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Paper 118

ON THE EFFECT OF FREEZE-DRYING ON PAPER-BORNE MICROORGANISMS

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Abstract
Today there is an ever-increasing interest in recovering flooded archival materials. Freeze-drying stands out as a valuable method as the water is frozen and the ice is then removed through sublimation. The present work is focused on the effect of freeze-drying on flooded paper contaminated with biodeteriogenic microorganisms (i.e. a bacterium Staphylococcus epidermidis, a yeast Rhodotorula mucilaginosa and a filamentous fungus Alternaria alternata). The effect of freezing and drying on microorganism survival and growth has been separately evaluated. In particular, different operative conditions for freezing (in a domestic freezer at about -20°C, at -40°C with a freezing rate of -1°C/min) and drying (200 µbar at 0°C and 20°C) have been tested. All the tested strains were able to survive after freezing and drying phases, nevertheless a different behaviour was observed: the growth of S. epidermidis was the same as that of the control, R. mucilaginosa showed a slight growth and carotenoid production increase, while A. alternata was inhibited, in particular after the freezing at -40°C, followed by a low temperature drying rate step.

Keywords*: freeze-drying, biodeterioration, filamentous fungi, flooded paper, process design.*

1. Introduction

Water damage to paper documents can occur by flooding or during a fire extinguishing. In these circumstances, a fast and non-invasive method to dry materials is required, aiming to prevent additional damages such as biodeterioration caused by microorganisms naturally present on the documents, or transferred by the contaminated water. In fact, contamination with biodeteriogenic microorganisms may occur when the paper is in contact with water and the probability of a microbial growth increases with the water contact time. The paper bioreceptivity, i.e. the ability of a material to be colonised by living organisms, is considered high, because cellulose could represent a primary carbon source for different microbial classes. Biodeteriogenic microorganisms, in particular filamentous fungi, can grow on paper in the presence of a suitable combination of humidity and temperature conditions (Sequeira *et al.*, 2012). When fungi biodegrade paper, stains and discolouration of the material can occur.

Freezing is a way to control microorganism growth and document deterioration, thus preserving the material in view of a successive treatment that can be programmed in a different place and time, after the emergency. Once the material has been frozen, it can be stored for a very long time with practically no consequences, and freeze-drying becomes an interesting possibility, since the first step of the process has already been carried out. As a matter of fact, drying of water-damaged archival and library materials by means of freeze-drying is a well-known technology, which can give very good results avoiding further damages because the operating temperatures are very low and there is no

presence of liquid water which can transport soluble components present in the material (McCleary, 1987; Fissore *et al*., 2017).

One of the open questions concerning the use of freeze-drying for the recovery of flooded archival and library material is its uncertain effect on the microorganism viability; for this reason, a post-drying treatment could be required to store safely the dried material. Different opinions have been stated, claiming that freeze-drying can significantly reduce the microbial load, and even proposing it as a treatment to significantly reduce the contamination, or being afraid that the lyophilisation process can even lead to an increase of it. Studies on the effect of freezing on microorganism viability report that increasing the freezing time, or decreasing the freezing rate, can reduce the viable microbial population (Florian, 1990). But there are few reports on the effect of freeze-drying on biodeteriogenic microorganisms; concerning filamentous fungi, spores are reported to be more resistant than hyphae (Troiano *et al*., 2012).

The aim of the present work is thus to evaluate the effect of freeze-drying on the viability of three different paper-borne microorganisms: the filamentous fungus *A. alternata*, the yeast *R. mucilaginosa* and the bacterium *S. epidermidis*. The effect of several operating variables of the freeze-drying process (i.e. freezing temperature, freezing rate, and sublimation temperature) and their influence on the paper recovery process will be investigated thus providing useful guidelines to freeze-drying practitioners facing this challenging task.

2. Materials and methods

Modern blank paper Double A (80 g/sheet) was used to prepare blocks (6 x 6 cm, height: 10 ± 1 mm) which were soaked in distilled water for 18 hours and then drained for 1 minute to simulate a real flooded and contaminated book.

Microorganisms were separately inoculated on a sterile single paper square (6 x 6 cm) deposited on Nutrient agar for *S. epidermidis*, Malt extract agar for *R. mucilaginosa* and Czapeck agar for *A. alternata*. The bacterium and the yeast were inoculated by tracing 3 parallel streaks, 4 cm long at a distance of 1 cm, applying a single withdrawal from a standardized cell suspension; they were incubated for 24 hours at the optimal temperature of 37 and 30°C respectively. The filamentous fungus *A. alternata* was inoculated by depositing a mycelial plug (5 x 5 mm), collected at the border of a 7 days old colony, and incubated at the optimal temperature of 30°C for 48 hours. Contaminated samples were composed as follows: inoculated squares were positioned on the surface and in the middle of the soaked paper blocks.

Aiming to assess the effect of the freezing rate and temperature, some samples were frozen inside plastic bags in a domestic freezer for 13 days at about -20°C, while other samples were frozen at -40°C, with a freezing rate of -1°C/min (using the same equipment where freeze-drying tests are carried out). Samples were then thawed, for 3 hours at 25°C, inside sealed glass jars.

With respect to the drying tests, after the freezing, samples were freeze-dried in a Millrock REVO freeze-drier at 200 µbar and 0°C (a test at 20°C was also carried out to evaluate the effect of the drying temperature). The temperature was monitored using thermocouples placed on the top and in the middle of the samples. Pressure was monitored by a capacitance manometer and a thermal conductivity gauge. The end of primary drying is determined from the ratio between the two sensors, as it becomes equal to one when there is no water vapour in the chamber (Patel et al., 2010).

At the end of the freezing and drying phases, contaminated sheets were recovered and reconditioned in sterile conditions, positioned on the same agar medium and incubated at the same temperature values as previously reported for the preparation of the paper sheet inoculum. As regard *R. mucilaginosa* and *S. epidermidis* are concerned, microbial growth was visually monitored and photographed at 0, 24 and 48 hours from the deposition of the treated paper sheets on the agar medium. At time 0 and then every 24 hours, *A. alternata* viability was checked visually, the colonies were photographed and the two perpendicular diameters were measured until the mycelium stopped to grow or reached the border of the Petri dish. For all the three microorganisms, the growth was compared to that of the untreated control, not frozen or dried, at the same incubation time as that of the treated samples.

3. Results and discussion

After soaking, the mean increase of the weight of the paper samples was 52%: almost all the water in the samples was removed at the end of the freeze-drying process, being the residual water content 2.77%. Drying duration was 28 hours at 0°C, and 20 hours at higher temperature, as expected: in both cases, the dried material reached the temperature of the heating shelf.

In order to evaluate the effect of the freezing modality on the microorganism viability and growth, thawed inoculated sheets were deposited on the proper agar medium plates and incubated at the optimal temperature value as indicated in the Material and Methods section.

In the two tested conditions (i.e. freezing in a domestic freezer and in the freeze-dryer at -40°C) no difference in growth for *S. epidermidis* and *R. mucilaginosa,* positioned on the surface and in the middle of the soaked paper blocks, was observed. A comparison of the treated sheets with the control (i.e. untreated inoculated sheet) showed that the growth of *S. epidermidis* was the same as that of the control (Figure 1, right hand side) while *R. mucilaginosa* showed a slight growth and carotenoid production increase (Figure 1, left hand side). With respect to the effect of drying on the bacterium and yeast survival and growth, no effects were observed, independently from the drying temperature value (data not showed).

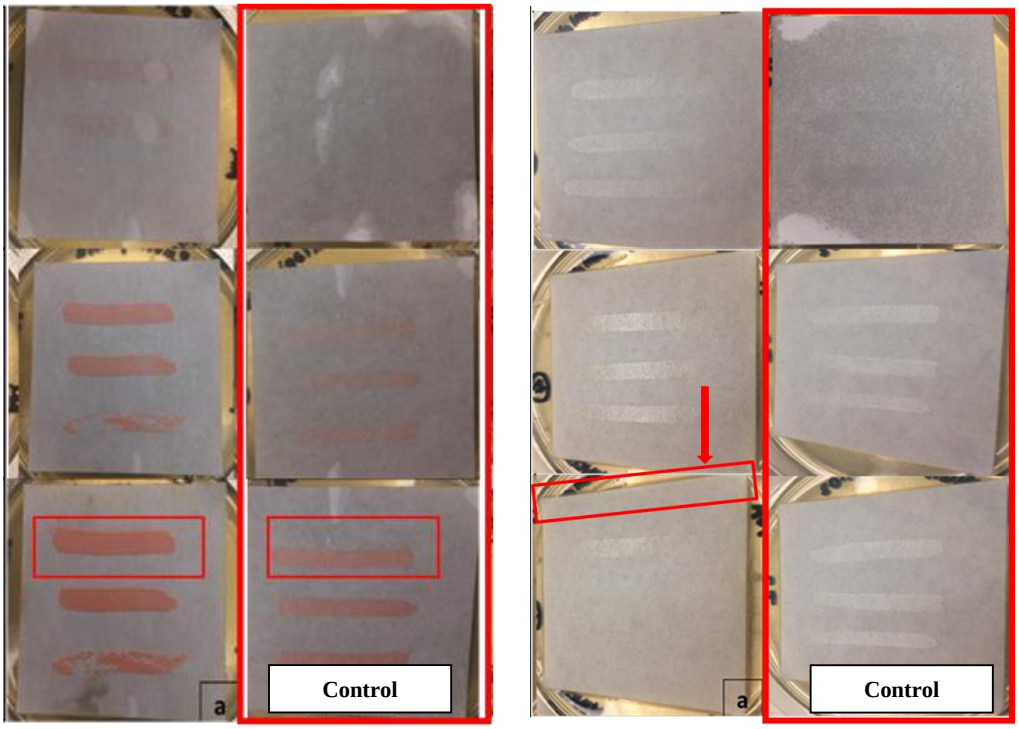

Figure 1*. R. mucilaginosa (on the left) and S. epidermidis (on the right) growth on a sheet after freezing in a domestic freezer (a) compared with a control at time 0 (top), 24 (middle) and 48 (bottom) hours.*

For *A. alternata* contaminated sheets, after the freezing in a domestic freezer (Figure 2a) and in the freeze-dryer at -40° (Figure 2b), only a slightly different behaviour in the colony diameter values (Figure 3), with respect to that of the control sheet, was observed. The main difference between treated and untreated inoculated sheets was a morphological one: after both freezing modality, at 24 hours of incubation, an abundant aerial mycelium growth was observed, along with the absence of sporulation.

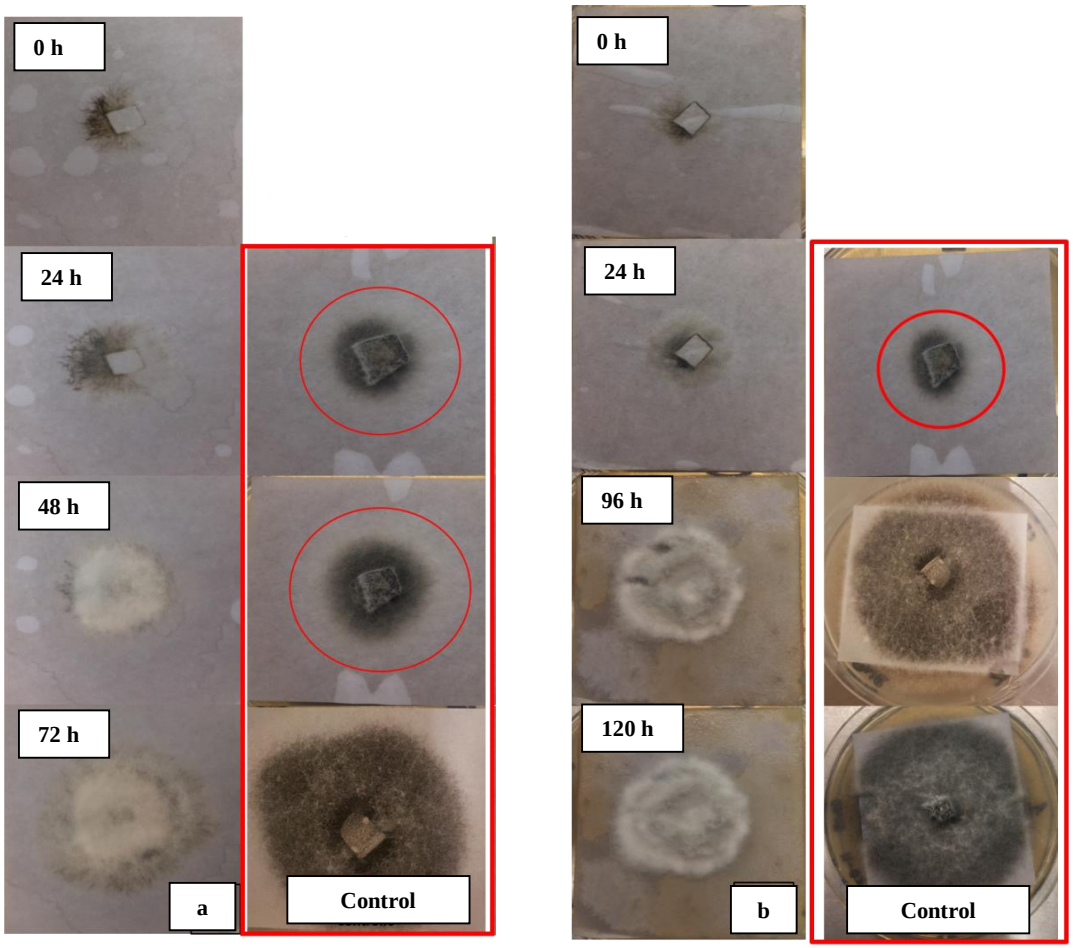

***Figure 2**. A. alternata growth on a sheet after freezing in a domestic freezer (a) and in a freeze-dryer at -40°C (b) at different times of incubation.*

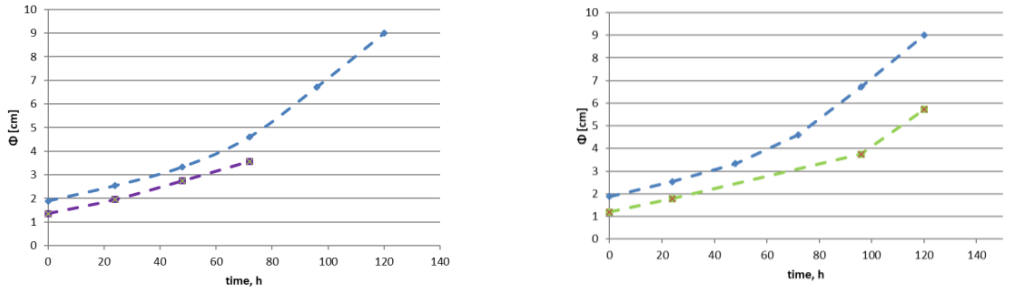

***Figure 3**. Mean diameter of A. alternata colony on a sample after freezing in a domestic freezer (on the left) and in a freeze-dryer at -40°C (on the right) at different times of incubation. Blue curves represent the control sheets.*

In Figure 4, the results obtained with *A. alternata* after the freeze-drying at -20°C are showed. In both the photos and the graphic, a growth inhibition up to 48 hours of incubation is evident. At 96 hours, the diameter of the freeze-dried mycelium (2.45 cm) is significantly

lower than that of the control (about 6.72 cm). Moreover, it is also possible to observe a conspicuous sporulation of the mycelium, probably due to a negative influence of the drying phase on the fungus.

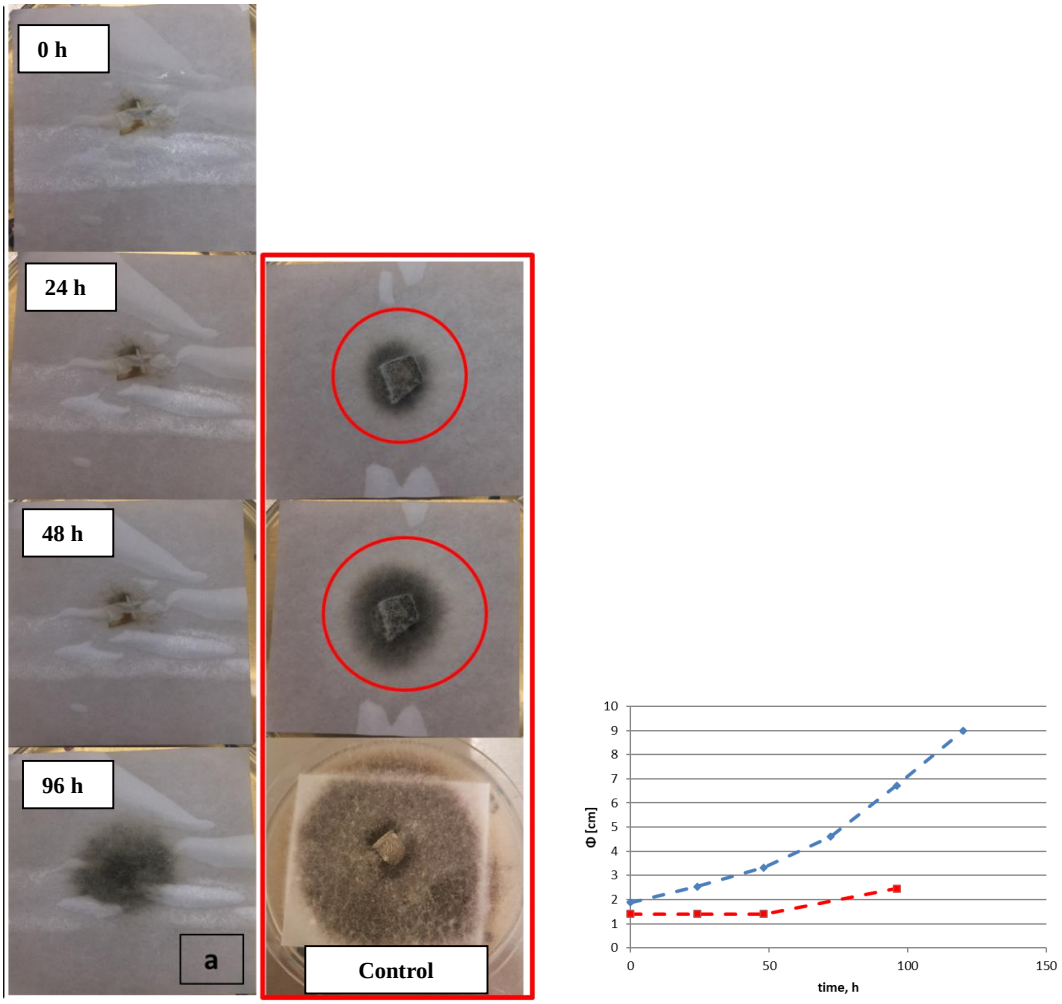

Figure 4. *A. alternata growth on a sheet after freeze-drying at -20°C (freezing was carried out in a domestic freezer) at different incubation times (on the left). Comparison between the mean diameter of the fungus on the treated sheet (red) and the control (blue) (on the right).*

A. alternata growth, after a freezing in the freeze-dryer at -40°C and freeze-drying at -20°C, was characterized by a clear lag phase until 72 hours of incubation. After that, the fungus starts to grow with a rate comparable to that of the control, in the first 48 hours of incubation. This behaviour is evident in both the pictures and the graphic reported in Figure 5. The obtained results suggest a higher efficacy of the freeze-drying process on the control of *A. alternata* growth, respect to that obtained after the one and only freezing step.

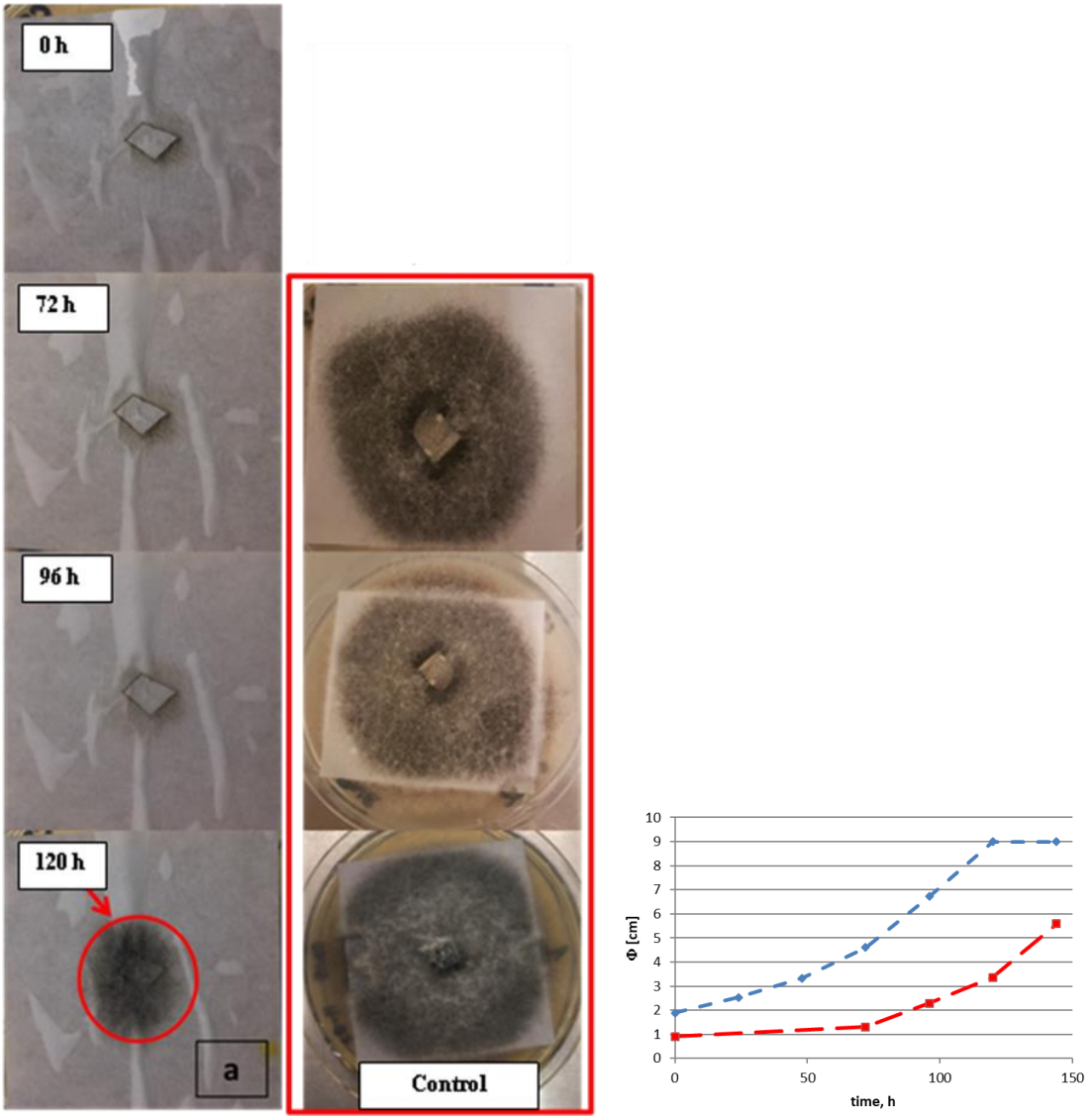

***Figure 5**. A. alternata growth on a sheet after freeze-drying at -20°C (freezing was carried out in the freeze-dryer, at a final temperature of -40°C) at different incubation times (on the left). Comparison between the mean diameter of the fungus on the treated sheet (red) and the control (blue) (on the right).*

4. Conclusions

In the present work, the effect of freezing and drying on the survival of some selected biodeteriogenic microorganisms on flooded paper was described. As expected, all the tested strains were able to survive, but a different behaviour was observed. In general, *S. epidermidis* did not show any clear difference respect to the untreated sheets, *R. mucilaginosa* was stimulated, while *A. alternata* was inhibited, in particular after the freezing at -40°C, followed by a low temperature drying rate step. In the case of filamentous fungi, freeze-drying may give a positive contribution to the control of biodeterioration; as a matter of fact, for *A. alternata*, tested in optimal cultural conditions, a lag-phase of about 3 days was observed. In a real case (e.g. a freeze-dried book arranged in a library), in the presence of temperature and humidity control, a longer duration of the lag-phase could be hypothesized.

It must be stressed anyway that the goal of the freeze-drying treatment is the removal of water, that is in general effectively reached. If the material is characterized by a significant microbial load, already present on the material or developed as a consequence of the flooding, freeze-drying may have an effect on it, marginally positive or sometime negative, as demonstrated in the present work. The application of post-freeze-drying treatments (e.g. essential oil) could be considered in order to delay or completely inhibit the growth of paper-born microorganisms. In any case, proper storage conditions must be provided to guarantee preservation of the restored material.

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