Succession of Microbial Decomposers Is Determined by Litter Type, but Site Conditions Drive Decomposition Rates

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ABSTRACT Soil microorganisms are diverse, although they share functions during the decomposition of organic matter. Thus, preferences for soil conditions and litter quality were explored to understand their niche partitioning. A 1-year-long litterbag transplant experiment evaluated how soil physicochemical traits of contrasting sites combined with chemically distinct litters of sedge (S), milkvetch (M) from a grassland, and beech (B) from forest site decomposition. Litter was assessed by mass loss; C, N, and P contents; and low-molecular-weight compounds. Decomposition was described by the succession of fungi, *Actinobacteria*, *Alphaproteobacteria*, and *Firmicutes*; bacterial diversity; and extracellular enzyme activities. The M litter decomposed faster at the nutrient-poor forest site, where the extracellular enzymes were more active, but microbial decomposers were not more abundant. *Actinobacteria* abundance was affected by site, while *Firmicutes* and fungi by litter type and *Alphaproteobacteria* by both factors. *Actinobacteria* were characterized as late-stage substrate generalists, while fungi were recognized as substrate specialists and site generalists, particularly in the grassland. Overall, soil conditions determined the decomposition rates in the grassland and forest, but successional patterns of the main decomposers (fungi and *Actinobacteria*) were determined by litter type. These results suggest that shifts in vegetation mostly affect microbial decomposer community composition.

IMPORTANCE Anthropogenic disturbance may cause shifts in vegetation and alter the litter input. We studied the decomposition of different litter types under soil conditions of a nutrient-rich grassland and nutrient-poor forest to identify factors responsible for changes in the community structure and succession of microbial decomposers. This will help to predict the consequences of induced changes on the abundance and activity of microbial decomposers and recognize if the decomposition process and resulting quality and quantity of soil organic matter will be affected at various sites.

KEYWORDS succession, enzyme activities, forest, grassland, organic matter

Microbial community structure and activity reflect changes of biotic (soil biota) and abiotic (climate, soil chemistry, and litter quality) factors as well as ecosystem functioning (1, 2). In particular, the complex environmental characteristics of a given site influence the pool of microbial species at the regional scale, while litter quality affects the dynamics of microorganisms coming from that pool at the local scale (3, 4).

It is still debatable whether environmental conditions or litter quality plays a larger role in decomposition (5). The transfer of litter between sites can differentiate between

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these two factors because site will have a predominant effect on decomposition if the local communities decompose the litter to which they are preadapted faster (6). Yet, if the litter type affects decomposition regardless of the site, the microbes are functionally redundant with respect to litter type (7). Therefore, to understand the factors driving microbial decomposition, connections between local and regional scales need to be considered over time, together with various litter types and origin (5, 8).

The roles of biotic and abiotic factors change over time as the litter is sequentially decomposed. As the chemical composition of litter changes, the associated microbial community and extracellular enzyme activities also change (9–11). The differential response of microbes to the changing litter chemistry is the basis for sorting microbes into specific guilds (10, 12). More specifically, a nutritious organic substrate is connected to a wide range of nonspecialized microbes, while recalcitrant organic substrates are decomposed only by a limited number of taxa, which can break the resistant organic structures (3, 13). It has been proposed that the identification of microbes from the late stage and/or specialized guilds should be the first step in predicting the consequences of environmental change connected to decomposition processes in temperate areas (5, 14).

Fungi have long been considered the main microbial decomposers catalyzing the decomposition of complex substrates (9, 15). Most studies focused on their decomposing abilities, while factors affecting them were found to be variable (10, 16, 17). However, the role of bacteria in decomposition has also been recently highlighted (7, 11). Particularly, *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* produce extracellular enzymes and are active in substrate degradation (5, 18). Moreover, *Actinobacteria* are able to produce lignolytic enzymes and secondary metabolites and form filaments, which are strategies similar to those of fungi (19–23). Therefore, *Actinobacteria* may compete with fungi (15), but those two groups have rarely been studied together during the decomposition process. Their coexistence patterns, such as niche overlap and/or separation (3, 15) under the influence of various abiotic factors, could be a missing biotic predictor of decomposition (5, 15, 24).

The aim of this study was to explore the interplay of factors affecting the abundances and succession of main decomposers. We hypothesized that main decomposers will be site specific but will selectively inhabit different litter and shift their enzyme activities during the decomposition stages. In particular, we wanted to determine the importance of cross-kingdom coexistence and successional patterns of microbial communities in influencing the effects of litter type and soil condition on decomposition processes.

RESULTS

Low-molecular-weight compounds (measured by high-performance liquid chromatography [HPLC]) shifted over time (Fig. 1A). In January and March 2011, compounds were separated according to litter type, while in later months they were separated according to sites. The HPLC profiles of the milkvetch (M) litter type in January and March 2011 significantly correlated with C content ($R = 6.377$, $P < 0.001$) and most extracellular enzymes, such as β -glucosidase ($R = 7.491$, $P < 0.001$), acid phosphatase ($R = 7.776$, $P < 0.001$), and chitinase ($R = 6.152$, $P < 0.001$), according to the permutation test. Of all bacterial groups, the abundance of *Actinobacteria* most strongly correlated with the late-stage HPLC profile ($R = 10.569$, $P < 0.001$). *Actinobacteria* significantly correlated with soil water potential ($R = 2.347$, $P = 0.004$) and temperature ($R = 4.871$, $P < 0.001$).

Bacterial communities on the beech (B) and sedge (S) litter types separated from that on the M litter type, especially in January and March 2011 (Fig. 1B). Bacterial communities also shifted from the initial to late stages of decomposition (confirmed by Sammon's multidimensional scaling; see Fig. S1 in the supplemental material). Bacterial communities from a later month of decomposition significantly correlated with the abundances of *Actinobacteria* ($R = 2.5940$, $P < 0.001$), *Firmicutes* ($R = 1.2363$, $P < 0.001$), and bacterial diversity ($R = 0.9875$, $P < 0.001$) according to the permutation test. From January

FIG 1 RDA of low-molecular-weight compounds (A, Manhattan distance) and bacterial communities (B, Bray-Curtis distance) from beech (circle), milkvetch (square), and sedge (triangle) litters at the forest (full symbol) and grassland (empty symbol) sites during the decomposition experiment (5 replicates). Vectors correspond to the statistically significant linear variables. Numbers 1 to 7 (1 to 4, respectively, for bacterial communities) correspond to sampling months (1, January 2011; 2, March 2011; 3, May 2011; 4, July 2011; 5, September 2011; 6, November 2011; 7, January 2012). Blue arrows represent extracellular enzyme activities.

2011, bacterial communities on the M litter type were significantly correlated with C ($R = 1.3441$, $P < 0.001$) and most extracellular enzyme activities, such as β -glucosidase ($R = 1.3134$, $P < 0.001$), cellobiohydrolase ($R = 0.5805$, $P < 0.001$), chitinase ($R = 0.5606$, $P < 0.001$), and acid phosphatase ($R = 0.4410$, $P = 0.007$). Bacterial communities on the B and S litter types in January 2011 significantly correlated with laccase ($R = 0.8082$, $P < 0.001$). The bacterial communities on the M litter from March significantly correlated with N ($R = 0.7699$, $P < 0.001$) and P ($R = 0.5463$, $P = 0.002$). Late-stage bacterial communities significantly correlated with soil water tension ($R = 0.7697$, $P < 0.001$) and temperature ($R = 0.3895$, $P = 0.007$).

Litter mass loss. Loss of dry mass significantly differed between sites in all decomposition stages. At the forest site (F), the average residual dry mass was about 0.5 g lower than the grassland site (G) (ANOVA, $P < 0.001$) (see Table S1 in the supplemental material). Milkvetch (M) had the highest average mass loss (ANOVA, $P < 0.001$) (Table S1), while that of sedge (S) was significantly higher than beech (B) (ANOVA, $P < 0.001$) (Table S1) at both sites. Time courses of dry mass loss were similar for the same litter type regardless of the site (Fig. 2, P_{perm} [i.e., the P value from permutational multivariate analysis of variance using distance matrices {PERMANOVA}] < 0.001 , Table S1), while dispersion did not differ between the litter types (Fig. 2; Table S1) (P_{Disp} [i.e., the P value from homogeneity of multivariate dispersions using dissimilarity measures] = 0.382). No significant differences were observed between the indigenous and exogenous origin of the litter (i.e., M and S litters did not decompose faster than B litter in the grassland and B litter did not decompose faster than M and S litters in the forest).

Carbon, nitrogen, and phosphorus contents in the litter. Carbon (C), nitrogen (N), and phosphorus (P) contents in litter significantly differed between the forest and grassland sites during decomposition (see Fig. S2 in the supplemental material). The N and C contents in all litter types were significantly higher at the forest site than the grassland site (ANOVA, $P < 0.001$) (Table S1), while P content showed the opposite relationship (ANOVA, $P = 0.001$) (Table S1). C content did not differ between litter types

FIG 2 Average dry mass of beech (brown), milkvetch (yellow), and sedge (green) litters at the grassland and forest sites during the decomposition experiment ($\pm$ SD, 5 replicates). The x axis indicates sampling months (i.e., initial, before litterbag burial in November 2010 and then in 2-month periods from January 2011; overall, 12 months). The P values indicate differences between profiles' distances (PERMANOVA; $P_{Perm.}$) and dispersion (dispersion test, $P_{Disp.}$).

at both sites, while N and P contents significantly differed, with the highest content measured for the M litter ($P < 0.001$ for N, $P < 0.001$ for P) (Table S1).

HPLC analysis of extractable low-molecular-weight compounds. HPLC profiles significantly differed between sites and litter types within each site ($P < 0.001$ for all sampling times and litter types) (Table S2).

Extracellular enzyme activities. Overall, the activities of extracellular enzymes were significantly higher in the forest than in the grassland (ANOVA, β -glucosidase, $P < 0.001$; cellobiohydrolase, $P = 0.001$; β -xylosidase, $P < 0.001$; chitinase, $P < 0.001$; glucuronidase, $P = 0.003$; acid phosphatase, $P < 0.001$; arylsulfatase, $P < 0.001$; α -glucosidase, $P = 0.001$; laccase, $P = 0.007$). Manganese peroxidase activity did not differ between the sites. Overall, higher enzyme activities were measured for the M than the S or B litters for β -glucosidase ($M > B$ and S , $P < 0.001$), cellobiohydrolase ($M > S$, $P < 0.005$; $M > B$, $P < 0.001$; $S > B$, $P < 0.001$), β -xylosidase ($M > S$, $P = 0.015$; $M > B$, $P < 0.001$; $S > B$, $P < 0.001$), and acid phosphatase ($M > B$ and S , $P < 0.001$). The activities on the M and S litters were not statistically different but were higher than those of the B litter for chitinase ($P < 0.001$), glucuronidase ($P < 0.001$), and α -glucosidase ($P < 0.001$). The S litter had the highest laccase activity ($P = 0.007$) (Table S1; Fig. 3).

Time courses of enzyme activities for chitinase, arylsulfatase, laccase, and manganese peroxidase were similar for the same litter type regardless of the site, while dispersion did not differ between the litter types (chitinase, $P_{Perm.} = 0.005$, $P_{Disp.} = 0.914$; arylsulfatase, $P_{Perm.} = 0.004$, $P_{Disp.} = 0.052$; laccase, $P_{Perm.} = 0.009$, $P_{Disp.} = 0.796$; and manganese peroxidase, $P_{Perm.} < 0.001$, $P_{Disp.} = 0.385$) (Table S1).

Bacterial communities. A total of 9,366,330 16S rRNA gene sequences were obtained, of which 6,898,021 (i.e., 73.6%) were mapped to 13,987 operational taxo-

FIG 3 Extracellular enzyme activities on beech (brown), milkvetch (yellow), and sedge (green) litters at the forest and grassland sites during the decomposition experiment ($\pm$ SD, 5 replicates). The x axes indicate sampling months (January 2011 to January 2012, i.e., 2 to 14 months after litterbag burial). The P values indicate differences between profiles' distances (PERMANOVA; P_{Perm}) and dispersion (dispersion test, P_{Disp}).

FIG 4 Average proportions of phyla in the bacterial 16S rRNA gene amplicon sequence libraries (5 replicates). Labels on the horizontal axis indicate sampling months (January to November 2011, i.e., 2 to 12 months after litterbag burial) and experimental site (G, grassland; F, forest).

nomic units (OTUs). The numbers per sample ranged between 12,630 and 78,406 sequences, with a median at 41,137 sequences.

Bacterial communities differed significantly between the forest and grassland sites in the first two sampling months for the M litter ($P = 0.009$ in January 2011, $P = 0.008$ in March) (Table S3) and in the last two sampling months for the B and S litters ($P = 0.008$ and $P = 0.006$, respectively, in July, and both species $P = 0.009$ in November 2011) (see Table S3 in the supplemental material). The bacterial community on the M litter significantly differed from that on the B and S litters (analysis of molecular variance [AMOVA], $P < 0.001$ and $P = 0.003$ at the grassland and forest sites, respectively, for both litter types) (see Table S4 in the supplemental material), with the bacterial communities not differing between the latter two litter types.

The proportions of phyla in the bacterial 16S rRNA gene amplicon sequence libraries were the highest for *Proteobacteria*, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Verrucomicrobia* (Fig. 4). These proportions increased over time for *Actinobacteria* (except on the B litter in November 2011 in the forest) and *Firmicutes* (especially from January to March 2011) and decreased for *Proteobacteria* and *Bacteroidetes* from January to November 2011 (Fig. 4). *Acidobacteria* proportions were the highest in the late sampling months on the M and S litters (Fig. 4) and in soil (see Fig. S3 in the supplemental material) in January and November 2011. *Verrucomicrobia* proportions were the highest in the middle sampling months (Fig. 4). Within the phylum *Actinobacteria* (see Fig. S4 in the supplemental material), the families *Streptomyetaceae* (mostly on S plants) and *Mycobacteriaceae* increased at both sites, while the proportions of *Pseudonocardiaceae*, *Gaiellaceae*, and *Promicromonosporaceae* increased mostly in the grassland, and the order *Frankiales* increased in the forest. The Trebon clade proportion increased in November 2011 on the S litter in the forest (Fig. 4).

The number of OTUs (cutoff, 97%) significantly contributing to differences between the sites was the lowest for the B litter and the highest for the M litter at both sites according to metastats (see Fig. S5 in the supplemental material). According to the rarefaction curves, the highest number of OTUs in January and March 2011 was found at the grassland site, especially for the B and S litter types. The number of OTUs was similar for all litter types for both sites in July, while the highest number of OTUs in November was measured for the M followed by S and B litter types at the grassland site (see Fig. S6 in the supplemental material). Rarefaction analysis showed that bacterial diversity differed between the sites (ANOVA; $F = 12.86$, $P < 0.001$). Bacterial diversity

FIG 5 Abundances of fungi (18S rRNA genes), *Actinobacteria*, *Alphaproteobacteria*, and *Firmicutes* (16S rRNA genes) assayed by qPCR on beech, milkvetch, and sedge at the forest and grassland sites during the decomposition experiment ($\pm$ SD, 5 replicates). The x axes indicate sampling months (i.e., initial in November 2010 and then in 2-month periods from January 2011; overall, 14 months).

increased on all litter types over decomposition except for the S and B litters in the forest, where the diversity declined from July to November 2011 (see Fig. S7 in the supplemental material).

Abundance of soil microorganisms. Overall, *Alphaproteobacteria* gene copies (16S rRNA) were more abundant in the grassland than at the forest but only for January and March 2011 (ANOVA, $P = 0.009$) (Table S1) and on the M than the B and S litters (both $P < 0.001$). *Actinobacteria* gene copies (16S rRNA) were more abundant in the grassland than at the forest (ANOVA, $P < 0.001$) (Table S1) but did not differ between litter types. Fungi (18S rRNA) and *Firmicutes* (16S rRNA) gene copies did not differ between the sites. *Firmicutes* were more abundant on the M than the B and S litters (ANOVA, both $P < 0.001$) (Table S1). Fungi were the least abundant on the B litter compared with both the S (ANOVA, $P < 0.001$) (Table S1) and M litters ($P = 0.015$) (Fig. 5).

The time courses of *Actinobacteria* ($P_{Perm} = 0.021$; $P_{Disp} = 0.387$) and fungi ($P_{Perm} < 0.001$; $P_{Disp} = 0.505$) were similar for the same litter type regardless of site, while dispersion did not differ between the litter types.

DISCUSSION

Decomposition rate, extracellular enzyme activities, and litter chemistry were specific according to sites and within both sites according to litter type but not litter origin. Fungi, *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Verucomicrobia* each had its own role in the decomposition process. Their successional patterns differed based on their requirements for site conditions or litter type. *Actinobacteria* were identified as late-stage site-specific substrate generalists, which was the opposite pattern to fungi. The dynamics of these two groups together with bacterial community composition were driven by litter type and were synchronized with the mass loss of the most nutritious litter of milkvetch (M).

Overall, the forest site had higher decomposition rates and enzyme activities than the grassland site. The beech forest soils are considered to be an environment limited by low soil N and P (25). Thus, nutrient limitations might stimulate microbes to produce enzymes to gain the deficient nutrients from complex substrates (26). This is also supported by our finding that P acquisition for all substrates, as well as enzyme activities, was significantly higher at the forest site (27, 28). In contrast, the nutrient-rich but warmer and drier soil with diverse sources of litter at the grassland site (29) probably sufficiently covered the demands of soil microbes (30). Consequently, a higher diversity of bacterial communities was found on the grassland litter. (31).

The profiles of the low-molecular-weight compounds derived from the litter substrates represented the molecular environment during decomposition and were characteristic of each site (29) and as well as each decomposition stage within a site (32). Over time, the litter types separated mostly according to the site as opposed to the litter types (redundancy analysis [RDA]), which demonstrates how decomposition transforms litter to a humus type specific for a given site (33).

The bacterial communities for the sedge (S) and beech (B) litters considerably diverged between sites but only in the later stages of decomposition when they were composed mostly of bacteria representing the indigenous soil communities of the sites. In contrast, the associated bacterial communities on the M litter at the two sites converged over time as the nutrient-rich M litter was sequentially exhausted (4, 10). A uniform community, therefore, reflected the final stage of decomposition for the M litter, while it possibly represented only the initial depolymerization of lignin for the S and B litters (34). Furthermore, the higher-quality M litter showed the largest shifts in bacterial OTUs over time, as well as the higher decomposition rates and highest activity of hydrolytic enzymes compared with the B and S litters across both sites (35). Thus, local-scale decomposition was driven by the local community according to the litter type (33) without any effect of endogenous or exogenous litter origin (i.e., M and S litters originating at the grassland site and beech at the forest site), contrary to the home-field advantage theory (31).

Quantitative analysis based on 16S and 18S rRNA gene abundances suggested discriminating driving factors for various microbial groups during litter decomposition. Specifically, *Firmicutes* and *Alphaproteobacteria* preferentially colonized the M litter, but colonized *Firmicutes* at both sites and *Alphaproteobacteria* (only for January and March 2011) at the grassland site. Fungi were affected more and *Actinobacteria* less by litter quality, similarly as in boreal peat soil (36). Therefore, *Actinobacteria* are substrate generalists influenced by environmental conditions, and consequently, they can specifically reflect site soil characteristics (37). In contrast, the site generalism of fungi supports this group's resiliency to environmental factors (17). However, that contrasts with the generally acknowledged assumption of fungi sensitivity to soil pH (38). Thus, in this study, the relationship between fungi and bacteria was probably neutral because fungi were pH dependent only if they grew in competition with bacteria (39).

The dynamics of *Actinobacteria* and fungi, certain enzymatic activities (arylsulfatase, chitinase, laccase, and manganese peroxidase), and mass loss were more similar for one

litter type between sites than the three litter types were similar within each site. Most of those enzymes target complex organic substrates and were found to be (except manganese peroxidase) produced by both *Actinobacteria* and fungi (20, 40–42). The ability of different soil communities to carry out decomposition of the same substrate over time regardless of the site implies functional redundancy (43). Such dynamics were, however, not the case for *Alphaproteobacteria* and *Firmicutes*, which are, therefore, likely functionally distinct (7). The results indirectly suggest that the dynamics of *Actinobacteria* and fungi were driven by the same factor, i.e., litter type (19).

The decomposition process is often considered to be separated into three stages characterized by typical microbial guilds and their functions (11). In the present study, the initial stage (from January to March 2011) was characterized by high extracellular enzyme activities, especially at the forest site. The bacterial communities on the M litter type were positively affected by available N and P, suggesting that bacteria require a lower C:N but higher N:P ratio (44), especially after intense enzyme production which likely leads to substantial losses of C and N (9). In this stage, a specific form of low-molecular-weight compounds was recorded on the M litter (45). In addition, the nutrient-rich M litter had the most dynamic bacterial communities, with high incidences of fungi and *Alphaproteobacteria* (according to quantitative PCR [qPCR]). Since fungi and *Alphaproteobacteria* are known for their ability to depolymerize recalcitrant compounds (46), their initial dominance may correspond with carbon depolymerization of the M litter. Meanwhile, *Bacteroidetes* (according to amplicon sequencing) occurred on all litter types at both sites. *Bacteroidetes* are also known for their decomposing activity, and their presence in all litter types suggested promotion by litter addition (47) regardless of its quality and origin.

The middle stage of decomposition was prevalently characterized by cellulose decomposition (48), even though in the current study it was documented only at grassland site from the increased activity of cellulolytic enzymes in May for the M litter and in July for the S litter. In this stage, *Verrucomicrobia* and *Proteobacteria* make up the highest proportion of taxa on all litter types, which suggests that they are substrate generalists and polysaccharide decomposers (49). Fungi decreased and were replaced by bacteria at both sites possibly because bacteria are scavengers benefitting from the initial cellulose degradation by fungi (48).

Finally, bacterial diversity increased especially on the M litter type over time, which suggested an increased number of ecological niches when the soil and litter communities combine (9, 50). The gradual incorporation of litter to the soil is supported by the relative increase of *Acidobacteria* and *Actinobacteria*, also increased later in the decomposition process, which corresponds to their relative abundance in soil, especially *Actinobacteria* (51). The increase of *Actinobacteria*, as well as *Firmicutes*, on the M and S litter types (according to both qPCR and relative proportion in sequence libraries) implies that late-stage decomposition is associated with Gram-positive K-strategist microbial groups (10, 52).

Actinobacteria were substrate generalists that adapt to litter of different decomposability (19, 53). *Actinobacteria* quantity, together with overall bacterial diversity, was positively correlated with the structure of the late-stage bacterial communities, suggesting they have a specific function in that decomposition stage. This conclusion was also supported by the late proportional increase of the *Actinobacteria* families *Mycobacteriaceae* and *Streptomycetaceae*, of which some genera are soil saprotrophs (54) or known for lignin breakdown (46). These families are also recognized for antimicrobial activity, which may be utilized for competitor suppression or as a synergic communication to overcome the small energetic return from recalcitrant compounds (15, 23). The relative abundance of *Frankiales* also increased in the late stages but only at the forest site, suggesting the advantage of the N fixation metabolism typical for this group in decomposing recalcitrant substrates in infertile soil (53). Interestingly, we also found an increase in the proportion of the Trebon clade later in decomposition at the forest site but only on the S litter, supporting previous findings of that clade's adaptation to low pH forest soil with recalcitrant humus (55). Finally, *Actinobacteria* might have a strong impact on litter-derived humus formation through modulation of the molecular environment and interactions within the decomposer community.

Niche partitioning is related to changes in environmental conditions (35) and might result from previous competition between species regulating their coexistence (56). This may be demonstrated by the different abundance maxima of the studied microbial groups, especially fungi and *Actinobacteria*, on the rich M litter (57). On the poorer B and S litters, the abundance curves of *Actinobacteria*, fungi, and *Alphaproteobacteria* were congruent, which may represent a synergistic interaction (58, 59). In spite of fungi being the initial decomposers, they dominated over *Actinobacteria* in the later stages at the forest site on the S litter, habitats favorable for fungi and unfavorable for *Actinobacteria*. This is in accordance with the higher fitness of specialists (fungi) at their optimal environmental conditions relative to generalists (*Actinobacteria*) at the same site (60). The S litter at the grassland site represents a niche overlap for *Actinobacteria* and fungi since it is the most favorable habitat of both. Interestingly, in this situation, *Actinobacteria* dominated over fungi, which might suggest a greater contribution of *Actinobacteria* to ecosystem functioning (61).

Site conditions had the biggest impact on the progress of decomposition, measured as litter mass loss, litter chemistry, and enzyme activities. The site conditions also determined bacterial diversity, although the litter seemed to create specific niches according to the type and stage of decomposition. Finally, the site conditions were most important for the total abundance of *Actinobacteria*. In contrast, litter type determined the abundance of fungi, while the abundance of *Alphaproteobacteria* was related to both factors. Also, litter type influenced the succession of fungi and *Actinobacteria* regardless of the site. Consequently, the observed patterns revealed that the decomposition process is independent of litter type and, therefore, may be relatively stable with respect to shifts of vegetation due to disturbance. However, the niches created by various litter types and decomposition stages may have an impact on the dynamics of the main microbial decomposers and that may lead to changes in organic matter quality and sequestration.

MATERIALS AND METHODS

Litterbag experiment. A grassland site situated on the western slope of Oblik Hill in the Bohemian Massif Central, Czech Republic, and a forest site in Dorotheer Wald, Neustift near Vienna, Austria, were chosen for the litterbag experiment. Soil humidity and temperature differed between the sites (see Fig. S8 in the supplemental material). The forest site was dominated by beech (*Fagus sylvatica*), with acidic soil (pH 5.3) and soil organic matter content of 11.7%. The grassland site was dominated by diverse forbs (29), with alkaline soil (pH 7.9) and soil organic matter content of 11.0%.

For the litter transplant experiment, we used beech leaves (B), collected in November 2010 at the forest site from beech trees before litter fall, and aboveground parts of a sedge (S; *Carex humilis*) and milkvetch (M; *Astragalus exscapus*) both cut from living plants in November 2010 at the grassland site. We chose these litter types according to their quality and decomposability as described by their C:N:P ratio. B had the highest, M the lowest, and S an intermediate C:N:P ratio (Fig. S2). Polyester litterbags of size 10 × 10 cm with a mesh size of 1 mm were filled with 7 g of dried litter, with 70 litterbags prepared for each litter type. The remaining litter was stored at −70°C for future analysis and was considered the initial sampling time (initial). Overall, 105 litterbags (35 litterbags from each litter type) were placed under the top 5 cm of the soil organic layer at each site in November 2010 (Fig. 6).

A total of 50 ml of surrounding soil and litterbag samples were collected in 2-month intervals; overall, 7 samplings were performed (January, March, May, July, September, and November 2011; and January 2012). Each litterbag was weighed to assess the weight loss during the decomposition process. The M litter was completely decomposed by January 2012 and no material remained in the litterbags. Therefore, analysis of the M litter type for this sampling time is missing (Fig. 6).

The soil and litter samples were cooled during transportation and homogenized and divided into subsamples upon arrival at the laboratory. Litter water content was assessed by the gravimetric method with oven drying (62). For microbial analysis, the soil and litter samples were placed in 2-ml sterile plastic tubes and stored at −70°C until further analysis. For high-performance liquid chromatography (HPLC) analysis, 100-ml glass bottles were filled with the soil and litter samples, overlaid with a methanol-water-acetic acid mixture (80:19:1 [vol/vol/vol]), and stored at −20°C. The remaining soil and litter samples were placed in plastic bags for an analysis of pH; organic carbon content (soil samples); and C, N, and P contents (litter samples) (Fig. 6).

Soil analysis. Soil moisture tension and temperature were recorded at both sites by a Microlog SP1 datalogger (EMS, Brno, Czech Republic) equipped with a gypsum block sensor. From the recorded values, mean temperature and humidity were calculated for every month from November 2010 to March 2012. Soil pH was measured by a Multi 350 WTW glass electrode in a soil water extract (5 g of soil was extracted with 20 ml of distilled water and set for 12 hours at room temperature). Soil organic matter content was estimated by combustion in an oven at 550°C to a constant weight.

FIG 6 Diagram showing the design of the litterbag experiment on decomposition of beech (B), milkvetch (M), and sedge (S) at the grassland (G) and forest (F) sites over 1 year (January to November 2011, i.e., 2 to 12 months after litterbag burial).

Carbon, nitrogen, and phosphorus analysis in the litter. Total phosphorus (P) content was determined colorimetrically in 20 mg of dried plant litter sequentially decomposed with HNO_3 and HClO_4 using the ammonium molybdate-ascorbic acid method on a QuikChem 8500 flow injection analysis system (Lachat Instruments, Hach Company, Loveland, CO, USA) at the Botanical Institute in Třeboň, Czech Republic (63). Total carbon (C) and nitrogen (N) contents were determined in 2 g of dried litter by high-temperature combustion, followed by gas separation in a Vario Max CNS element analyzer (Elementar Analysensysteme, Hanau, Germany) by Aquatest a.s. (Prague, Czech Republic).

HPLC analysis of soluble low-molecular-weight soil metabolites. Low-molecular-weight compounds of the soil and litter samples were analyzed by high performance liquid chromatography (HPLC) (64). The compounds were extracted in methanol-water-acetic acid (80:19:1 [vol/vol/vol]) and then filtered and vacuum concentrated. The concentrated solution was fractionated using Amberlite XAD

TABLE 1 Primers used for qPCR and for MiSeq Illumina sequencing

Procedure	Primer	Sequence, 5'–3'	Concn (μM)	Target	Annealing temp (°C)	Reference
qPCR	EUB518 ^a	ATTACCGCGGCTGCTGG	0.2	Bacteria, 16S rRNA	56	76
	Act235	CGCGGCCTATCAGCTTGTTG	0.2	Actinobacteria, 16S rRNA	56	77
	S-C-aProt-0528-a-S-19	CGGTAATACGRAGGGRGYT	0.4	Alphaproteobacteria, 16S rRNA	61	67
	S-C-aProt-0689-a-A-21	CBAATATCTACGAATTTCACCT	0.4	Alphaproteobacteria, 16S rRNA	61	67
	S-P-Firm-0352-a-S-18	CAGCAGTAGGGAATCTTC	0.2	Firmicutes, 16S rRNA	57	67
	S-P-Firm-0525-a-A-18	ACCTACGTATTACGCGG	0.2	Firmicutes, 16S rRNA	57	67
	FF390	CGATAACGAACGAGACCT	0.6	Fungi, 18S rRNA	50	78
	FR1	AICCATTCATCGGTAIT	0.6	Fungi, 18S rRNA	50	78
Sequencing	CS1-515F	ACACTGACGACATGGTTCTA- CAGTGCCAGCMGCCGCGGTAA		Bacteria, 16S rRNA		68
	CS2-806R	TACGGTAGCAGAGACTTGTT CTGGACTACHVGGGTWTCTAAT		Bacteria, 16S rRNA		68

^aEub518 was used as a reverse primer for Act235.

1180 (5 g aqueous suspension; 2-cm-diameter glass column, equilibrated with water). A total of 50 ml of water (MilliQ quality) was used as the effluent for the first (hydrophilic) fraction and 50 ml of absolute ethanol for the second (lipophilic) fraction. The ethanolic fraction was evaporated and diluted in methanol at a concentration of 5 mg ml⁻¹. The analysis was performed using the Acquity ultraperformance liquid chromatography (UPLC) system with a 2996 photodiode array (PDA) detector (Waters, Milford, MA, USA) with a Phenomenex Luna column (5 mm, C18[2], 100 Å, 100 × 4.6 mm) equipped with a SecurityGuard cartridge (C18, 4 × 3.0-mm internal diameter [ID]; Phenomenex, Torrance, CA, USA). The separation was run in an air-conditioned room at 20°C, with a flow rate of 1.0 ml min⁻¹. Solvent A was ultrapure water, while solvent B was acetonitrile. The gradient started with 10% of solvent B for 1 min and then increased linearly to 100% of B within 8 min. The final concentration was held for a further 3.5 min. A total of 20 μl of each sample was injected. The spectra were recorded from 194 to 800 nm in 1-nm intervals. Data points were recorded twice per second and downloaded using MassLynx software (Waters) in text format for further processing.

Enzyme extraction and assays. The activities of the extracellular hydrolytic enzymes α-glucosidase (EC 3.2.1.3), 1,4-β-xylosidase (EC 3.2.1.37), 1,4-β-glucosidase (EC 3.2.1.21), acid phosphatase (EC 3.1.3.1), chitinase (EC 3.2.1.52), cellobiohydrolase (EC 3.2.1.91), glucuronidase (EC 3.2.1.31), and arylsulfatase (EC 3.1.6.1) were directly measured using methylumbelliferone-derived fluorogenic substrates in aliquots of litter samples homogenized in 50 mM sodium acetate buffer at pH 5.0 (65).

For the extracellular oxidative enzymes laccase (EC 1.10.3.2) and manganese peroxidase (EC 1.11.1.13), aliquots were desalted to separate inhibitory molecules. The activity of laccase was measured by monitoring the oxidation of 2,20-azino-3-ethylbenzothiazoline-6-sulfonic acid in a citrate-phosphate buffer (pH 5.0) (66). Manganese peroxidase activity was assayed in a succinate-lactate buffer (100 mM, pH 4.5) using 3-methyl-2-benzothiazolinone hydrazone and 3,3-dimethylaminobenzoic acid, which were oxidatively coupled by the enzyme. The results were compared to the activities of samples without manganese (for manganese peroxidase, the addition of manganese sulfate was substituted by an equimolar amount of EDTA). One unit of enzyme activity is defined as the amount of enzyme forming 1 μM reaction product per minute. Enzyme activities were measured in three analytical replicates and expressed per gram of plant litter dry mass (48).

DNA extraction and microbial community analysis. DNA was extracted from litter and soil samples according to Sagova-Mareckova et al. (67). The method is based on bead beating and phenol-chloroform extraction. The samples were purified by incubation with acetyltrimethylammonium bromide, followed by chloroform extraction and incubation with CaCl₂, and finally cleaned with the GeneClean turbo kit (MP Biomedicals, Santa Ana, CA, USA). DNA extracts were stored at -70°C until further analysis.

Illumina MiSeq and data processing. Bacterial 16S rRNA gene fragments, including the variable region V4, were amplified by PCR using universal primers with overhang adapters (Table 1) (68). The construction of amplicon libraries and sequencing using the MiSeq sequencer (Illumina, San Diego, CA, USA) were done at the DNA Services Facility, Research Resources Center, University of Illinois (Chicago, IL, USA). The resulting paired sequence reads were merged, filtered, and aligned using a reference alignment from the Silva database (69) and chimera checked using the integrated Vsearch tool (70) according to the MiSeq standard operation procedure (MiSeq standard operating procedure [SOP], February 2018 [68] in Mothur v. 1.39.5 [70]). A taxonomical assignment of sequence libraries was performed in Mothur using the nonredundant subset of the SILVA small subunit rRNA database, release 132 (71), adapted for use in Mothur (https://mothur.org/w/images/3/32/Silva.nr_v132.tgz), as the reference database. Sequences of plastids and mitochondria and those not classified in the domain *Bacteria* were discarded.

The sequence library was clustered into OTUs (97%) using Usearch v. 10.0.240 (72), and the OTU table was further processed using tools implemented in Mothur. Distance matrices describing the differences in community composition between individual samples were calculated using Bray-Curtis dissimilarity (73). Analysis of molecular variance (AMOVA) (74) was based on a matrix of the Bray-Curtis distances. Metastats (75) analyses were used to detect differentially represented OTUs. Rarefaction analysis was performed with the rarefaction.single function in Mothur, and areas under the rarefaction curves were

calculated using the trapezoidal rule in R version 3.4.4 (76). Figures were created using Inkscape (v. 0.92; <http://www.inkscape.org>).

Quantitative real-time PCR. Quantification of the 16S rRNA gene in *Actinobacteria*, *Alphaproteobacteria*, and *Firmicutes* and the 18S rRNA gene of fungi in the DNA extracted from litter samples was performed with the primers listed in Table 1 (77–79).

Quantitative PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using 96-well plates with GoTaq qPCR master mix (Promega, Madison, WI, USA) containing SYBR green as a double-stranded DNA (dsDNA) binding dye. The reaction mixture contained in total a volume of 15 μ l with 1 $\times$ GoTaq qPCR master mix, primers (for concentrations see Table 1), and 2-ng DNA sample. For all microbial groups, the PCR cycling protocol consisted of initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation (95°C for 15 s), annealing (for respective temperature see Table 1), and elongation (72°C for 30 s). Standards containing a known number of copies of the target genes 16S rRNA from *Streptomyces europaeiscabiei* DSM 41802 (for *Actinobacteria*), *Bartonella*-like clone (for *Alphaproteobacteria*), and *Paenibacillus* clone (for *Firmicutes*) and 18S rRNA from a *Penicillium* sp. clone (for fungi) were serially diluted to calibrate each qPCR melting curve and were recorded to ensure qPCR specificity. Baseline and threshold calculations were performed with StepOne v. 2.2.2. Inhibition was tested by serial DNA dilutions from each site. All qPCR measurements were done in duplicate.

Statistical analysis. All statistical calculations were done in the R computing environment (76). The effect of sites and litter types on bacterial community composition and the litter molecular parameters during the decomposition process were measured as the relationship between the distance matrices of the HPLC profiles of low-molecular-weight compounds (HPLC) and bacterial communities using a generalized distance covariance (80). The HPLC profiles were baseline corrected using the PTW package (81), and distances between profiles were calculated using Manhattan metrics. The distance matrices of the HPLC profiles and bacterial communities (exported from Mothur) were plotted by RDA projection and Sammon's multidimensional scaling using the MASS package (82). Correlations between linear variables, bacterial community, and HPLC profiles were analyzed by permutation test for capscale under a reduced model using the Bray-Curtis (bacterial communities) and Manhattan (HPLC) dissimilarities (with 999 permutations) in the VEGAN package in R (83).

To analyze the overall quantitative differences of linear variabilities (dry mass loss; abundances of microbial groups; enzyme activities; and C, N, and P contents in litter) between sites and litter types (within sites), a linear model with the additive effect of time, site, and litter type was calculated (84).

We used two tests based on the distance between the profiles as measured by correlation coefficients (see p. 319 of reference 85) to analyze the qualitative similarities between the linear variables. Permutational multivariate analysis of variance (PERMANOVA; significance values denoted here as P_{perm}) (86) tests whether the within-group distances (same litter type) are on average shorter than the between-group distances (between different litter types, aimed at detection of different mean profiles). The dispersion test (significance values denoted here as P_{disp}), introduced in Gijbels and Omelka (87), checks whether the average within-group distances differ among groups (aiming at detecting different dispersions of the samples). For manganese peroxidase, a Manhattan distance instead of the distance based on the correlation coefficient was used due to zero values in some profiles.

All linear variables (dry mass loss; microbial gene quantities; C, N, and P content; and enzyme activities, except manganese peroxidase) were logarithmically transformed (with negative values of enzymes replaced by 1 before transformation) to make their distribution more similar to a normal distribution.

Data availability. The Illumina MiSeq 16S rRNA gene amplicon sequences have been deposited in the NCBI Sequence Read Archive under accession no. [SRP158384](https://www.ncbi.nlm.nih.gov/sra/SRP158384) and are available under BioProject accession number [PRJNA485178](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA485178).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01760-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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We declare no conflict of interest.

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