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► **To cite this version:**

Dmytro Kosiachevskyi, Kamilia Abahri, Isabelle Trinsoutrot-Gattin, Lisa Castel, Anne Daubresse, et al.. New Refined Experimental Analysis of Fungal Growth in Degraded Bio-Based Materials. Processes, 2024, 12 (10), pp.2188. 10.3390/pr12102188 . hal-04839677

**HAL Id: hal-04839677**

**<https://normandie-univ.hal.science/hal-04839677v1>**

Submitted on 16 Dec 2024

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## Article

# New Refined Experimental Analysis of Fungal Growth in Degraded Bio-Based Materials

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**Abstract:** When exposed to different building environmental conditions, bio-composite materials, such as hemp mortars, represent a risk of mold proliferation. This later plays a critical role in the biodeterioration of the materials when their physical properties are locally modified by the natural aging process. The primary objectives of the present work are first to assess the evolution of the surface of contaminated mortar; second, to investigate an accurate DNA extraction method that could be used for both bio-composite mortars and their fiber sources collected in situ; then, to understand the process of the proliferation of mold strains on both hemp shives and hemp mortar; and finally, to compare mold strains present in these phases to show their relationship to mold contamination and their impact on human health. In situ hemp mortar contamination behavior was investigated in the region of Pau (France) two months after hemp mortar application in extreme conditions (high humidity, low temperature, no aeration), which did not match the standard conditions under which hemp mortar must be used. The SEM observations and FTIR and pH analyses highlighted the decrease in pH level and the presence of organic matter on the mortar surface. DNA sequencing results showed that hemp shives were the main source of fungal contamination of hemp mortar. A mold population analysis showed that the most dominant phylum was Ophistokonta, which represented 83.6% in hemp shives and 99.97% in hemp mortar. The Acrostalagmus genus representatives were the most abundant, with 42% in hemp shives and 96% in hemp mortar. The interconnection between the mold strain characteristics (particularly the ability to grow in extreme environments) and the presence of hemp mortar was emphasized.

**Keywords:** hemp mortar; durability; biodeterioration; ribosomal deoxyribonucleic acid sequencing; mold contamination; in situ behavior; DNA



**Citation:** Kosiachevskiy, D.; Abahri, K.; Trinsoutrot-Gattin, I.; Castel, L.; Daubresse, A.; Chaouche, M.; Bennacer, R. New Refined Experimental Analysis of Fungal Growth in Degraded Bio-Based Materials. *Processes* **2024**, *12*, 2188. <https://doi.org/10.3390/pr12102188>

Academic Editors: Andrzej Pacana and Dominika Siwec

Received: 11 September 2024

Revised: 2 October 2024

Accepted: 4 October 2024

Published: 9 October 2024



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## 1. Introduction

Today, the use of materials with a low environmental impact, such as bio-composite mortars, which are already recognized for their thermal and acoustic insulating qualities, is gaining increasing attention in the construction building sector. Unlike conventional building materials, they present a renewable resource derived from plant biomass. They are also low-cost energy-saving materials and are responsible for low CO<sub>2</sub> emissions, which respects the life cycle.

Thermally, hemp shives have low thermal conductivity, making them suitable as insulating materials that are well adapted to function with mineral binders [1]. In addition, hemp mortar exhibits double porosity (having both intra- and inter-particles), which offers better insulation performance [2]. Simultaneously, the durability of these materials in terms

of internal and external environmental conditions is essential. In fact, during their life cycle, bio-based mortars are exposed to various factors that can cause their degradation [3–5]. One such factor is microorganisms of different natures that interact in different ways [6]. Examples of these organisms are molds, which are known for their ability to provoke hemp mortar deterioration by locally modifying the properties and chemical composition of hemp mortar. In fact, several adverse effects are derived from the exposure of hemp mortar to molds. For example, molds are known for their ability to decrease the pH level [7] and are thus responsible for the degradation of critical phases of material, such as those of ettringite or portlandite. This is even more critical when highly hygroscopic, organic composite materials such as hemp mortars are considered. The presence of mold in hemp mortars degrades their thermal and hydric performances. Thus, overcoming these fungal phenomena, which may present risks to the long-term life cycle, is necessary.

Molds are filamentous eukaryotic, heterotrophic, and generally aerobic, thallophytic, sporogenous, non-chlorophyllous, and acidophilic (pH between 3 and 7) microorganisms with a septate filamentous (septate) or siphoned (non-septate) thallus (hyphomycetes and zygomycetes) [8]. Mold proliferation and growth are ensured by thallus growth or by issuing spores, depending on the species involved. This proliferation depends on environmental conditions such as temperature, relative humidity [9], pH level [10], nutrient abundance, oxygen [8], and light presence.

In recent years, the effects of molds on the degradation of bio-based mortars have piqued the interest of research communities, particularly in civil engineering. For example, Sinka et al. [7] showed that mold proliferation decreased pH levels. In fact, molds, during growth, use specific metabolites (enzymes, pigments, toxin synthesis, and organic acids) to uptake nutrients from the environment. Among these metabolites, organic acids can be distinguished by their extreme ability to degrade different materials [11]. Indeed, the natural fungal production of organic acids plays a critical role in nature and may give a competitive advantage to acid-tolerant filamentous fungi over other organisms by decreasing the ambient pH [11,12]. In addition, decreasing the pH can improve mineral alteration by solubilizing soil minerals, thus releasing nutritive ions for absorption (e.g., ectomycorrhizas) [13] or can lead to acid-catalyzed holocellulose hydrolysis [14]. Regarding their direct interaction with the environment, organic acids participate in metal detoxification by complexation. Similarly, oxalic acid plays a significant role in the degradation of biomass [14,15], partially explaining the biodeterioration of bio-composite materials derived from plant biomass such as hemp mortars.

However, a lack of information exists regarding mold population identification in general and mold species in hemp mortars, particularly with respect to their origin and how they survive under highly alkaline mortar conditions with an average pH level of 12.5. Of the few studies available on this topic, Viel et al. [16] used a non-destructive method based on an optical evaluation of bio-composite mortar durability toward mold growth. However, this technique does not enable all mold species present in the material to be distinguished and quantified. This method does not detect the presence of bacteria and sources of external contamination related to the experimental environment, which may prevent true species related to contamination or fungal growth from being identified.

Accurate identification of mold species is necessary to characterize the interaction and possible influence of a mold's identified type on both mortar and hemp durability. Therefore, the main objective of this study is to evaluate the mold growth of contaminated hemp mortar collected in situ from a house located in the region of Pau in France. The study identifies mold species present in hemp shives before they are incorporated into the mortar, examines the contaminated hemp mortar, and compares the identified species. Particular attention is devoted to choosing the deoxyribonucleic acid (DNA) extraction method and the subsequent metagenomics analysis of the fungal community. A trophic cycle analysis is then conducted to determine how molds adapt to extreme environments such as that of fresh mortar with an average pH of 12.5.

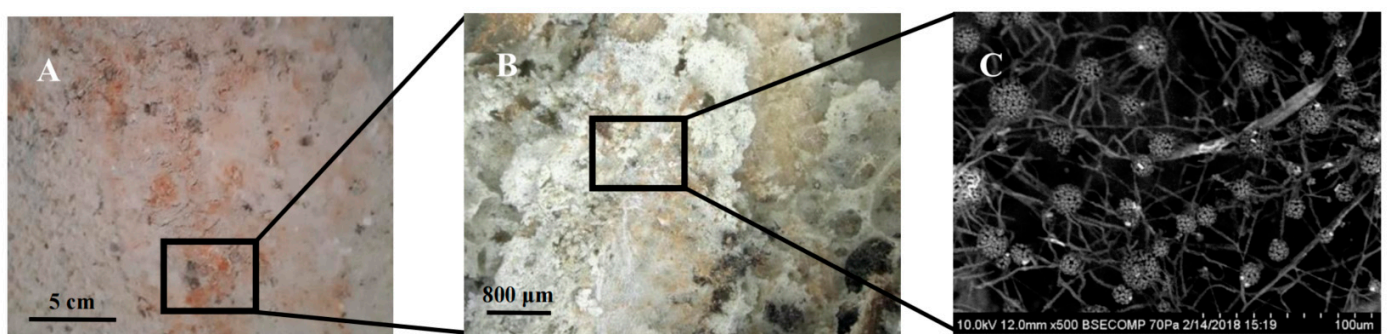
## 2. Materials and Methods

### 2.1. General Strategy of Experiment

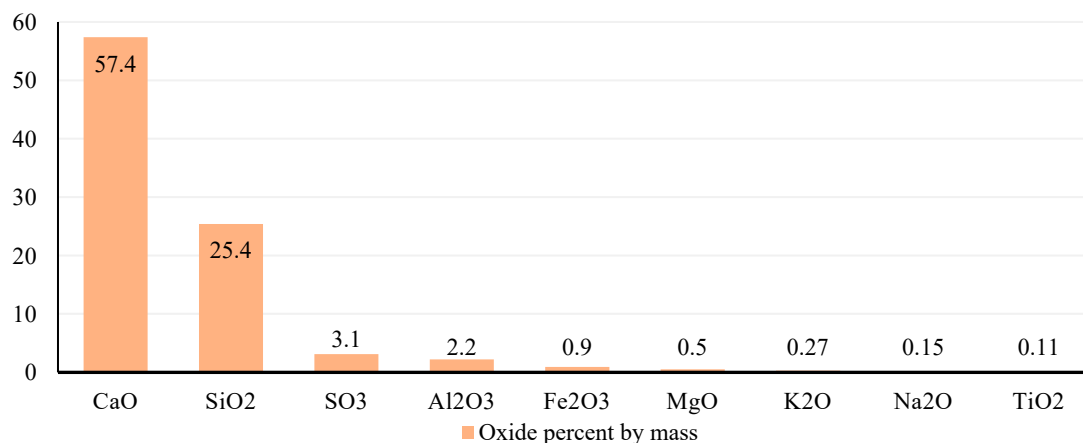
To better understand the nature of hemp shives and the biodeterioration of whole hemp mortar, we carefully analyzed the behaviors of both materials. The experimental protocol consisted of two parts. The first part included the analyses of the surface of hemp mortar contaminated with mold. Scanning electron microscope (W-SEM) observations and an Attenuated Total Reflectance-Fourier-Transform InfraRed spectroscopy (ATR-FTIR) analysis enabled the study of the surface and of the presence of organics on the mortar sample. Measurements of the pH level allowed us to analyze the influence of molds on this parameter. The second part embodied the fungal type of identification using DNA sequencing. More specifically, hemp shive contamination was studied separately to identify mold strains already present before incorporation into the mortar. We then followed and evaluated the evolution of these fungal species after integration into the cement matrix (in hemp mortar). Analyzing how hemp fibers interact with the mortar and evaluating whether these natural fibers were the main sources of contamination were critical in this study. In addition, we identified whether mortar affects the expansion of these fungi and if any species were already present in hemp mortars and not in hemp fibers.

### 2.2. In Situ Conditioning and Sample Preparation

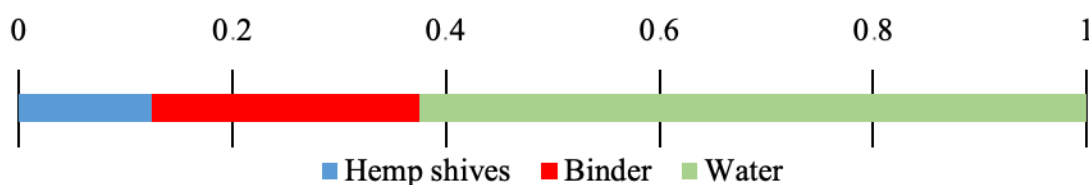
Two types of samples were studied. The first was hemp naturally contaminated one month after its in situ insulation application in November 2017 from a house in the region of Pau in France. The first fungal proliferation was observed in December after a single month's exposure to extreme conditions that were incompatible with the standard conditions under which hemp mortar is used [17]. Proliferation was visually determined by the fact that the mortar was covered with red spots and then analyzed by microscopic observation (Figure 1) and surface analyses. The second sample was of hemp shives that were previously used in the application of hemp mortar in Pau. In order to protect hemp particles from external contamination, they were stocked for one month in laboratory conditions (23 °C and 50% RH) in closed boxes. The "hemp mortar-raw material" link allowed us to compare the obtained results. The lime-based binder with hydraulic additions that we used is a commercial mix designed by the Sika group; its chemical composition is presented in Figure 2. The mix proportion ratios of the hemp mortar are presented in Figure 3.



**Figure 1.** Images of mold proliferation on hemp mortar taken from contaminated areas in January 2018 (A) with the optical microscope (B) and with the electronic microscope (C).



**Figure 2.** Oxide analysis of used lime-based binder with hydraulic additions.



**Figure 3.** Mass ratios of hemp mortar composition.

### 2.3. Analyses of Contaminated Hemp Mortar and Mold Proliferation

First, the surface of contaminated hemp mortar was scanned using an ATR-FTIR. It was performed using a PerkinElmer Spectrum on the contaminated sample. Four measurements were accumulated with a resolution of  $4\text{ cm}^{-1}$ . The test was conducted by studying vibratory frequencies in the middle Infrared ( $4000\text{--}500\text{ cm}^{-1}$ ).

Second, the W-SEM observations were conducted to investigate the presence of molds on the hemp mortar samples. The Hitachi S-3400N scanning electron microscope was used. All samples were previously covered with carbon in order to improve the image quality. Secondary electron detectors with an acceleration voltage of 15 kV in high-vacuum mode (pressure  $< 1\text{ Pa}$ ) were used.

Finally, the pH level of the specimens was examined both on the surface and inside at a depth of 2 cm. A WTW Sentix electrode was employed, providing a reliable result, particularly on different parts of the sample [18]. For this assay, samples taken in situ were examined. As pH measurements are only possible in solution, a constant volume of demineralized water (0.2 mL) was applied to the surfaces under investigation. The electrode was then held on the sample surface so that the membrane remained in contact with the hemp mortar. At equilibrium, the WTW Sentix delivers a constant pH value, which is later recorded. Three measurements were taken at different points on the sample for each pH value for reasons of repeatability.

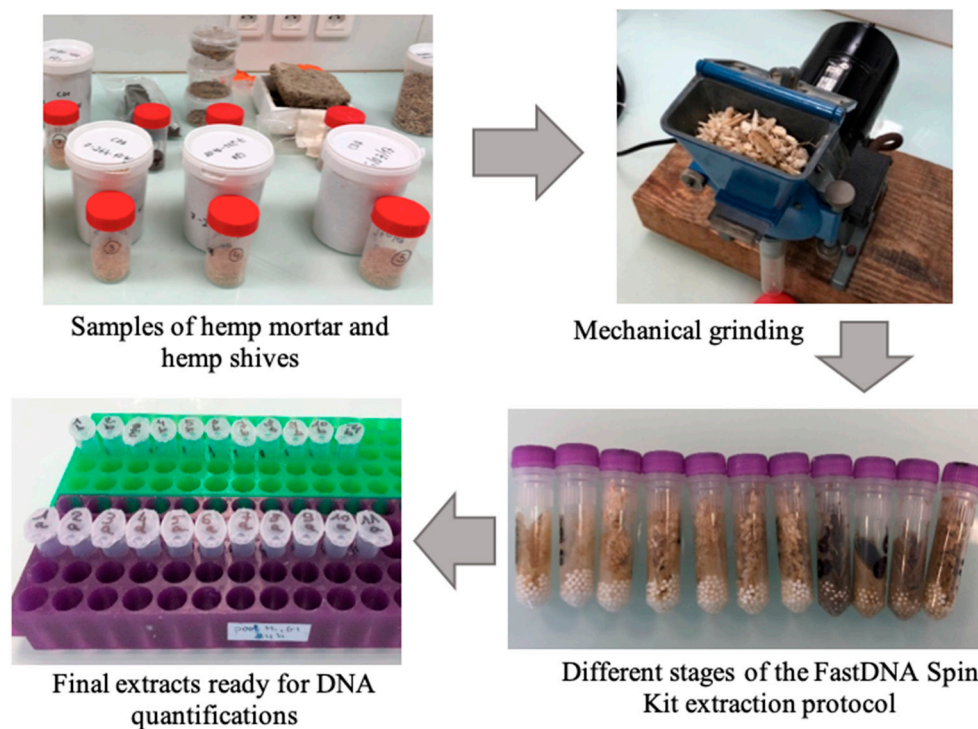
### 2.4. Metagenomic Analyses of Microbial Community

The experimental protocol of metagenomic analysis consisted of different treatments of naturally contaminated in situ collected samples of hemp mortar and hemp shives. First, the DNA was extracted using a specific protocol. A DNA quantification was then conducted to determine whether the extraction method functioned well. Finally, DNA sequencing was performed to identify the mold species. All these stages are detailed below.

The DNA extraction experimental protocol (Figure 4) consisted of grinding samples to obtain fine particles. A benchtop mechanical grinder was used for grinding. Prior to each grinding session, all grinder surfaces were treated with acetone to avoid cross-contamination of the samples with possible DNA residues. Then, we proceeded to the DNA extraction using specially dedicated kits. The following three kits were used after all



precautions of the suppliers for extraction were considered: the FastDNA SPIN Kit, which is dedicated to soils (MP Biomedicals, Irvine, CA, USA), the EZNA High-Performance Fungal DNA Kit (Omega Bio-Tek, Norcross, GA, USA), and the DNeasy PowerPlant Pro Kit (Qiagen, Germantown, MD, USA). To verify the repeatability of the results, two replicates were performed for each kit. After each extraction, total DNA and fungal DNA quantification were performed to ensure the kit's ability to extract DNA from our material matrix. Finally, the FastDNA SPIN Kit (MP Biomedicals, Irvine, CA, USA) was used for further treatments because it allowed the extraction of a greater total, particularly fungal DNA. This step was completed by the sequencing of 18S rDNA, which allowed us to identify mold strains in both hemp mortar and hemp shives.



**Figure 4.** Scheme of the DNA extraction protocol.

Nucleic acids were extracted from 0.1 g of hemp and 0.2 g of hemp mortar using a FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA) according to manufacturer instructions. Two replicates were performed for each sample. DNA was quantified by a fluorimetric measurement [19]. The 18S rDNA amplifications for fungal biomass estimation were conducted by 18S rDNA real-time quantitative polymerase chain reaction [20]. The amplification protocol (40 cycles of PCR, 40 s at 95 °C, 45 s at 64 °C, and 30 s at 72 °C) was performed using a thermocycler LightCycler 480 (Roche Diagnostics, Penzberg, Germany). The efficiency of the qPCR ranged from 90% to 100%.

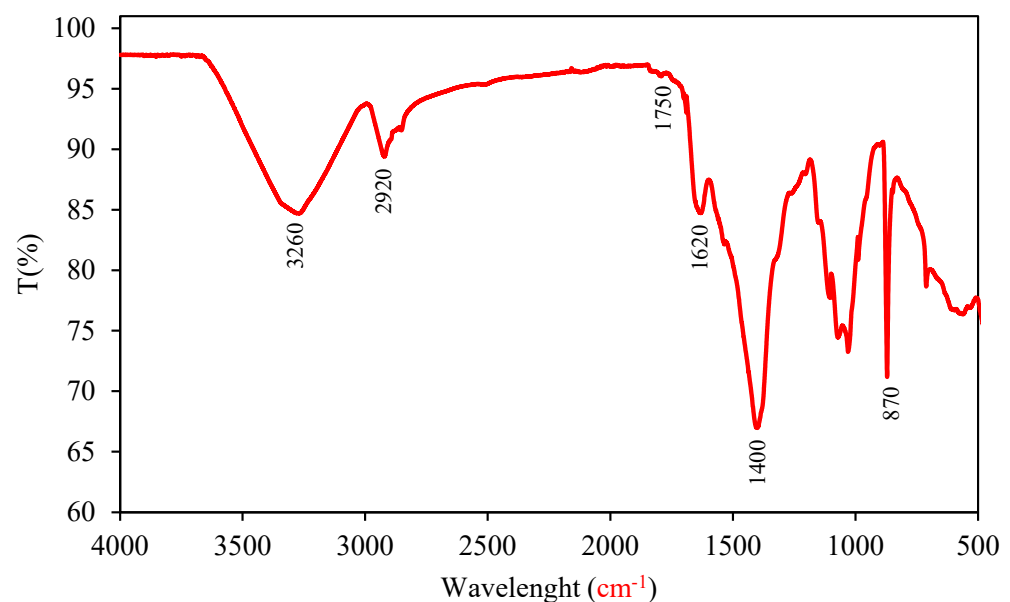
The two highest DNA concentrations extracted from hemp and hemp mortar samples were analyzed using a metagenomic approach targeting the 18S region at the GenoScreen platform (Lille, France). A clone library was generated using the MetaBiotec<sup>®</sup> kit. Amplicons were sequenced on a MiSeq sequencer, and the quality of the generated sequence readings was evaluated using the Quantitative Insights into Microbial Ecology (QIIME) program (version 1.9.1) based on the Metabiote<sup>®</sup> pipeline [21,22]. The similarity data and phylogenetic analysis were combined to define the operational taxonomic units (OTUs). An OTU was defined as when the percentage of similarity exceeded 97%. These sufficient and high-quality 18S full-length sequences enabled us to achieve affiliation to obtain the taxonomic profile of eukaryotic populations identified within the studied samples. As the DNA of the negative control was sequenced, the fungal genera present in the con-

trol sample were subtracted from the hemp shive and hemp mortar extraction. Further identification and classification of sequences were conducted by Greengenes, version 13.8, [www.greengenes.gov](http://www.greengenes.gov) (accessed on 10 September 2020), using v2.2 of the RDP classifier [23]. A Venn diagram was plotted to compare the obtained results from a source/material point of view. This represented the number of genera present in hemp shives, in hemp mortar, and in both materials.

### 3. Results and Discussion

#### 3.1. Hemp Mortar Surface Analysis

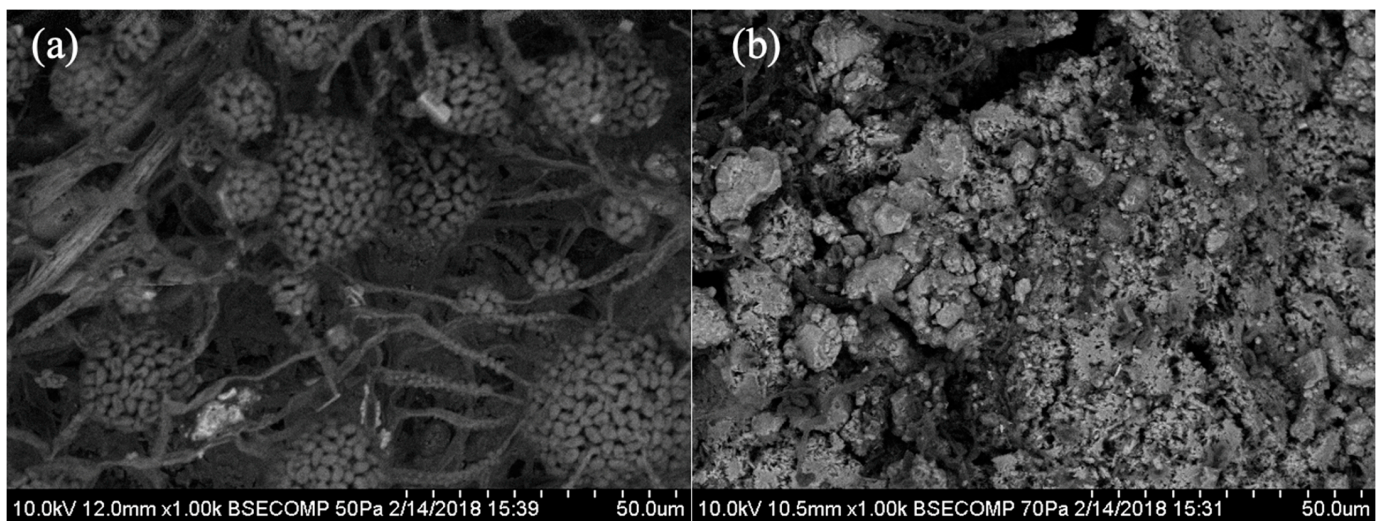
First, an analysis of the mold proliferation on the hemp mortar surface was conducted using ATR-FTIR scanning. Figure 5 shows the ATR-FTIR spectrum for the contaminated sample collected in situ. The intense peak at around  $3260\text{ cm}^{-1}$  is assigned to the stretching vibration of hydroxyl and -NH groups. Other peaks at  $2920\text{ cm}^{-1}$ ,  $1750\text{ cm}^{-1}$ , and  $1620\text{ cm}^{-1}$  represent the  $\text{CH}_2$  methylene group,  $\text{C}=\text{O}$ , and N-H stretching vibrations, respectively, that demonstrate the presence of organic material on the surface of the sample. Also, the peaks at  $1400$  and  $870\text{ cm}^{-1}$  are attributed to the deposit of calcium carbonate.



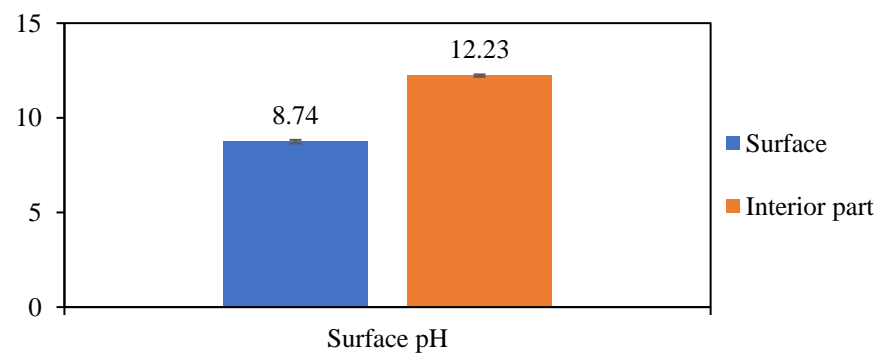
**Figure 5.** ATR-FTIR spectrum obtained from the contaminated hemp mortar sample.

Second, the W-SEM observations demonstrate the presence of molds on the hemp mortar sample surface. The fungal proliferation is represented by mold hyphae that are flattened on the mineral matrix and on the hemp particles. It highlights the capacity of molds to proliferate on both phases of the material (Figure 6).

Finally, the pH level analysis shows that the pH of the hemp mortar at 2 cm of the depth of the sample remains high at 12.23, but the value at the surface is lower at 8.74 (Figure 7). It is explained by the carbonation of calcium hydroxide, which is accompanied by a decrease in pH. In fact, the diffusion of the air dioxide carbon in the hemp mortar provokes its reaction with the portlandite and decreases the pH level. As the surface of the sample is exposed to the air, the pH level is lower because of the carbonation. The results demonstrated the correlation of the mold growth with the low pH level.



**Figure 6.** W-SEM observations of fungal growth on the contaminated hemp mortar samples.



**Figure 7.** Diagram of pH level evolution on the surface and in the middle of the samples before and after the weathering aging cycles.

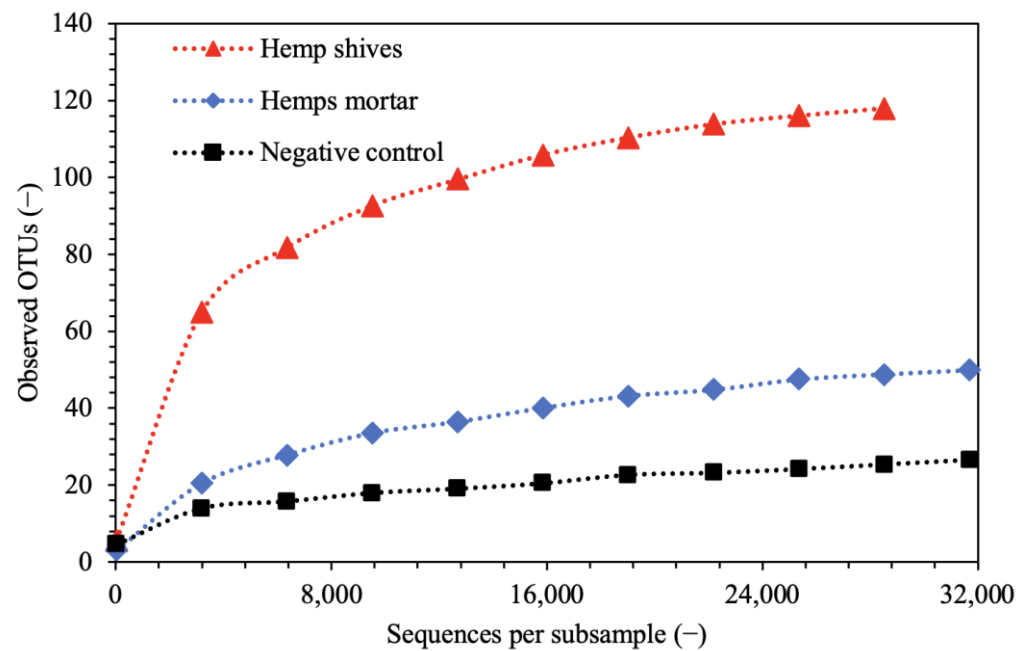
### 3.2. DNA Extraction

Following the DNA extraction step, a fluorimeter analysis was conducted to determine whether the DNA extraction kit was well chosen. Two replicates were performed for each sample. The samples with the highest total DNA concentration were 7.53, 46.49, and 0.23 ng/ $\mu$ L for the hemp shives, hemp mortar, and the control sample, respectively. These samples were used for DNA sequencing. Based on the total concentrations, it was determined that the extraction method worked well, and a sufficient quantity of DNA was extracted. Simultaneously, because the FastDNA SPIN Kit used for DNA extraction is designed to extract all DNA from the soil (i.e., plant, bacterial, and fungal DNA) in the resulting mixture, all were extracted. The qPCR analysis of the extracts from hemp shives and hemp mortar was conducted to identify the mold DNA concentration. The number of copies of  $5.42 \times 10^5$  and  $3.94 \times 10^6 \mu\text{L}^{-1}$  for hemp shives and hemp mortar, respectively, were obtained. Total DNA extracts were used after rDNA sequencing.

### 3.3. Rarefaction Analysis

To validate the representativeness of our hemp mortar and hemp shive samplings across the entire sample, the diversity was studied. To do this, the OTUs observed for hemp shives, hemp mortar, and negative control samples as a function of an increasing number of sequences were plotted (see Figure 8). Thus, we considered a detailed description of the sample diversity when the curve reached the asymptote. We note that the asymptote was reached for all samples by demonstrating a detailed description of eukaryotic diversity.





**Figure 8.** Rarefaction analysis of the observed OTUs for the hemp shives, the hemp mortar, and the negative control as a function of an increasing number of sequences.

However, we should note that the diversity of hemp shives was more significant than what was possible due to less auspicious conditions in the hemp mortar. This could also be explained by the nature of the vegetal fibers, which are very heterogeneous from a dimensional point of view (aggregate hemp size) and where we risk finding traces of dust and contamination (due to the industrial process of retting).

### 3.4. Mold Species Identified

Following the subtraction of fungal genera present in the negative control, four phyla remained in the studied samples of hemp shives and hemp mortar. As Figure 9 shows, Opisthokonta was the major phylum in both cases, with 83.6% in hemp shives (Figure 9a) and 99.97% in hemp mortar (Figure 9b). This suggested that the Opisthokonta phylum species, initially present in the hemp shives, could survive under the fresh hemp mortar's aggressive conditions and dominate following the application.

The second most abundant phylum was Archaeplastida, representing 16.3% of hemp shives (Figure 9a). However, it decreased to 0.004% in the mortar (Figure 9b). This showed that the conditions of fresh mortar are not suitable for these species. A decrease in abundance was also noted for SAR phylum strains (from 0.13% to 0.02%). However, it could be observed that incertae sedis phylum microorganisms were present at 0.007% in the hemp mortar samples and were not found in hemp shives. This may have been due to external contamination following the hemp mortar application (Figure 9).

Next, mold strain abundances from hemp shives and hemp mortar were analyzed separately. Thirty-seven and twenty-five genera of mold were identified in hemp shives and hemp mortar samples, respectively. Some fungi have not been identified up to the genus, but they are reported to be in the phylum represented in Figure 9. For example, eight undefined (17% total abundance) and four undefined (0.05% total abundance) fungi exist in hemp shives and hemp mortar, respectively, which had not been previously identified and are represented by "other/unclassified" (Figures 10 and 11).

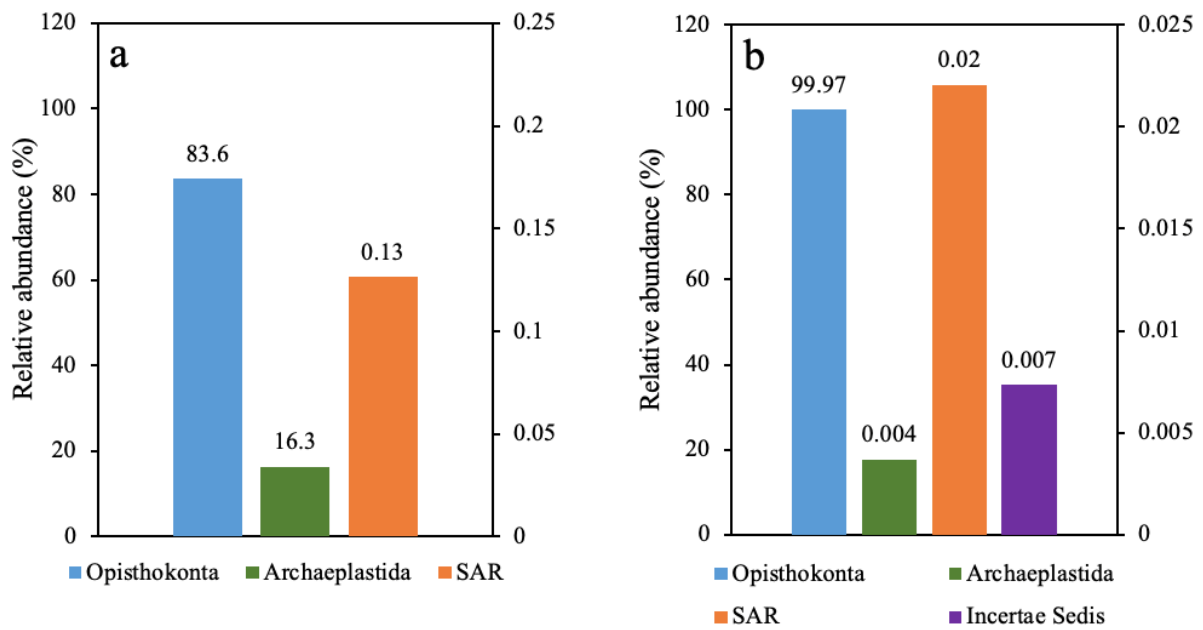


Figure 9. Relative abundance levels of each fungal phylum for hemp shives (a) and hemp mortar (b).

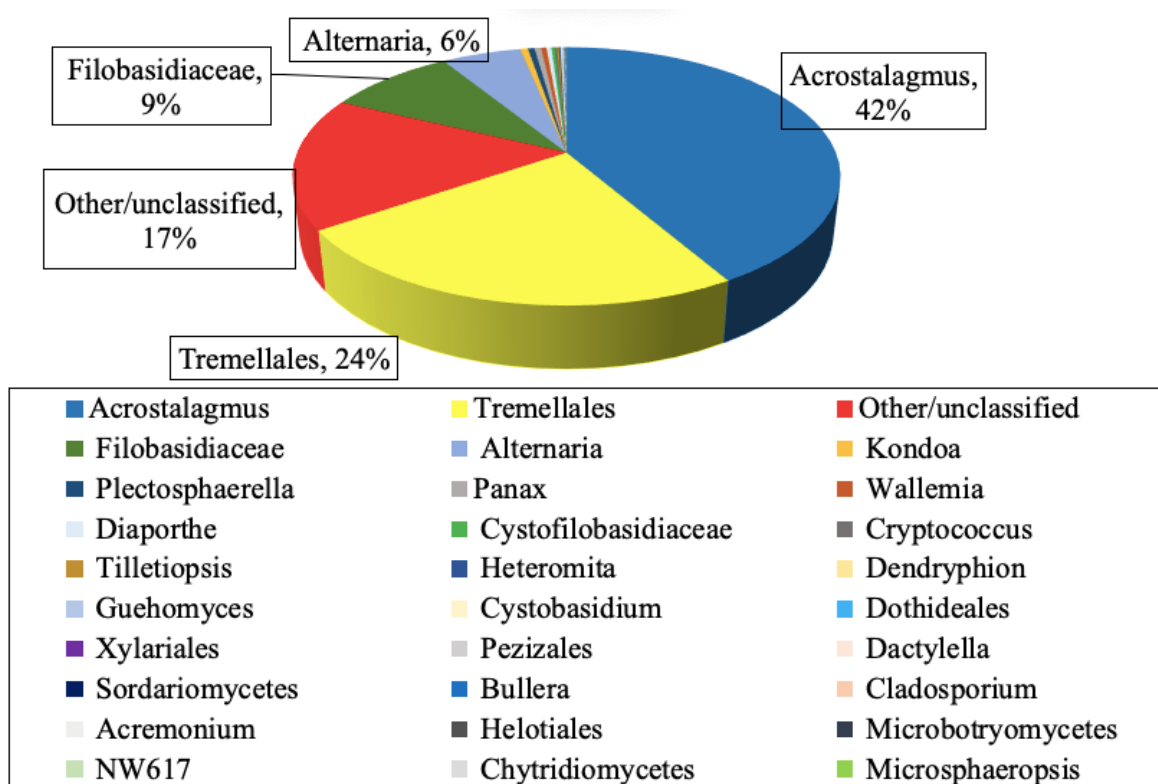
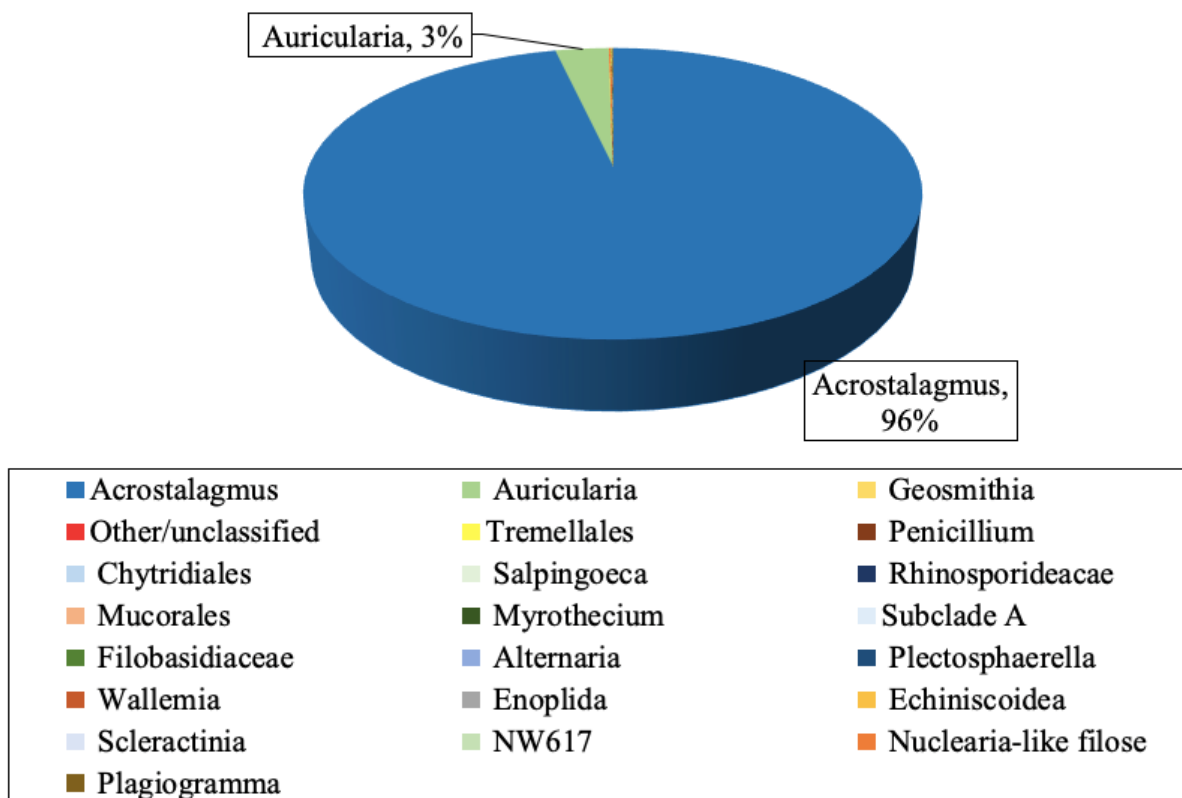


Figure 10. Fungal community analysis in hemp shives (18S rDNA).



**Figure 11.** Fungal community analysis in hemp mortar (18S rDNA).

In hemp shives, the majority of identified genera (abundance greater than 1%) are representatives of the Opisthokonta phylum: *Acrostalagmus* (42%), *Tremellales* (24%), *Filobasidiaceae* (9%), and *Alternaria* (6%). In the case of the hemp mortar samples, *Acrostalagmus* (96%) and *Auricularia* (3%) genera are major (also belonging to the Opisthokonta phylum) (Figures 10 and 11). A trophic cycle analysis of these genera was conducted and is described later in this section.

In addition, the Venn diagram shows that a greater number of genera exist in hemp shives than in hemp mortar, which can be explained by the extreme conditions of the alkaline medium, such as mortar after mixing. There are 87.4% sequences that have not been identified up to the genus in hemp shives. They are represented by “other/unclassified” (Figure 12). Simultaneously, 95% of these sequences belong to the Charophyta order of Archaeplastida phylum and 5% to the fungi order of the Opisthokonta phylum. The most abundant genera identified are *Kondoa* (2.8%), *Diaporthe* (1.9%), and *Cystofilobasidiaceae* (1.2%) of the Opisthokonta phylum and *Panax* (2.5%) of the Archaeplastida phylum.

Among the genera that are present only in hemp mortar, we distinguished two major ones: *Auricularia* (96%) and *Geosmithia* (2%) (Figure 12). These genera belong to the Fungi order. Regarding the fungal genera present in both hemp shives and hemp mortar, we distinguished seven genera; six genera belong to the Fungi order and one to the phylum SAR, order Cercozoa. Four genera in hemp shives are considered major: *Acrostalagmus* (51%), *Tremellales* (30%), *Filobasidiaceae* (11%), and *Alternaria* (7%) (Figure 10). By contrast, only one genus dominates in hemp mortar, namely, *Acrostalagmus* (99.9%). We note that the *Acrostalagmus* genus representatives are the majority of mold species in hemp shives and dominate in hemp mortar (Figure 12).

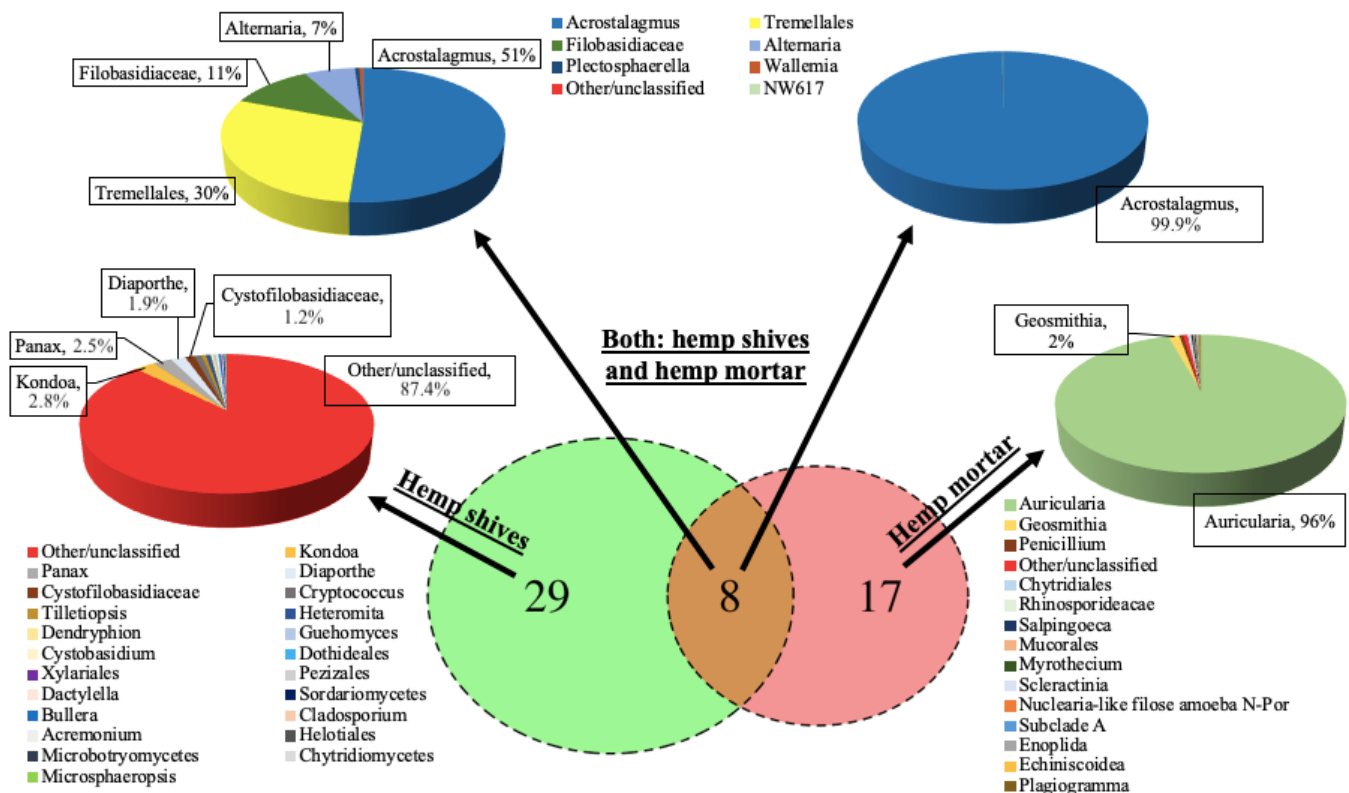


Figure 12. Venn diagram evaluating the mold contamination of hemp shives, hemp mortar, and both.

#### 4. Discussion

To better understand the proliferation of mold strains in hemp mortar, a major genera representative trophic cycle analysis is proposed. As previously noted, the major genera of hemp shives are represented by the *Acrostalagmus*, Tremellales, Filobasidiaceae, and *Alternaria*. For hemp mortar, the two major genera of *Acrostalagmus* and *Auricularia* exist.

According to [24], 54 records are listed in the nomenclatural databases Index Fungorum and MycoBank under the name *Acrostalagmus* with 29 included species, six forms, and eight varieties. The authors [24] also reported that the representatives of the *Acrostalagmus* genus are cosmopolitan fungi and could be isolated from various soil types, including alkaline soils in Europe, Russia, Turkey, Sri Lanka, Nepal, Japan, Australia, Hawaii, Canada, and the continental USA, or from a great variety of types of plant debris as well as from dung (for example, *Acrostalagmus luteoalbusis*). They could also be saprophytic on wood, bark, leaves, and herbaceous stems. They are occasionally isolated from tropical and subtropical soils (for example, *Acrostalagmus annulatusis* [25]).

The Tremellales genus representatives are characterized by dolipore septa with vesiculate parenthesomes [26]. This order includes more than 50 lichenicolous species [27]. They are cosmopolitan fungi that have been isolated in different countries (Brazil, Europe, Russia, Mexico, Panama, and Greenland).

Using Bayesian analysis on the seven-gene dataset for the Filobasidiaceae, we could distinguish five strongly supported clades named *Aerius*, *Albidus*, *Cylindricus*, *Filobasidium*, and *Gastricus* [28]. The representatives of this order are mostly different species and varieties of *Cryptococcus*. It has been reported that Filobasidiaceae species can grow in extreme conditions. For example, three species of *Gastricus* were isolated from acid rock drainage from a pyrite mine in Portugal [29], and another three species were isolated from hypersaline soil of the Urmia Lake National Park in Iran, which also showed the possibility of growing in saline environments (10–15% of NaCl) [30].

*Alternaria* species are fungi distributed worldwide as saprophytes, endophytes, and plant pathogens in soil, atmosphere, plant materials, and food commodities because of



their ability to adapt and survive in environmental conditions that are far from optimal [31]. As plant pathogens, *Alternaria* spp. have been reported on crops, including cereals, oil crops, ornamentals, vegetables, and fruits [32]. *Alternaria* spp. are also known to produce many secondary metabolites, such as host-specific toxins required for pathogenicity, a vast number of mycotoxins, and several allergens [33].

The *Auricularia* genus representatives are cosmopolitan fungi reported in eastern Asia, North America, Europe, and elsewhere [34]. *Auricularia* is a major genus among the jelly fungi because of its widespread consumption and medicinal (antibacterial) properties. For example, *Auricularia heimuer* is one of the most famous traditional Chinese foods and medicines and is the third most cultivated mushroom worldwide [35].

Thus, results of rDNA sequencing showed that the major fungal genera from hemp shives and hemp mortar are cosmopolitan and widely present in the world. Some of them (*Acrostalagmus*, *Filobasidiaceae*) can reportedly grow in extreme environments. Mortar (high pH, high salinity) is one example. We should also note that the number of representatives of the two orders Tremellales (mostly parasitic fungi) and *Alternaria* (mostly saprophytic fungi) was greatly reduced in hemp mortar as compared to hemp shives. We could hypothesize that this was due to environmental conditions and the inaccessibility of food because of enrobing hemp shives by mortar. By contrast, the *Acrostalagmus* representatives accustomed to alkaline environments preserved their viability and maintained their ability to grow. As a result, they had an advantage over other mold strains. Strains from the *Auricularia* genus proliferated after mortar casting, as they were present only in the hemp mortar, which could be explained by further external contamination. In addition, we observed that environmental conditions played a critical role in determining the durability of hemp mortars and particularly fungal growth because of the conditions of mold proliferation (humidity, temperature, pH level, etc.) and even greater external contamination.

These results offer a detailed analysis of the contamination and mold growth process of bio-composite materials using a refined approach based on the metagenomic DNA method. In fact, the mold growth was considered in the plant fiber alone, then in the cementitious matrix, followed by fine identification and quantification of the fungal species and community in each phase, along with an explanation of the interactions between them. Also, the affinity of the DNA method is new in the field of construction materials, unlike the classical methods used in the literature, which are based on the phenotypical identification approach [11,16]. This method can be generated using other bio-based materials. It can be improved by an enlarged analysis area of the fungal and bacterial DNA sequencing, as the microbiological contamination depends on the region.

## 5. Conclusions

This study conducted a refined analysis and identification of fungal communities present in in situ-collected hemp mortar and its raw material, that is, hemp shives. Thus, this study developed a detailed protocol for DNA extraction and sequencing for hemp shives and hemp mortar that could be used for other composite cementitious materials. A real case involving in situ-collected samples of hemp mortar and its source (hemp shives) was studied separately to understand the interaction between the mold contamination of raw hemp shives and final hemp mortar as follows:

- First, the surface of contaminated hemp mortar was analyzed with ATR-FTIR and W-SEM, which confirmed the presence of organics. Second, a DNA extraction method was carefully selected based on total and fungal extracted DNA quantification;
- Then, the diversity of studied samples using rarefaction analysis was evaluated to ensure the representativeness of the results;
- Finally, the mold genera present in hemp shives and hemp mortar were then accurately identified to analyze the trophic cycle of major fungi.

The results of DNA sequencing showed a difference between mold contamination of hemp shives and composite hemp mortar. The relative abundance levels of fungal phyla present in both hemp shives and hemp mortar revealed that the most abundant phylum

was Opisthokonta. It represented 83.6% of hemp shives and 99.97% of hemp mortar mold contamination. Analysis of the identified species emphasized the interconnection between the mold strain characteristics (particularly the ability to grow in extreme environments) and their presence in hemp mortar. For example, the most abundant identified genus was *Acrostalagmus*, which represented 42% of hemp shives and 96% of hemp mortar mold contamination. This was explained by its ability to survive in alkaline environments (e.g., alkaline soils). The representatives of other genera, such as Tremellales, Filobasidiaceae, and *Alternaria*, were present in high abundances in hemp shives, but their presence in hemp mortar was in the range of 0.1–0.2%. It shows their difficult adaptation to the highly alkaline environment of the mortar with its high alkalinity. The abundance of representatives of other genera was lower than 1%, which is why they are not mentioned. Because more genera exist in hemp shives than in hemp mortar (37 against 25, respectively) and because the *Acrostalagmus* genus present in hemp shives is the most abundant in hemp mortar, we could conclude that the hemp shives are the main source of hemp mortar contamination. However, the environmental conditions were also found to be significant. As a result, only eight species exist in both hemp shives and hemp mortar, and 17 species are not present in hemp shives (but instead derive from external contamination).

The chosen DNA extraction method could be used to characterize other types of mortars and their raw materials under different conditions. The present study opens the possibility of using a molecular rDNA screening method for building materials that could help in understanding the consistent patterns of proliferation of different microbiological organisms. The obtained results should be considered when studying the durability of hemp mortars and their interaction with fungal communities. In addition, we observed that the most crucial origin of fungal contamination was hemp shives. This adds to the urgency of further studies on protecting hemp mortars from initial or external contamination, for example, by applying different treatments of hemp shives or by using essential oil additives.

**Author Contributions:** D.K.: Conceptualization, Methodology, Formal analysis, Data Curation, Writing—Original Draft, Visualization; K.A.: Conceptualization, Methodology, Writing—Review and Editing, Formal analysis, Supervision; I.T.-G.: Conceptualization, Resources, Review and Editing, Supervision; L.C.: Methodology, Validation, Formal analysis, Resources; A.D.: Review and Editing, Supervision, Project administration; Evelyne Prat: Review and Editing, Supervision, Project administration; M.C.: Review and Editing, Supervision, Project administration; R.B.; Writing—Review and Editing, Formal analysis. All authors have read and agreed to the published version of the manuscript.

**Funding:** The authors acknowledge SIKA France group for supporting this project and the platform AGROBIOTECH where the analysis of microbial abundance and diversity was achieved. The microbial community analysis work was carried out at the AGROBIOTECH platform on the UniLaSalle Rouen campus.

**Data Availability Statement:** All data are available.

**Conflicts of Interest:** Authors Dmytro Kosiachevskyi and Anne Daubresse were employed by the company SIKA France. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The SIKA France group had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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