These two species are widespread in the temperate eastern Asia, including Japan (Kitao et al., 2000). The soil type classified as brown forest soil in this region (Kanda et al., 2016).

A 15 m × 15 m plot was established in both the oak and birch forests (42°59'N and 141°19'E, 130-140 m elevation). The two forests were adjacent to each other. In each plot, stem height and diameter at breast height (DBH) were measured on each tree taller than 5 m. The stem volumes were calculated from the height and diameter with an assumption of cylindrical shape. The results were as follows: The oak forest had 818 m^3/ha with 800 stems/ha. The oak explained 75% of the stem volume. The birch forest had 877 m^3/ha with

1200 stems/ha. The birch explained 80% of the stem volume. The species composition of the herbaceous layer below 2 m was similar between the two forests.

Soil moisture was measured at nine points in each forest at two-month interval from April to November during snow-free periods by a time domain reflectometry with 12 cm electrode (Hydrosense, Campbell Scientific Australia, Queensland). The soil moisture was 28-29% in both the forests (Table 1). The mean ground-surface temperatures were slightly higher in the oak forest. The mean soil pH was measured by a glass electrode pH meter (PH-222, Kenis Limited, Osaka). and was slightly acidic, with pH of 6.5 ± 0.8 (mean ± standard deviation).

Litter supply in the birch and oak forests was measured by litter collected from nine 25 × 25 cm^2 plots in each forest in November 2014 soon after the defoliation. The litter was air-dried for two weeks and weighed. The litter supply averaged 192 ± 54 $g/m^2/yr$ in the oak forest and 190 ± 46 $g/m^2/yr$ in the birch forest (Table 1). The compositions of birch and oak litter were 50% and 38% in the birch forest and 13% and 60% in the oak forest, respectively. Since the oak forest was adjacent to the birch forest, the birch litter should be easily immigrated to the oak forest. Other species in the litter were *Kalopanax pictus*, *Cornus controversa*, etc. with low dominance, less than 27% share.

Table 1 (a) Environmental characteristics (canopy openness, light intensity, temperature and soil moisture) in the birch and oak forests during snow-free periods from 2010 to 2012. Means are shown with standard deviations. Different letters show significant difference between the two forests at $p < 0.05$ (GLMM) (b) Litter supply (g/m^2) in the birch and oak forests. Mean is shown with standard deviation. Different letters show significantly different at $P < 0.05$ (GLM)

(a)

Forest	Canopy openness (%)		PPFD (mmol m^{-2} sec^{-1})		Temperature (°C)		Soil moisture (%)	
Birch	23 ± 2	a	64.6 ± 99.1	a	11.9 ± 9.1	a	29 ± 10	a
Oak	22 ± 3	a	84.9 ± 136.9	b	12.4 ± 10.6	b	28 ± 9	a

(b)

	Litter			
Forest	Birch	Oak	Others	Total
Birch	94 ± 33 a	73 ± 29 c	24 ± 12 e	190 ± 46 g
Oak	25 ± 20 b	114 ± 35 d	53 ± 25 f	192 ± 54 g

175

176 *2.2. Litter decomposition*

177

178 The litter decomposition was measured by litterbags made of black sheer nets (2 mm
179 pore) with 20 cm length and 12 cm width. The litter produced in the autumn of 2009 were
180 collected from the ground surface adjacent to the two surveyed sites in late November 2009
181 when most leaves defoliated to avoid disturbance. The samples were air-dried for more
182 than two weeks. Three litter types were formed to investigate litter-mixing effects on litter
183 decomposition: 5 g of oak litter, 6 g of birch litter and 6 g of litter mixed with 3 g of birch
184 and 3 g of oak litter. In total, 288 litterbags were set up on the ground surface of each forest
185 in mid-December 2009. During snow free periods from April 2010 to August 2012, three
186 litterbags were randomly collected from each litter type from both forests. The total number
187 of litterbag recoveries was 11 times during the three years, i.e. April, June, August and
188 October in 2010, April, June, August and December in 2011, April, June and August in
189 2012.Litter decomposition constant (k) at each sampling date on each sample was
190 calculated by $\log(X/X_0) = -kt$, where X is litter remaining mass at t days after setting
191 litterbags and, X_0 is the litter mass at $t = 0$ (Olson, 1963).

192

193 *2.3. Measurements of chemical properties in litter*

194 The litter samples recovered from the fields were freeze-dried over seven days soon
195 after the collection and were weighed to measure the remaining litter mass that was
196 calculated as: (litter remaining weight) divided by (initial litter weight). Then, the litter
197 samples were grinded in a mill with a metal blade. Carbon (C) content and nitrogen (N)
198 content were measured with a stable isotope ratio mass spectrometer (Finnigan MAT252,
199 Thermo Fisher Scientific, Yokohama) (Coetsee et al., 2010). Carbon-to-nitrogen ratio
200 (C/N) was calculated, as this ratio often decreases with proceeding litter decomposition
201 (Aerts and de Caluwe, 1997).

202

203 *2.4. PLFA analysis*

204 To estimate the bacterial and fungal biomass in each litterbag, PLFAs in the litter were
205 identified and quantified by using phospholipids extracted from the litter under a gas
206 chromatography (G-3000 Gas Chromatograph, Hitachi, Tokyo) and a gas chromatograph-
207 mass spectrometer (Varian Saturn 2200, Agilent Technologies, Santa Clara). The detailed
208 procedures are described in a previous paper (Otaki et al., 2016).

209 The taxon-specific PLFAs are: i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10Me-
210 16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0 produced only by
211 bacteria and 18:2 only by fungi (Šnajdr et al., 2011). Almost all of 18:2 in litter is derived
212 from fungi, because 18:2 derived from plants vanishes soon after defoliation (Laczko et al.,
213 2004). PLFA of 16:1ω7 was treated as the production of bacteria, as it is characteristic to
214 bacterial production (Graham et al., 1995).

215 The bacterial PLFAs were divided into three groups by the origins: 10Me-16:0, 10Me-
216 17:0 and 10Me-18:0 produced by actinomycetes, i15:0, a15:0, i17:0 and a17:0 by gram-
217 positive bacteria and cy17:0 and 16:1ω7 by gram-negative bacteria (Moore-Kucera and
218 Dick, 2008). The PLFAs produced by gram-positive bacteria, gram-negative bacteria and
219 actinomycetes were summed and were used as total bacterial PLFAs. The other PLFAs
220 produced by plants, bacteria and/or fungi were treated as PLFAs produced by
221 miscellaneous organisms. Bacteria to fungi (B/F) ratio was calculated based on the
222 composition of PLFAs to investigate the microbial dominance of litter decomposition.
223 Because a few samples showed no bacterial PLFAs, B/F ratio was used instead of F/B ratio.

224

225 *2.5. Statistical analysis*

226 The differences and interactions in remaining litter mass, C, N, C/N ratios and PLFAs
227 were examined between the litter types and between the forests by a generalized linear
228 mixed-effects model (GLMM) with number of days passed after litterbag establishment as
229 a random effect. Steel-Dwass test was used for multiple comparisons of the differences in
230 remaining litter mass and litter decomposition rates between the surveyed dates. χ^2-test was

used to confirm the differences in the amounts of PLFAs of fungi and bacteria with increasing time. All the significance levels were set at $P < 0.05$.

Canonical correspondence analysis (CCA) was used to investigate the distribution characteristics of PLFAs in the litter. A taxon matrix was made according to the PLFAs produced by fungi and bacteria in each litterbag. The environmental matrix consisted of four factors: litter types, forest types, days after establishing litterbags and litter remaining ratios. The days and liter remaining ratios were numerical variables and litter types and forest types were categorical. Monte Carlo Permutation test was conducted to confirm the significance of CCA. All the statistical analyses were performed by a software package R (ver. 3.1.3) (Ihara and Gentleman, 1996) with vegan library (Oksanen et al., 2015).

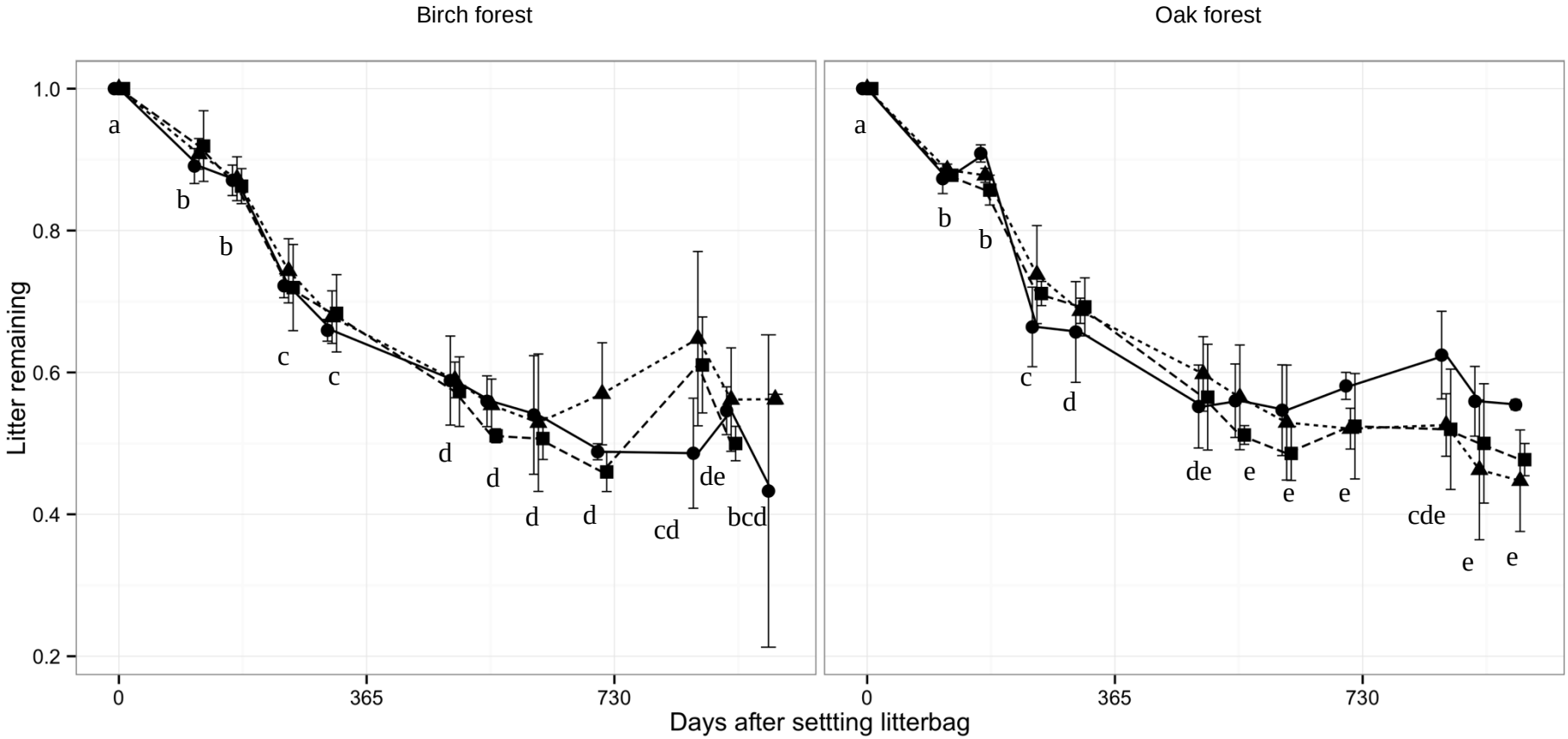
3. Results

3.1. Litter decomposition

N content of the initial litter prior to the decomposition, i.e., 0 days, was 0.81% and 0.68% in birch and oak litter, respectively. N was significantly higher in the birch litter than in the oak litter (GLM, $P < 0.05$) in the initial litter the C concentration was higher in the birch litter (42.7%) than in the oak litter (38.7%) ($P < 0.05$). C/N ratios were 52.8 and 57.3 in the litter of birch and oak, respectively, and were not different between the litter types ($P > 0.05$). The quantitative differences in the initial litter between the two species were derived mostly by the N concentration.

At the end of the first year, 66-69% of the initial litter mass remained (Fig. 1). The litter mass further decreased to 43-56% in the second and third years. The mixed litter in the birch forest decomposed slower than the monoculture litter (GLMM, $P < 0.05$), showing that the mixing effects slowed the litter decomposition in the birch forest. However, the mixed litter did not slow the decomposition in the oak forest 1.7 years later, resulting in a significant interaction between forests and litter species ($P < 0.05$). The litter decomposition constants (k) decreased from 0.038 to 0.013/day during the first year and

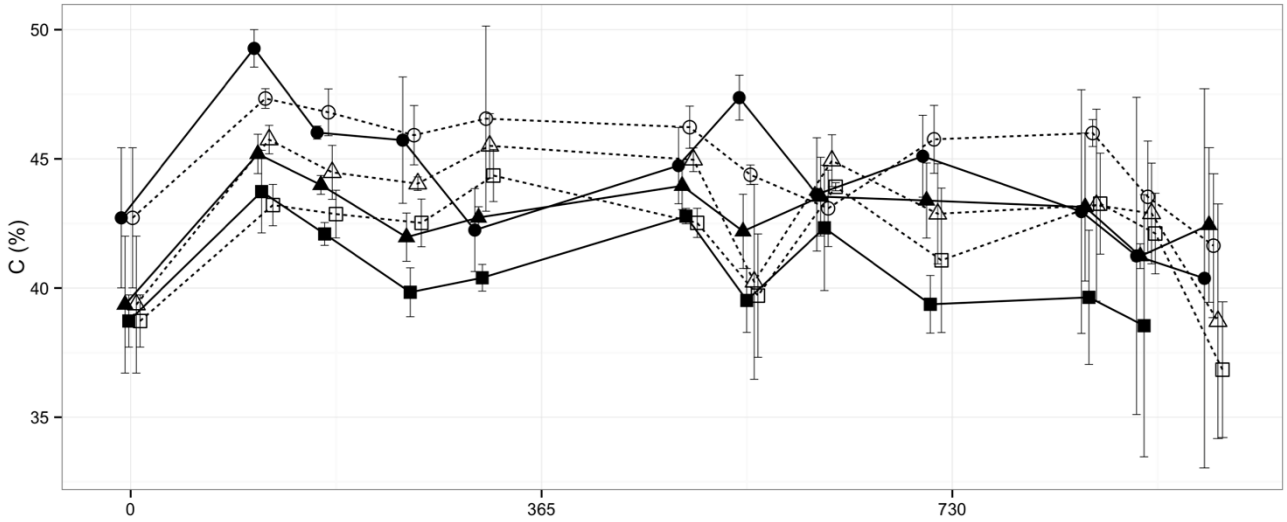
then further decreased to 0.004/day by the third year ($P < 0.05$), showing that the litter mass decreased faster in the early stages in both the forests. The *k* was not different among the litter types and between the forests ($P > 0.05$).



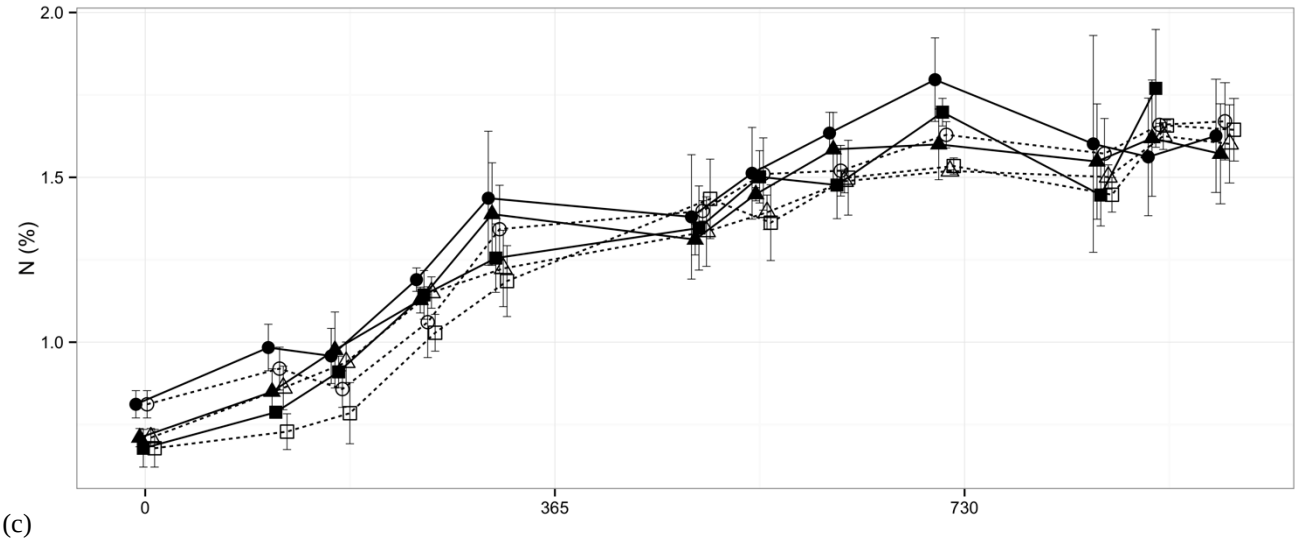
264 **Fig. 1.** Temporal changes in the ratios of remaining litter mass consisting of birch (circles), oak (squares) and mixture (triangles) in the birch
265 and oak forests. Mean (symbols) is shown with standard deviation (error bars). The rates showed significant difference as the number of days
266 increased after setting the litterbags (GLMM, $P < 0.05$) and between the mixture and monoculture litter. The interaction between litter and
267 forest is significant at $P < 0.05$.The different letters show significant differences (Steel-Dwass test, $P < 0.05$)

C concentration of the litter was different between the forests and among litter types; viz. C was highest in the birch litter placed in the oak forest (Fig. 2a). N concentration of the litter increased with time and was significantly different between the two forests (GLMM, $P < 0.05$) (Fig. 2b). The litter types also affected N, i.e., N in birch litter was higher than N in the oak and mixed litter ($P < 0.05$). C/N decreased with time and was higher in the oak forest than in the birch forest (Fig. 2c). Since C did not change significantly while N increased with time especially in the first year, the C/N ratio was determined mostly by changing N content in the litter. No interaction was detected between the forest and litter quality on C, N and C/N ($P > 0.05$).

279 (a)


50
45
40
35
C (%)
0
365
730

(b)


2.0
1.5
1.0
N (%)
0
365
730

(c)

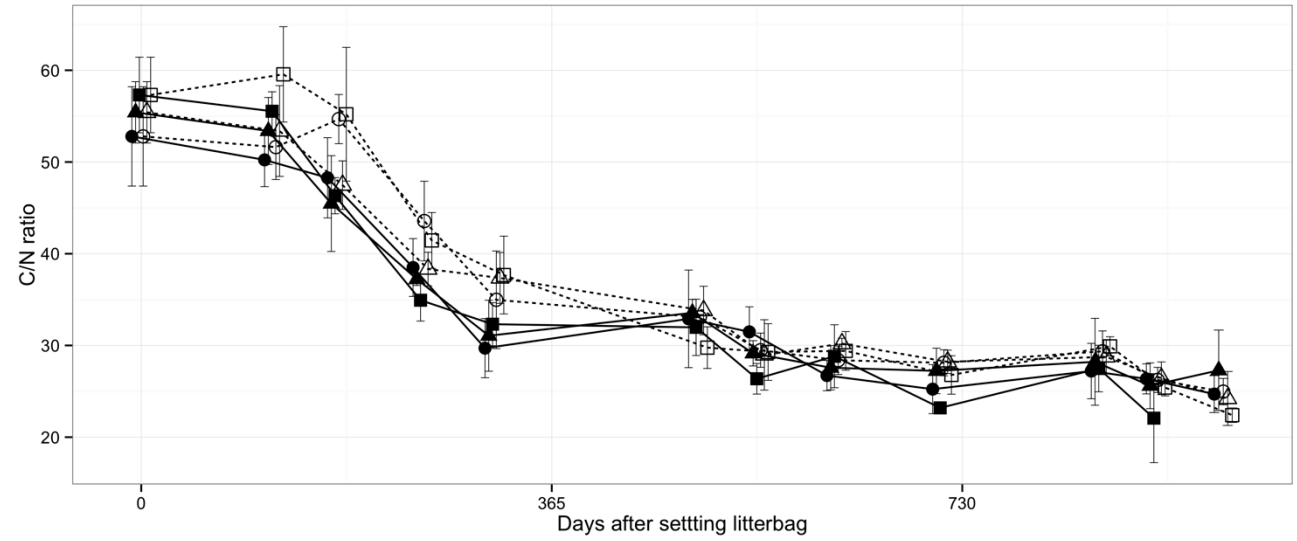

60
50
40
30
20
C/N ratio
0
365
730
Days after settting litterbag

Fig. 2. Fluctuations of C (a), N (b) and C/N (c) in litter during the three years. Litter: birch (circles), oak (squares) and mixture (triangles). Forest: birch (filled) and oak (open). Means (symbols) are shown with standard deviations (error bars). The C, N and C/N are significantly different between the forests (GLMM, $P < 0.05$). The C and N in birch litter are significantly different from the oak and mixed litter.

288 *3.2. PLFAs in litter*

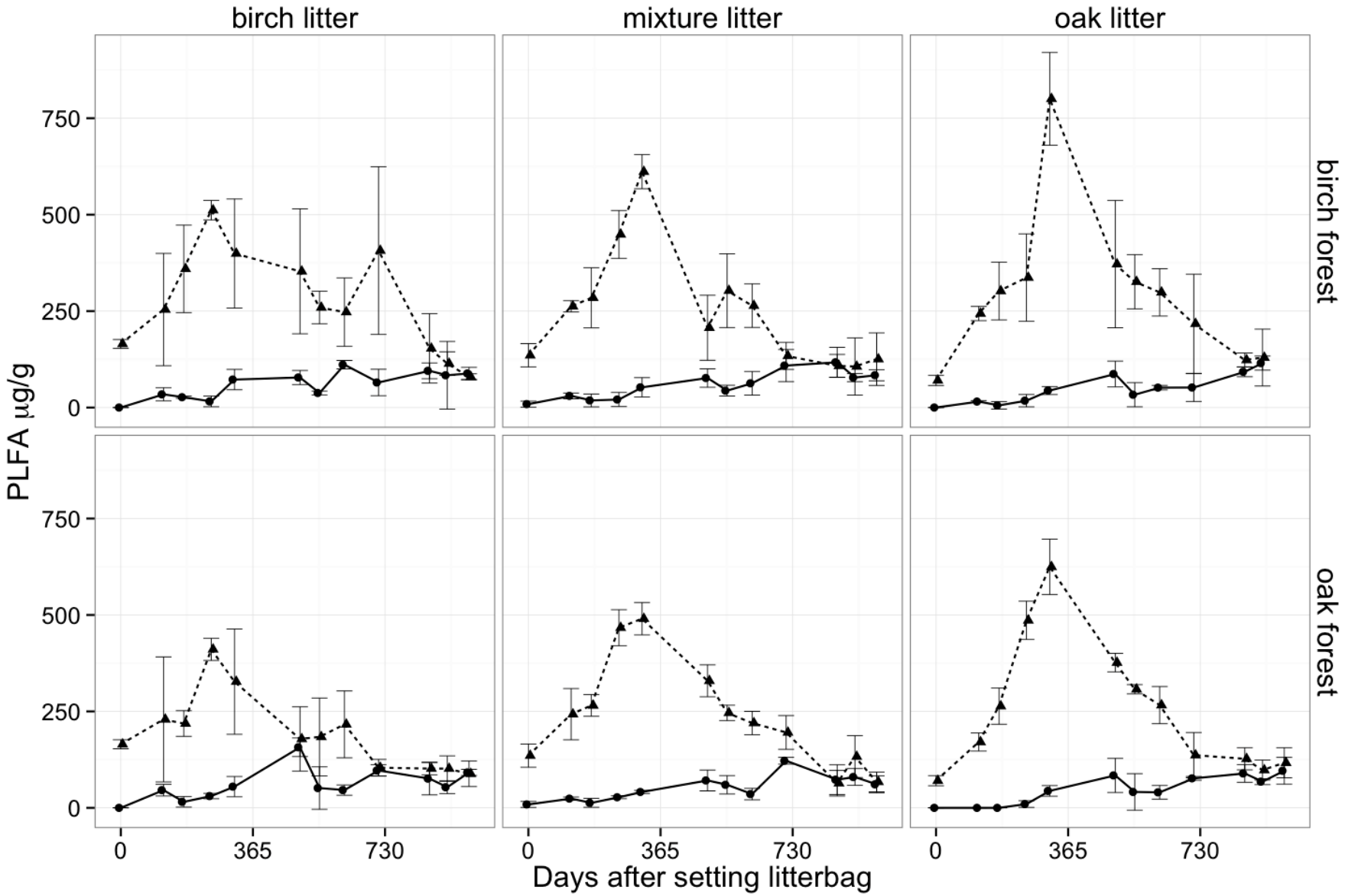
289 Nine bacterial PLFAs, i15:0, a15:0, 16:1ω7, i17:0, a17:0, cy17:0, 17:0, 10Me-18:0
290 cy19:0, were detected from the recovered litter samples. The fungal PLFA, 18:2, showed
291 higher concentration than the total bacterial PLFAs, in particular, until 314 days after
292 setting the litterbags, independent of the litter species (GLMM, $P < 0.05$) (Fig. 3).

293 The amounts of fungi and bacterial PLFAs were different after setting the litterbags
294 (χ^2-test, $P < 0.05$). The fungal PLFA concentrations peaked in the summer and autumn of
295 the first year, i.e., 250-320 days (Fig. 3), showing that the litter decomposition was
296 undertaken mostly by fungi in the early successional stages. The concentrations of fungal
297 PLFA were lower in the oak forest than in the birch forest (GLMM $P < 0.05$), although the
298 concentrations of fungal and bacterial PLFAs did not differ between the litter types ($P >$
299 0.05). The fungal PLFA in the oak forest was interacted with the litter mixture ($P < 0.05$),
300 implying that the fungal biomass increased more in the oak forest. In contrast to the fungal
301 PLFA, the bacterial PLFAs increased steadily until the end of this study. The
302 concentrations of bacterial PLFAs were not different between the forests and among the
303 litter types (GLMM, $P > 0.05$).

304 The B/F ratios increased gradually with time (GLM, $P < 0.05$), although the average
305 was less than one even in the late stages. B/F ratios were zero in the early stages because
306 the bacterial PLFAs were not detected. These indicated that fungi were more dominant than
307 the bacteria during the three years while the bacteria steadily increased their dominance.
308 The B/F ratios were higher in the oak forest than in the birch forest (GLMM, $P < 0.05$),
309 because the increase reflected the reduction of fungi biomass without the interaction
310 between the forest and litter types ($P > 0.05$).

311 The following bacterial PLFAs were detected for the three years: 10Me18:0 by
312 actinomycetes, cy17:0 and 16:1ω7 by gram negative-bacteria and i15:0, a15:0, i17:0 and
313 a17:0 by gram-positive bacteria. The PLFAs derived from actinomycetes were low
314 throughout the surveyed period (Fig. 4). The content of PLFAs in each of the gram-negative
315 and gram-positive bacteria varied over time (GLM, $P < 0.05$). The gram-positive bacteria

gradually increased their PLFAs during the three years, while the gram-negative bacteria reached their peak of PLFAs production around 500 days after starting the decomposition in the two forests. Therefore, successional replacement from gram-negative bacteria to gram-positive bacteria occurred in both the forests. The composition of PLFAs in these three taxa differed between the birch litter from oak and mixture litter (GLMM, $P < 0.05$), showing that the bacterial succession was affected by the litter types.


birch litter
mixture litter
oak litter
birch forest
oak forest
PLFA μg/g
0
250
500
750
0
365
730
Days after setting litterbag

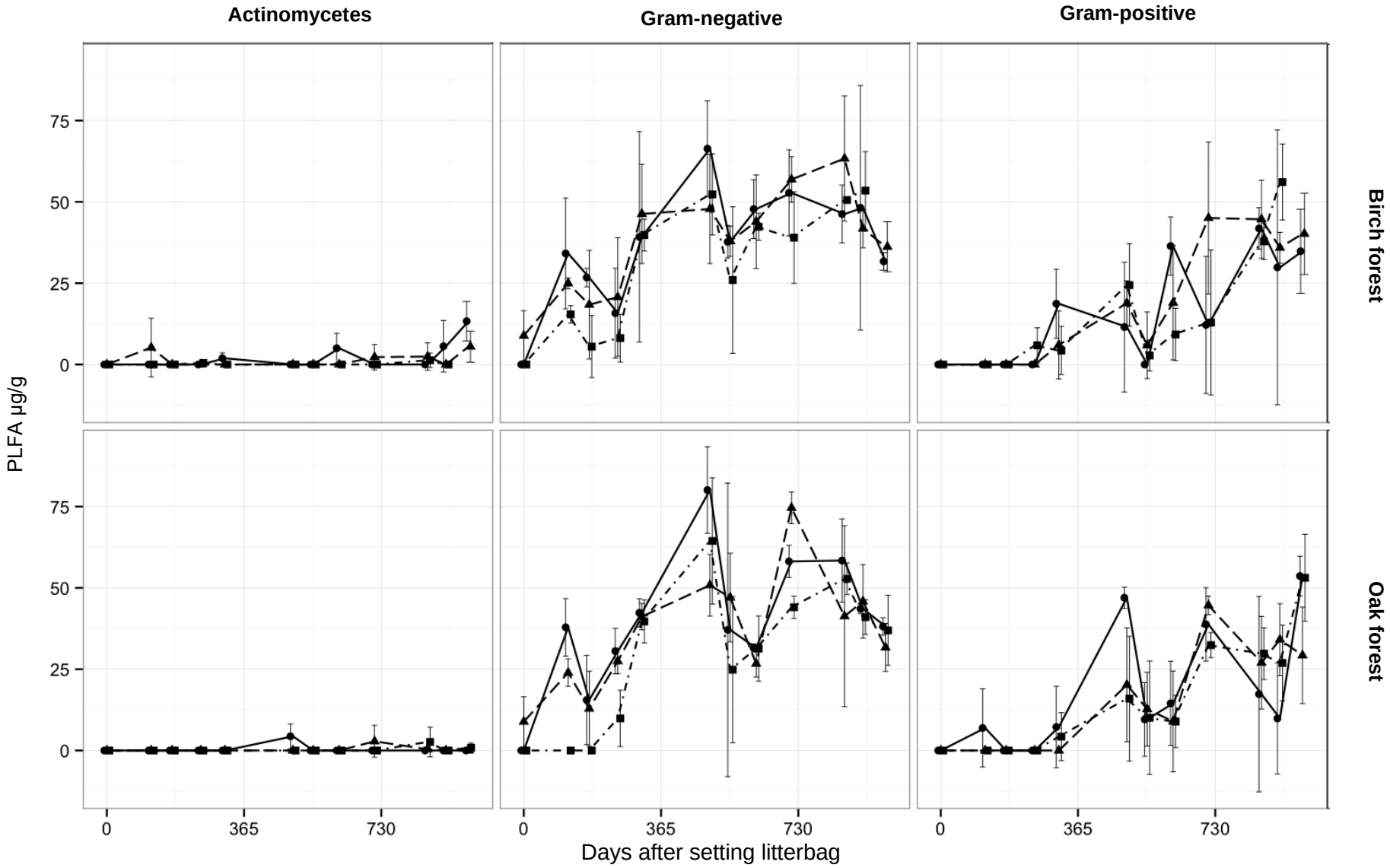
325

326

327 **Fig. 3.** Fluctuations of PLFAs produced by bacteria (solid lines) and fungi (dashed lines). Means are shown with standard deviations (error

328 bars)

329


Actinomycetes
Gram-negative
Gram-positive
Birch forest
Oak forest
PLFA µg/g
75
50
25
0
0
365
730
Days after setting litterbag

345

346

347

348 **Fig. 4.** Fluctuations of PLFAs produced by actinomycetes (10Me18:0), gram-negative bacteria (cy17:0 and 16:1ω7) and gram-positive
349 bacteria (i15:0, a15:0, i17:0 and a17:0) in the birch and oak forests. Litter: birch = circles, oak = squares, and mixture triangles. Means
350 (symbols) are shown with standard deviations (error bars)

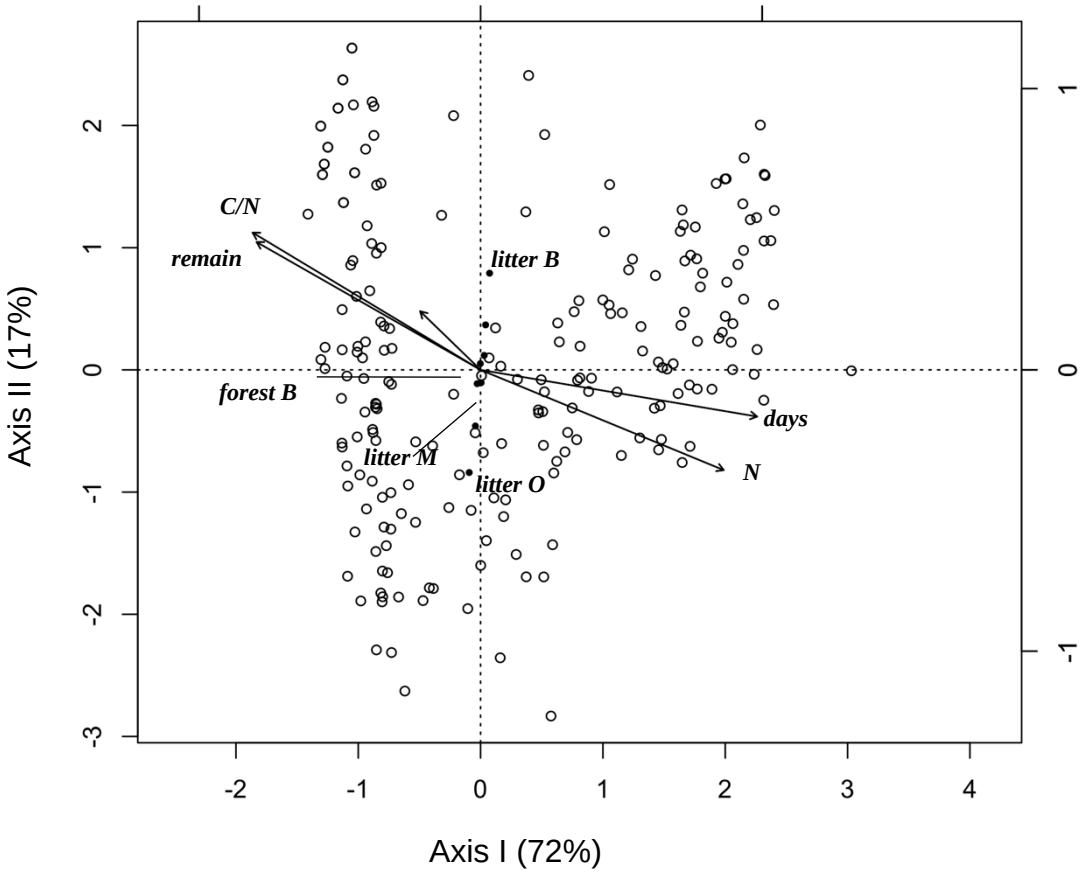
3.3. Distribution patterns of PLFAs

The CCA performed on the composition of PLFAs showed that 72% and 17% of variations were explained by the first and second axes respectively. Monte Carlo permutation test confirmed that these results were significant ($P < 0.05$). The days after setting the litterbags were negatively correlated to the litter mass remaining and were correlated highly to the first axis (Fig. 5). The litter mass remaining rate was primarily related to the C/N and N of the litter. Moreover, the scores of C/N and litter mass remaining showed that these two factors had comparable impacts on the composition of PLFA.

The fungal PLFA was scored to the opposite side of the bacterial PLFAs along the first axis of CCA, showing that the litter used by the fungi was spatial-temporally different from the litter used by the bacteria (Fig. 5). The fungal PLFA peaked one year after the decomposition and then decreased (Fig. 3), while the bacterial PLFAs increased steadily during the three years. These indicated that the major litter decomposers changed from fungi to bacteria over time, although B/F ratios were still below one during the three years. The scores of PLFAs produced by bacterial decomposers were broadly distributed along the second axis, showing that the bacterial composition was influenced more by the litter quality than by the years after litter decomposition.

Litter quality expressed by litter species composition was correlated to the second axis ($P < 0.05$) but were not related to the first axis (Fig. 5), showing that litter species, including litter mixture, had little influence on litter decomposition. The forest types were not correlated either to the first or the second axes ($P > 0.05$), showing that the habitat differences were not the prime factor in litter decomposition. The mixed litter showed intermediate scores between the birch and oak litter on the second axis (Fig. 5).

(a)


C/N
remain
litter B
forest B
litter M
litter O
days
N
Axis II (17%)
Axis I (72%)
-3
-2
-1
0
1
2
3
4

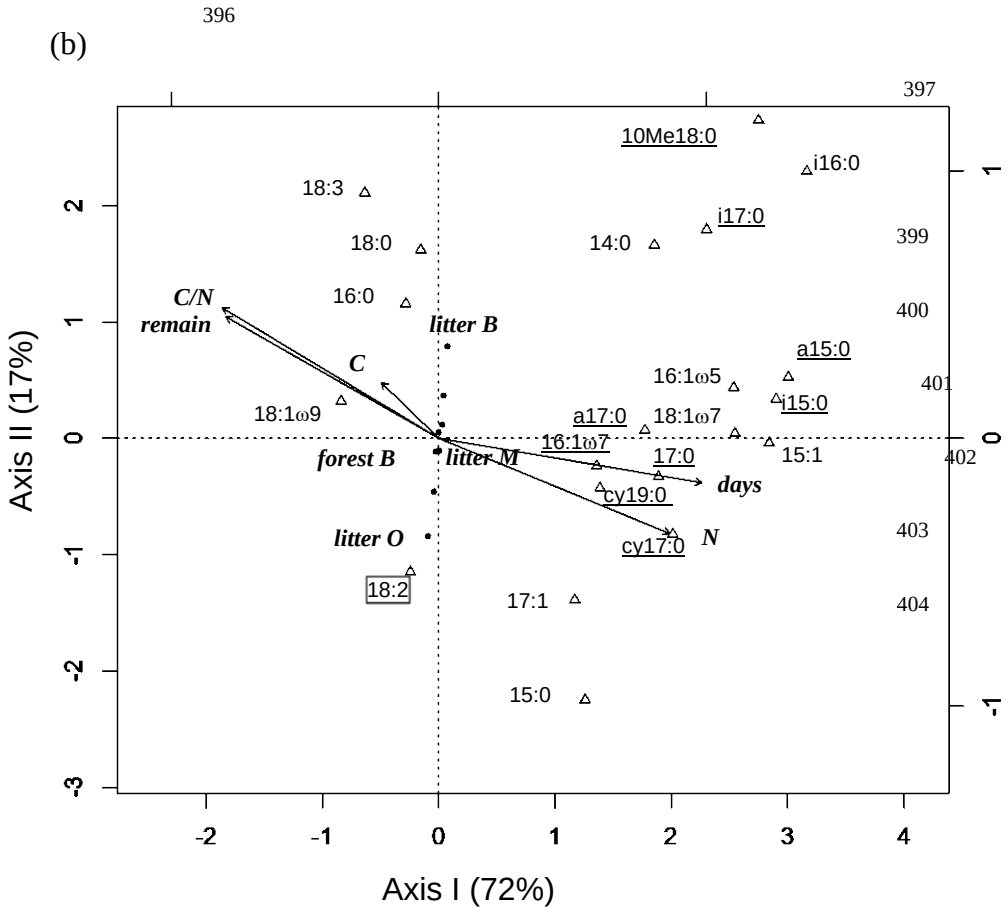


Fig. 5. CCA scores of PLFAs produced by microorganisms in litter. (a) The site scores and environmental factors are shown on the first two axes of CCA. (b) The PLFA scores and environmental factors are shown. PLFAs of i15:0, a15:0, i16:0, 16:1ω7, i17:0, a17:0, 17:0, cy17:0 10Me-18:0 and cy19:0 (underlined) are produced by bacteria while PLFA of 18:2 (enclosed) is produced by fungi. Environmental factors examined: Birch litter = *litter B*, Oak litter = *litter O*, Mixed litter = *litter M*, amount of remained litter = *remain*, days after setting litterbag = *days*, the forest dominated by birch = *forest B.*

4. Discussion

4.1. Forest structures and litter decomposition

The diversity of litter types affected the litter decomposition, i.e. the mixed litter showed different decomposition patterns from the monotonic litter. Total PLFA concentrations were higher in mixed litter than in single species litter in a mixed conifer forest (Chapman et al., 2013). However, the litter mixed from two broad-leaved tree species, *Tilia americana* and *Acer saccharum*, slows the decomposition in well-developed forests (Madritch and Cardinale, 2007). In a forest consisting of birch and oak, central Hokkaido, litter decomposition rates are determined more by litter types than by forest types (Ono et al., 2013). The present study showed the possibility that litter types also affected the litter decomposition rates.

The home-field advantage (HFA) hypothesis suggests that the interaction between litter decomposition and the environment determines the litter decomposition rate (Asplund et al., 2018; Ayres et al., 2009). The hypothesis predicts that litter decomposes faster the inside of its own ecosystem than the outside of it, because of the predictable and suitable environments for microbial activities (Prescott and Grayston, 2013). Because the litter decomposition was not affected by the forest types and litter quality, this hypothesis was rejected in this study. HFA is likely to occur more strongly for mono-specific litter than for the mixed litter. The plant species composition of the litter was diverse in the forests *in situ*. In fact, the HFA is hindered by the presence of diverse species in litter in cool temperate mixed forest (Jewell et al., 2015).

4.2. Temporal changes in fungi and bacteria

The litter mixture containing both birch and oak showed higher fungal PLFAs than bacterial PLFAs. The fungal PLFAs were dominant particularly in the early stages of litter decomposition, showing that the fungi contributed to the litter decomposition. This pattern

was also detected in Austrian forests (Schneider et al., 2012) and in subarctic broad-leaved forests (Urbanová et al., 2015). The litter of *Fagus crenata* Blume (Fagaceae), one of the common deciduous trees in Japan, is dominated by fungi in the first year of litter decomposition (Osono and Takeda, 2001).

The oak forest showed higher C/N ratio and lower fungi amount than the birch forest. C/N ratio is negatively correlated to the fungal activities in the early litter decomposition stages (Romaní et al., 2006), because N content in litter is determined by the amount of fungi (Hobara et al., 2014). The present study suggests that the successional changes of microorganisms from the dominance of fungi to that of bacteria are expected during long-term litter decomposition processes with increasing N concentration in the litter.

Although the N concentrations of initial oak litter were lower than those of initial birch litter, the decomposition of mixed litter was not different from that of single-species litter. HFA hypothesis assumes that N transportation is promoted by fungi (Lummer et al., 2012), while the N transportation between litter and fungi is not detected in the litter mixed with broad-leaved and needle leaved leaves (Steffen et al., 2007). Such relationship seems to be more common even in litter mixed with early and late successional tree species However, the bacterial biomass increased slowly and gradually during the surveyed period. Microbial succession is promoted by changes in C and N content of the litter, as the nutrient demands of bacteria are different from those of fungi (Schneider et al., 2012). Litter decomposed by fungi in the early stages should facilitate litter decomposition by bacteria.

4.3. Bacterial succession

The bacterial biomass in the litter increased and the composition of bacterial taxa changed for over the surveyed years. The bacterial composition changed from gram-negative to gram-positive bacteria along the succession. The environments surrounding the litter changed annually, because the current yearly litter overlaid the litterbags every autumn. Fungi and gram-negative bacteria are distributed in shallower soil layers than

gram-positive bacteria, depending on the decline of carbon availability with increasing soil depth (Fierer et al., 2003). The biomass of actinomycetes was totally low during the three years. PLFAs derived from actinomycetes increased in deep soil layers in coniferous forest with an increase of the pH in the humus due to the nitrogen limitation (Fritze et al., 2000; Frostegård, 1993). Therefore, the biomass of actinomycetes should increase when litter is buried deeper into the soil layer. Bacteria responded more to the environmental conditions, such as temperature and pH, than fungi (Yuste et al., 2011). Fungal exudates are a major source of nutrients for bacteria (Andrade et al., 1997). The present study together with other studies suggests that fungi in litter facilitate the immigration and colonization of bacteria. The succession of microorganisms is often promoted by facilitation, i.e., early colonizers provide suitable habitats for late colonizers (Blagodatskaya and Kuzyakov, 2008).

Nutrient transfer among litter consisting of multiple species causes in mixing effect (Gartner and Cardon, 2004). However, the litter decomposition was lowest in the mixed litter in the third year, while it did not differ in the first and second years. The amount of bacterial PLFAs increased annually. These show that litter decomposition is affected more by bacteria than by fungi with increasing time (Tláskal et al., 2016). The role of bacteria in litter decomposition should be clarified well to characterize litter decomposition processes in regions where litter decomposition is slow.

5. Conclusions

The litter decomposition was slow, i.e., 50% of the litter remained even after three years. The succession of microbial taxa in the litter occurred from fungi to bacteria and from gram-negative to gram-positive bacteria, independent of the forest structure for the three years. The succession was not affected greatly by the litter mixture, probably because the litterbags had already been enclosed by diverse litter. The present study suggests that the successional changes of microorganisms from fungi to bacteria were a key to understand the mechanisms of long-term litter decomposition processes with increasing N concentration in the litter.

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