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Original article

The impact of termite sheetings age on their fungal communities

M. Diouf, E. Miambi, P. Mora, S. Delgarde, C. Rouland*

Laboratoire d'écologie des sols tropicaux (LEST), UMR 137, université Paris-XII, 61, avenue du Général-de-Gaule, 94010 Créteil, France

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Abstract

In previous studies, we have shown that fungal communities in soil are quantitatively and qualitatively modified when termite sheetings are constructed, but the reasons for these changes are unknown. In this work we described the succession of fungal communities in the sheetings of three fungus-growing termite species by combining cultivation techniques and culture-independent methods specially DNA extraction, PCR and denaturing gradient gel electrophoresis (DGGE). We also estimated the abundance of fungi by using plate-count technique. The fungal communities of these sheetings were sampled at different ages. The potential functions of fungal communities was assessed by their capabilities of degrading different substrates including proteins, polysaccharides and tannins. Significant differences were found between the fungal communities of termite sheetings and those of surrounding soil. These differences were observed at quantitative, qualitative and functional level as well and were marked particularly in the fresh sheetings of termites. The characteristics of fungal communities of the surrounding soil were quite similar with those observed in the old sheetings of termites. These results show that the fungal communities are modified at once in freshly built sheetings, rather than by a progressive succession, as had been expected.
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Keywords: Fungus-growing termites; Foraging sheetings; Age; Fungal communities

1. Introduction

Sheetings built by termites of the Macrotermitinae sub-family are one of the most important among the bio-structures built by macrofauna in tropical savanna. Termites sheetings are made from a mixture of soil particles and saliva, and provide a protective enclosure around potential food items, within which feeding and foraging take place. They constitute very important amounts of moved soil, reaching in some areas 47 T ha^{-1} [8]. Moreover, because of their low stability, these types of bio-structure are quickly recycled in soils. In previous studies, we have shown that in these sheetings, the fungal component of soil microbial communities is different from that of the control soil [2]. These modifications may have great impact on soil biological process, considering the significant role of fungi on microbially-mediated functions such as organic matter cycling processes and stability of soil structure. However, mechanisms involved in the building process of sheetings and through which soil fungal communities are modified remain unknown. In this work, we have assessed fungal communities in sheetings of fungus-growing termites in relation to age, what would provide an insight about the origin and also the evolution of observed differences.

* Corresponding author. Fax: +33 1 45 17 15 05.
E-mail address: rouland@bondy.ird.fr (M. Diouf).

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2. Materials and methods

2.1. Sampling

Samples were collected in an experimental area of a sahelian savannah in Senegal (West Africa). They consisted of sheetings built by the three most abundant fungus-growing termite species in this ecosystem: *Macrotermes subhyalinus*, *Odontotermes nilensis* and *Ancistrotermes guineensis*. For *M. subhyalinus* and *O. nilensis* species, we sampled early in the morning sheetings built during the night. This was considered as starting time (T_0). Subsequent sampling was performed at 3 h (T_1), 6 h (T_2), 30 h (T_3) and 198 h (T_4) later. For *A. guineensis* whose structures are built inside stems, only the fresh (T_0) and dry stages (198 h) were considered. These sheetings of different age were then compared to the control soil taken at three depths: 0–1, 1–3 and 3–4 cm. Analyses were performed on composite samples which consisted in five samplings. The moisture was determined for each sample.

2.2. Methods

2.2.1. Enumeration of culturable fungi

The density of culturable fungi was compared between fresh (T_0) and dry (198 h) structures of the three species, whereas its variation in the course of time was followed only in sheetings built by *O nilensis* species. Ten-fold dilutions obtained from suspension of each sample were used to inoculate a non-selective but fungus-specific Sabouraud solid culture medium. For each composite sample, three repetitions were made and five Petri dishes were inoculated for each dilution. After 5 days of incubation at 30 °C, colony forming units were counted and the fungal strains were morphologically distinguished and their frequencies in each sample were used to compare the mycoflora at the qualitative level.

2.2.2. Comparison of metabolic profiles

Metabolic profiles were determined using the Most Probable Number (MPN) technique [5] on five substrates. Cultivation was performed in a Raulin mineral medium containing an antibiotic (chloramphenicol 50 mg l^{-1}) and 0.2% (p:v) of one polysaccharide (cellulose, xylan or starch), or 2% (p:v) of bactosoytone. This medium was inoculated with 10% (v:v) of 10-fold dilutions of the soil suspensions. Three flasks were inoculated per dilution and incubated in a rotative shaker at 150 rev. min^{-1} at 29 °C. After a week, "positive" flasks (i.e. with fungal growth) were used to determine the MPN from the McCrady table [5].

2.2.3. Assessment of fungal communities in termites sheetings

The assessment of the genetic structure of fungal communities was carried out on sheetings of *M. subhyalinus* and *O. nilensis* species sampled at different times, compared to the control soil at 0–1 and 1–3 cm depth. The molecular method used is PCR-denaturing gradient gel electrophoresis (DGGE, [6]). It allows the separation of PCR-amplified DNA fragments of a similar size according to the nucleotides composition of their sequences. DNA fragments with different G–C contents yield distinct bands. DNA extraction was carried out using a method described by Porteous et al. [7]. Then 20 ng of DNA were used for a specific PCR amplification a 260 bp fragment of the fungal rDNA large sub-unit (28S) using the primers 403-f/662-r; [10]). The PCR was performed with *Taq Qbiogene* according to the manufacturer instructions and 500 ng of the resulting PCR products were used for electrophoresis onto a 8% (w/v) polyacrylamide/bisacrylamide gel with a denaturing gradient from 30% to 60% (100% denaturant correspond to 7 M urea and 40% v:v deionized formamide). Gels were stained with Sybr Green (Bioproducts) and images were acquired under an UV transilluminator. Banding pattern was analyzed using the Bio-Profil image analysis software (Vilbert Lourmat).

2.2.4. Statistical analyses

Qualitative comparison between samples was carried out using Principal Component Analysis (PCA) of the ADE-4 software [11] which enables the comparison of several samples through different variables. Variables are DGGE band intensities, frequencies of fungus "morphotypes", fungi MPN on different substrates.

3. Results

3.1. Evolution of water content in termite sheetings with age

The water content was determined in sheeting of the three termite species. The results shows that the moisture is clearly higher in new sheetings than in old ones. Changes in water content was followed up only in sheetings of *O. nilensis* at different times (Fig. 1a). Moreover, sheetings dry quickly since after only 3 h, they lose more than 72% of their initial water content

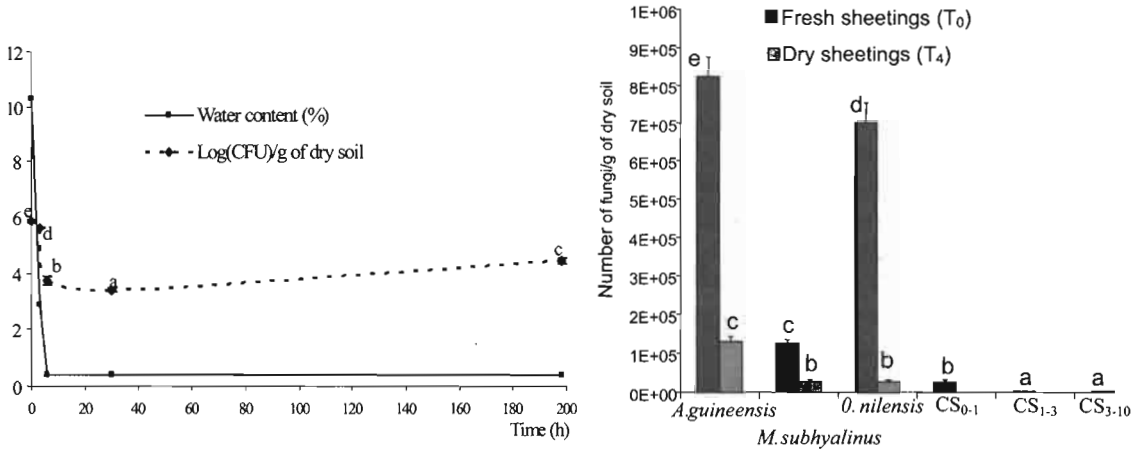

Fig. 1. Change of water content and density of culturable fungi (a) in sheetings of *O. nilensis* at different times and comparison of fungal density between sheetings of the three species at the fresh (T_0) and dry (T_4) stages and (b) CS_{0-1}, CS_{1-3}, CS_{3-10}, corresponding to depths of 0–1, 1–3 and 3–10 cm of the control soil, respectively.

and after 6 h, their moisture reaches an almost constant value equivalent to that of the control top soil.

3.2. Evolution of fungal density

Fungal density (as CFUs g^{-1} dry weight) is higher in fresh structures of *O. nilensis* species. However, this high fungal density decreases with the age of sheetings and even becomes lower than that of the control 0–1 cm after 6 h, and equivalent to that of the 1–3 and 3–10 cm horizons after 30 h (Fig. 1a). The density of culturable fungi is higher in the sheetings of *O. nilensis* collected after 1 week than in those sampled at T_1, T_2 and T_3, but remains largely lower than that of fresh sheetings. Moreover, all fresh structures (T_0) are significantly more colonized than those of 1 week and the control soil, whatever the sheeting type (Fig. 1b). This higher fungal colonization decreases with sheetings age since after 1 week, fungal densities are similar between biostructures built by *M. subhyalinus* and *O. nilensis* species and the control top soil (0–1 cm). It is interesting to notice that the dry sheetings of *A. guineensis* have a higher fungal density than sheetings of the other termite species studied and the control soil.

3.3. Comparison of culturable morphotypes

Diversity and distribution of the "morphotypes" in sheetings of different age built by *O. nilensis* species were compared to those of "morphotypes" found in the control soil (Fig. 2a). Five fungal "morphotypes" were identified in fresh structures (T_0), six in those sampled at T_1. Then, this number increases appreciably (9 and 11 morphotypes) in sheetings sampled at following times whereas seven morphotypes were found in each of the depths 0–1 and 1–3 cm and six morphotypes in the 3–10 cm depth. In sheetings collected in the first day a single morphotype largely dominated all the others (77.8 and 85.9 at T_0 and T_1) but its frequency decreases in old structures (only 10.7% at 198 h). In a similarity dendrogram (Fig. 2b), groups of morphotypes isolated from sheetings and the parent soil formed two clusters, the first consisting of isolates from fresh structures and the second of isolates from older sheeting and the parent soil. Old sheeting is therefore more similar to the control soil in respect of fungal morphotypes than to young sheeting. *O. nilensis* sheetings of less than 2 days age (from T_0 to T_2) are closely related in the same cluster which is segregated from the cluster containing oldest structures (T_3 and T_4) and the different control soils

3.4. Metabolic profiles of fungal community

Comparison of MPN on different substrates, between fresh structures (T_0) of *M. subhyalinus* and *O. nilensis* and dry ones (T_4) results in a higher number of fungi in fresh than in dry structures whatever the substrate and the structure type, except the sheetings of *M. subhyalinus* on tannins (Table 1). As seen in the plate-count, this difference is due to the higher fungal density in fresh than in old sheetings. Thus, for metabolic profiles comparison between samples by PCA, we have used the MPN relative values on each substrate (compared to the total MPN on all the substrates). Correlation circle (Fig. 3a) shows that the distribution of

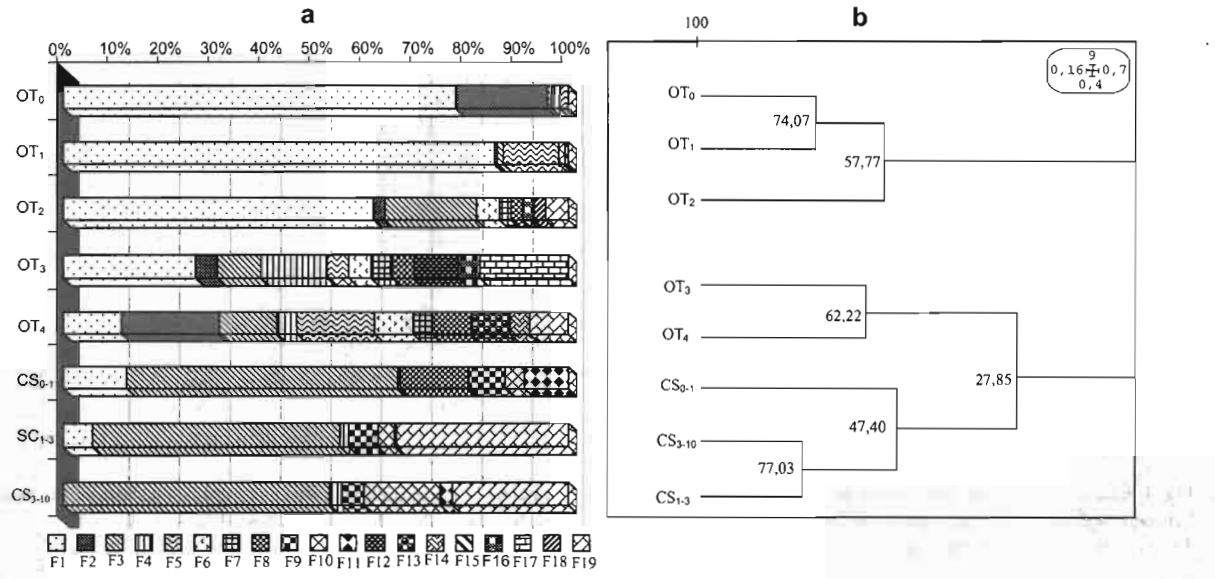

Fig. 2. Fungal "morphotypes" distribution between sheetings of *O. nilensis* of different age and different depths of the control soil (a) and similarity dendrogram obtained by PCA from this distribution (b). OT_0, OT_1, OT_2, OT_3 and OT_4 correspond to sheetings of *O. nilensis* sampled at 0, 3, 6, 30 and 198 h, respectively; CS_{0-1}, CS_{1-3} and CS_{3-10} as in Fig. 1.

Table 1
MPN of culturable fungi recovered from fresh and dry structures of termites and from control soil after growth on three polysaccharides (starch, xylan, cellulose) tannins and a vegetal derived protein (bactosoytone)

Samples	Starch	Xylan	Cellulose	Tannins	Bactosoytone
OT_0	9.5	45	25	0.45	250
OT_4	0.45	0.95	4.5	0.25	1.5
MT_0	9.5	45	4.5	2.5	250
MT_4	2.5	2	0.95	4.5	9.5
AT_0	9.5	25	0.75	25	250
AT_4	nd	nd	nd	nd	nd
CS_{0-1}	0.95	0.95	0.95	0.095	0.95
CS_{1-3}	0.45	2.5	4.5	0.025	2.5
CS_{3-10}	0.95	0.25	0.25	0.25	4.5

Given values correspond to means of the number of fungi g^{-1} of dry soil $\times 10^3$. OT_0, MT_0 and AT_0 correspond, respectively, to sheetings of *O. nilensis*, *M. subhyalinus* and *A. guineensis* collected at initial time while OT_4, MT_4 and AT_4 correspond to the same structured collected after a week (198 h).

samples on axis 1 (50% of the total inertia) is mainly determined by proteolytic, cellulolytic and xylanolytic components whereas on axis 2, samples are mainly segregated by abundance of fungal populations able to degrade starch and tannins. The projection of samples on these PCA axes (Fig. 3b) reveal that fresh sheetings differ from the other samples mainly through their higher percentage of proteolytic fungi. However, this feature of fresh foraging structures disappears with age since old sheetings have clearly different metabolic profiles and are even close to those of control soils.

3.5. Genetic structure of fungal communities

PCR-DGGE analysis of the structure of fungal communities according to sheetings age was performed on those of *O. nilensis* (Fig. 4a) and *M. subhyalinus* species (Fig. 4b), compared to the control soil at 0–1 and 1–3 cm depths. From DGGE banding pattern, it is apparent that whatever the termite species, lowest genetic diversity is found sheetings of 1 week and control topsoil (0–1 cm). The PCA (Fig. 4c,d) carried out from the band intensities matrix leads to the same observations for the two kinds of sheeting. Indeed, fresh sheetings sampled at T_0 contain a fungal community definitely different from that of the other samples. In structures collected at T_1, T_2 and T_3 fungal populations are close the ones to others whereas after 1 week, they are closer to those of the top soil (0–1 cm).

4. Discussion

Plate-count showed a higher fungal density in fresh sheetings than in old ones and control soils, whatever the termite species. This high fungal density seems to be a main feature of newly built foraging structures of Macrotermitinae since it is common for three species. There is not any other known study of the microflora of

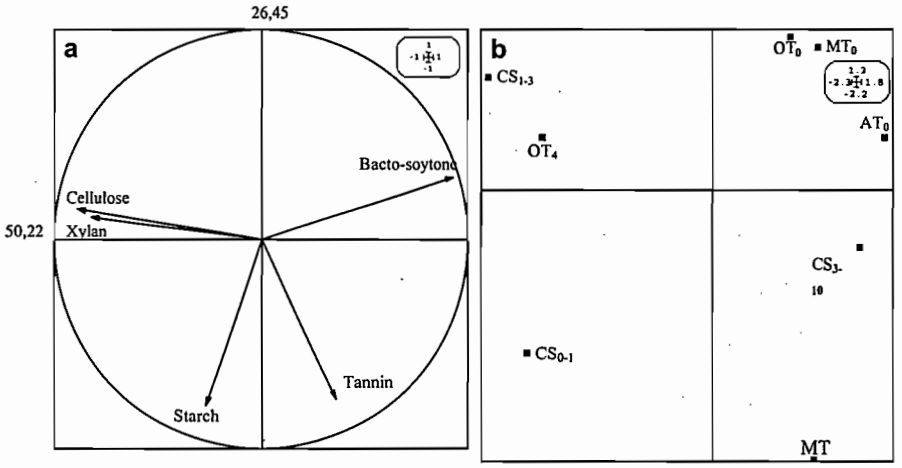

Fig. 3. Principal Component Analysis carried out from relative abundance of fungal populations able to grow on different substrates. (a) correlation circle; (b) Distribution of samples on the plane defined by the axes 1 and 2.

fresh structures still soaked with termite saliva. The only known study of a similar kind was carried out on bacterial communities in the nest of a soil-feeding termite *Cubitermes niokolensis* and found that bacterial density differed between the inner and outer walls, the inner walls having both the higher density and the higher moisture content [3]. However, it should be stressed that *C. niokoloensis* belong to another trophic functional group (soil-feeder) and comparison of biogenic structures across group may be misleading.

The qualitative studies conducted with a combination of culture-dependent and molecular methods is the first, dealing with the dynamics of the fungal communities in the foraging structures made by termites. The differences observed between fresh sheetings and the parent soils extend to fungal genotypes, morphotypes and functional groups as well as CFU density. These results corroborate also those of our first study performed on not-dated sheetings of fungus-growing termites and which highlighted such modifications of fungal communities [2]. Moreover, the modification of microbial communities in sheetings seems not to be gradual, but occurs very early. In other words, special fungal populations which characterize the fresh structures do not likely colonize them progressively as one could suppose it. The result raises the question of the source of these fungi found very early in new sheetings.

However, all these differences tend to disappear when sheetings have aged. Moreover, unlike the fresh sheetings, the mycoflora found in the oldest sheetings built by *O. nilensis* and *M. subhyalinus* as well is very similar to that of the control soil at 0–1 cm depth. Two assumptions can be formulated to explain these similarities:

- The first hypothesis is that when termites are building their foraging structures, they may use surface soil from 0 to 1 cm layer. Then mycoflora of this soil layer is temporarily activated by termites saliva, but fast-growing fungi that colonize sheetings are not adapted to natural environmental conditions and their number decreases in few hours, while autochtonous populations remain unaffected. This hypothesis of activation is plausible according to the high ratio of proteolytic fungi in the new sheetings, which suggests a selective activation due to the high protein content of termite saliva [4]. The high drying speed of structures attests severity of the environmental conditions (low moisture, high temperature, intense solar radiations) to which growing microorganisms are particularly sensitive [1]. Such a temporary activation, followed in a short term by a decrease of activity and abundance of microorganisms at a level sometimes lower than that of the reference soil was often found in casts of some earthworms [9,12].
- The second assumption is that the surface soil considered as a control mainly consists of old sheetings eroded. The status of this soil horizon is unclear for two main reasons:
  - a similarity between its fungal communities and those of old sheetings;
  - the difference between this layer and deeper layers. Our hypothesis is that the surface 1 cm is largely derived from termites sheeting and in that sense is not the genuine parent material [8].

The sheetings built by *A. guineensis* have a different evolution: in old structures the fungal communities are clearly different from those of the other samples. These

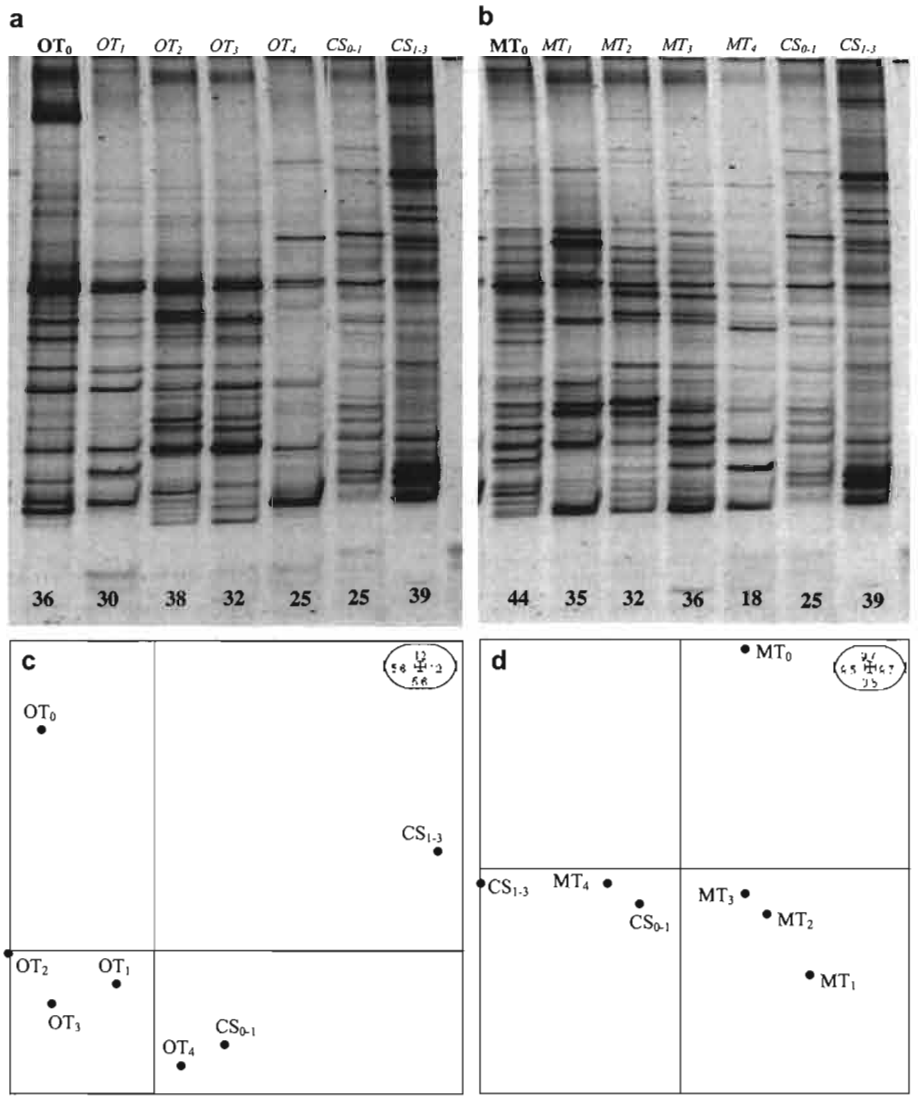

Fig. 4. DGGE separation of PCR-amplified partial 28S rDNA from sheetings of *O. nilensis* (a) and *M. subhyalinus* (b) sampled at different times as compared to control soil from 0–1 and 1–3 cm depths and PCA carried out from DGGE-band intensities between samples (c and d). Numbers below the electrophoresis gels indicate the total number of band detected for each sample. MT_0, MT_1, MT_2, MT_3, MT_4 indicate, respectively, sheetings of *M. subhyalinus* species collected at 0, 3, 6, 30 and 198 h.

structures, built into, and not on, the plant material, are protected from solar radiations and some fungal colonies could then be maintained.

5. Conclusion

Differences are observed between the fungal communities of termite sheetings and control (parent) soil extending to CFU density, morphotypes and functional groups. The differences are large in fresh sheetings but diminish as the sheetings age and are least noticeable when old sheetings and the control soil are compared. These results suggest that the change of microbial community in sheetings is not gradual but occurs since beginning of building. One can consequently wonder about the source of these original fungal populations which colonize instantaneously fresh sheetings and which are not found in the control soil.

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