Evaluating the xerophilic potential of moulds on selected egg tempera paints on glass and wooden supports using fluorescent microscopy

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Highlights

- 11 fungal isolates from cultural heritage institutions' interiors were screened for xerophilic trait
- Specially designed incubators were constructed to hold specific RH levels of 55 %, 63 % and 74 %
- Fungal growth was monitored at low relative humidity on wood and glass supports painted by traditional artists' paints
- Effects of pigments, support materials and strain variability are discussed

Abstract

Even though contamination of painted artwork by xerophilic moulds frequently causes aesthetical, physical and/or biochemical biodeterioration, mould growth on paints, prepared from assorted traditional artists' pigments, has yet to be systematically evaluated especially with regard to low relative humidity (RH) levels and painted support materials. Therefore, we investigated 11 fungal strains isolated mostly from cultural heritage institutions' interiors for their potential to grow on egg tempera paint films prepared with different colouring agents and applied on wooden and glass supports which were maintained in monoculture in specially designed incubators at three different RH levels of 55 %, 63 % and 74 %. The growth rate of mould over the surface was assessed using fluorescent microscopy after Calcofluor White staining. Additionally, these stains were screened for their xerophilic and hydrolytic potential using standard microbiological assays. Results show that when comparing growth rates on egg tempera paint films, 6 isolates grew exclusively on wood, exemplifying the greater susceptibility of this supporting material to mould attack. Prussian blue paint also stimulated the growth of 6 isolates, and the maximum overall expansion (38 %) was observed on Prussian blue painted wood. RH was the key factor limiting growth, and at RH of 55 % only a slight growth of 2 isolates was observed on Prussian blue painted wood. On the same samples incubated at RH of 63 %, 10 isolates exhibited a moderate to strong growth and 4 of these showed an additional increase in growth at 74 % RH. Paints consisting of artists' pigments carmine lake or lead white in general completely prevented the development of moulds. Nevertheless, tolerance was species/strain dependant and the growths of isolates *Cladosporium halotolerans* EXF-15333, *Aspergillus niger* EXF-14897 and *Aspergillus creber* EXF-15148 on lead white paint (containing ions and salts of heavy metal lead (Pb^{+2})) even exceeds 11 %. Standard microbiological tests showed that all stains had hydrolytic potential and proved positive for xerophilic trait,

nevertheless their ability to develop on egg tempera paint films was mostly dependant of very specific conditions.

Keywords: Cultural heritage, egg tempera paints, artists' pigments, fluorescent microscopy, xerophiles, fungal overgrowth

1. Introduction

In museums, fungi can grow and thrive on a wide range of art objects [1–3] and their dispersion is effected by the regular movement of employees and by the ventilation system. Moreover, temperature, RH, and/or light intensity, greatly impact the development of fungi [4–7]. Objects of art, such as oil paintings, works on parchment, painted wood etc., are composed of an array of different organic materials, such as binders, supporting cellulose or collagen based material, glues, pigments and varnishes which represent a rich media prone to fungal colonization [8]. Fungal species can cause aesthetical (stains), physical (hyphal penetration and cracking) and/or biochemical biodeterioration (enzymes and organic acids) [9,10]. Moreover, they are constantly present in the indoor air [11–13], and are considered a potential agent responsible for sick building syndrome, respiratory problems, allergies and certain opportunistic infections at higher spore counts [14–17].

Existing directives on art repository standards are not adequate, because the guidelines do not consider the relevance of micro-niches and the fact that most occurring fungi are microbial extremophiles [6,18]. The majority of fungi need a high RH to develop (water activity $a_w \approx 1$), however extremophiles are able to survive at low water activities and these are classified as xerophilic fungi [19,20]. In fact, some xerophilic species (mainly *Aspergillus* and *Penicillium* spp.) are considered to be primary colonizers since they are capable of growing at $a_w < 0.8$ (RH below 80 %), while some are secondary colonizers (a_w 0.8 - 0.9) [21,22]. Primary colonizers produce metabolic water and thereby increase water activity of the substrate, converting it to a more favourable medium which is suitable for the development of a stable biofilm [23].

Conditions on painted cultural heritage items stored in museums comprise of temperatures of around 25 ^{o}C and of RH lower than 60 % [24]. In art depos and archives however (usually located below ground level), stored items are subjected to a lower temperature range (around 22 ^{o}C) and to a greater fluctuation in RH (can rise above 60%), especially during the summer periods for which HVAC (Heating, ventilation, and air conditioning) system failures are more common and can last up to 2 weeks. Frequent failures include leaking refrigerant, clogged air filter, frozen coils and faulty motor capacitors [25]. Therefore, in such conditions (RH between 55 % and 70 %), the xerophilic potential of a fungal species is an important advantage, enabling its establishment on valuable art objects. Nevertheless, for fungal isolates emerging from cultural heritage institutions, this trait has never been addressed properly, and most studies have only focused on strains isolated from mural paintings [26–31], their molecular identification, defining their enzymatic activities for protein (test for gelatin hydrolysis) [32–39] and cellulose biodegradation [36,40–43], as well as traits which define their pathogenicity to human health (growth at 37^{o}C, phospholipase and hemolytic activities) [44,45]. The xerophilic potential has only been described by standard assays, which employ simple selective solid media with high concentrations of sodium chloride or glucose, which lower the a_w of a medium at an optimum temperature range for mould growth (28 ^{o}C) [46]. Additionally, to our knowledge, no study has systematically investigated the influence of different artists' paints and their individual components (e.g. various pigments) on mould growth, especially in relation to low RH levels and different painting supports.

2. Research aim

The aim of this study was to see if fungal species, with standardly proven xerophilic trait (standard microbiological assay on high osmolarity media with reduced water activity), can develop and grow on selected materials comprising painted cultural heritage items in conditions which prevail in museums or in art depos (temperature of around 23 ^{o}C and RH levels between 50 % and 70 %). With this aim, we devised special incubators with saturated salt solutions, which can hold painted model samples at specific RH levels. Different support (inert glass or hygroscopic wood) and paint (whole egg tempera paints employing 3 historically popular artists' pigments) materials were tested for their impact on mould growth. Finally, the ability of these fungal species to utilise nutrients contained within the whole egg tempera paint film was assessed using standard microbiological Petri plate assays (for proteolytic and lipolytic activities).

3. Material and methods

3.1 Fungal isolates

Strains isolated from the following artefacts and environments were used to inoculate the surfaces of prepared model samples: the Celje ceiling (Slovenia), air in the depot of the restoration centre, oil paintings on canvas, a 17th century parchment, a wooden African sculpture, Sečovlje salterns (Gulf of Trieste), fruits and nursing cream (see Table 1 for details). Isolates designated as ZIM were supplied from the Collection of Industrial-Microorganisms-Slovenia and isolates designated as EXF were supplied from the Infrastructural Centre Mycosmo-Culture-Collection, Slovenia. All were grown to sporulation on malt extract agar (MEA, 30 g/L of malt extract (Sigma Aldrich) and 15 g/L of agar (Sigma Aldrich)) solid medium at 26 °C.

Table 1: Eleven fungal strains isolated mostly from cultural heritage institutions' interiors.

Isolate designation	Identified species	Source	Isolation procedures	Identification method	Collected by/ Reference
EXF-10689	*Engyodontium album*	The Celje Ceiling in the main chamber of the Old Counts' Mansion, CRM	Dilution plates (MEA) after swabbing as described by Jurjević et al. [47], and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) amplification and sequencing described in Sklenář et al. [48]	P. Zalar[A], M. Matul[A] (May 2014)
EXF-15047	*Aureobasidium melanogenum*	Air in the depot of the RC of IPCHS	Bio-aerosol impaction sampler as described by Peterson & Jurjević [49], plating on MEA and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) amplification and sequencing described in Sklenář et al. [48]	P. Zalar[A], M. Matul[A] (May 2014)
ZIM-F94	*Penicillium crustosum*	Fruits from the DFST, BFL	Dilution plates (MEA) of homogenized matter and DNA isolation from colonies	18S-ITS1-5.8S-ITS2 rDNA (primers NS1 and ITS4) amplification and endonuclease restriction analysis described in Raspor et al. [50]	N. Čadež[B]
EXF-7651	*Aspergillus destruens*	Oil painting on canvas of the NGS	Dilution plates (MEA) after swabbing as described by Jurjević et al. [47], and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) and benA (primers Bt2a, T10 and Bt2b) amplification and sequencing described in Sklenář et al. [48]	[48]
EXF-10623	*Aspergillus halophilicus*	17th century nobel diploma written on parchment from the ARS	Dilution plates (MEA) after swabbing as described by Jurjević et al. [47], and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) and benA (primers Bt2a, T10 and Bt2b) amplification and sequencing	P. Zalar[A], M. Matul[A] (May 2014)

				described in Sklenář et al. [48]	
EXF-10201	*Wallemia* sp.	Oil painting on canvas from the RC of IPCHS	Dilution plates (MEA) after swabbing as described by Jurjević et al. [47], and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) amplification and sequencing described in Sklenář et al. [48]	P. Zalar[A], M. Matul[A] (May 2014)
EXF-150	*Aureobasidium pullulans*	Sečovlje salterns (Gulf of Trieste)	Dilution plates (MEA) and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) amplification and sequencing described in Sklenář et al. [48]	[51]
EXF-14897	*Aspergillus niger*	Sečovlje salterns (Gulf of Trieste)	Dilution plates (MEA) and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) and benA (primers Bt2a, T10 and Bt2b) amplification and sequencing described in Sklenář et al. [48]	P. Zalar[A], M. Matul[A] (May 2014)
EXF-15333	*Cladosporium halotolerans*	Oil painting on canvas (Vittore Carpaccio) from the RC of IPCHS	Dilution plates (MEA) after swabbing as described by Jurjević et al. [47], and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) and Act (primers ACT-512F and ACT-783R) amplification and sequencing described in Sklenář et al. [48]	P. Zalar[A], M. Matul[A] (May 2014)
EXF-15148	*Aspergillus creber*	Religious wooden sculpture from Mali, (first half of the 20th cent., SEM)	Dilution plates (MEA) after swabbing as described by Jurjević et al. [47], and DNA isolation from colonies	ITS (primers ITS1, NL4 and ITS4) and benA (primers Bt2a, T10 and Bt2b) amplification and sequencing described in Sklenář et al. [48]	P. Zalar[A], M. Matul[A] (May 2014)
ZIM-F42 = [52]	*Aspergillus niger*	Nursing cream from the DFST, BFL	Dilution plates (MEA) of homogenized matter and DNA isolation from colonies	18S-ITS1-5.8S-ITS2 rDNA (primers NS1 and ITS4) amplification and endonuclease restriction analysis described in Raspor et al. [50]	N. Čadež[B]

Abbreviations: National Gallery of Slovenia (NGS); Restoration Centre (RC) of the Institute for the protection of Cultural Heritage of Slovenia (IPCHS); Archives of the Republic of Slovenia (ARS); The Department of Food Science and Technology (DFST); Biotechnical Faculty of Ljubljana (BFL); Slovene Ethnographic Museum (SEM); Internal transcribed spacer (ITS); β-tubulin (benA); Actin (Act); Fungal ribosomal operon (18S-ITS1-5.8S-ITS2); Chloramphenicol and dichloran-glycerol agar medium (DG18); Collection of Industrial-Microorganisms-Slovenia (ZIM, https://www.zim-collection.si/); and Infrastructural Centre Mycosmo-Culture-Collection, Slovenia (EXF, https://www.ex-genebank.com/). [A]Microbiology, Department of Biology; Chair of Molecular Genetics and Microbiology, Večna pot 111, Ljubljana, Slovenia; [B]DFST, Chair of Biotechnology, Microbiology and Food Safety, Jamnikarjeva 101, Ljubljana, Slovenia.

3.2 Determination of the xerophilic and hydrolytic potential

The xerophilic trait was determined using two petri dish assays, one containing saturated salts (NaCl) and the other one containing saturated sugars. For the first test, 10 µL of spore inoculation suspension was carefully pipetted onto a sterile Potato Dextrose Agar plate (PDA; 200 g/L of potato infusion, 20 g/L of dextrose and 15 g/L of agar) supplemented with 12 % NaCl (120 g/L) [53]. For the second test, the same was performed, however, a sterile high sugar

osmolarity CY20S medium containing 1 g/L of K_2HP0_4, 3 g/L of $NaNO_3$, 0.5 g/L of $MgSO_4.7H_2O$, 0.5 g/L of KCl, 0.01 g/L of $FeSO_4.7H_2O$, 5 g/L of yeast extract, 15.0 g/L of agar and 200 g/L of sucrose was used instead [53]. Petri plates from both tests were then incubated for 14 days at 28 ^oC, and after incubation the fungal growth was assessed. For the gelatin hydrolysis test (standard test for proteolytic activity), agar plates were prepared by mixing autoclaved Reasoner's 2A agar (R2A; contains 0.05 % of proteose peptone, 0.05 % of casamino acids, 0.05 % of yeast extract, 0.05 % of dextrose, 0.05 % of soluble starch, 0.03 % of dipotassium phosphate, 0.005 % of magnesium sulfate, 0.03 % of sodium pyruvate and 1.5 % of agar, Sigma Aldrich) with 0.4 % of sterilized gelatin (Sigma Aldrich). After a 10 µL spore inoculation and a 14 days long incubation at 28 ^oC, the hydrolysis zone was visualized by using a 10% tannin solution, which was flooded onto the agar plates [54]. For lipolytic activity testing, Spirit Blue agar was prepared by suspending 32.15 g of Spirit Blue agar (Spirit Blue being the indicator of lipolysis) (Sigma-Aldrich) in 1000 mL distilled water, autoclaved, cooled down to 50 °C and supplemented with 30 ml of lipase substrate (1 mL of Tween 80, 400 mL of warm distilled water and 100 mL of olive oil; sterilized by autoclaving). This was slowly mixed and poured into Petri dishes [54]. After a 10 µL spore inoculation and a 14 days long incubation at 28 ^oC, the halos around the grown mycelia indicated lipolysis.

3.3 Construction and testing of incubators

In figure 1, an air-permeable (perforated) incubator is presented, which is basically a 370 ml household glass jar (cat. no. 3029550 Merkur, Slovenia) with 3 holes (2 mm in diameter) drilled in the middle of its metal cover. The holes were covered on top with a 4-layer laboratory paper towel which was attached onto the cover with an adhesive tape. This perforation was necessary for

the growth of fungi, as it allows the exchange of respiratory gases. The bottom of the jar was filled with 20 ml of the appropriate saturated salt solution for maintaining specific RH, and a plastic stand was inserted (Avacom cat. no. 561934 Merkur, Slovenia). A saturated solution of NaCl, KI or K_2CO_3 was used. For KI (Sigma Aldrich, 207969) 190.5 g of salt was added to 100 ml of MilliQ water; for NaCl (Sigma Aldrich, 7760) 50 g of salt was added to 100 ml of MilliQ water; and for K_2CO_3 (Sigma Aldrich, 209619) 155.5 g of salt was added to 100 ml of Milli-Q water. For each solution, RH within the enclosed (cover attached) incubator was measured by using a household RH meter (Techno Line, Spar Slovenia) which was placed on top of the plastic stand. The measurement was taken after a two day long incubation at 23 °C.

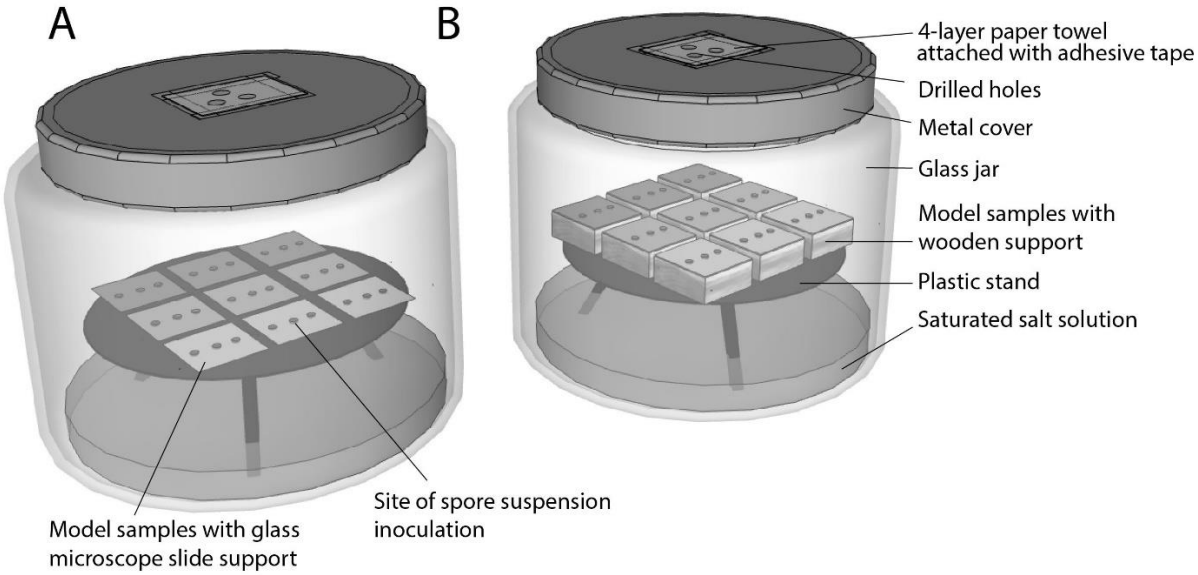

Figure 1: Incubator with paint samples on glass (A) and wood (B).

3.4 Preparation of model samples

Two series of model samples were prepared, the first represented paints applied on glass supports (non-hygroscopic material), the second the same paints on wooden supports (hygroscopic material). Glass supports were made from objective glass slides (Art No. 42401010; Glasswarenfabrik Karl Hecht GmbH & Co KG) that were cut down into sizes of 25 x 25 x 1 mm and cleaned with distilled water and 96% (v/v) ethanol (ACS Reagent) subsequently. Wooden supports were prepared from naturally aged untreated (no coatings present) spruce boards, which were cut to rectangular blocks of approximate dimension 25 x 25 x 13 mm. The blocks were then sanded with sand paper and cleaned with compressed air to remove any of the remaining dust particles.

For the preparation of paints, we chose plain whole egg as a tempera binder without any other possible additives (no water and no preservatives). Although traditional tempera painting has always been associated mainly with egg yolk, recipes, comprehensively recorded since the Middle Ages in the treatises such as by Cennino Cennini, Giorio Vasari and Giovanni Armenini among others, and hitherto put into practice in many variations, frequently involve the use of whole egg as the sole or the main binder of the tempera paints as well [55]. The reason to employ whole egg as the binder was also microbiological. Whole egg is a rich source of both proteins (egg white) and fat (egg yolk), and therefore, by choosing whole egg, we allowed for the development of both proteolytic and lipolytic fungal isolates on the painted surfaces. Binder preparation was as follows: 2 free-range chicken eggs were separated from the shell and then thoroughly mixed using a household mixer. After the setting of the liquid, the obtained binder was further mixed with each of the three selected pigments into three paints using a metal spatula

293 and grinded with a glass muller on a glass plate to an adequate paint consistency (all tools were
294 cleaned with tap water and detergent, and washed with distilled water). The binder to pigment
295 weight ratio in the paint was ≈ 1 : 0.8. The following pigments were used: for paint containing
296 lead white type of pigment (basic lead carbonate, $(PbCO_3)_2 \cdot Pb(OH)_2$; PW1, C.I. 77597)
297 Cremnitz White (Kremer Pigmente GmbH & Co.KG; Art.No. 46000.13010.120), for paint
298 containing pigment Prussian blue (iron (III) hexacyanoferrate (II), $Fe_4[Fe(CN)_6]_3$; PB27, C.I.
299 77510) Prussian blue LUX (Kremer Pigmente GmbH & Co.KG; Art.No. 45202.12100.136) and
300 for paint containing carmine lake type of pigment (red lake (aluminium lake) of carminic acid
301 (carmine lake) of cochineal origin; NR4, C.I. 75470) Carmine Naccarat (Kremer Pigmente
302 GmbH & Co.KG; Art.No. 42100.12100.136). Lead white was chosen as the principal European
303 colouring agent of white colour from antiquity until modern times. We selected Prussian blue as
304 a leading representative of traditional blue pigments in European painting since its discovery in
305 the beginning of the 18th Century, while carmine lakes (with the exception of vermillion) were
306 probably the most used red artists' pigments between the 16th Century and the discovery of
307 synthetic alizarin in the 19th Century. The colouring agents were also selected according to
308 colour, chemical composition and possibility to be more or less susceptible to mould growth
309 (dark/light colour, involving heavy metals, organic/inorganic constituents, iron ions etc.).
310 Prepared paints were applied with brush (Utrecht Manglon 2630-B No. 10) directly on the
311 frontal planes of glass or wooden supports in a single layer application. To better evaluate the
312 effects of pigments within paints, control samples with egg binder alone were prepared as well,
313 applied to glass and wooden supports without the presence of the pigments. All of the prepared
314 samples were left to dry at room temperature on covered plastic stands for a period of 2 weeks.
315 After drying, one series of 10 glass and one series of 10 wooden samples (3 repetitions of model

samples for each of the three paint varieties: lead white, Prussian blue and carmine lake; plus one control sample) were each placed within the prepared incubators with saturated salt solutions. The enclosed incubators were sterilised in autoclave (121 °C, 1.1 bar, 15 min).

3.5 Inoculation of model samples

Spores of fungal monocultures on MEA plates were collected and were stored at 4 ^{o}C in skimmed milk (1 mL) which was instantly dehydrated by mixing it with 10 mL of anhydrous granular silica gel. Prior to mixing, the latter was dry-heat sterilised at 175 °C for 2 h in 15 mL glass vials [56], turning it into yellow granules. To prepare for the inoculation, 0.5 mL of granules containing spores were suspended in 0.5 ml of Milli-Q water, spore count was determined under the microscope Zeiss LSM 800 using a standard Bürker-Türk counting chamber (hemocytometer) [57] and the final spore concentration was adjusted to around $1x10^7$ spores/mL by additional dilutions in Milli-Q water.

For the final inoculation, sterile incubator was opened in aseptic conditions and 10 µL of spore inoculation suspension (monoculture) was carefully pipetted onto each painted surface (three inoculation spots for each model sample; see Figure 1). The incubator was then sealed with the lid and placed in the dark at 23 ^{o}C. Each incubator contained model samples which were inoculated with only one fungal monoculture (isolate). The entire inoculation period of the samples spanned over 120 days.

3.6 Sampling from incubators

Examination of mould growth rate on model samples was performed three times for each incubator and through the entire incubation time (120 days). First sampling took place after 28 days, second

after 68 days, and third after 120 days of incubation. When sampling, the incubator was opened in aseptic conditions and 1 model sample was removed from the incubated series. Immediately after, the incubator was resealed and placed back in the dark at 23 ^{o}C. The removed sample was further analysed under the fluorescent microscope to determine the percentage of mould expansion over the painted surface.

3.7 Analysis of mould growth using fluorescent microscopy

The exact percentage of inoculated mould formation on the surface of the model samples was determined using the fluorescent microscope analysis, for which the sample was firstly stained using a fluorescent dye Calcofluor White, which specifically binds to the chitin cell wall of the mould (Figure 2). For this purpose, 10 μL of Calcofluor White dye, previously mixed in a 1:1 ratio with 10% KOH, was pipetted onto the surface of a model sample at the inoculation spots. Staining was analysed using the Zeiss LSM 800 confocal fluorescence microscope with a fluorescence filter with an emission maximum of 460 nm and a 50x magnification. The individual images for each slide were analysed with an open source image processing package of ImageJ called Fiji (version 1.52c) [58], to obtain the mould surface coverage using a triangle method and an ImageJ measuring function [59]. The images were converted to binary format with a black background and white biofilm, with an automatically determined threshold value that separates the biofilm from the background. Visual noise was removed from all the images in the set, using the “despeckle” function. Considering the entire field of view of the image, we then calculated the mould surface coverage percentage using the "measure" function, and from 3 images the average percentage of coverage was calculated.

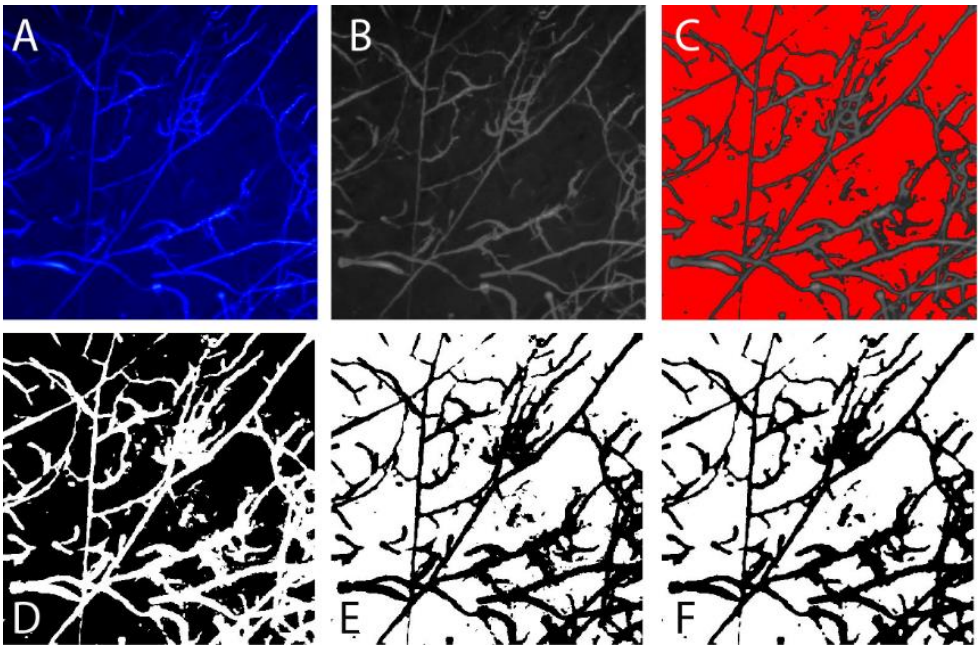

Figure 2: Fluorescent microscopic photographs used for determination of percentage of mould surface coverage (on the photographs, *A. destruens* after 68 days of incubation on wood painted with Prussian blue at an RH level of 63 % is presented). A: original photograph; B: black and white 8 bit photograph; C: boundary focus determination between the mould and the background; D: binary format with black background and white mould; E: inverted image; and F: noise removal.

4. **Results**

Initially, within each incubator containing a saturated solution of either NaCl, KI or K_2CO_3, RH was measured after a two day long incubation at 23 ^oC. The established humidity levels within the enclosed incubators (measured using the Rh meter) were 55 %, 63 % and 74 % for K_2CO_3, KI and NaCl, respectively.

Standard screening of the 11 fungal isolates for xerophilic and hydrolytic potential revealed that all isolates could tolerate NaCl or sucrose induced osmolarity and demonstrated at least one biodegradative property (Table 1). Most of the isolates, with the exception of *A. halophilicus* EXF-10623, could easily grow in 12 % NaCl. However, when the medium was supplemented with 20 % of sucrose, the growth of 6 isolates was severely delayed. Isolates with the most active proteolytic activity were *E. album* EXF-10689, *C. halotolerans* EXF-15333, *P. crustosum* ZIM-F94 and *A. niger* ZIM-F42; and isolates exhibiting the highest lipolytic activity were *Wallemia* sp. EXF-10201, *Aureobasidium melanogenum* EXF-15047, *Aspergillus niger* ZIM-F42 and *E. album* EXF-10689. Interestingly, no proteolytic reaction was observed for *Wallemia* sp. EXF-10201.

Table 2: Xerophilic and hydrolytic potential of examined fungal isolates.

Isolates	NaCl induced osmolarity	Sucrose induced osmolarity	Proteolytic activity	Lipolytic activity
Engyodontium album EXF-10689	+++	+	++	++
Aureobasidium melanogenum EXF-15047	+++	++	+	++
Penicillium crustosum ZIM-F94	+++	+++	++	+
Aspergillus destruens EXF-7651	+++	++	+	+
Aspergillus halophilicus EXF-10623	+	+++	+	+
Wallemia sp. EXF-10201	+++	+	-	+++
Aureobasidium pullulans EXF-150	+++	+	+	+
Aspergillus niger EXF-14897	+++	+	+	+
Cladosporium halotolerans EXF-15333	+++	+	++	+
Aspergillus creber EXF-15148	+++	+	+	+
Aspergillus niger ZIM-F42	+++	++	++	++

Legend: +++: quick and extensive positive reaction for hydrolytic activities (for osmolarity: extensive growth and sporulation); ++: strong positive reaction (for osmolarity: obvious growth); +: weak positive reaction (for osmolarity: observable micelium); -: no reaction (for osmolarity: no growth).

The effect of a specific RH, established within the incubator, on inoculated mould growth kinetics is presented in figure 3. Mould growth is represented in the form of mould expansion percentage, covering the surface of painted model samples and calculated from images obtained under fluorescent microscopy.

For wood painted with Prussian blue, at RHs of 63 % and 74 %, a steady growth of *E. album* EXF-10689 (Figure 3, Graph A), *Aureobasidium melanogenum* EXF-15047 (Figure 3, Graph B) and

Aspergillus halophilicus EXF-10623 (Figure 3, Graph E) reaching between 5-10 % was observed (after 120 days of incubation). For the same model samples and RH conditions, the growth of *P. crustosum* ZIM-F94 (Figure 3, Graph C) and *A. destruens* EXF-7651 (Figure 3, Graph D) after 68 days of incubation reached around 20 % of surface coverage and the growth of *Wallemia* sp. EXF-10201 exceeded 35 % (Figure 3, Graph F). On all other model samples (lead white and carmine lake on wood and glass supports), regardless of the employed humidity, strains *E. album* EXF-10689, *A. melanogenum* EXF-15047 and *P. crustosum* ZIM-F94 exhibited no surface growth. At RHs of 63 % and 74 %, *Aspergillus destruens* EXF-7651 (Figure 4) and *Aspergillus halophilicus* EXF-10623 steadily grew to 5-10 % surface coverage on wood painted with carmine lake.

Regardless of the supporting material used, at RH of 74 %, *A. pullulans* EXF-150 grew well (around 20 % expansion) on Prussian blue paint, and also grew slightly (around 5 % growth) on lead white paint (Figure 3, Graph G). However, at RH of 63 % growth was only detected on wood covered by Prussian blue tempera. Similar results were obtained for the formation of *A. niger* EXF-14897 (Figure 3, Graph H). The main differences in comparison to the growth of *A. pullulans* EXF-150 were that *A. niger* EXF-14897 reached an overall lower surface coverage (11 %) on wood-Prussian blue samples at RH of 74 %, and that its growth was non-existent on glass-Prussian blue samples at the same RH.

Interestingly, even at RH of 63 %, *C. halotolerans* EXF-15333 exhibited a moderate (10 % coverage on glass) to strong (20 % coverage on wood) surface growth on lead white paint (Figure 3, Graph I). As with most isolates, *C. halotolerans* EXF-15333 only grew if Prussian blue was applied to wood (RHs of 63 % and 74 %).

Aspergillus creber EXF-15148 grew strongly on every single model sample exposed to RHs of 63 % or 74 %. Furthermore, on all model samples containing wood (at 74 % RH) its growth exceeded

33 % of surface coverage (Figure 3, Graph J). In contrast to *A. creber* EXF-15148, *A. niger* ZIM-F42 exhibited no surface growth and even at RH of 74 %, the conditions were still too dry for its development (Figure 3, Graph K).

When RH was as low as 55 %, only isolates *Wallemia* sp. EXF-10201 and *A. creber* EXF-15148 exhibited a slight development of around 1 % surface coverage (exclusively on wood-Prussian blue samples).

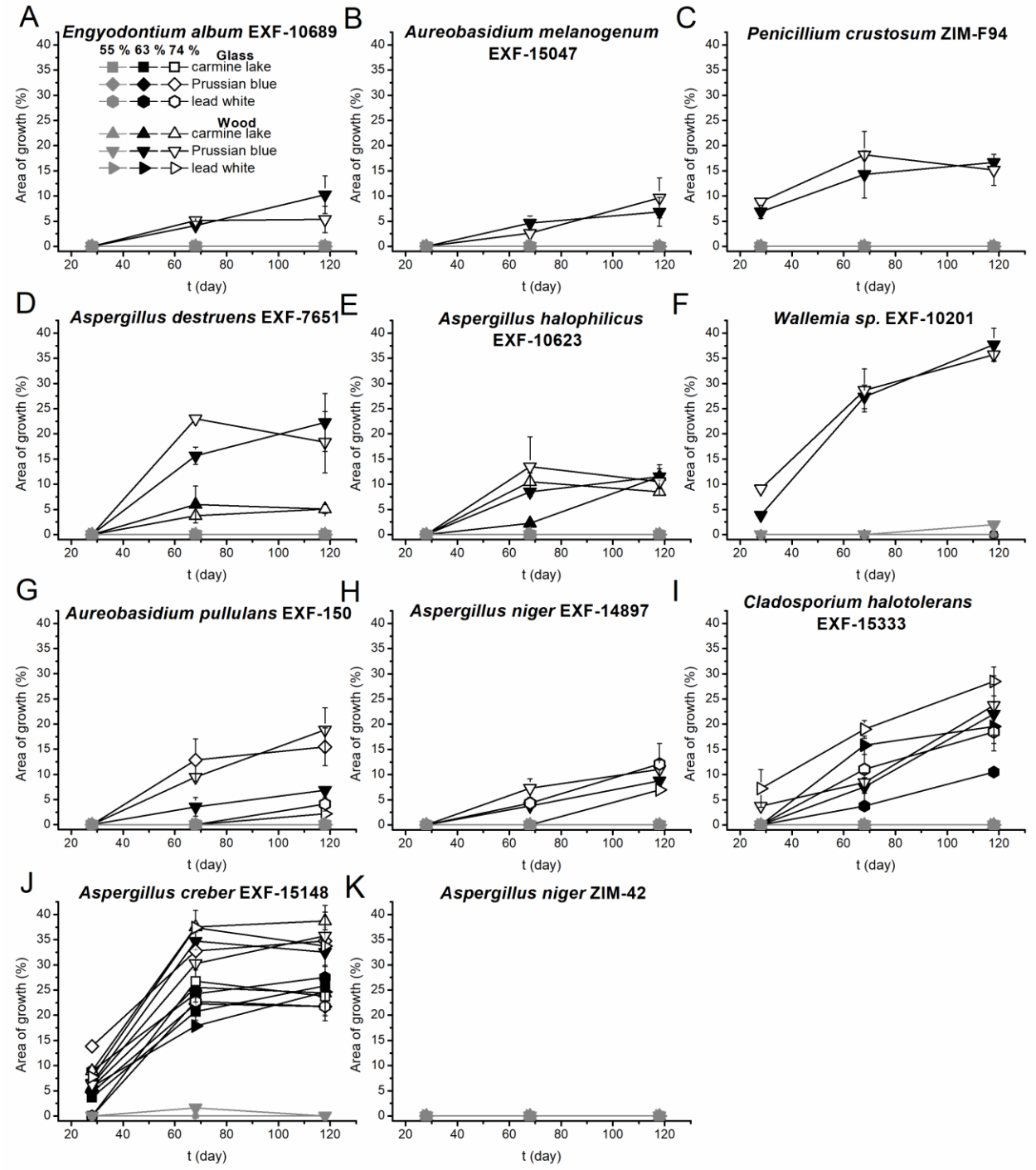

Figure 3: Percentage of growth of 11 fungal isolates on wooden and glass model samples painted with egg temperas containing three different pigments (lead white, Prussian blue, carmine lake). Incubation continued for 120 days at a specific RH (55 %, 63 % or 74 %) and percentage of fungal expansion over the surface was determined under the fluorescent microscope.

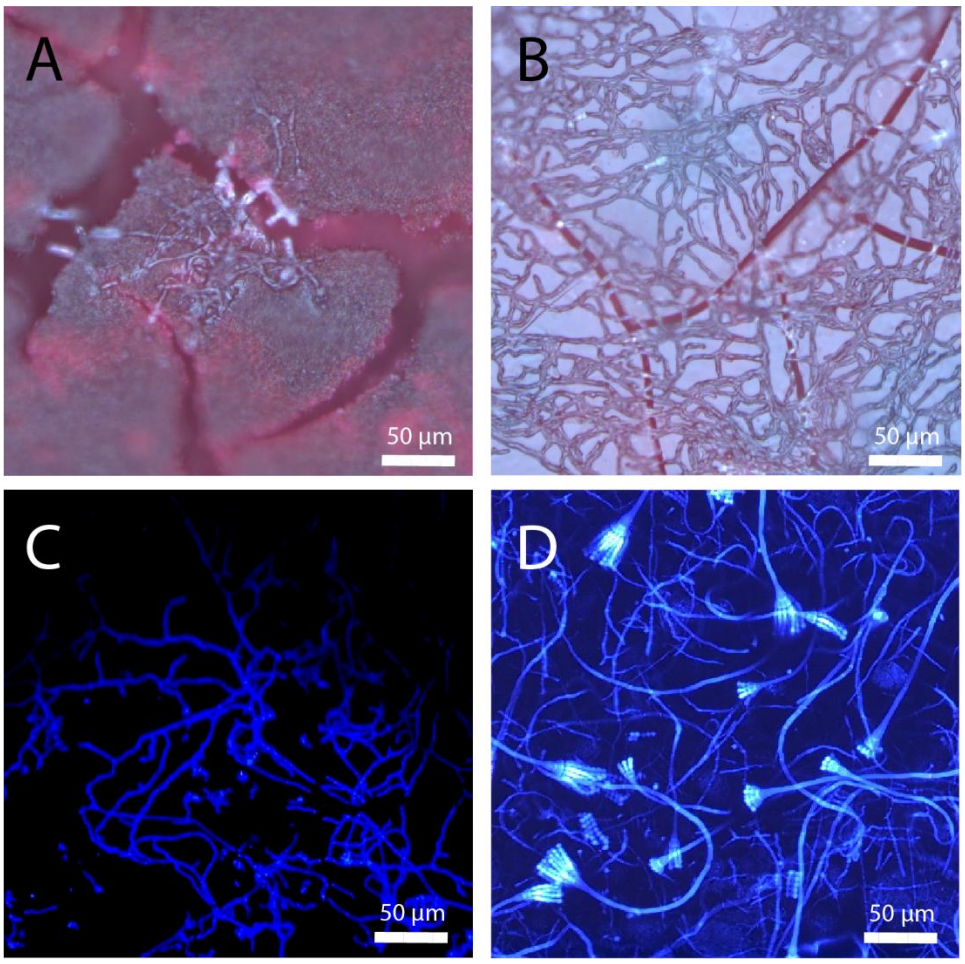

Figure 4: Fungal growth on wood based model samples under optical and fluorescent microscopy. Initial (A; 28 days on incubation) and developed (B; 68 days on incubation) growth of *A. destruens* EXF-7651 on wood with carmine lake egg tempera at RH of 74 % and analysed under optical microscopy. Overgrowth of EXF-7651 (C) and *P. crustosum* ZIM-F94 (D) (68 days on incubation at RH of 74 %; carmine lake egg tempera on wood) under fluorescence microscopy after Calcofluor White staining.

To evaluate how pigments within paint films effect mould growth, 11 fungal isolates were also inoculated onto glass and wood control samples containing only a layer of the egg binder (egg white and egg yolk mixture). These control samples were then incubated at an RH of 74 % for 68

days (at 23 °C) and the percentage of fungal surface overgrowth was measured using the fluorescent microscope (Figure 5). RH of 74 % was chosen for incubation because we initially thought that at lower RH levels (55 % and 63 %) surface mould growth would be minimal or non-existent, and therefore the inhibiting effects of pigments on mould growth would be difficult to measure.

On glass based control samples, isolates *Aureobasidium pullulans* EXF-150 (1.3 %), *Aspergillus niger* EXF-14897 (3.6 %), and *C. halotolerans* EXF-15333 (4.8 %) exhibited a slight surface growth and isolate EXF-15148 showed a moderate rate of growth (21.5 %) (Figure 5, Graph A). However, when wood control samples were used for incubation, all isolates, with the exception of *A. niger* ZIM-F42, were able to exhibit low (*E. album* EXF-10689, *Aureobasidium melanogenum* EXF-15047, *Aspergillus destruens* EXF-7651, *Aureobasidium pullulans* EXF-150 and *Aspergillus niger* EXF-14897) to moderate (*P. crustosum* ZIM-F94, *Aspergillus halophilicus* EXF-10623, *Wallemia* sp. EXF-10201, *C. halotolerans* EXF-15333 and *Aspergillus creber* EXF-15148) surface growth (Figure 5, Graph B).

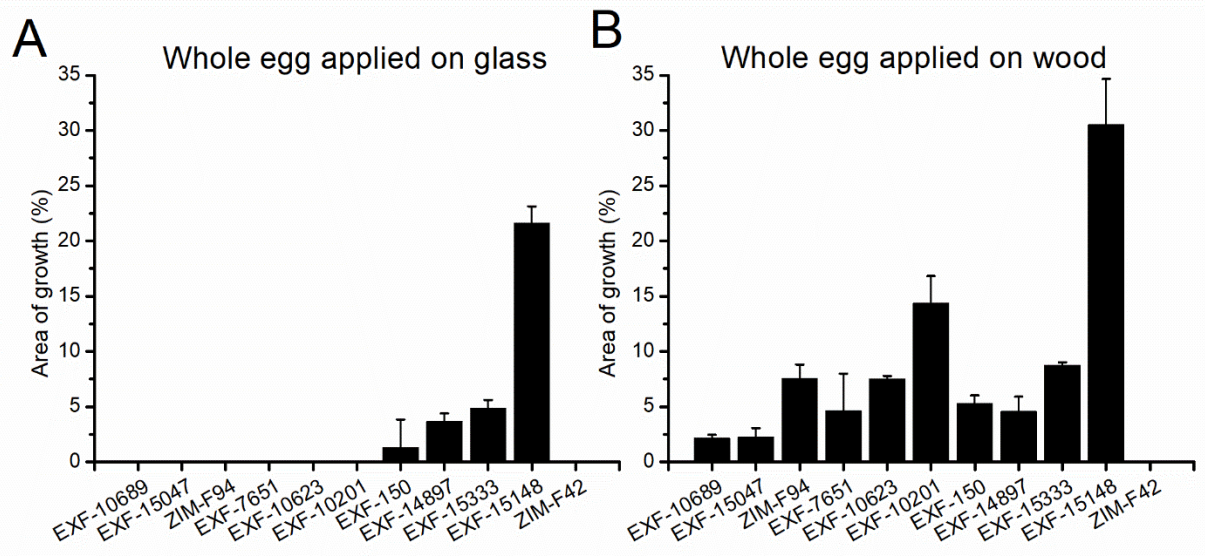

Figure 5: Percentage of growth of 11 fungal isolates on glass (A) and wooden (B) control samples containing only a layer of the whole egg binder. Incubation at RH of 74 % was terminated after 68 days and the percentage of fungal formation on the surface was determined under the fluorescent microscope.

5. Discussion

Standard screening for xerophilic potential revealed that all of the isolates could easily tolerate and show extensive growth in high osmolarity medium induced by NaCl. For species such as *Aureobasidium pullulans* [60], *A. melanogenum* [61], *E. album* [62], *Aspergillus* spp., *Cladosporium* spp. [63], *P. crustosum* [64], *Wallemia* spp. [65], *Aspergillus niger* [66], and *A. creber* [67] this is a commonly observed trait. When sucrose was used to induce osmolarity, the growth of 6 isolates was delayed and only the growth of *P. crustosum* ZIM-F94 and *A. halophilicus* EXF-10623 was non-restricted. In contradiction to the observations of Smolyanyuk and Bilanenko [68], that *A. halophilicus* is a salt tolerant species, our tests show a clear preference of *A. halophilicus* EXF-10623 to sucrose based osmolarity.

Standard screening for hydrolytic potential revealed that all isolates (with the exception of *Wallemia* sp. EXF-10201 (only lipolytic)) exhibited both proteolytic and lipolytic acitvities. The most proteolytic isolates proved to be *E. album* EXF-10689, *C. halotolerans* EXF-15333, *P. crustosum* ZIM-F94 and *A. niger* ZIM-F42. The proteolytic activities for these species were also confirmed by Chellappan et al. [69] (for *E. album*), by Borrego et al. [45] (for *A. niger*), by Jaouani et al. [63] (for *C. halotolerans*) and by Park et al. [70] (for *P. crustosum*). Furthermore, we observed a strong lipolytic activity for *Wallemia* spp. EXF-10201, *Aureobasidium melanogenum* EXF-15047, *E. album* EXF-10689 and *Aspergillus niger* ZIM-F42, and similar observations were made by Chamekh et al. [71] (for *Wallemia* spp.), Vitisant et al. [72] (for *Aureobasidium melanogenum*), Alapont et al. [73] (for *E. album*) and by Mahadik et al. [74] (for *Aspergillus niger*).

The effect of a specific RH, established within the incubator, on mould growth kinetics on painted model samples, revealed that at RH of 55 % almost no growth was observed and only a slight development was observed for isolates *Wallemia* sp. EXF-10201 and *A. creber* EXF-15148. Nevertheless, it is surprising that for 6 (*E. album* EXF-10689, *Aureobasidium melanogenum* EXF-15047, *P. crustosum* ZIM-F94, *Aspergillus destruens* EXF-7651, *A. halophilicus* EXF-10623 and *Wallemia* sp. EXF-10201) out of 11 isolates fungal formation percentage was similar between RH levels of 63 % and 74 %. This is in contridiction to Strang [75], who made an extensive literature review on mould development in relation to RH, and noticed a clear linear progression in mould growth from 55 % to 100 % RH. Nonetheless, for isolates *Aureobasidium pullulans* EXF-150, *Aspergillus niger* EXF-14897, *A. creber* EXF-15148 and *C. halotolerans* EXF-15333 growth in general was promoted when RH was increased from 63 % to 74 %.

Regardless of the RH levels tested (even at 55 % RH), our results show that the bioreceptivity (the ability of a material to be colonised by living organisms [76]), of glass or wood support materials within laboratory samples is vastly different. In fact, when comparing glass and wood based control samples, glass did not prove to be bioreceptable for 6 isolates (*E. album* EXF-10689, *Aureobasidium melanogenum* EXF-15047, *P. crustosum* ZIM-F94, *Aspergillus destruens* EXF-7651, *A. halophilicus* EXF-10623 and *Wallemia* sp. EXF-10201) which otherwise exhibited significant growth (colonisation) on wood supports. Higher bioreceptivity of wood in comparison to glass was also observed on model samples containing pigments (Prussian blue: 3.4 % higher growth of *Aureobasidium pullulans* EXF-150; lead white: 10.0 % higher growth of *C. halotolerans* EXF-15333 and *Aspergillus creber* EXF-15148; carmine lake: 10.0 % higher growth of *A. creber* EXF-15148).

Wood is a hygroscopic material which changes mass and volume depending on the surrounding RH, meaning it is a material that absorbs water [77]. Water gets into wood in three ways: as a fluid through the cell lumens through capillary tension, as vapour through the cell lumens, and as molecular diffusion through the cell walls. This makes wood a highly bioreceptable material on which mould mediated biodegradation of dried substrates (organic binders) can occur even at low RH levels [78].

Pigments strongly inhibited or increased the growth of moulds. This was especially evident when incubations on wood based control samples (without pigment) were compared with incubations on wood based model samples (with pigment). For instance, at RH of 74 %, Prussian blue increased the growth of isolates *P. crustosum* ZIM-F94, *Aspergillus destruens* EXF-7651, *A. halophilicus* EXF-10623, *Wallemia* sp. EXF-10201 and *Aureobasidium pullulans* EXF-150 by 10.6 %, 18.3 %, 6 %, 14.2 % and by 4.2 %, respectively. In contrast to Prussian blue, however, pigments carmine lake and lead white generally completely prevented the growth of moulds. The exceptions were isolates *A. halophilicus* EXF-10623 and *A. creber* EXF-15148, of which formation was boosted by carmine lake (*A. halophilicus* EXF-10623 by 3 % and *A. creber* EXF-15148 by 7 %; RH of 74 %), and isolates *C. halotolerans* EXF-15333 and *A. creber* EXF-15148, which were boosted by the pigment lead white (*C. halotolerans* EXF-15333 by 10.2 % and *A. creber* EXF-15148 by 6.8 %; RH of 74 %).

Studies focusing on the identifiation of protein binders within painted model samples using the enzyme-linked immunosorbent assay (ELISA) have shown that paints consisting of cochineal lakes (carmine lakes), lead white, natural chalk, bone black, raw sienna or Verdigris contain binding proteins (ovalbumin or casein) which are almost fully degraded [79,80], sometimes to the

extent that even antibodies, which are specific for these binding proteins, could not result in a positive ELISA reaction. This high level of protein degradation was not detected for other kinds of pigments. Therefore, in the case of lead white, important fungal proteins could undergo enhanced degradation (preventing fungal development) due to the heavy metals within this paint. As a matter of fact, interaction of lead (Pb^{+2}) with proteins (protein misfolding disorders and aggregation of nascent proteins) represents a fundamental mechanism by which Pb^{+2} exerts toxicity [81,82].

Carminic acid within the pigment carmine lake can even enhance fungal biomass growth and even though its nutrimental role is unclear it may be involved in fungal melanin synthesis as it directly elevates the activity of the enzyme laccase (catalyses the formation of melanin by oxidizing L-DOPA) [83–85]. Moreover, Fernandes et al. [86] have shown that the inhibition of the melanin synthesis in the fungus *Alternaria infectoria* results in the accumulation of the pigment carmine red rather than in an albino phenotype. Therefore, it is most probable that aluminium and calcium salts, which are added to complex the carminic acid into a natural carmine pigment [87,88] are responsible for protein degradation. Aluminium reacts via the hexa-aqua trivalent species $Al^{3+}_{(aq)}$, [89] and ligates to oxygen-based functional groups, principally through aluminium substitution for competitive cations such as Ca^{2+} and Mg^{2+}, disrupting the functionality of various proteins [90–92]. For example, even at low concentration (10 μM), $Al^{3+}_{(aq)}$ stimulates the level of ubiquitin within murine neuroblastoma (NBP2) cell cultures [93]. The ubiquitin proteasome pathway is highly conserved within eukaryotes (from fungi to mammals) and its function is to tag proteins for their final destruction mediating the levels of cell cycle regulators and transcription factors involved in morphogenesis [94]. The link between aluminium and the ubiquitin-mediated protein degradation has also been observed in plants [95,96] and although similar responses in fungi have

not yet been investigated, various fungal species show intolerance of aluminium at concentrations of as low as 500 ppm [97,98].

On the other hand, Prussian blue, an inorganic microcrystalline iron(III) hexacyanoferrate(II), may represent a rich and easy source of iron which is essential for many biochemical processes within a fungal cell, such as the synthesis of deoxyribonucleotides, respiration, the tricarboxylic acid cycle, and the synthesis of numerous small molecules such as amino acids, lipids and sterols [99]. Recently, Planý et al. [100] observed fungal-induced atmospheric iron corrosion within the Natural History Museum in London. The mechanism they describe includes the establishment of a fungal based biofilm, the production of a corrosive electrolyte within the biofilm, transport of ions and biomineralisation of iron-rich minerals.

Our observations confirm that tolerance to paint is species or strain dependent. For example, isolates EXF-14897 and ZIM-F42, both identified as *A. niger*, had remarkably different growth responses. Isolate EXF-14897 grew on Prussian blue as well as on lead white paint, however, the formation of ZIM-F42 on any of the paints was non-existent. In accordance, for strain dependent tolerance to lead, Sayer et al. [101] observed that some strains of *A. niger* could produce lead oxalate dihydrate via the pyromorphite transformation process, which is the first recorded biogenic solubilised formation of lead.

Overall, our results show that even at RH level as low as 55 %, which some experts regard as the border line for fungal growth in museum environments [18], certain xerophilic fungal species can still contaminate a painted surface, especially if it is wood supported and if the pigment present is

609 iron based. Considering the whole, however, the comparison between standard microbiological
610 tests (for xerophilic trait) and our incubator based tests indicates that fungi with a standardly
611 proven xerophilic trait may still fail to develop on painted model samples subjected to a low RH.
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6. Conclusions

In this study we have investigated the xerophilic trait of 11 fungal isolates which were mostly obtained from cultural heritage institutions. These were inoculated onto painted model samples and incubated in monoculture in specially designed incubators with RH levels of 55 %, 63 % or 74 %. The following conclusions can be drawn:

1. Six isolates (*E. album* EXF-10689, *Aureobasidium melanogenum* EXF-15047, *P. crustosum* ZIM-F94, *Aspergillus destruens* EXF-7651, *A. halophilicus* EXF-10623 and *Wallemia* sp. EXF-10201) grew exclusively on wood based control samples.
2. At RH of 55 %, 2 isolates exhibited a slight growth; at RH of 63 %, the growth of 10 isolates was moderate to strong; 4 of these showed an additional increase in growth at RH of 74 % (all examined on Prussian blue painted wood).
3. In comparison to the control samples (only binder), Prussian blue pigment within the painted model samples increased the growth of isolates *P. crustosum* ZIM-F94, *Aspergillus destruens* EXF-7651, *A. halophilicus* EXF-10623, *Wallemia* sp. EXF-10201 and *Aureobasidium pullulans* EXF-150. In contrast, pigments carmine lake and lead white generally completely prevented fungal growth with some isolates being tolerant exceptions.
4. Fungi with a standardly proven xerophilic trait may still fail to develop on painted model samples subjected to a low RH at 23 ^{o}C for a period of 120 days.

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Figure captions

Figure 1: Incubator with paint samples on glass (A) and wood (B).

Figure 2: Fluorescent microscopic photographs used for determination of percentage of mould surface coverage (on the photographs, *A. destruens* after 68 days of incubation on wood painted with Prussian blue at an RH level of 63 % is presented). A: original photograph; B: black and white 8 bit photograph; C: boundary focus determination between the mould and the background; D: binary format with black background and white mould; E: inverted image; and F: noise removal.

Figure 3: Percentage of growth of 11 fungal isolates on wooden and glass model samples painted with egg temperas containing three different pigments (lead white, Prussian blue, carmine lake). Incubation continued for 120 days at a specific RH (55 %, 63 % or 74 %) and percentage of fungal expansion over the surface was determined under the fluorescent microscope.

Figure 4: Fungal growth on wood based model samples under optical and fluorescent microscopy. Initial (A; 28 days on incubation) and developed (B; 68 days on incubation) growth of *A. destruens* EXF-7651 on wood with carmine lake egg tempera at RH of 74 % and analysed under optical microscopy. Overgrowth of EXF-7651 (C) and *P. crustosum* ZIM-F94 (D) (68 days on incubation at RH of 74 %; carmine lake egg tempera on wood) under fluorescence microscopy after Calcofluor White staining.

Figure 5: Percentage of growth of 11 fungal isolates on glass (A) and wooden (B) control samples containing only a layer of the whole egg binder. Incubation at RH of 74 % was terminated after 68

700 days and the percentage of fungal formation on the surface was determined under the fluorescent
701 microscope.

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703 **Table captions**

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705 Table 1: Eleven fungal strains isolated mostly from cultural heritage institutions' interiors.

706 Table 2: Xerophilic and hydrolytic potential of examined fungal isolates.
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