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ANOXIC ERADICATION OF FUNGI IN WOODEN OBJECTS

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ABSTRACT This work examined the effects of low-oxygen conditions, achieved with either argon or nitrogen gas, on the viability of the wood-decay fungi *Antrodia vaillantii, Coniophora puteana, Gloeophyllum trabeum, Serpula lacrymans,* and *Trametes versicolor* cultivated on infested wood samples. The fungal cultures were exposed to low oxygen concentrations, below 20 ppm, for one to five weeks in hermetically sealed vessels or gas-impermeable double-enveloped plastic enclosures.

After treatment, CO_2 production of cultures was measured to determine if mycelia regenerated. In addition, the ability of hyphae to resume growth was also assessed by transferring the treated blocks to fresh nutrient agar. The effect of the anoxic conditions on the mycelia of treated blocks was expressed as either a complete absence of regrowth of the fungus or as a delay in the mycelia's recovery.

Results clearly showed that argon was more effective than nitrogen gas at reducing or eliminating fungal activity. Anoxic treatment by either argon or nitrogen gas did not affect *T. versicolor* cultures in the time span of the experiment. *G. trabeum* was only slightly affected, and *A. vaillantii* mycelial cultures proved to be less sensitive to anoxic treatment than those of *S. lacrymans* and *C. puteana,* which lost their viability completely after one month of treatment.

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INTRODUCTION Objects of cultural and historical heritage are irreplaceable and should therefore be protected from damage caused by insects and wood-decay fungi. Wooden objects may be attacked by various wood-boring insects, e.g., the furniture beetle or the powderpost beetle, which can, over long periods, cause severe damage. The furniture beetle, in particular, likes to attack wood that has been infested by fungi. Wood-attacking fungi can decay wooden objects very rapidly and in a short period completely destroy them.

The anoxic treatment method has been developed to eliminate insect infestations with no side effects to the object (Gilberg, 1991; Koestler, 1992, 2001). This approach has proven very successful, especially when coupled with measurement of insect respiration (Koestler et al., 2000; Stusek et al., 2000).

Using an FTIR system for CO_2 detection, even a single organism in a 10-liter sealed enclosure can be detected (Koestler et al., 2000). Since an infestation in an art object generally involves many more insects, their presence can be readily detected in volumes greater than 10 liters. Measurement of respiration is also important in demonstrating the effectiveness of any treatment, anoxic or chemical.

Another important capability of the FTIR respiration system is that by measuring the loss of respiration activity after anoxic treatment, accurate length of treatment (LOT) can be established for different insects, their life stages, object types and sizes, and temperature and humidity conditions. In practice it has been found that LOT values for insects in art objects sometimes differ significantly from those given in published studies of insects in artificial situations, e.g., in jars.

Anoxic treatment for insects is typically performed using argon or nitrogen gas. Valentin et al. (1992) have shown that argon is 25–50% faster at killing insects than is nitrogen. They speculated that the argon dried out the insects faster than nitrogen did, and that loss of moisture was the reason for the faster deaths with argon. A more plausible explanation is that since the argon-treated insects die faster they have more time to desiccate than do nitrogen-treated ones. Argon, being heavier than oxygen, sinks to the bottom of the enclosure and pushes any remaining oxygen out of the object and to the top of the enclosure. Nitrogen, on the other hand, is lighter than oxygen and rises to the top, while the oxygen sinks to the bottom of the enclosure, and remains within the object longer than under argon.

Eradicating a fungal infestation is more difficult than eliminating insects. Dry wood is nearly completely protected against germination of fungal spores, but if the object is already infested with fungal mycelia, drying will not always eradicate the infestation. For example, the dry-rot fungus *S. lacrymans* cannot be eliminated by drying, and a more active procedure should be employed (Pohleven, 2000).

Virtually all of the procedures to eradicate fungi involve chemical treatment that can potentially damage the art, and that can be dangerous for conservators and the environment. Since the anoxic treatment has proven to be safe and successful for insect control, it was decided to test its effectiveness for fungal control too.

While there have been a few physiological studies on the effect of different oxygen concentrations on growth and survival of fungi, and on the extent of decay that they cause, none have specifically addressed using low-oxygen environments to kill them. Jensen (1967) reported that fungal biomass production was significantly lower at an oxygen concentration of 15% (as compared to the normal oxygen concentration of 20.9%) and that it ceased in the absence of oxygen. Otherwise, if the oxygen concentration was kept at a fixed level and the carbon dioxide concentration was varied, the amount of dry weight produced decreased as carbon dioxide concentrations increased. The ability of several fungal species to degrade wooden blocks was severely retarded when oxygen content was as low as 1% or CO_2 levels were raised to 10% (Highley et al., 1983). On the other hand, delignification was not affected by an atmospheric CO_2 content of 14%, and was only slightly retarded at 7% of O_2 in the bulk gas phase, concentration inside the wood particles could be lower (Reid, 1985). The ability of fungi to survive in an environment with a low oxygen concentration is best described by Scheffer (1986). There it was shown that the fungi that survived the longest were more often heart-rot rather than the sap- and products-decay fungi. Some of the fungi were even able to survive in sealed vessels for more than two years. However, most of the fungal species studied died within three months, and cultures of two of the species died within a week.

None of the above-cited authors used pure argon or nitrogen gas to replace the oxygen and carbon dioxide atmosphere in experimental vessels. Tavzes et al. (2001) have shown that some species of brown-rot fungi lost significant viability when grown in pure culture medium (PDA) under extremely low oxygen concentrations generated with argon or nitrogen gas. Optimization of the asphyxiation method might shorten the time needed for eradication of these fungi. In insects, argon gas has been shown to be more effective than nitrogen in killing insects—25 to 50% faster (Valentin et al., 1992). The aims of the current research were to ascertain whether fungi in wood blocks responded the same way as those same fungi in nutrient medium (PDA), and to see whether argon was more effective than nitrogen as the replacement gas in anoxic eradication of fungi. If the process reported by Tavzes and co-workers (2001) could be improved upon, then it could be useful in treating fungal-infested art objects.

MATERIALS AND METHODS

Organisms, Media, Material, and Inert Gases Strains of wood-decay basidiomycetes *Antrodia vaillantii* (DC. ex Fr.) Ryv. (*A.v.4*), *Coniophora puteana* (Schum. ex Fr.) Karst. (*C.p.*), *Gloeophyllum trabeum* (Pers.) Murr. (*G.t.2*), *Serpula lacrymans* (Wulf. ex. Fr.) Bond. (*S.l.5*), and *Trametes versicolor* (L. ex Fr.) Pilat (*T.v.*) were obtained from the fungal collection of the Department of Wood Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia. The mycelia were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI). Wood blocks used in the experiment were Standard SIST EN 113, dimensions 1.5 × 2.5 × 5 cm (longitudinal direction) Norway spruce (*Picea abies*) sapwood and beech (*Fagus sylvatica*) (ECS, 1989).

Ventilation lids were made using Metalwork valves (Metalwork, Italy), Manesmann-Rexroth (Germany) adaptors, and Rectus (Germany) connectors. All of the tubes in the system were made of polyurethane plastic. To improve sealing, silicon vacuum paste (silicon high vacuum grease medium, Merck, Germany) was used on all connections and contact surfaces.

Argon (99.98% pure) and nitrogen (99.999% pure) gas were used to generate the anoxic environments within the experimental jars and plastic enclosures, and synthetic air (20.5% O_2 and 79.5% N_2) was used to perform respiration measurements.

Equipment for O_2 and CO_2 Concentration Measurements Two devices were used for measuring the concentration of gases within the experimental jars. One of them was supplied by Art Care International, Inc. (Orangeburg, NY) for measurements of O_2 concentrations in the range from 0 to 2000 ppm (with a relative accuracy of 2%). It consisted of a membrane pump, an oxygen perfusion sensor, and a digital display. The other one was produced by ECHO d.o.o. (Slovenia), and consisted of a membrane pump (flow rate 0.5 l/min), an electrochemical oxygen sensor that measures in the range from 0 to 25% (absolute accuracy 0.01%), an IR carbon dioxide sensor (0–3000 ppm, accuracy 5 ppm) and a 16-bit A/D converter for computerized data acquisition. The ECHO system was a closed-circuit system and permitted measurements of changes in O_2 and CO_2 concentrations over time.

Design of the Experiment Fungi were inoculated in jars onto previously autoclaved PDA. Mycelia were allowed to grow in a normal atmosphere; i.e., jars were covered with punctured lids for one week (apertures were filled with sterile cotton to prevent contamination). In that time mycelia overgrew approximately two-thirds of

the nutrient medium diameter and formed vital and fast-growing mycelia. Steam-sterilized beech wood blocks were put onto *T. versicolor* cultures and spruce samples onto *A. vaillantii, C. puteana, G. trabeum,* and *S. lacrymans* cultures (on a plastic net over the cultures) for eight weeks. During that period the blocks were completely colonized. Infested wood blocks were carefully removed from underlying mycelia and put either in empty experimental jars or double-enveloped plastic enclosures, and were subjected to asphyxiation. The jars, sealed with ventilation lids, were flushed either with argon or nitrogen gas, until the concentration of oxygen in the exhaust air was below 10 ppm. The double-enveloped plastic enclosures used a heat-sealable, triple-layer EVA-nylon-EVA material. The inner bag had two valves inserted into it to allow gas exchange to the required level (less than 20 ppm). To reduce potential oxygen leakage into the inner bag, an outer bag was constructed around it and then flushed for ten minutes with argon. Oxygen scavenger was placed inside the outer bag, which was then heat-sealed.

All of the inoculations, re-inoculations, exchanges of the lids, and sealing of the inner bags were performed in a laminar flow hood, in sterile conditions. The temperature in growth chambers was maintained at 25°C throughout the experiment.

Observations on the jars were performed over a five-week period. Four experimental groups were formed. The first group was tested after one week of exposure to anoxic conditions; the second, third, and fourth after two, three, and five weeks, respectively. Each group contained six experimental jars. Three of them contained four wood blocks infested with *A. vaillantii, C. puteana,* or *T. versicolor* and then treated with argon gas. The other three were treated with nitrogen gas and contained three wood blocks infested with their respective fungal strain.

After the period of anoxic treatment, wood blocks were removed from experimental jars and placed onto sterilized PDA in jars covered with punctured lids (apertures were filled with sterile cotton to prevent contamination). Mycelial cultures infesting the wood blocks were incubated in normal O_2 conditions in a growth chamber. Visual monitoring for possible growth of hyphae was done every day, for at least four weeks.

Five double-enveloped bags were constructed, one for each tested species (*A. vaillantii, C. puteana, G. trabeum, T. versicolor,* and *S. lacrymans*). Five infested wood block samples of each species were placed in their respective bag, flushed with argon as described above, and incubated for four weeks in a growth chamber. After the four-week treatment, recovery of respiration was measured. To measure viability of the fungus, the bags were opened and the blocks were placed in experimental jars containing fresh, sterilized PDA. Ventilation lids were placed on each

jar. Jars were flushed with synthetic air and the concentrations of O_2 and CO_2 were measured for five minutes using the ECHO measuring apparatus. Then the valves on the jar lids were closed and the cultures incubated for one hour. After the incubation period, another five-minute measurement was performed. A CO_2-respiration value was calculated as the difference between the average values obtained during the first five-minute reading and the final one. After respiration measurements, samples were tested for regeneration as described above for samples treated in jars.

RESULTS

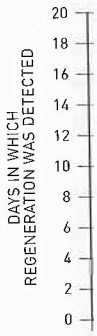
Mycelial Cultures, Anoxically Treated in Experimental Jars Figure 1 shows regeneration results of *A. vaillantii* cultures from wood blocks asphyxiated with either argon or nitrogen in experimental jars. [FIG. 1] It should be noted that the higher the bar in the graph the longer it took for the culture to recover. Bars that reach the top of the graph represent cultures that did not regenerate in the timeframe of the experiment. As can be seen in the figure, argon-treated samples started to lose viability earlier and to a greater extent than the ones treated with nitrogen. Argon treatment had an effect on the viability of the cultures as early as the second week, and by the fifth week 50% of the cultures failed to regenerate. Nitrogen treatment did not exhibit any effect after three weeks and by the fifth week only 33% of the cultures did not regenerate.

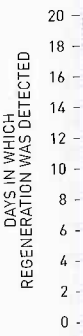
Figure 2 shows that after just one week of treatment with argon, *C. puteana* cultures were not able to regenerate, whereas treatment with nitrogen caused only a short-term loss of viability even after five weeks of treatment. [FIG. 2]

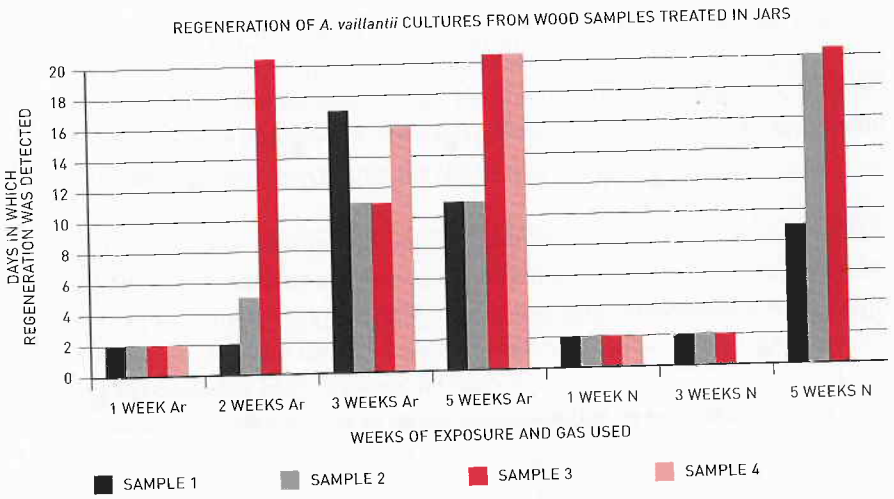
Treatment with either argon or nitrogen had no apparent effect upon the viability of *T. versicolor* cultures infesting wood blocks within the timeframe of the experiment. All of the cultures regenerated within two days after removal from treatment jars, the same response as exhibited by the controls.

Mycelial Cultures, Anoxically Treated in Double-Enveloped Plastic Enclosures Three of the brown-rot fungi, *A. vaillantii*, *C. puteana*, and *S. lacrymans*, were unable to regenerate after four weeks of asphyxiation in argon. The fourth brown-rot fungus, *G. trabeum*, commenced growth three days after being placed in ideal growth conditions. Cultures of the white-rot fungus, *T. versicolor*, were also unaffected by anoxic treatment and resumed growth as soon as control cultures did ([FIG. 3], lower section).

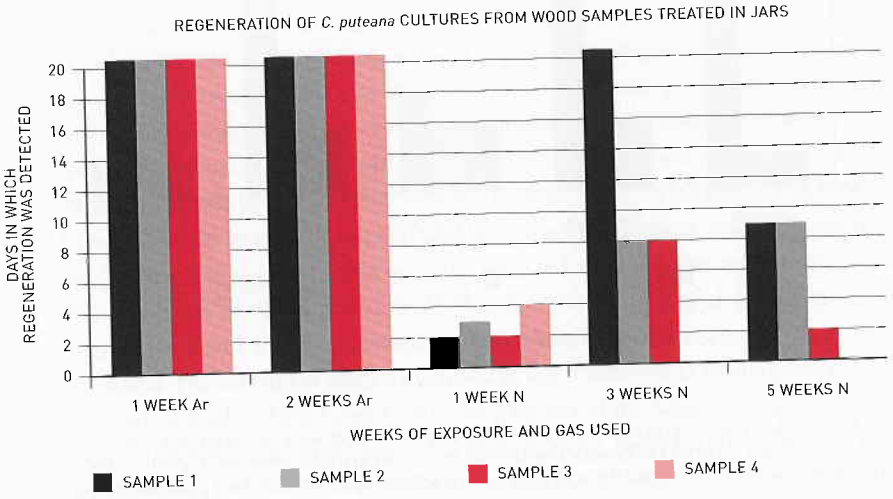
The upper part of figure 3 summarizes the data for one hour of CO_2 production, measured immediately after removal from anoxic conditions. The cultures of the three brown-rot species that did not regenerate did not have any detectable CO_2



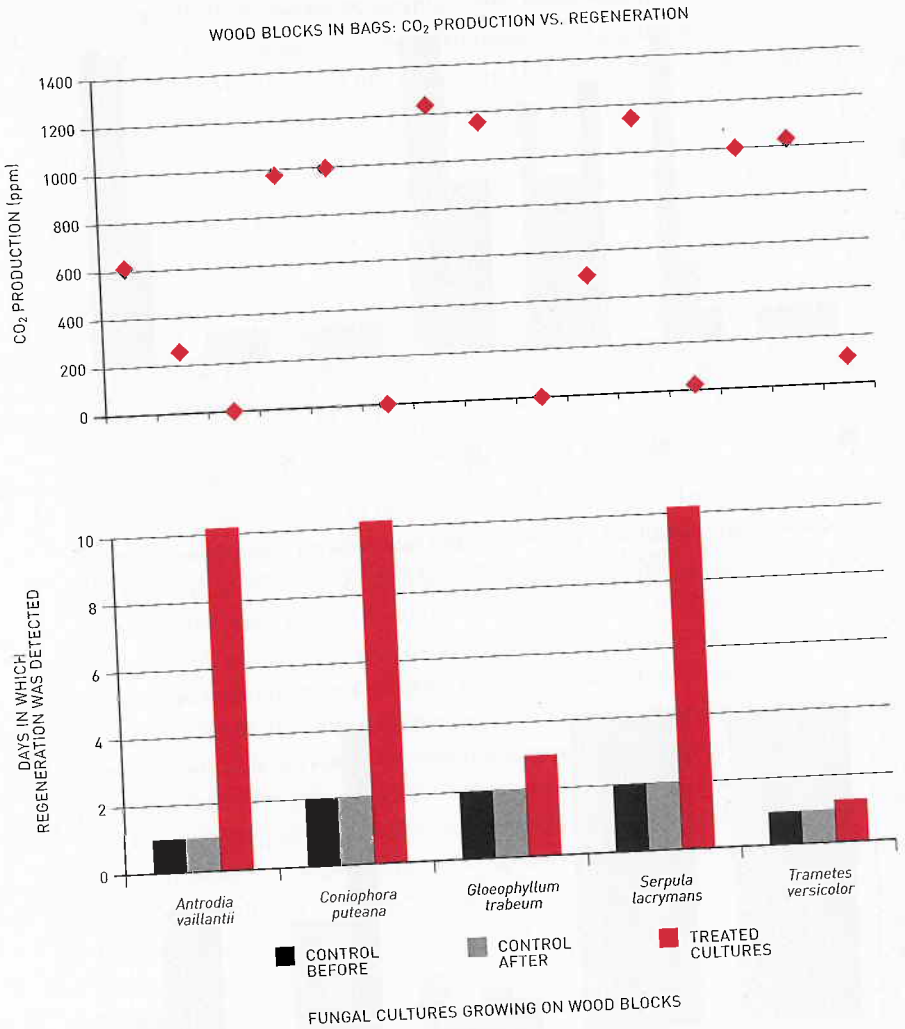




[1] Regeneration of *A. vaillantii* cultures, grown on Norway spruce sapwood blocks and treated with either argon or nitrogen gas for selected period. If the bar showing days in which regeneration was observed exceeds 20 days, it means that this culture did not regenerate.



[2] Regeneration of *C. puteana* cultures, grown on Norway spruce sapwood blocks and treated with either argon or nitrogen gas for selected period. If the bar showing days in which regeneration was observed exceeds 20 days, it means that this culture did not regenerate.



[3] Regeneration and CO_2 production of *A. vaillantii*, *C. puteana*, *G. trabeum*, *S. lacrymans*, and *T. versicolor* cultures, cultivated on wood blocks and asphyxiated for four weeks in double-enveloped enclosures. If the bar showing days in which regeneration was observed exceeds ten days, it means that this culture did not regenerate.

production, especially when compared to their respective controls. The white-rot fungus that recovered quickly gave CO_2 readings four to ten times greater than those of any of the brown-rot fungi. The fourth brown-rot fungus, *G. trabeum*, which took three days to show detectable growth, did not have any CO_2 production immediately after removal from the anoxic environment.

DISCUSSION The experimental results clearly demonstrate that at least some fungal infestations in wood can be eradicated by anoxic treatment within four weeks. Furthermore, there was a clear difference in treatment effectiveness between argon and nitrogen gas, with argon being able to eradicate *C. puteana* infestations in wood blocks completely while nitrogen did not.

Prior work by Tavzes et al. (2001) on suffocation of fungal cultures on PDA with either argon or nitrogen did not show a significant difference in effectiveness between the two gases. That work showed that even in 16 weeks of treatment at least some samples of the species tested were still viable. The reason was believed to be that the fungi could absorb readily accessible nutrients from the PDA medium. However, this is not the case in the wood blocks because there the fungi had to secrete oxygen-requiring enzymes in order to break down this heterogeneous and complex polymer and thus derive the needed nutrients. In the absence of oxygen, fungi may not produce enough energy to create and/or secrete these enzymes.

A. vaillantii and especially *C. puteana* cultures infesting wood blocks started to lose viability much earlier and the effect was much more pronounced if argon, rather than nitrogen gas, was used to create an anoxic environment in experimental jars. That difference was also observed for insect control in wooden objects, where argon gas proved to have higher efficiency (Koestler, 1993, 1996) because its higher specific weight enabled it to penetrate into wood cell lumens and push out the oxygen trapped within. Results on suffocation of fungal cultures on PDA showed that there was no significant difference in the loss of viability of treated mycelia regardless of which gas—argon or nitrogen—was used in a treatment (Tavzes et al., 2001). The amount of oxygen dissolved in water within agar media is obviously not high enough to help fungi to defy the adversity of the oxygen-depleted atmosphere. Therefore, one has to be careful when making direct analogies between experiments on nutrient growth media and treatment of fungi-infested wood.

Asphyxiation of fungi-infested wood blocks in plastic enclosures was the procedure most similar to practical conservation measures taken for insect control. Since argon has proven to have higher efficiency than nitrogen in lowering the viability of fungal cultures infesting wood blocks (Tavzes et al., 2002), it was decided

to use only this gas for the test with plastic enclosures. For this refined experiment, five fungal species, well-known destroyers of wooden art and historic objects, known to differ in their sensitivity to anoxia (Scheffer, 1986), were chosen.

Three of the brown-rot fungi, *A. vaillantii*, *C. puteana*, and *S. lacrymans*, died within a month of exposure to anoxia achieved with argon. [FIG. 3] In the experiments described by Scheffer (1986), these same or similar species responded in an analogous manner. In general, the period of time needed to kill the fungi was shorter in our tests than in Scheffer's study. This is probably because in our experiments O_2 was eliminated rapidly, putting the fungi under immediate stress rather than waiting as Scheffer did for them to consume the residual O_2 in the vessels. In the case in which the fungi did not die there was a measurable effect, either in a lengthening of time needed to recover (e.g., *A. vaillantii* in jars) or in lowered CO_2 respiration reading (*G. trabeum* in bags). Scheffer indicated that *T. versicolor* survived 6–12 months and *G. trabeum* 3–6 months. This is borne out in our study, as *T. versicolor* recovered in one day and *G. trabeum* in three. Another indication of *T. versicolor* being more resilient than *G. trabeum* is seen in CO_2 production measured immediately after exposure to normal air (119 vs. 1 ppm). The results clearly show a species-specific ability of fungi to survive low oxygen concentrations. Therefore to ensure effective use of the argon-anoxic procedure it is necessary to identify the species infesting the object. Rapid identification of species would be important to speed up the conservation effort on an art object. Molecular biology techniques of DNA identification (spores or hyphae) offer the promise of doing this (Adair et al., 2002; Di Bonaventura et al., 2002).

Dry wood is nearly completely protected against infestation (germination) by fungal spores, but if the object is already infested with fungal mycelia, drying will not always eradicate the infestation. If all cells in the fungal mycelium that are causing damage to a wooden object die, and the object is maintained properly afterwards and stored in dry conditions that prevent recurrence of an infestation, the treatment is completed and there is no more threat to its integrity and appearance (Pohleven, 2000). Anoxically treated cultures of *S. lacrymans* completely lost their viability after a month of exposure. Since *S. lacrymans* infestations of wood are the most obstinate and cannot be eradicated with drying, this bodes well for further development of anoxic treatment for control of fungal infestations of wooden art objects. Future studies will focus on testing more species and assessing the effectiveness of the argon-anoxic procedure for treating fungus-infested art and cultural heritage objects.

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ART, BIOLOGY, AND CONSERVATION: BIODETERIORATION OF WORKS OF ART

The Metropolitan Museum of Art, New York