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Analysis of Microbial Diversity and Greenhouse Gas Production of Decaying Pine Logs

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Abstract: In Sustainable Forest Management, decaying wood plays an important role in forest biodiversity, carbon balance and nutrient cycling. The management of this important component of forest ecosystems is limited by the fact that little is known about relationships between substrate quality and community structure of wood-inhabiting microorganisms. During decomposition, carbon stored in deadwood is lost either in the atmosphere or in the soil, but to our knowledge, limited information on the quantities of CO_2 and other greenhouse gases (GHG) emitted is available. In the present research we investigated the correlation between the decay of logs, the decomposer microorganisms and their activities, in terms of GHG production and enzymes, in a black pine (*Pinus nigra* Arnold ssp. *nigra*) degraded forest. The decomposition of deadwood was visually assessed using a five-class system, and for each decay class four wood samples were collected. CO_2, CH_4 and N_2O potential production from each decay class was measured in closed systems by means of gas chromatography. Enzyme activities related to carbon, nitrogen, sulphur and phosphorus cycling were measured fluorometrically. The composition of decomposer microbial communities (fungi, bacteria and actinobacteria) was assessed by using polymerase chain reaction-denaturing gradient gel electrophoresis fingerprinting. CO_2 production and enzyme activities were significantly higher in the last decay classes of deadwood. The molecular approach highlighted differences in microbial community structure both at species and abundance levels, depending on the rate of decay.

Keywords: deadwood; decay classes; GHG potential production; microbial biodiversity; peri-urban forests; Monte Morello (Italy)

1. Introduction

Sustainable Forest Management (SFM) is a dynamic concept focused on the maintenance of the economic, social and environmental value of forests, for the benefits of present and future generations [1–4]. From a practical point of view, SFM contributes to maintain the functionality of forest ecosystems as sources of products (i.e., timber, fuel wood, fodder, non-wood forest products), while simultaneously contributing to maintain biodiversity and to supply several ecosystem services useful for the society such as water and carbon cycles, natural hazards protection and recreation [5]. This new paradigm has allowed the spread of forest management practices aimed at considering all components of forest ecosystems including deadwood [6]. Deadwood is defined as all non-living woody biomass not contained in the litter, either standing, lying on the ground, or in the soil [7] and

it can be classified in three main components [8,9]: lying deadwood or logs, standing dead trees or snags, and stumps.

Lying deadwood (or logs) is a fundamental component of forest biodiversity because a large number of organisms are dependent on decaying wood for nutrients or habitat [10–13]. Saproxylic fungi and wood-inhabiting bacteria are particularly important in consideration of their role in wood decomposition and, consequently, in nutrient and carbon cycling [14,15]. The process of microbial wood decay is related to the degradation of polysaccharides and biopolymers, cellulose, hemicelluloses and lignin, which are the components of plant cell walls. Saproxylic fungi modify the deadwood logs both chemically and structurally, and generate new habitats and food resources for other species [16]. The fungal decomposer community (i.e., fungal phyla *Ascomycota* and *Basidiomycota*) play a crucial role for the diversity of other organisms associated with deadwood (i.e., saproxylic insects) and for deadwood carbon dioxide (CO_2) emission rates [17]. In particular, white-rot fungi are particularly important in lignin mineralization because these fungi secrete a set of extracellular oxidative enzymes to oxidize the recalcitrant lignin polymer, while brown-rot fungi oxidize lignin via a mechanism relying on hydroxyl radicals [18]. Conversely, bacteria are capable to colonize wood under aerobic and anaerobic conditions and the erosion bacteria are even able to attack the cell wall [19]. Hydrolytic enzymes produced by fungal and bacterial species mediate the breakdown of the polysaccharide compounds, but few studies address their relationship with deadwood decay classes and CO_2 emission rates [20,21]. In the international literature, many studies focus on the decomposition rate constants for several tree species to estimate fluxes of carbon from the deadwood decay classes to the atmosphere and factors that influence deadwood decomposition [22–24]. In addition, several studies highlight that two attributes of forests influence the saproxylic fungal community and its species richness [25–29]: the amount (volume of deadwood) and the quality of deadwood. Conversely, the diversity of bacterial communities in deadwood and the relationships between amount and quality of deadwood and bacterial species richness are little explored [17]. Currently, the knowledge gap is in the relationship between saproxylic fungal and bacterial communities and decay classes of deadwood. In fact, few studies have investigated the bacterial and fungal abundances in the different decay classes of deadwood [29].

Starting from these considerations, the study aims at analyzing saproxylic fungal and bacterial community diversity in different decay classes of lying deadwood in a black pine (*P. nigra* Arnold ssp. *nigra*) forest. The study has been focused on the relationships between saproxylic fungal, bacterial and actinobacterial biodiversity and decomposition rates of deadwood. The investigation was conducted in a case study in Central Italy (Monte Morello peri-urban forest in Tuscany region). The composition of microbial communities (fungi, bacteria and actinobacteria) was assessed by using the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting method. DGGE has been extensively used to study microbial community composition in various environments and although this method tends to bias towards the predominant microbial groups within a community [30], it may as well provide indications on the relative changes in abundance and genetic community structure between samples.

The potential decomposition rate of deadwood was assessed by measuring the CO_2 production in closed systems by means of gas chromatography. With this technique at the same time the potential output of methane (CH_4) and nitrous oxide (N_2O) was also measured, assessing the potential involvement of deadwood in GHG production. Moreover, the potential microbial activity in deadwood was also evaluated by fluorimetric assay to measure the activity of enzymes related to the main nutrient cycles.

2. Materials and Methods

2.1. Study Area

The study area is Monte Morello (43°51′20″ N; 11°14′23″ E)—a morphologically well-circumscribed mountain range—located in the Tuscany region in Central Italy. Monte Morello consists

of a series of hills maintained at around 700 m. and reaches a maximum altitude at 934 m. It is formed from geological formations of the Paleocene and Eocene, composed of alternating white and gray limestone marls, clayey schists and calcareous sandstones. The area is characterized by very different climates because it is subjected to geographical conditions of relative isolation, which means that in the southern and western zones, the climate is Mediterranean—with mild winters and dry summers—whereas in the northern and eastern zones the climate is affected by the continental features of the neighboring Mugello valley that makes summers cooler and winters colder. During the last decades (from the early 1980s) the total annual rainfall is 1003 mm and the average annual temperature is 13.9 °C.

With regard to land uses, the study area offers a predominantly forest landscape, consisting of a mosaic of deciduous and coniferous forests alternated with few agricultural fields (olive groves, meadows and arable lands). The main native deciduous species are Downey oak (*Quercus pubescens* Willd.), Turkey oak (*Q. cerris* L.) and hop hornbeam (*Ostrya carpinifolia* Scop.). Regarding conifers, the main species are black pine (*P. nigra* Arnold ssp. *nigra*), Calabrian pine (*P. laricio* Poir.) and Mediterranean cypress (*Cupressus sempervirens* L.), growing in part as a result of reforestation measures carried out from 1909 to 1980 for the restoration of degraded forests [31]. Monte Morello reforestation was realized with a density of more than 2500 trees per hectare, but during the rotation period the necessary silvicultural treatments were not applied, and the stands have been largely abandoned, with important consequences on tree stability, mortality and increase of fire risk [32]. Currently, Monte Morello forest can be considered a degraded forest characterized by poor regeneration, huge quantity of deadwood and a high degree of flammability.

2.2. Experimental Design and Deadwood Sampling

The relationship between fungal and bacterial community diversity and deadwood was analyzed on 20 samples of lying deadwood distributed among five decay classes based on a visual classification system. The five decay classes were assigned considering the main visual characteristics of deadwood such as the structure of the bark, the presence of small branches, the softness of wood, the rot extension and the development of fungal mycelium [33–35]. The visual assessment of rates of decay was executed by two forest technicians. The two forest technicians worked together during the visual assessment of decomposition rate of deadwood. In addition, with the aim of harmonizing their evaluations and to minimize bias in visual estimates, technicians were previously trained in field for two days. The main characteristics used to assign the decay class in the field are shown in Table 1.

Table 1. Description of visual characteristics used to assign decay class of deadwood in a black pine (*P. nigra* Arnold ssp. *nigra*) forest in Monte Morello, Italy.

Decay Class	Description
1	Recently dead—bark intact, small branches, wood texture intact, visible small rotten areas under bark
2	Weakly decayed—bark intact but not attached, twigs partly present, wood texture intact, rotten areas <3 cm
3	Medium decayed—trace of bark, no twigs, wood texture partly broken, rotten area >3 cm
4	Very decayed—no bark, no twigs, wood soft, texture with blocky pieces, large rotten areas
5	Almost decomposed—no bark, no twigs, wood soft, powdery texture, very large rotten areas with musk and lichens

The samples of lying deadwood were collected in 18 circular fixed-area plots (531 m^2) randomly located in the Monte Morello study area where field measurements for dendrometric data and

deadwood assessment were realized [36]. Deadwood samples were subjectively selected in the different plots in order to have in total 4 samples in each decay class, with a total of 20 deadwood samples.

The samples of lying deadwood (cylindrical core of deadwood) were collected using a battery drill (20.4 V) with a modified bit following the procedure proposed by Paletto and Tosi [35]. The modified bit is designed to remove standard cylinders of wood with a volume comprised between 15.30 to 56.52 cm^3. The diameter of the cylinder was fixed (3 cm), while the length was variable and it was measured by a caliper with an accuracy of 1/10 mm.

The samples were drawn from the middle of the log piece with the drill bit towards the ground. Each sample of lying deadwood was placed in a sterile plastic bag and collected samples were transported to laboratory for subsequent analysis.

2.3. GHG Potential Production from Deadwood

The potential production of GHGs (CO_2, N_2O, CH_4) from the different decay classes was estimated by gas chromatography using a closed system and measuring the gas produced by each deadwood sample in the arc of 48 h.

Each deadwood sample was weighed and put into a 1-L Erlenmeyer flask closed with a silicone gas-tight cap and incubated at controlled moisture and temperature conditions to assess potential production of GHGs. Within each flask, a plastic tube containing 3 mL of water was added to keep the internal moisture constant during the test. Two empty flasks of the same size were used as a negative control (blank). Flasks were incubated at 20 °C and gas production was monitored in the arc of 48 h through measurements performed at the beginning of the experiment (T_0) and after 6, 9, 24 and 48 h (T_1, T_2, T_3 and T_4, respectively). At each incubation time, 25-mL headspace gas samples were collected with an air-tight 30-mL propylene syringe and immediately pressurized into a pre-evacuated 12-mL glass Exetainer® vial (Labco Ltd., Buckinghamshire, UK) with a septa screw cap (Labco Ltd.). After each air sampling, flasks were opened, and closed again with the silicone gas-tight cap. Concentrations of CO_2, N_2O and CH_4 were analyzed using a GC-2014 gas chromatograph (Shimadzu Scientific, Kyoto, Japan) with a thermal conductivity detector (TCD) for CO_2, a ^{63}Ni electron capture detector (EDC) for N_2O and a flame ionization detector (FID) for CH_4. The GC detection limits were 8357 $ng \cdot L^{-1}$, 84.8 $ng \cdot L^{-1}$ and 11.6 $ng \cdot L^{-1}$ for CO_2, CH_4 and N_2O, respectively. Gas samples were analyzed within one week of collection. At each incubation time, blank controls concentration was subtracted from the samples concentrations, to remove background noise of atmospheric concentrations of CO_2, N_2O and CH_4. The difference between blank and samples was 5,018,500 $ng \cdot L^{-1}$, 303.5 $ng \cdot L^{-1}$ and 37 $ng \cdot L^{-1}$, for CO_2, CH_4 and N_2O respectively and was always higher than instrumental detection limits.

Gas concentrations were converted to mass per volume units using the Ideal Gas Law and measuring air temperatures and volumes, then expressed in $mg \cdot g^{-1}$ considering the headspace of each flask and the weight of each deadwood sample. The cumulative curve was calculated for each gas by summing the concentrations at each incubation time.

After the last gas sampling, deadwood samples were removed from flasks and dried to determine the dry-weight and percentage of moisture as described below.

2.4. DNA Extraction and PCR-DGGE Analysis of Microbial Communities

At the end of the GHG production experiment, the deadwood samples were processed for the analysis of deadwood microbial (fungi, bacteria, and actinobacteria) community structure by PCR-DGGE technique. Firstly, deadwood samples were coarsely ground in an IKA A11 analytical mill for 30 s (IKA, Staufen, Germany) and successively the genomic DNA was extracted by using the Fast DNA SPIN kit for soil (Biomedicals, Santa Ana, CA, USA). Afterward, 0.25 g of sawdust of each deadwood sample was homogenized with a FastPrep cell disrupter instrument (Bio101, ThermoSavant, Qbiogene, Carlsbad, CA, USA) for 40 s at 6.0 speed and then processed according to the manufacturer's guidelines. DNA was eluted in sterile water and its integrity was verified by agarose gel electrophoresis (1% w/v). For DGGE analysis, the extracted DNA was amplified using specific primers for

V7–V8 region of fungal 18S rDNA (EF390–GCFR1; [37]), for V6–V8 region of bacterial 16S rDNA (GC986f–UNI1401r; [38]), and for V2–V3 region of actinobacterial 16S rDNA (F243–R513GC; [39]). PCR reactions were carried out using a T100 Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK) in 25 µL volumes containing 1× Flexi PCR buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 250 µM deoxynucleotide triphosphates (dNTPs), 400 nM each primer, and 1U GoTaq®Flexi DNA polymerase (Promega). Amplifications were performed under the following conditions: an initial denaturation of 94 °C for 5 min followed by 35 cycles of 94 °C for 20 s, annealing (48 °C, 55 °C and 60 °C for fungi, bacteria, and actinobacteria, respectively) for 30 s and 72 °C for 45 s, and a final extension of 72 °C for 10 min. Three independent PCR amplifications were performed for each primer set and each deadwood sample, and the triplicate amplification products were pooled to minimize the effect of PCR biases. After PCR, amplified products were verified by agarose gel electrophoresis (1.2% w/v) and amplicon yields were estimated by comparison of amplified DNA to Low DNA mass ladder (Invitrogen, Carlsbad, CA, USA) using the Chemidoc Apparatus (Bio-Rad). The DGGE analysis was carried out using a Dcode DGGE System (Bio-Rad). Amplicons (600 ng) were loaded on a polyacrylamide gel (40% acrylamide/bis 37.5:1; Serva Electrophoresis GmbH, Heidelberg, Germany) containing a linear chemical denaturant gradient obtained with a 100% denaturant solution consisting of 40% v/v deionized formamide, 7 M urea. In particular, 18S-DGGE for fungal community was carried out in an 8% polyacrylamide gel and a 35–65% denaturing gradient; 16S-DGGE for bacterial community was carried out in a 6% polyacrylamide gel and a 45–65% denaturing gradient; and 16S-DGGE for actinobacterial community was carried out in an 8% polyacrylamide gel and a 42–75% denaturing gradient. The gels were run in 1× TAE buffer for 17 h at 60 °C and constant voltage (75 V). After electrophoresis, gels were stained with SYBR®Gold (Molecular Probes, Eugene, OR, USA) diluted 1:1000 in 1× TAE buffer, and images were digitally captured under UV light (λ = 302 nm) using ChemiDoc XRS apparatus (Bio-Rad).

Evaluation of band migration distance and intensity within each lane of the DGGEs was performed using Gel Compare II software v. 4.6 (Applied Maths, Saint-Martens-Latem, Belgium). Although amplification products coming from the different microbial species can co-migrate, each band was considered as corresponding to a single microbial group and band intensity (relative surface of the peak compared to the surface of all the peaks in the profile) as corresponding to the relative abundance of the corresponding microbial group [40]. The number of bands and their relative abundance were used as a proxy of taxon richness [41] and diversity (Shannon index, H′) of microbial communities within each DGGE profile, as described by Pastorelli et al. [42].

2.5. Analysis of Physical and Chemical Properties

Moisture was determined immediately after the GHG production experiment by measuring fresh weight and dry weight after incubation at 50 °C for 48 h.

After the first coarse ground described above, deadwood samples were homogenized with a cutting mill (Retsch SM 100, Haan, Germany), at rotor speed of 1500 min^{-1}, until a final fineness of 0.25 mm. After sieving, nitrogen and carbon contents of homogenized samples were measured by dry combustion on a Thermo Flash 2000 NC soil analyzer (Fisher Scientific, Waltham, MA, USA). To this aim, 10 to 20 mg samples were weighed into Ag-foil capsules and % of N and C was measured by thermal conductivity detector.

2.6. Enzyme Assays

Enzyme activity was measured according to Marx et al. [43], based on the use of fluorogenic methyl-umbelliferyl (MUF) substrates. The deadwood sawdust fractions, obtained as previously described, were analyzed for β-cellobiohydrolase (exo-1,4-β-glucanase, EC 3.2.1.91), acid phosphatase (EC 3.1.3.2), β-glucosidase (EC 3.2.1.21), *N*-acetyl-β-glucosaminidase (EC 3.2.1.30), xylanase (EC 3.2.1.8), butyrate esterase (EC 3.1.1.1.), acetate esterase (EC 3.1.1.6), and arylsulphatase (EC 3.1.6.1). The respective substrates were 4-MUF-β-D-cellobioside, 4-MUF-phosphate, 4-MUF-β-D-glucoside,

4-MUF-*N*-acetyl-β-glucosaminide, 4-MUF-7-β-D-xyloside, 4-MUF-butyrate, 4-MUF-acetate, and 4-MUF-sulfate. A moist deadwood sawdust sample (equivalent to 1 g of oven-dried material) was weighed into a sterile jar and 50 mL of Na-acetate buffer, pH 5.5, were added. A homogeneous suspension was obtained by homogenizing with UltraTurrax at 9600 rpm for 3 min. Aliquots of 50 µL were withdrawn and dispensed into a 96-well microplate (three analytical replicates per sample per substrate). Finally, 50 µL of 1 mM substrate solution was added, yielding a final substrate concentration of 500 µM. Fluorescence (excitation 360 nm; emission 450 nm) was measured with an automated fluorimetric plate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA) after 0, 30, 60, 120, and 180 min of incubation at 30 °C.

Enzyme activity was expressed as absolute activity in nmoles of MUF g^{-1} oven-dry deadwood h^{-1}.

2.7. Statistical Analysis

The results of chemical analysis, GHG cumulative emissions, microbiological data (richness and Shannon diversity index), and enzymatic activity were analyzed by one-way analysis of variance (ANOVA) followed by Fisher least-significance difference (LSD) *post-hoc* test to assess the significance of differences between mean values ($p < 0.05$) by using Statistica software (Palo Alto, CA, USA). Pearson correlation analysis was performed between chemical, biochemical and microbiological data by PAST3 software (Oslo, Norway) [44].

The banding patterns of each DGGE, based on position and presence/absence of the bands in the different profiles, were imported into PAST3 for multivariate statistical analysis. Non-metric multidimensional scaling (MDS) was used to represent the distance between each DGGE profile in a two-dimensional space. The accuracy of the MDS plots was determined by calculating a 2D stress value. Analysis of similarity (ANOSIM) was performed to determine whether the distances between the microbial communities of the different decay classes that were observed in the MDS plots were statistically significant. An ANOSIM *R* value of 1 indicates that replicates within a decay class are more similar to each other than to any samples from another class, whereas an *R* value of 0 indicates that there is as much variation within a group as among groups being compared. To complete the ANOSIM, a permutational analysis of variance (PERMANOVA) was also performed to determine the extent of the differences between DGGE profiles according to the different decay classes. In deciding whether deadwood decomposition status influenced microbial community structure, both *R* and *F* values and the significance level were considered. MDS, ANOSIM, and PERMANOVA were conducted using the Dice distance measure and 9999 permutation tests.

3. Results

3.1. GHG Potential Production from Deadwood

Cumulative values of CO_2 potential production showed a typical trend towards a plateau for the five decay classes. CO_2 production started to differentiate significantly among the five decay classes after the first hours of incubation (Figure S1A). At the end of 48 h, CO_2 production showed the following ranking: decay classes 4 and 5 > decay classes 2 and 3 > decay class 1 (Figure 1A).

N_2O potential production showed a different trend during the incubation time, with a fast increase in the first 9 h, which reduced at 24 h and stabilized until the end of incubation (Figure S1B). Decay classes 1 and 2 showed minimum values and decay classes 4 and 5 maximum values from the beginning. Overall, maximum values reported in Figure 1B were found between 9 and 48 h of incubation, without a clear trend among decomposition classes. The ranking was similar to that of CO_2, with decay class 5 $\geq$ decay class 4 $\geq$ decay class 3 $\geq$ decay classes 1 and 2.

CH_4 potential production showed a slow increase at the beginning of incubation, not significantly different in the first 24 h (Figure S1C). The highest values were recorded at the end of incubation and are reported in Figure 1C. The maximum values were found in the decay class 5, significantly different from decay classes 1–3.

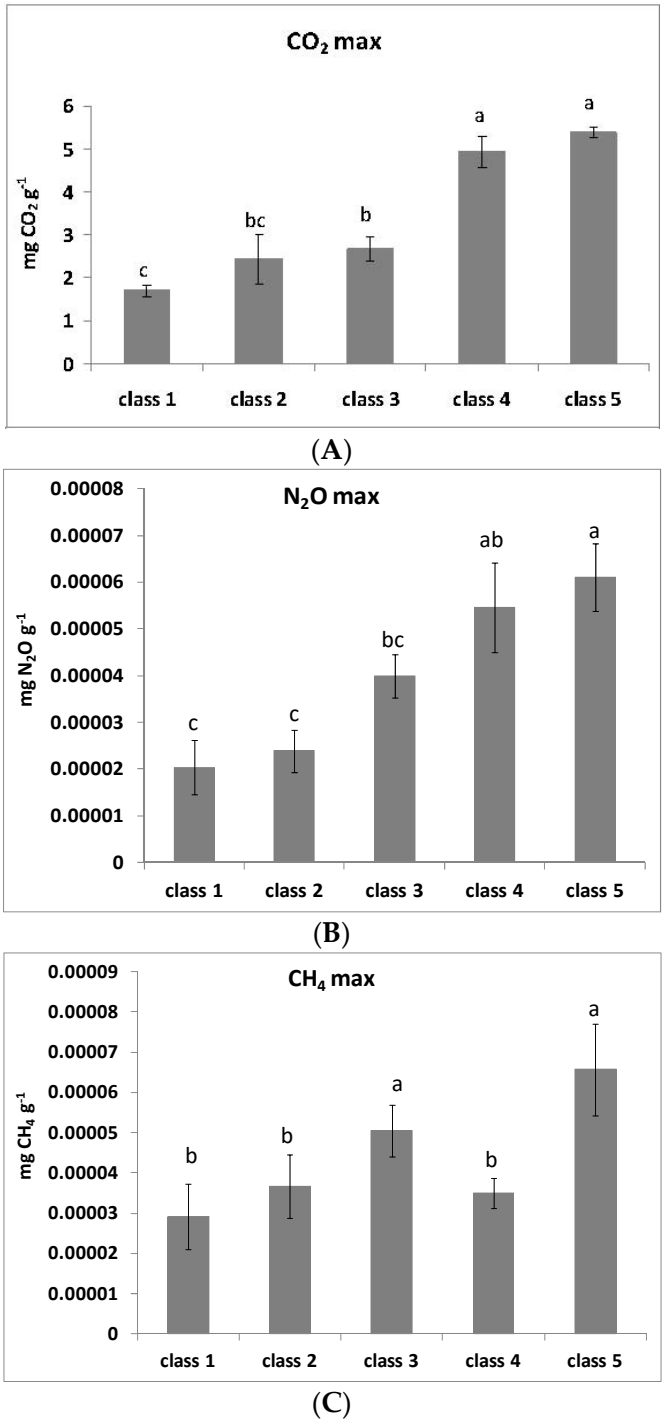

Figure 1. Maximum values CO_2 (**A**); N_2O (**B**); and CH_4 (**C**) production from the black pine deadwood of the five different decomposition classes. Vertical bars indicate standard errors of means (n = 4). Different letters indicate significant differences at $p < 0.05$ among means.

3.2. PCR-DGGE Analysis

The DGGE methodology generated fingerprints (Figure S2) that highlighted the dominant microbial representatives of fungal, bacterial, and actinobacterial communities of deadwood. The DGGE band profiles of bacterial community showed the highest values of both richness and Shannon-Weiner index (27.8 ± 0.9 and 3.28 ± 0.03, respectively), those of actinobacterial community

showed the lowest values (13.1 ± 0.93 and 2.50 ± 0.06, respectively), whereas those of fungal community showed intermediate values (17.7 ± 0.7 and 2.83 ± 0.045, respectively). Significant differences among means were observed for both indices.

Evaluating the abundance (richness) and diversity (Shannon-Weiner index) of DGGE bands according to the different decay classes, significant differences were found in fungal and actinobacterial DGGE profiles of decay classes 1 and 5, which showed the highest and lowest values, respectively (Table 2). A comparison of these indices calculated on total bacterial 16S-DGGE profiles showed a different trend, and the highest values were found in samples of decay class 3 and the lowest values were found in samples of decay classes 1 and 4 (Table 2).

Table 2. Average species richness and Shannon-Weiner diversity index calculated on 18S- and 16S-DGGE band profiles from fungal, bacterial, and actinobacterial communities in black pine deadwood, on the basis of decay class; standard errors in parentheses.

DecayClass	Fungi (18S-DGGE)				Bacteria (16S-DGGE)				Actinobacteria (16S-DGGE)			
	Richness		Shannon		Richness		Shannon		Richness		Shannon	
1	20.2 (0.5)	a	2.99 (0.02)	a	25.0 (1.1)	b	3.19 (0.04)	b	16.7 (1.9)	a	2.69 (0.05)	a
2	16.7 (0.2)	ab	2.80 (0.02)	ab	29.2 (1.8)	ab	3.33 (0.06)	ab	13.7 (1.8)	ab	2.58 (0.12)	a
3	18.2 (0.8)	ab	2.88 (0.05)	ab	31.5 (0.9)	a	3.42 (0.03)	a	14.0 (2.0)	ab	2.62 (0.14)	a
4	17.5 (0.9)	ab	2.83 (0.06)	ab	25.0 (1.8)	b	3.18 (0.07)	b	12.5 (1.9)	ab	2.48 (0.14)	ab
5	15.7 (2.9)	b	2.67 (0.20)	b	28.2 (2.8)	ab	3.29 (0.11)	ab	9.0 (0.7)	b	2.18 (0.08)	b

Different lowercase letters in a column indicate significant differences at $p < 0.05$ among means.

In all MDS ordinations (Figure 2), the replicates from decay class 1 resulted distinctly separated from the others' ribotype profiles. This separation was more evident for fungal community and it is possible to observe a rank of fungi > bacteria > actinobacteria. For fungal communities, replicates of decay classes 2 and 3 grouped separately whereas replicates of decay classes 4 and 5 formed a single group, clearly separated from the other decay classes (Figure 2A). For bacterial communities, replicates of decay classes 2–5 exhibited slight separation, although MDS plots clearly indicate that those DGGE profiles were more similar within each decay class than to replicates from other classes (Figure 2B). For actinobacterial community, the DGGE profiles of middle and late stages of decomposition (decay classes 2–5) were loose and overlapping and no particular grouping could be highlighted (Figure 2C). The relative positions of the points in the MDS were weakly reliable, in particular for actinobacteria (stress = 0.344), and DGGE profiles were further analyzed by multivariate analysis.

The outcomes of the ANOSIM global test revealed significant differences in the genetic community structure of all microbial groups analyzed, according to decay class (Table 3). The larger R values for fungal community structure revealed that wood decomposition status had a greater effect on the fungal community than on the bacterial or actinobacterial community structure.

For fungal community, pair-wise comparisons between the different decay classes were significantly different ($p < 0.05$), except for decay classes 4 and 5 (Table S1). For bacterial community, all pair-wise comparisons were significantly different ($p < 0.05$). For actinobacterial community, DGGE profiles of decay class 1 resulted significantly different ($p < 0.05$) from all other classes, whereas it is to be underlined the lack of any significant difference between the middle decay classes (classes 2 and 3) and between the two late decay classes (classes 4 and 5). Same significant results were obtained by PERMANOVA analysis (Table 3 and Table S1).

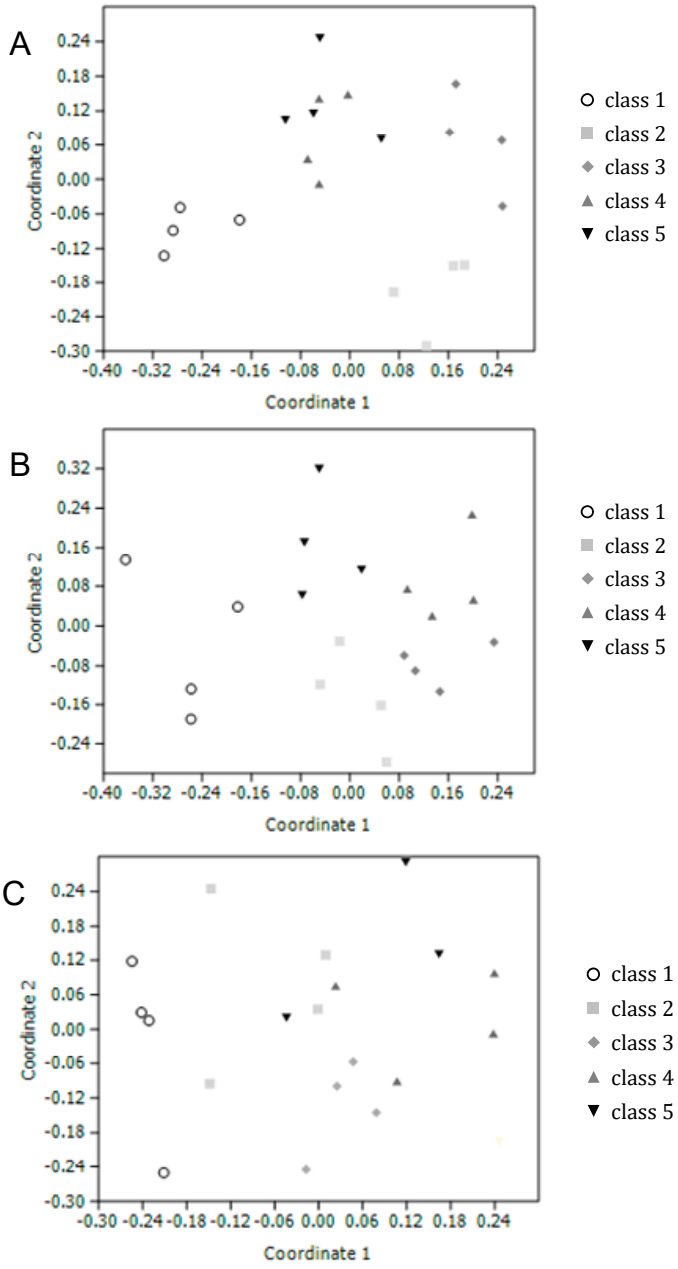

Figure 2. MDS ordination plots of fungal 18S rDNA (**A**, stress = 0.270); bacterial 16S rDNA (**B**, stress = 0.261); and actinobacterial 16S rDNA (**C**; stress = 0.344) obtained from black pine deadwood.

Table 3. ANOSIM and PERMANOVA global tests based on the Dice similarity matrices of 18S- and 16S-DGGE, for microbial groups in decaying black pine deadwood.

Microbial Group	ANOSIM		PERMANOVA	
	R	*p*<	*F*	*p*<
Fungi, 18S-DGGE	0.827	0.001	4.67	0.001
Bacteria, 16S-DGGE	0.699	0.001	3.55	0.001
Actinobacteria, 16S-DGGE	0.381	0.001	1.91	0.001

3.3. Physical and Chemical Analysis

The results of determination of moisture and total C and N contents conducted on the deadwood samples are reported in Table 4. Data were averaged on the basis of the different decay classes. Moisture values ranged from 56.4% of decay class 1 to 72.4% of decay class 5, but any significant differences were found between means. N content significantly increased with the progress of the decomposition status, from decay class 1 to decay class 5 with an exponential trend (R^2 = 0.91) from averaged 0.134% of decay class 1 to 0.890% of decay class 5. Samples of the early decay classes did not show significant differences in averaged N content whereas samples of the last decay stages (classes 4 and 5) showed significantly higher values comparing to each other and comparing with values of the early decay classes. The percentage of C was on average 47.9%, without a clear trend among decomposition classes, except decay class 4, which showed the highest value. Consequently, the C/N ratio significantly decreased from the early decay class 1 (366.1) to the late decay classes 4 and 5 (74.3 and 56.5, respectively) through intermediate averaged values of decay classes 2 and 3 (239.9 and 220.7, respectively).

Table 4. Average values of moisture, total C and N contents, and C/N ratio of the cores from the different decay classes of black pine deadwood; standard errors in parentheses.

Decay Class	Moisture (%)		Total N (%)		Total C (%)		C/N Ratio	
1	56.4 (1.9)	a	0.134 (0.011)	c	47.85 (0.50)	ab	366.1 (33)	a
2	67.4 (10.6)	a	0.200 (0.011)	c	47.52 (0.38)	b	239.9 (13)	b
3	60.2 (4.5)	a	0.214 (0.012)	c	46.78 (0.31)	b	220.7 (11)	b
4	62.5 (5.4)	a	0.690 (0.078)	b	49.28 (0.75)	a	74.3 (9)	c
5	72.4 (3.2)	a	0.890 (0.102)	a	48.14 (0.43)	ab	56.5 (7)	c

Different lowercase letters in a column indicate significant differences at $p < 0.05$ among means.

3.4. Potential Enzymatic Activities

The deadwood samples from decay class 1 did not show assessable fluorescence for any enzymatic activity taken into consideration (Table 5). Decay class 5 showed the highest activity (Table 5) for all enzymes tested and in general, the enzyme activity increased with the progression of deadwood decomposition status, from decay class 1 to decay class 5. The most evident increment was registered in β-cellobiohydrolase and β-glucosidase activity which resulted about 10-times higher in decay class 5 than decay classes 4 and 3, respectively. NAG and butyrate esterase showed a linear increment (R^2 = 0.91 and 0.90, respectively), from decay class 1 to decay class 5.

No significant differences were found between means of NAG and arylsulphatase activities of decay classes from 2 to 5, whereas the decay class significantly affected the other enzymatic activities. β-cellobiohydrolase, β-glucosidase, xylanase, and acetate esterase were significantly higher in decay class 5 with respect to the other intermediate decay classes. Acid-phosphatase significantly increased starting from decay class 3 (3-times higher than decay class 2) and any significant differences were found in the late decay classes (classes 3–5). Decay classes 4 and 5 did not show any significant differences in butyrate esterase which resulted 2- and 4-times higher than the early decay classes (classes 2 and 3), respectively.

Table 5. Average values of enzymatic activities (nmol MUF $g^{-1} \cdot h^{-1}$) measured in black pine deadwood samples from the different decay classes; standard errors in parentheses.

Class	β-Cellobiohydrolase		Acid-Phosphatase		β-Glucosidase		NAG		Xylanase		Butyrate Esterase		Acetate Esterase		Arylsulphatase	
1	0 (0)	b	22.1 (22.1)	c	0.1 (0.1)	b	0 (0)	b	0 (0)	b	0.7 (0.7)	b	0 (0)	b	0 (0)	a
2	4.9 (4.9)	b	796.5 (272.6)	bc	59.2 (35.6)	b	29.3 (15.5)	ab	18.2 (14.5)	b	245.9 (137.8)	b	193.5 (106.6)	b	1.1 (1.4)	a
3	22.3 (13.2)	b	2326.7 (500.4)	ab	117.6 (17.7)	b	57.1 (14.0)	ab	18.5 (2.6)	b	282.4 (70.2)	b	431.5 (111.1)	b	5.3 (2.1)	a
4	37.0 (20.1)	b	1236.5 (592.2)	abc	346.9 (180.4)	b	185.4 (122.8)	ab	38.7 (27.7)	b	556.5 (371.9)	ab	820.5 (542.5)	b	19.6 (17.6)	a
5	353.1 (112.7)	a	2867.1 (916.2)	a	1763.8 (383.8)	a	197.2 (63.0)	a	95.2 (21.1)	a	1060 2 (227.3)	a	2944.4 (962.3)	a	20.5 (10.3)	a

Different lowercase letters in a column indicate significant differences at $p < 0.05$ among means. NAG, N-acetyl-β-D-glucosaminidase.

3.5. Correlation of Chemical, Microbiological and Biochemical Data

Most of the measured enzymatic activities resulted significantly and positively correlated with N content of deadwood samples, with the exception of acid phosphatase. C content did not show any significant correlation with any measured microbiological or biochemical parameter (Table S2). Moisture resulted negatively correlated with abundance of deadwood fungal community and positively correlated with xylanase and butyrate esterase (Table S2). CO_2 and N_2O production resulted positively correlated with most enzyme activities. Moreover, fungal community richness resulted negatively correlated with xylanase, butyrate esterase, acetate esterase and CO_2 production. Actinobacterial abundance resulted negatively correlated with N content, C/N, moisture, all measured enzymatic activity, CO_2 and N_2O production (Table S2). Finally, bacterial richness didn't show any significant correlation with chemical or biochemical characteristics of deadwood (Table S2).

4. Discussion

The results obtained from De Meo et al. [36] on Monte Morello study area (59.9 $m^3 \cdot ha^{-1}$) show a high amount of lying deadwood in accordance with other studies that highlight a volume of lying deadwood in unmanaged forests at more than 50 $m^3 \cdot ha^{-1}$ [45–47]. With regard to the decay class, they found that the lying deadwood volume is concentrated in the third decay class (54% of total volume), while in the first two decay classes there is 30% of total volume. Generally, in unmanaged forests for over 50 years the amount of lying deadwood is fairly equally distributed in all decay classes but with a slightly greater concentration in the first decay classes. Kraigher et al. [45] have estimated that more 70% of lying deadwood volume is concentrated in the first three decay classes in two virgin forest reserves in Slovenia. Lombardi et al. [48] point out that in the unmanaged forests the amount of deadwood in the last decay classes is higher than in the managed forests in a case study in Central Italy. The authors show that more than 75% of deadwood volume is concentrated in the first two decay classes using a 4-class classification system.

In this study, the PCR-DGGE fingerprinting method was used as a culture-independent approach to investigate microbial communities in deadwood logs. The richness and evenness of bacterial bands in DGGE profiles resulted higher than those in fungal and actinobacterial DGGE profiles. Fungi are considered key players in deadwood decomposition in forest ecosystems thanks to the secretion of a battery of oxidioreductase and hydrolase enzymes that contribute to completely mineralize most of wood residues [20,29]. However, our study suggests that bacteria colonizing deadwood might play a more important role than previously thought [29,49].

Taken each microbial community individually, the abundance and diversity of fungal DGGE bands, clearly decreases with deadwood decomposition stage. Our results are not consistent with others previously described that showed a general increase in fungal richness with the progression of decomposition [50–52]. Maybe not all the fungal species present in the late stages of decomposition were detected by the couple of primers used. Furthermore, it is well known that DGGE evidences only numerically dominant species in a community [53], suggesting as an alternative that early stages of wood decay are accomplished by a restricted number of dominant fungal taxa while advanced stages may be accomplished by a wider range of fungal species present in low number.

As for fungi, also actinobacterial DGGE bands decreased in abundance and diversity from decay class 1 to decay class 5. Accordingly, Hoppe et al. [54] found that actinobacterial relative abundance decreased significantly with progressive wood decay, conferring to this bacterial group a potential role in the early colonization and decomposition of deadwood logs. Differently, the average bacterial DGGE band richness and Shannon index per decay class were slightly highest in the middle stages of decomposition and lowest in the early and late stages. Bacteria have a limited ability to decompose polymeric lignocelluloses [20,55] and fungal decay activity weakens lignin barriers and releases easily degradable oligomers, providing opportunities for bacterial access and growth [56]. Thanks to their great metabolic versatility and redundancy, bacteria may be favored in a heterogeneous environment consisting of a greater amount of easily degradable carbon compounds derived from ligninocellulolitic

material partially decomposed [57]. In the late decay classes, competitive interactions between microbial groups may occur, leading to a slight decrease of the bacterial species. These results suggest that wood decomposition implies a successional pathway of different microbial taxa. In confirmation of this assumption, we found that each deadwood decay class harboured a quite distinct microbial community. The robustness of the MDS ordinations has been corroborated by multivariate results that highlighted significant variability of microbial community composition between decay classes but a relative low variability between sample replicates of each class.

The most remarkable differences in community composition were found in decay class 1, where the microbial groups analyzed were clearly separated from those of the intermediate and late decay classes. A strong shift of fungal community structure was evident among the decay classes, although patterns of decay class 4 and decay class 5 grouped together. Patterns of decay class 1 resulted more closer to patterns of decay class 4 and decay class 5 than decay class 3, evidencing the greater importance of the environmental conditions in the earlier and intermediate classes rather than in the later stage of decomposition.

Fungi have been assumed to be pioneer colonizers of deadwood and through decomposition, directly modulate the availability of resources not only for themselves but also for other microbial groups [58,59]. They use two main colonization strategies: spore dispersal in the atmosphere and mycelial filaments in the surrounding soil [29,60]. Following these stochastic events [60], the capabilities of some fungal groups to degrade different substrates leads to temporary changes in fungal community, with the death of certain species and the colonization by others [50,56,61]. Fungal communities in forest ecosystems are dynamic, implying different assemblages and association of species, and complex interactions with their surrounding abiotic and biotic environments [62]. Bacteria extensively interact with wood-rot fungi during the decomposition process, and they may potentially confer negative effects, as they compete for easily degradable substrates that are necessary for fungal colonization and degradation activities [29,63]; consequently fungi may affect bacterial proliferation by producing toxic secondary metabolites [56]. However, bacteria can provide to fungi additional nitrogen via N-fixation [20,54,64]. Thus, as wood decomposition progresses, the microbial species are replaced by those more suited to the new substrates or that survive via competitiveness [27,61].

Our results suggest that changes in environmental conditions likely due to changes in substrate availability may have strong impacts on fungal and bacterial community compositions, whereas slight effects were found for actinobacteria that revealed low species diversity within the intermediate and the later stages of deadwood decay. Actinobacterial group may be less influenced by environmental changes with the progression of decomposition than the other microbial groups or alternatively, may be less competitive. The importance of actinobacteria in wood degradation is still unclear. They have good capabilities for degrading insoluble organic polymers, such as cellulose, and presumably they can degrade lignin-model compounds [57]. Nevertheless their contribution to wood decay is thought minor, giving us reason for the lack of a clear successional pathway of this microbial group with the progression of wood decomposition.

Significant changes at community level along decay classes correspond to concurrent and interdependent changes in N and C contents of deadwood. In our findings, N content in deadwood logs significantly increases as decay stage progresses, with a slight increment in the early decay classes (from 1 to 3) and a strong rise in the late decay classes (4 and 5). Conversely, the C concentration remains quite constant in the early decay classes with an increment observable in the decay class 4 followed by a slight decline in the decay class 5. These trends are consistent with results already obtained for other species such as beech, silver fir, white birch, trembling aspen, jack pine, and spruce [50,65–67], evidencing that during the decay, the chemical quality of deadwood gradually changes, giving reason that the different microbial groups of deadwood have to cope with a transient environment with an ever-changing number, quality and spatial arrangement of substrates over time [51]. In addition, the observed modifications in microbial communities and nutrient contents (N and C) support the hypothesis that low N content of wood promotes the selective lignin removal

whereas high N concentration stimulates the polysaccharide break-down [68], evidencing the important role of fungi in the early stage of deadwood decomposition and the presumable increasing involvement of bacteria as the decay progresses.

What is more, as the N increases, the C/N ratio of deadwood samples significantly declines along the decay classes. The C/N ratio of organic matter inputs is important to trigger a shift in the saprotrophic community, altering the competitiveness of different species and their decomposition activity [69]. Our study shows a significant positive correlation between C/N ratio and fungal and actinobacterial relative abundance, highlighting once more the important role of these two microbial groups in the early stages of decomposition.

The N increment has direct effects on microbial physiology and enzyme activity, and this may be associated also with the increment of deadwood respiration [69]. In fact, our study added evidences on the increase of CO_2 production with decomposition, as already observed by Wang et al. [70] and Bond–Lamberty et al. [71]. The large CO_2 production during wood decomposition process provided new insights on the multiple roles of deadwood and its environmental implications. Commonly, deadwood is considered as C stock [72]. However, deadwood is a transient C pool [17] and the amount of this C loss and its relevance at the ecosystem level has been rarely addressed. Few studies on the GHG emissions from deadwood are available in literature and are mainly related to CO_2 from boreal [70,71] and temperate forests [72–74]. Even if our attempt was limited to controlled laboratory conditions, we found evidences of a large flux of CO_2 from deadwood, highlighting its role as a C source. Furthermore, from our results, C losses from deadwood increased during the decomposition process, reflecting also enzyme activity patterns among decay classes. Except for acid-phosphatase, all enzymes correlated with CO_2 emissions show a similar trend among decay classes. A positive correlation of hydrolytic enzymes with decay rates of deadwood has been found by Kahl et al. [21]. Activities of lignin-related oxidoreductases were found in the less decomposed deadwood [20,21]. Our study highlighted the contribution of hydrolytic enzymes, starting from decay class 2. A significant increase was however found only in the more decomposed deadwood (decay classes 4 and 5), reflecting the relative abundance of cellulose and hemicellulose substrates. Differently, only enzymes related with hemicellulose and chitin degradation were correlated with N_2O production, suggesting different processes and microbial groups involved.

Moreover, an increase of N_2O and CH_4 production has been observed starting from decay classes 4 and 5, respectively. To our knowledge, CH_4 and N_2O production from deadwood had never been assessed in this manner, except in the study of Covey et al. [75], who assessed GHG concentrations in deadwood samples. The authors found a near-anaerobic environment in deadwood samples, with CH_4 concentrations 24 times higher than ambient and N_2O approx. 25% lower than ambient. Mukhin and Voronin [76] proposed a symbiotic association between wood-degrading fungi and methanogenic archaea, where fungi cleave wood carbohydrates and lignin and the products formed are fermented to produce CO_2 and hydrogen, the substrate for biogenic methane. It is well known that accumulation of N in deadwood may involve several processes such as translocation of N compounds from soil by fungal hyphae, mineralization of organic matter by microbial activity [77], and N-fixation by non-symbiotic microorganisms [54,64,78]. Thus, the increase of N concentration with decomposition progress might also explain the N_2O trend, as confirmed by the positive correlation. This was not the case of CH_4, which was naturally correlated with samples' moisture content. Our findings, therefore, support the evidence that microbial inhabitants of deadwood might act as sources of N_2O and CH_4.

Thus, deadwood logs constitute a complex and heterogeneous environment where abundance, composition and activity of all decomposer communities change substantially over time due to deterministic succession of species accompanying the wood decomposition [51,55].

5. Conclusions

This study provided information on changes in composition, abundance, and potential activity of microbial communities inhabiting deadwood, with the progress of decomposition. We chose to

analyze the richness and community structure of three main microbial groups—fungi, bacteria, and actinobacteria—by a PCR-DGGE approach. The microbial activity was evaluated in terms of GHG (CO_2, N_2O, CH_4) production and enzyme activity.

Despite the limits of DGGE methodology, our study highlighted the complexity of the different microbial components of deadwood, each one properly influenced by decaying status, and providing valuable new knowledge of the microbial successional pathway occurring during wood decomposition. Having deadwood used as an indicator of forest biodiversity, our research can contribute in providing biodiversity information which cannot be gathered with structural indicators, such as the volume per hectare, usually derived from forest field monitoring.

Besides the high increase of CO_2 production with decomposition due to microbial activity, our study added evidences on the potential involvement of deadwood microorganisms in GHG emissions in forest ecosystems. We also highlighted a positive correlation between hydrolytic enzymes and decay rates of deadwood.

Few studies on GHG emissions from deadwood are available in literature, and even though many study have been focused on wood-decaying fungi, the contribution of bacteria to wood turnover is still largely unexplored. Studies on interactions between bacteria and wood-rot fungi, and their involvement in forest nutrient cycling, are essential to understand forest ecosystem functioning and for solving environmental issues such as mitigating adverse effects of carbon emissions. Thus, objectives for future studies are to investigate the functional microbial communities involved in specific processes of nitrogen- and carbon-cycling such as nitrification, denitrification, and methanogenesis, as well as diazotrophic and methanotrophic bacteria, to lead to a deeper insight into the processes occurring in deadwood decay.

The five-class system used was based on visual assessment of rates of decay assigned in field by experts, and is consequently affected by subjectivity of the operator. An important challenge for future researchers is to define a system of classification of deadwood decomposition stages based on objective and measurable variables, such as wood resistance, with the final aim of establishing clearly-defined boundaries among decay classes.

Supplementary Materials: The following are available online at //www.mdpi.com/1999-4907/8/7/224/s1, Table S1: *p* values from ANOSIM (upper right side) and PERMANOVA (lower left side) pair-wise comparison of band compositions of 18S- and 16S-DGGE, Table S2: Pearson correlation matrix of chemical, microbiological and biochemical parameters measured in deadwood samples from the different decay classes, Figure S1: Cumulative production of CO_2, N_2O and CH_4 from the black pine deadwood of the five different decomposition classes, in the arc of 48 h, Figure S2: DGGE profiles of 18S rDNA fungal community, 16S rDNA bacterial community, and 16S rDNA actinobacterial community.

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