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

Biological litter decomposition and the litter-associated microbial organisms were 23 monitored for three years to characterize litter decomposition in early and late 24 successional stages. Two forests were used for the investigation: pioneer a forest 25 dominated by birch (Betula platyphylla var. japonica) and a climax forest by oak 26 (Quercus mongolica var. grosseserrata) in the cool-temperate region of northern Japan. 27 Three types of litter were used: birch, oak and mixed litter. The litter decomposition was 28 effective during the first year but 50% of the original litter remained even after three 29 years. Carbon-to-nitrogen ratios in the litter decreased largely in the first year and became 30 stable thereafter. The litter decomposition rates were not different among the litter types 31 and between the forests. The temporal changes in phospholipid fatty acids (PLFAs) 32 showed that fungal biomass reached its peak in the first year and the bacterial biomass 33 increased steadily until the end of the experiment. The concentrations of fungal PLFAs in 34 the litter did not differ between the litter types but were lower in the oak forest. The litter 35 decomposition was performed mostly by fungi, in particular in the early stages, while 36 bacterial decomposition depended on the litter types and/or the forest types. Gram-37 negative bacteria reached their peak of PLFAs in the second year while gram-positive 38 bacteria PLFAs increased gradually during the three years. Therefore, the succession of 39 microorganisms in the litter occurred from fungi to bacteria and from gram-negative 40 bacteria to gram-positive bacteria in the two forests. Unlike in the case of coniferous or 41 monotonic forests, the effects of forests and litter types on litter decomposition for the 42 first year were weak. The forest types on litter decomposition appeared only for the long-43 term litter decomposition. The successional changes of microorganisms occurred from 44 fungi to bacteria for long-term litter decomposition processes with increasing N 45 concentration in the litter. 46

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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

56 1. Introduction

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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, 63 influenced by temperature, precipitation, vegetation and litter type (Parton et al., 2007). In 64 addition, forest structures affect the composition of fungal and bacterial communities in 65 the soils (Prescott and Grayston, 2013) and in the litter (Urbanová et al., 2015). Litter 66 decomposers regulate C and N consumption in the early litter decomposition stages, while 67 moisture and legacy in the soils play a major role in the later stages (García-Palacios et al., 68 2016). Since litter decomposition is slow in cool-temperate region, to investigate the 69 microbial activities in the litter, long-term monitoring is required. However, few studies 70 have been conducted for long term in this study area so far. 71

The composition of microorganisms involved in litter decomposition varies depending 72 on the stage of decomposition. The fungi/bacteria ratio decreases greatly in the first eight 73 months in the oak forests of Czech Republic (Voříšková et al., 2013). The diversities of 74 bacteria and fungi change with succession (Tláskal et al., 2016; Purahong et al., 2016), 75 although the relationships between bacteria and fungi are unclear. In the cool temperate 76 regions of Japan, the dominant species along a successional sere change from birch to oak. 77 This implies that litter mixed with birch and oak are often developed. During vegetation 78 succession in temperate and cool-temperate regions, including Japan, birches (Betula) often 79 develop pioneer forests and oaks (Quercus) form later and/or climax forests (Ishikawa and 80 Ito, 1988). These two forest types are broadly distributed in Japan (Krestov et al., 2015). 81 Therefore, the comparisons of these forests are invaluable to understand successional 82 changes in litter decomposition and microbial composition. Furthermore, the conservation 83

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 89 encouraged by litter mixed from only a few species. This hypothesis is examined well by 90 litter mixed with broad-and needle-leaved leaves (Gartner and Cardon, 2004; Asplund et 91 al., 2018; Ayres et al., 2009). However, this hypothesis has not been examined well in 92 broad-leaved forests where the litter should be made of various broad-leaved plants (Gao 93 et al. 2016). Since the litter components are different between broad-leaved and mixed 94 forests, the different HFA effects are expected in broad-leaved forests. Also, the succession 95 of microorganisms in these forests has not been investigated well. Therefore, the 96 decomposition of litter consisting of birch, oak and their mixture were monitored. 97

Litterbag experiments are often used to investigate litter decomposition and its related 98 factors (Wieder and Lang, 1982; Kazakou et al., 2006). In addition, biological litter 99 decomposition is commonly estimated by indicators of biomass and composition of 100 microorganisms, represented by quality and quantity of phospholipid fatty acids (PLFAs) 101 (Chapman and Newman, 2010; Šnajdr et al., 2011). The concentrations of carbon (C) and 102 nitrogen (N) in litter show the litter quality and N/C fluxes (Berg and McClaugherty, 2008). 103 The present study provides new insights into microbial succession by using these 104 techniques with considering differences in litter quality in cool-temperate regions. We 105 monitored the successional changes in the biomass of each microbial taxon and litter 106 decomposition at two forest sites of mixed forest examining birch and oak litter to 107 investigate how the litter decomposition and the microbial succession develops and 108 changes over time. Using these surveys, two hypotheses were examined: (1) HFA effects 109 were weak to decompose litter in broad-leaved forests, as compared with mixture of broad-110 and needle-leaved litter, because of the similarity of litter species. (2) Microbial succession 111

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

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115 **2. Materials and methods**

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117 2.1. Study sites
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The study was conducted in a lowland at the foot of Mount Toishi (826 m a.s.l.) in the 118 city of Sapporo, Hokkaido, northern Japan. The survey was conducted for three years (33 119 months) from December 2009 to August 2012. The mean annual temperature was 9.3-120 9.8°C during the surveyed period at the distance of 8 km from the study site (Japan 121 Meteorological Agency, 2012). The maximum daily temperature was recorded as 34.1°C 122 in August 2010 and the minimum was at -12.6°C in February 2010. The annual 123 precipitation during the three years was between 1254 mm and 1325 mm, of which 21% 124 was supplied by snow during November and April. 125

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 ($42^{\circ}59$ 'N and 134 141°19'E, 130-140 m elevation). The two forests were adjacent to each other. In each plot, 135 stem height and diameter at breast height (DBH) were measured on each tree taller than 5 136 m. The stem volumes were calculated from the height and diameter with an assumption of 137 cylindrical shape. The results were as follows: The oak forest had 818 m³/ha with 800 138 stems/ha. The oak explained 75% of the stem volume. The birch forest had 877 m³/ha with 139 1200 stems/ha. The birch explained 80% of the stem volume. The species composition of
140 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 \pm standard deviation).

Litter supply in the birch and oak forests was measured by litter collected from nine 148 25×25 cm² plots in each forest in November 2014 soon after the defoliation. The litter 149 was air-dried for two weeks and weighed. The litter supply averaged 192 ± 54 g/m²/yr in 150 the oak forest and $190 \pm 46 \text{ g/m}^2/\text{yr}$ in the birch forest (Table 1). The compositions of birch 151 and oak litter were 50% and 38% in the birch forest and 13% and 60% in the oak forest, 152 respectively. Since the oak forest was adjacent to the birch forest, the birch litter should be 153 easily immigrated to the oak forest. Other species in the litter were Kalopanax pictus, 154 Cornus controversa, etc. with low dominance, less than 27% share. 155

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160**Table 1** (a) Environmental characteristics (canopy openness, light intensity, temperature161and soil moisture) in the birch and oak forests during snow-free periods from 2010 to1622012. Means are shown with standard deviations. Different letters show significant163difference between the two forests at p < 0.05 (GLMM) (b) Litter supply (g/m²) in the164birch and oak forests. Mean is shown with standard deviation. Different letters show165significantly different at P < 0.05 (GLM)

166 (a)

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

167

168 (b)

	Litter			169
Forest	Birch	Oak	Others	Total
Birch	94 ± 33 a	73 ± 29 c	24 ± 12 e	$\frac{171}{190 \pm 46 \text{ g}}$
Oak	25 ± 20 b	114 ± 35 d	$53 \pm 25 \text{ f}$	$192 \pm 54 g^{172}$
				173

176 2.2. Litter decomposition

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

192

193 2.3. Measurements of chemical properties in litter

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

203 2.4. PLFA analysis

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

The taxon-specific PLFAs are: i14:0, i15:0, a15:0, $16:1\omega7t$, $16:1\omega9$, $16:1\omega7$, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0 produced only by bacteria and 18:2 only by fungi (Šnajdr et al., 2011). Almost all of 18:2 in litter is derived from fungi, because 18:2 derived from plants vanishes soon after defoliation (Laczko et al., 2004). PLFA of $16:1\omega7$ was treated as the production of bacteria, as it is characteristic to bacterial production (Graham et al., 1995).

The bacterial PLFAs were divided into three groups by the origins: 10Me-16:0, 10Me-215 17:0 and 10Me-18:0 produced by actinomycetes, i15:0, a15:0, i17:0 and a17:0 by gram-216 positive bacteria and cy17:0 and 16:1w7 by gram-negative bacteria (Moore-Kucera and 217 Dick, 2008). The PLFAs produced by gram-positive bacteria, gram-negative bacteria and 218 actinomycetes were summed and were used as total bacterial PLFAs. The other PLFAs 219 produced by plants, bacteria and/or fungi were treated as PLFAs produced by 220 miscellaneous organisms. Bacteria to fungi (B/F) ratio was calculated based on the 221 composition of PLFAs to investigate the microbial dominance of litter decomposition. 222 Because a few samples showed no bacterial PLFAs, B/F ratio was used instead of F/B ratio. 223

224

225 2.5. Statistical analysis

The differences and interactions in remaining litter mass, C, N, C/N ratios and PLFAs were examined between the litter types and between the forests by a generalized linear mixed-effects model (GLMM) with number of days passed after litterbag establishment as a random effect. Steel-Dwass test was used for multiple comparisons of the differences in 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 233 234 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 235 four factors: litter types, forest types, days after establishing litterbags and litter remaining 236 ratios. The days and liter remaining ratios were numerical variables and litter types and 237 forest types were categorical. Monte Carlo Permutation test was conducted to confirm the 238 significance of CCA. All the statistical analyses were performed by a software package R 239 (ver. 3.1.3) (Ihara and Gentleman, 1996) with vegan library (Oksanen et al., 2015). 240

241

242 **3. Results**

243

244 3.1. Litter decomposition

²⁴⁵ N content of the initial litter prior to the decomposition, i.e., 0 days, was 0.81% and ²⁴⁶ 0.68% in birch and oak litter, respectively. N was significantly higher in the birch litter ²⁴⁷ than in the oak litter (GLM, P < 0.05) in the initial litter the C concentration was higher in ²⁴⁸ the birch litter (42.7%) than in the oak litter (38.7%) (P < 0.05). C/N ratios were 52.8 and ²⁴⁹ 57.3 in the litter of birch and oak, respectively, and were not different between the litter ²⁵⁰ types (P > 0.05). The quantitative differences in the initial litter between the two species ²⁵¹ 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).



Fig. 1. Temporal changes in the ratios of remaining litter mass consisting of birch (circles), oak (squares) and mixture (triangles) in the birch and oak forests. Mean (symbols) is shown with standard deviation (error bars). The rates showed significant difference as the number of days increased after setting the litterbags (GLMM, P < 0.05) and between the mixture and monoculture litter. The interaction between litter and 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; 269 viz. C was highest in the birch litter placed in the oak forest (Fig. 2a). N concentration of 270 the litter increased with time and was significantly different between the two forests 271 (GLMM, P < 0.05) (Fig. 2b). The litter types also affected N, i.e., N in birch litter was 272 higher than N in the oak and mixed litter (P < 0.05). C/N decreased with time and was 273 higher in the oak forest than in the birch forest (Fig. 2c). Since C did not change 274 significantly while N increased with time especially in the first year, the C/N ratio was 275 determined mostly by changing N content in the litter. No interaction was detected between 276 the forest and litter quality on C, N and C/N (P > 0.05). 277 278





- **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

Nine bacterial PLFAs, i15:0, a15:0, $16:1\omega7$, i17:0, a17:0, cy17:0, 17:0, 10Me-18:0 cy19:0, were detected from the recovered litter samples. The fungal PLFA, 18:2, showed higher concentration than the total bacterial PLFAs, in particular, until 314 days after setting the litterbags, independent of the litter species (GLMM, P < 0.05) (Fig. 3).

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

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

The following bacterial PLFAs were detected for the three years: 10Me18:0 by actinomycetes, cy17:0 and 16:1 ω 7 by gram negative-bacteria and i15:0, a15:0, i17:0 and a17:0 by gram-positive bacteria. The PLFAs derived from actinomycetes were low throughout the surveyed period (Fig. 4). The content of PLFAs in each of the gram-negative 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.

322



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



- Fig. 4. Fluctuations of PLFAs produced by actinomycetes (10Me18:0), gram-negative bacteria (cy17:0 and 16:1ω7) and gram-positive
- 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)

351 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 359 first axis of CCA, showing that the litter used by the fungi was spatial-temporally different 360 from the litter used by the bacteria (Fig. 5). The fungal PLFA peaked one year after the 361 decomposition and then decreased (Fig. 3), while the bacterial PLFAs increased steadily 362 during the three years. These indicated that the major litter decomposers changed from 363 fungi to bacteria over time, although B/F ratios were still below one during the three years. 364 The scores of PLFAs produced by bacterial decomposers were broadly distributed along 365 the second axis, showing that the bacterial composition was influenced more by the litter 366 quality than by the years after litter decomposition. 367

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).

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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, 16:1 ω 7, 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*.

419 **4. Discussion**

420

421 4.1. Forest structures and litter decomposition

The diversity of litter types affected the litter decomposition, i.e. the mixed litter 422 showed different decomposition patterns from the monotonic litter. Total PLFA 423 concentrations were higher in mixed litter than in single species litter in a mixed conifer 424 forest (Chapman et al., 2013). However, the litter mixed from two broad-leaved tree species, 425 Tilia americana and Acer saccharum, slows the decomposition in well-developed forests 426 (Madritch and Cardinale, 2007). In a forest consisting of birch and oak, central Hokkaido, 427 litter decomposition rates are determined more by litter types than by forest types (Ono et 428 al., 2013). The present study showed the possibility that litter types also affected the litter 429 decomposition rates. 430

The home-field advantage (HFA) hypothesis suggests that the interaction between 431 litter decomposition and the environment determines the litter decomposition rate (Asplund 432 et al., 2018; Ayres et al., 2009). The hypothesis predicts that litter decomposes faster the 433 inside of its own ecosystem than the outside of it, because of the predictable and suitable 434 environments for microbial activities (Prescott and Grayston, 2013). Because the litter 435 decomposition was not affected by the forest types and litter quality, this hypothesis was 436 rejected in this study. HFA is likely to occur more strongly for mono-specific litter than for 437 the mixed litter. The plant species composition of the litter was diverse in the forests *in situ*. 438 In fact, the HFA is hindered by the presence of diverse species in litter in cool temperate 439 mixed forest (Jewell et al., 2015). 440

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442 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 longterm litter decomposition processes with increasing N concentration in the litter.

Although the N concentrations of initial oak litter were lower than those of initial 456 birch litter, the decomposition of mixed litter was not different from that of single-species 457 litter. HFA hypothesis assumes that N transportation is promoted by fungi (Lummer et 458 al., 2012), while the N transportation between litter and fungi is not detected in the litter 459 mixed with broad-leaved and needle leaved leaves (Steffen et al., 2007). Such 460 relationship seems to be more common even in litter mixed with early and late 461 successional tree species However, the bacterial biomass increased slowly and gradually 462 during the surveyed period. Microbial succession is promoted by changes in C and N 463 content of the litter, as the nutrient demands of bacteria are different from those of fungi 464 (Schneider et al., 2012). Litter decomposed by fungi in the early stages should facilitate 465 litter decomposition by bacteria. 466

467

468 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 gramnegative 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 474 depth (Fierer et al., 2003). The biomass of actinomycetes was totally low during the three 475 years. PLFAs derived from actinomycetes increased in deep soil layers in coniferous forest 476 with an increase of the pH in the humus due to the nitrogen limitation (Fritze et al., 2000; 477 Frostegård, 1993). Therefore, the biomass of actinomycetes should increase when litter is 478 buried deeper into the soil layer. Bacteria responded more to the environmental conditions, 479 such as temperature and pH, than fungi (Yuste et al., 2011). Fungal exudates are a major 480 source of nutrients for bacteria (Andrade et al., 1997). The present study together with other 481 studies suggests that fungi in litter facilitate the immigration and colonization of bacteria. 482 The succession of microorganisms is often promoted by facilitation, i.e., early colonizers 483 provide suitable habitats for late colonizers (Blagodatskaya and Kuzyakov, 2008). 484

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.

492

493 **5. Conclusions**

The litter decomposition was slow, i.e., 50% of the litter remained even after three 494 years. The succession of microbial taxa in the litter occurred from fungi to bacteria and 495 from gram-negative to gram-positive bacteria, independent of the forest structure for the 496 three years. The succession was not affected greatly by the litter mixture, probably because 497 the litterbags had already been enclosed by diverse litter. The present study suggests that 498 the successional changes of microorganisms from fungi to bacteria were a key to 499 understand the mechanisms of long-term litter decomposition processes with increasing N 500 concentration in the litter. 501

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511				
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