COMBINED EFFECTS OF GAMMA-IRRADIATION, DOSE RATE AND MYCOBIOTA ACTIVITY ON CULTURAL HERITAGE – STUDY ON MODEL PAPER

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Abstract

Cellulose, the main constituent of paper-made objects of cultural heritage, is a favorable substrate for fungal growth. Gamma irradiation is a well-established low-cost treatment convenient for decontamination of such objects. Since side-effects to paper-based CH are always a concern the aim of this work was to investigate if a synergism of microbiological contamination and gamma irradiation effects exists and if it induces changes in paper's appearance and structure. The dose rate plays an important yet generally ignored role in the efficacy of the radiation treatment so another goal was to assert its influence on decontamination efficiency and paper properties.

Irradiation conditions for treating the highly resistant secondary colonizer *Cladosporium sphaerospermum*, as well as the naturally occurring mycobiota were evaluated. Untreated and inoculated samples of paper were irradiated with doses commonly applied to CH objects, as well as to significantly higher doses, at two dose rates that differ for two orders of magnitude. Microbiological analysis of irradiated samples was conducted. Colorimetric analysis, UV-vis and FTIR measurements were performed after short lived reaction species have decayed.

The results have shown that in the case of high contaminations (10^4 CFU/g) the applied dose needs to be adjusted and that the corresponding dose rate needs to be high enough. While at the dose rate of 31 kGy/h the irradiation dose of 7 kGy seems to be effective to obtain proper reduction of mycobita, at the dose rate that was two orders of magnitude lower the required dose increased approximately ten times. Thus the reevaluation of the recommended dose of 8 ± 2 kGy is needed. Considering the side effects of radiation treatment dose rate effect has also been observed. At the higher of the investigated dose rates the irradiation doses needed for decontamination did not alter the appearance of paper, while at the lower one the changes were *hardly perceptible*. The main species showing their contribution to color change were the carbonate anion-radicals that were formed on the $CaCO_3$ paper filler. No oxidation or change in crystallinity of cellulose was detected. Overall changes were too insignificant to make any conclusion on the contribution of mycobiota to the irradiation side-effects on the paper under the studied conditions.

Keywords: *Cladosporium sphaerospermum*; radiation treatment; D_{10} value; CIE Lab colorimetry; FTIR; carbonate anion-radical

1. Introduction

A great part of cultural heritage (CH) is made of paper. Books and documents are a valuable and priceless source of historic information. Paper constitutes of cellulose which is a favorable substrate for fungal growth thus such CH objects are highly susceptible to fungal contamination that may cause detrimental paper decay. CH objects are frequently stored in inappropriate conditions where fungal contamination can be transferred from their surroundings. Afterwards these objects can serve as a source of fungal contamination to other stored objects.

Fungal indoor colonizers are divided into three categories based on water activity (a_w), i.e. amount of free water in the substrate (WHO, 2009). Primary colonizers (a_w< 0.80; relative humidity-RH <80%) are many species of *Aspergillus* and *Penicillium*, *Paecilomyces* spp., and *Wallemiasebi*. Secondary colonizers (a_w 0.80 – 0.90; RH 80–90%) include species of *Cladosporium, Mucor* and *Rhizopus*. Tertiary colonizers, which prefer high water content (a_w> 0.90; RH >90%) include species of *Stachybotrys*, *Trichoderma*, *Exophiala*, *Phoma*, *Ulocladium* and *Fusarium*. Fungal species produce degradative enzymes that hydrolyze cellulosic materials. Particularly active cellulolytic fungi include *Cladosporium* species commonly found in outdoor and indoor environments. As a result of biodegradation unwanted changes in the appearance and loss of mechanical strength of the object occur. In some cases, it results in partial or even complete disintegration. Besides biodegradation, handling the paper contaminated by fungi can be a serious health risk since many species, including *Cladosporium* spp., have allergenic potential. Some species of *Aspergillus* and *Penicillium* produce mycotoxins or may cause opportunistic infections in humans (Sterflinger and Pinzari, 2012; Kaarakainen et al., 2009).

To prevent or at least to reduce biodegradation of CH objects various methods like fumigation, anoxia or biocide treatments are used. The effects of all these methods are diffusion-limited and some of them can induce serious side-effects to the material of the treated object, to the environment, or to conservators and curators or even to museum visitors. On the other hand, ionizing radiation is a universal biocide that permanently damages DNA molecules of biodegrading agents thus offering a safer and more effective treatment. Radiation treatment of CH started in the 1960s and today is a well-established low-cost decontamination technique. Since it is fast (several hours to several days), it is particularly suitable for treating large volumes of material in a short time (Katušin-Ražem et al. 2017). It is temperature independent, leaves no residues and at least partially inactivates enzymes that would otherwise damage the object even after the initial attack of bio-degrading agents was treated. Gamma radiation is high-energy electromagnetic radiation emitted by radioactive nuclei and is not inducing secondary radioactivity. Its penetration is very high so the treated objects may be packed and shall remain protected while the packaging is intact. After several studies were performed to establish the irradiation dose for paper decontamination (Moise et al. 2012) a group of experts engaged by the International Agency of Atomic energy recommended 8±2 kGy as the absorbed dose range for treatment of mold infected paper materials (Havermans, 2017). Yet, other studies reported that these doses were insufficient in some cases. For example Gonzales et al. (Gonzales et al. 2002) studied the influence of gamma-rays on paper heavily contaminated by mold and established that 14.4 kGy was needed for decontamination.

Early research on radiation stability of cellulose and paper (Charlesby 1981) were not aimed at CH objects but were studying high-dose effects on cellulose and have shown that cellulose may undergo degradation or crosslinking, depending on the irradiation conditions. The first studies using gamma radiation for treatment of paper-based CH infected by fungi in the early 1960s were done by Belyakova (Belyakova, 1961). For several decades studies focused on the effects of radiation on the bio-burden and singled out some molds as being quite radiation resistant, *Cladosporium sphaerospermum* was one of them. As the interest into radiation treatment of CH increased the research focused on the side-effects to the paper. While irradiation conditions

for disinsection are quite well established and it had been confirmed that some materials like wood can be repeatedly irradiated if needed, for fungal decontamination and more fragile materials like paper the situation is not so simple. Many considerations need to be taken into account, like the age and state of the treated object, the type and degree of infestation, etc. Adamo et al. (Adamo et al. 1998) showed that with increasing doses of irradiation the degree of polymerization increases in the same percentage on new and aged paper. Since ageing itself causes a drastic fall in the degree of polymerization they concluded that the older the paper the less polymerization decreases. It was also shown that at the doses used for CH treatment the changes in paper induced by irradiation and ageing do not negatively predispose it to a new insect or fungi attack (Magaudda et al. 2001, Adamo and Magaudda, 2003, Adamo et al. 2003).

An important and often neglected aspect of radiation treatment is the dose rate effect. Prolonged irradiation at low dose rate is known to result in changes caused by diffusion-limited oxidation (Gillen et al., 1986, Baccaro et al. 1993). Because of that we studied the influence of the dose rate on highly resistant *C. sphaerospermum*. Another objective of this study was to assess whether there is a synergistic effect of the microbiological contamination and gamma irradiation that could enhance changes to selected properties of model Vergé paper, a type of paper common for restoration and bookbinding.

2. Materials and methods

2.1. Materials

The investigations were performed on acid-free Vergé paper weighting 120 g/m^2. It is composed of pure cotton fibers and buffered with calcium carbonate to a pH value between 7.5 and 9.5.

A single mold C. *spaherospermum* was used for inoculation. The strain was taken from the microbial collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb (Croatia). C. *spaherospermum* was grown on a Malt Extract Agar (MEA) and after 10 days of incubation at 25 °C from the grown sporulating culture suspensions of conidia were prepared in sterile water to the concentration 1x10^6 CFU/mL using a densitometer (DEN-1 McFarland Densitometer, Kisker Biotech GmbH).

2.2. Sample preparation

Paper was cut into squares sized 3.5 x 3.5 cm and left to homogenize in a plastic bag. For each type of sample, dose and dose rate the samples were prepared in duplicate.

Paper for *inoculated samples* was first sterilized in autoclave for 15 min at 121°C and 1.2 bar. Two random samples were microbiologically analyzed by plating on MEA to check their sterility (these samples were not used in the following experiments). Then the samples were inoculated with a suspension of *C. spaherospermum* culture (1x10^2 CFU/g), kept for 7 days at 25 °C and 70-80 % RH and then irradiated.

Referent samples for assessment of alterations in paper were prepared and treated in the same manner as the ones used for analysis of microbiological growth but were not inoculated.

In order to determine naturally occurring mycobiota of non-irradiated *inoculated* and *referent* samples (Table 1), both types of samples were analyzed immediately without incubation in humidity and after 7 days at 25 °C and 70-80 % RH. For further experiments with gamma radiation we used *referent* and *inoculated* samples that were incubated for 7 days at 25 °C and 70-80 % RH.

2.3. Gamma irradiation

Irradiation was performed at the panoramic Co-60 γ-source of the Radiation Chemistry and Dosimetry Laboratory, Division of Materials Chemistry, Ruđer Bošković Institute at room temperature, in contact with air. The samples were irradiated to doses D = 2, 7, 20 and 50 kGy. The samples were irradiated either at a dose rate of 0.4 kGy/h designated *lower dose rate* (*low-DR*) or at 31.0 kGy/h i.e the *higher dose rate* (*high-DR*).

The irradiation times were calculated on basis of dose mapping of the γ-source previously determined by ECB dosimetric system (ISO/ASTM 51538:2017) and recently confirmed using calculation simulations (Majer et al. 2019). An ECB dosimeter was used for control.

The *referent* and *inoculated* paper samples were incubated for 7 days at 25 °C and 70-80 % RH prior to irradiation. During the post-irradiation period the samples were kept in the dark at room temperature.

2.4. Determination of the mycobiota composition

There were four groups of specimens (not to be confused with paper samples) for micologycal analysis. The first group was prepared immediately after irradiation (0^{th} day) and plated on MEA, while the rest were kept for 14, 28 and 56 days at room conditions in a dark and dry place. After these periods the concentration (CFU/g) and composition of mycobiota that survived irradiation were determined by plate count method on MEA. All specimens were analyzed in duplicate and mean values are presented as the results. The specimens were briefly homogenized by vortexing in 2 mL of pepton water with Tween 80 (0.5%) and further diluted to obtain dilutions ranging from 10^{-1} to 10^{-4}. From each dilution 100 µL was applied on the MEA surface. Plates were incubated for 7 days at 25 °C. All specimens were analyzed in duplicate and average values are presented as the results.

The number of colony-forming units per gram of material is calculated according to the formula:

$$\text{CFU/g} = \frac{\sum C}{V(n_1 + 0.1 n_2)d} \quad (1)$$

where ΣC is the number of colonies counted on all selected plates; V is the volume of the inoculums applied on the MEA; n_1 is the number of plates kept for counting after the first dilution; n_2 is the number of plates kept for counting after the second dilution; and d is the dilution from which the first numbers were obtained.

The decimal reduction dose (D_{10}), i.e. the dose required to inactivate 90% of the initial number of molds in a sample was determined according to the formula:

$$D = D_{10} (\log N_0 - \log N) \quad (2)$$

where N_0 is the initial number of microorganisms; N is the final number of microorganisms; and D is the decimal reduction dose.

2.5. UV-vis measurements and color evaluation

Kodama et al. (Kodama et al., 2016) irradiated contemporary paper at room temperature with a doses of 22 - 24 kGy and a dose rate of 16 kGy/h. They showed that the half-life of cellulose radicals in paper is 2.5 days in these conditions. Thus, to ensure that most cellulose radicals decayed both UV-vis and FTIR measurements were performed 2 months after irradiation.

A Konica Minolta® spectrophotometer model CM-2600d was used to measure color, as well as UV-vis spectra in the range from 400 to 740 nm. The instrument was programmed with the following settings: measurement geometry d/8° (diffuse illumination, 8-degree viewing), selecting an area of 3 mm in diameter (SAV, Small Average Value), CIE Standard Illuminant

D65 corresponding to the average daylight, CIE 1964 Standard Observer (10° standard observer). The spectrophotometer was set to take four sequential measurements, giving the means of the obtained color coordinates. To ensure precise color evaluation four measurements were performed at two different points on each sample.

The radiation induced color change was investigated as described by Marušić et al. (Marušić et al. 2016) using CIE colorimetry, in the CIE 1976 Lab $L^*a^*b^*$ system (CIE 1978). Parameter L^* represents the lightness of a color, where L^* is from 0 (black) to 100 (white). The chromaticity of a color is represented in a two-dimensional diagram where axis a^* determines the ratio of green (negative) to red (positive), and axis b^* specifies the ratio of blue (negative) to yellow (positive).

The overall color change is estimated by the CIE 1976 $L^*a^*b^*$ color difference:

$$\Delta E^*_{a,b} = \sqrt{(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2} \quad (3)$$

where the colors of the two compared samples are specified by [L_1*, a_1*, b_1*] and [L_2*, a_2*, b_2*] parameters.

2.6. FTIR measurements

Fourier transform infrared spectra (FTIR) were recorded using Bruker's Tensor II equipped with ATR module at room temperature in frequency range 400-4000 cm^{-1} and processed by Opus and Spectragryph™ software. Up to 5 spectra were recorded on each particular sample.

3. Results and discussion

3.1. Effects of gamma radiation on mycobiota composition

Both microbiological contamination and irradiation may influence the properties of paper particularly its stability and color. It is known that irradiated paper may become yellowish although this effect cannot be observed by naked eyes at doses usually used in CH treatment (Otero D'Almeida et al. 2009). On the other hand, some strains of fungi that are common on old paper may produce a variety of colorants (Melo et al. 2019, Caneva et al. 1991). To our knowledge there has not been a systematic study of the symbiotic effect of bioburden and irradiation on the color change of paper.

The results on naturally occurring mycobiota of the investigated paper, i.e. of the non-incubated referent and inoculated samples are given in Table 1. To assess the effect of incubation itself part of the samples were incubated for 7 days. It can be seen that the natural contamination of the investigated paper consists of *Aspergillus* spp., *Penicilium* spp., *Cladosporium* spp., unidentified white mycelia and yeasts. These fungi are typically found on paper materials in libraries and archives (Pinzari et al. 2011). The fungi were unevenly dispersed on paper in form of so-called spot contamination. The initial contamination was $2x10^2$ CFU/g. Upon 7 days of incubation (at 25 °C and 70% RH) the mycobiota of the *referent* samples and Cladosporia contamination on *inoculated* samples increased 10 to 100 times compared to the initial values.

Table 1. Results of the microbiological analysis of non-irradiated paper samples both non-incubated and incubated for 7 days.

Type of sample	Incubation for 7 days	CFU/g	Fungi
Referent	NO	1.8×10^2	*Cladosporium* Yeasts
	YES	3.8×10^2	*Penicilium* *Aspergillus*
Inoculated	NO	1.0×10^3	*Cladosporium*

	YES	3.2×10^4	*Cladosporium*

Table 2 presents the microbiological growth on specimens prepared from the *referent* and *inoculated* samples upon irradiation with the *high-DR*, while Table 3 shows the growth after irradiation with the *low-DR*. It is obvious that the antifungal effect of gamma irradiation depends on both the applied dose and dose rate.

The applied radiation doses are not only in the range that is common in treatment of cultural heritage, but also significantly higher, up to 50 kGy. From Table 2 it can be seen that the mycobiota of the *referent* samples recovered up to the value of the same order of magnitude as the initial concentration (10^2 CFU/g). The absorbed dose of 2 kGy did not provide decontamination even at the *high-DR*. The same was observed for the *low-DR* (Table 3) where the values were even greater and all of the four species initially found on paper grew again in concentrations 100-2000 CFU/g. Irradiation to D = 7 kGy at the *high-DR* initially provided efficient decontamination, but after 14 days white mycelia were recovered and after 56 days yeasts recovered. While the concentrations of 46 CFU/g are lower than the initial values before irradiation (Table 1.) that is not satisfactory. Irradiation to D = 7 kGy at the *low-DR* was also initially efficient, but already after 14 days the mycobiota recovered to relatively high values. Doses D = 20 and D = 50 kGy inhibited the recovery of most fungal species except for white mycelia and yeasts, at both of the dose rates applied. As can be seen in Table 3 all the species initially found on paper grew again in concentrations 100-2000 CFU/g.

Table 2. Growth of the mycobiota on the *referent* and *inoculated* paper samples irradiated at the *high-DR* (31.0 kGy/h).

Day		**0th**	**14th**	**28th**	**56th**	**0th**	**14th**	**28th**	**56th**
***D*/kGy**		***Referent***				***Inoculated***			
2	CFU/g	2.3×10^2	0	0	9.1×10^1	4.0×10^4	5.5×10^3	1.1×10^4	9.5×10^2
	Fungi	Wm	-	-	Wm	Clad	Clad	Clad	Clad Wm
7	CFU/g	0	4.6×10^1	0	4.6×10^1	4.6×10^1	0	0	4.6×10^1
	Fungi	-	Wm	-	Y	Wm	-	-	Wm
20	CFU/g	0	0	0	0	0	4.6×10^1	0	0
	Fungi	-	-	-	-	-	Clad	-	-
50	CFU/g	9.1×10^1	0	0	9.1×10^1	0	0	0	0
	Fungi	Wm	-	-	Wm	-	-	-	-

Wm - White mycelia; Clad - *Cladosporium*; Y- yeasts

Table 3. Growth of the mycobiota on the *referent* and *inoculated* paper samples irradiated at the *low-DR* (0.4 kGy/h).

Day		**0th**	**14th**	**28th**	**56th**	**0th**	**14th**	**28th**	**56th**
***D*/kGy**		***Referent***				***Inoculated***			
2	CFU/g	4.1×10^2	1.4×10^2	2.0×10^3	9.1×10^1	3.4×10^4	2.8×10^4	5.1×10^3	1.4×10^2
	Fungi	Fus Pen Clad	Y	Y	Wm	Clad Wm	Clad	Clad Y	Clad Wm
7	CFU/g	0	1.4×10^3	9.1×10^2	9.1×10^2	2.7×10^3	2.6×10^3	8.2×10^3	2.8×10^3

	Fungi	-	Alt	Fus	Wm	Clad	Clad	Clad	Clad
22	CFU/g	0	0	0	0	1.3×10^3	6.6×10^3	1.9×10^4	4.4×10^3
	Fungi	-	-	-	-	Clad	Clad	Clad	Clad
50	CFU/g	0	2.3×10^2	0	0	0	1.4×10^4	2.0×10^3	2.0×10^3
	Fungi	-	Y	-	-	-	Clad	Clad	Clad

Fus- *Fusarium*; Pen- *Penicillium*; Clad- *Cladosporium;* Y- yeasts; Wm- White mycelia; Alt- *Alternaria*

Fig. 1 presents dependence of the logarithm of the survived molds on the absorbed dose immediately after irradiation. Concentration of Cladosporia 3.2×10^4 CFU/g (Table 1) was used as the initial concentration. The D_{10} values, i.e. the dose needed for inactivation of 90 % of molds, were determined as the negative reciprocal values of the slope of the linear fit of the data:

$$D_{10} = -1/a \tag{4}$$

where a is the slope of the lines. The determined values after 0th and 56th day are presented in Table 4.

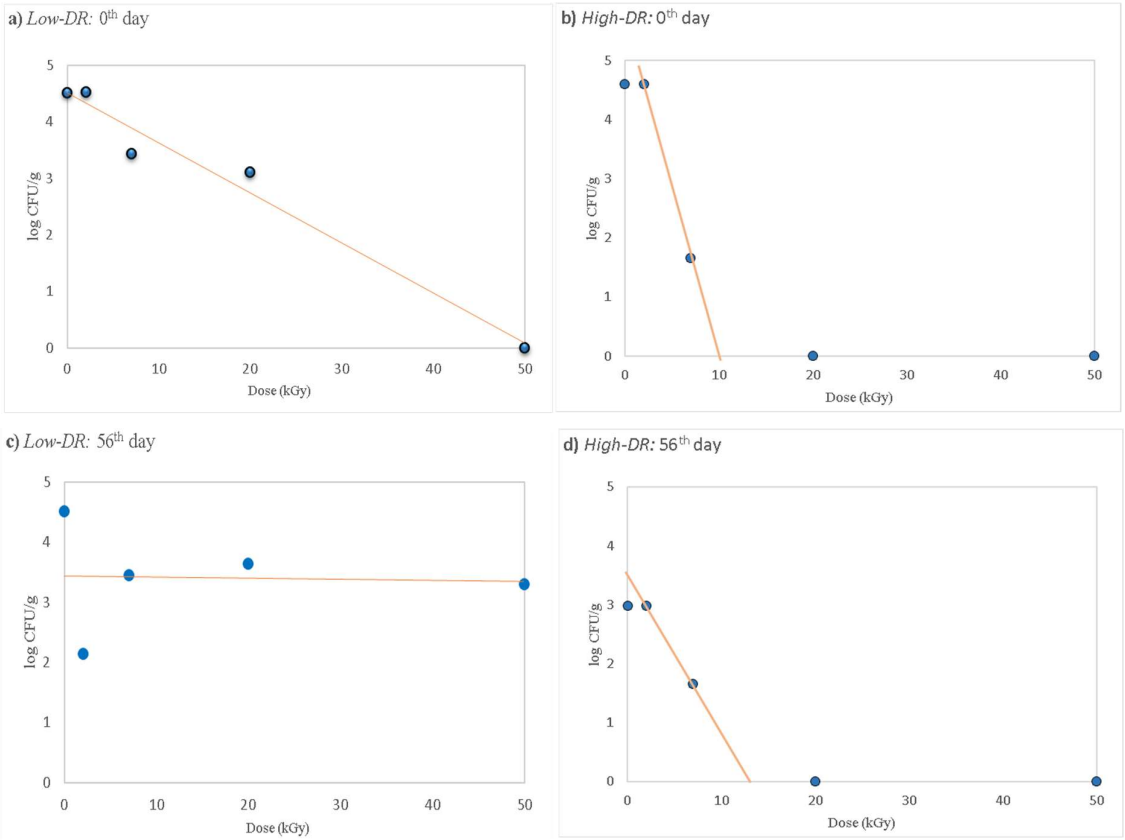

Fig. 1. Dependence of the logarithm of the survived *C. sphaerospermum* on the absorbed dose immediately after irradiation: a) at *low-DR* (0.4 kGy/h) and b) *high-DR* (31.0 kGy/h); and after 56 days of incubation: c) at *low-DR* (0.4 kGy/h) and d) *high-DR* (31.0 kGy/h);

Table 4. The determined D_{10} values at both dose rates and after the 0^{th} and 56^{th} day.

D_{10} / kGy	*Low-DR*	*High-DR*
0^{th} day	11.30	1.70
56^{th} day	588.24	4.95

The D_{10} value for the *high-DR* was obtained by linear fit of only two points for both plots (Figs. 2b and 2d). This suggests that the D_{10} values obtained at the *high-DR* are only approximations of the real values, so further investigations are needed in the dose ranges below 20 kGy to determine the exact value, as well as to be able to propose a minimum dose rate. Although the D_{10} values were obtained on very limited data set they are indicative of the order of magnitude of the dose needed. At *high-DR* D_{10} is an order of magnitude lower (1.70 kGy) than at *low-DR* (11.30 kGy) on the 0^{th} day. On the 56^{th} day the values differ even more, for two orders of magnitude, what leads to conclusion that decontamination was not efficient. As can be seen *Cladosporium* is highly resistant and the efficiency of its decontamination highly depends on both the irradiation dose and dose rate. Plausible explanation for such resistance is presence of melanin in its cell wall. Since some other melanized fungi were found in areas of extremely high radioactivity like the Chernobyl reactor Dadachova et al. (Dadachova et al., 2008) have concluded that melanin provides radioprotection as a "result of a combination of physical shielding and quenching of cytotoxic free radicals". Kortei et al. (Kortei et al. 2015) reported D_{10} values of 5.94 and 5.64 kGy at the dose rate of 1.7 kGy/h for reducing a mix of molds including *Aspergillus, Mucor, Rhizopus* and *Fusurium* species. Recent studies (Sharma et al., 2017; Shuryak et al., 2019) showed several fungi and yeasts such as *Cryptococcus* spp., *Saccharomyces* spp. are also radiation resistant when irradiated to dose rates of 50 Gy/h and lower. Radiation resistance of the yeasts strongly correlates with the intracellular content of Mn antioxidants, which renders antioxidant enzymes such as superoxide dismutase (SOD). Manganese is important among redox active transition metals in cells; Mn redox-cycling favors O_2 scavenging without the release of very reactive hydroxyl (HO•) radicals, while redox-cycling of other metals such as Fe and Cr produces HO• radicals. Accumulation of high concentrations of Mn antioxidants could also be a plausible explanation for radiation resistance of the white molds. In a recent study Shuryak et al. (Shuryak et al., 2017) presented that during exposure to high dose rates the total dose is delivered to cells over a time that is too short for substantial damage repair. They showed that mixed yeast cultures exposed to low dose rates over longer periods of time share antioxidative enzymes which at high cell concentrations can suppress oxidative stress. Taken together that leads to conclusion that antifungal effect of gamma radiation strongly depends on both dose and dose rate, because resistant fungi (e.g. Cladosporia and/or yeasts) present in high concentrations (e.g. 10^4 CFU/g) exposed to high doses but at low dose rates could suppress oxidative stress by intercellular sharing of reactive oxygen species scavenging enzymes enabling simultaneous growth and repair.

3.2. Colorimetric and spectroscopic studies of paper properties

Dose-rate effects become significant whenever some step in the kinetics underlying degradation occurs on a time scale comparable to the sample exposure time (Gillen et al. 1986). They are caused by diffusion-limited oxidation. At high dose rates dissolved oxygen is used up faster than it can be replenished from the surrounding atmosphere. This results in more oxidation near air-exposed surfaces and less in the interior. As the dose-rate is lowered, the oxidation will proceed further into the sample, leading eventually to a homogeneously-oxidized material. Because of that special attention is given to corresponding physical and chemical changes in studied model paper.

Fig. 2 presents the total color change (ΔE^*) in paper samples as a result of the irradiation treatment with different doses at the two dose rates that differ for about two orders of magnitude. The values presented in the plots are the mean values of L^*, a^* and b^* values.

There are different interpretations of the ΔE^* value that should be taken as a threshold value above which an observer will perceive a color difference. This value is the so called Just Noticeable Difference (JND) value. Recently two approaches have been introduced. Mahny et al. (Mahny, 1994) defined the value $\Delta E^* = 2.3$ as the limit for JND since this is the value below which not even a trained observer's eye can notice the difference. According to Hardeberg (Hardeberg, 2001) the values are: $\Delta E^* < 3$ the effect is *hardly perceptible*, $3 < \Delta E^* < 6$ *perceptible, but acceptable* and $6 < \Delta E^*$ *not acceptable*. Upon irradiation at the *high-DR* the ΔE^* value of all paper samples was below 1.5 at all absorbed doses (Fig. 2) so the color change can be considered insignificant. On the other hand, in the samples irradiated at the *low-DR* ΔE^* became greater than 2.3 already at $D = 7$ kGy. The difference could be visually perceived according to Mahy's interpretation of the JND although according to Hardeberg's categorization it was still *perceptible, but acceptable*. The highest ΔE^* values were achieved at $D = 20$ kGy. Interestingly upon irradiation to $D = 50$ kGy at both dose rates ΔE^* of both the *referent* and *inoculated* samples decreased to the value observed in samples irradiated to $D = 2$ kGy. The unexpected decrease of colorimetric parameters values at 50 kGy is not a result of an error. The effect is present in the samples that have been handled and irradiated separately like those that were irradiated at different dose rates. Inoculation had no influence.

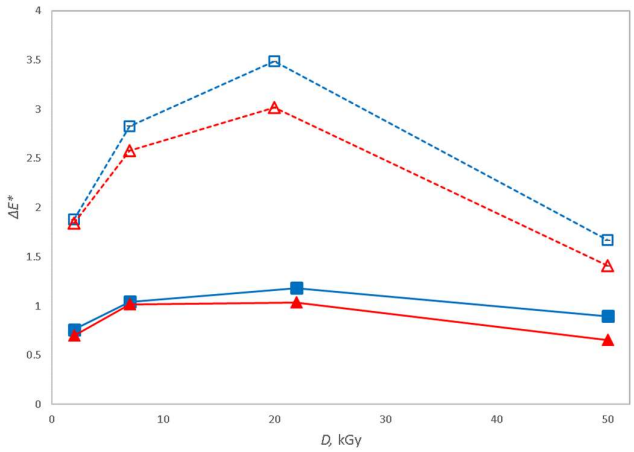

Fig. 2. The Overall color change in colorimetric CIE-$L^*a^*b^*$ system observed on both *referent* and *inoculated* samples at both dose rates, where squares (■) represent *referent* samples; triangles (▲) represent *inoculated* samples; full lines (—) *high-DR*; and dashed lines (---) *low-DR*.

The ΔE^* value is only an estimation of the overall color change so for a precise interpretation of the data each colorimetric parameter needs to be examined separately. Colorimetric parameters ΔL^*, Δa^* and Δb^* of irradiated *referent* and *inoculated* papers samples are shown in Fig 3. In all samples the ΔL^* values became negative indicating darkening. As already determined, upon irradiation at the *high-DR* all changes in color parameters are insignificant. On the other hand, in both *referent* and *inoculated* samples irradiated at the *low-DR* to 20 kGy observable colorimetric change is observable. The inoculated samples in this case became slightly reddish and slightly yellowish so both Δa^* and Δb^* values increased up to D=20 kGy.

In all other samples and irradiation conditions Δb^* values were negative as bluing of samples was measured. The Δa^* values were negative in samples irradiated at the *high-DR* since the samples acquired an extremely light greenish tone but in samples irradiated at *low-DR* they were positive, indicating redding of the samples. A similar effect was observed on colorimetric properties of irradiated nacre (Marušić et al. 2016) that were shown to arise of increased concentration of the carbonate anion-radical. To confirm this, visual reflectance spectra were analyzed. In Fig 4. a part of differential reflectance spectrum obtained by subtracting the spectra of non-irradiated *referent* sample from that of the sample irradiated to D = 20 kGy is shown. The differential spectrum shows a wide absorption band at around 600 nm that is characteristic for the carbonate anion radical (Behar et al. 1970).

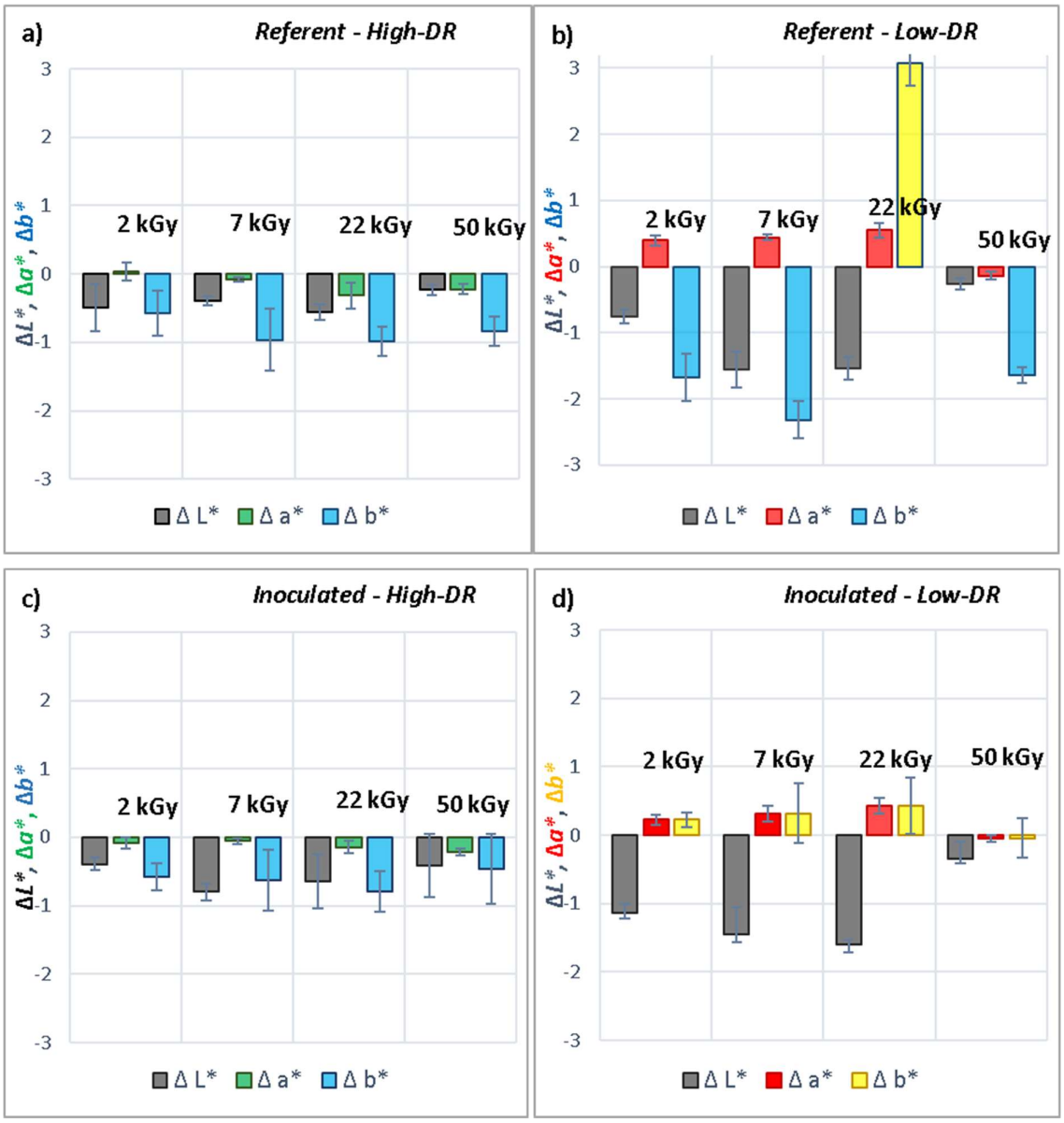

Fig. 3. The change in colorimetric CIE-*L*a*b** system parameters of the paper samples irradiated to selected doses, where: a) presents the *referent* samples at *high-DR;* b) *referent* samples at *low-DR;* c) *inoculated* samples at *high-DR*; and d) *inoculated* samples at *low-DR.*

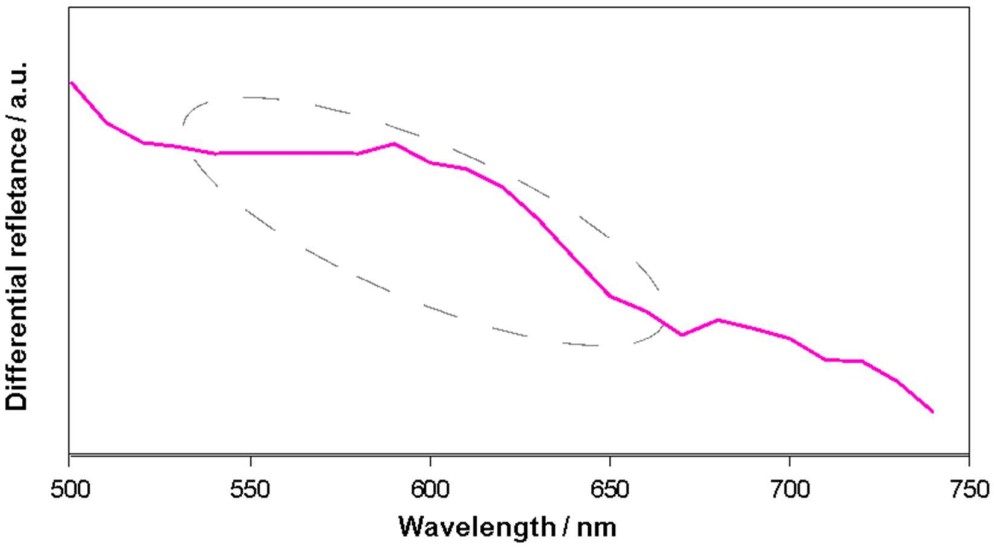

Fig 4. A part of the reflectance spectrum obtained as a subtraction of spectra of the *referent* sample irradiated to D = 20 kGy and the non-irradiated sample. A wide absorption characteristic for carbonate anion radical.

To assess whether any chemical or structural changes occurred upon irradiation of paper samples ATR-FTIR spectra were recorded. Several spectra were recorded in each sample what made it possible to recognize two distinct types of spectra. Both types were recorded in all *inoculated* and *referent* samples irrespective of dose. Selected partial spectra of each type are shown in Fig. 5. In both spectral types cellulose absorptions dominate so the spectra were normalized to pyranose ring absorption at 1160 cm^{-1}. In the spectral range 3660 to 2900 cm^{-1} where -OH and -CH bonds absorb no changes were observed thus were not shown. In the fingerprint region (1800 – 600 cm^{-1}) of both types of the spectra characteristic cellulose absorptions appear at 1426 cm^{-1}, 1369 cm^{-1}, 1315 cm^{-1}, 1027 cm^{-1} and 897 cm^{-1}. They arise from stretching and bending vibrations of -CH_2 and -CH, -OH and C-O bonds (Garside and Wyeth 2003, Abidi et al. 2014). Cellulose is a semicrystalline polymer so absorptions in the region between 1420 and 1430 cm^{-1} are associated with its crystalline part, while the peak at 897 cm^{-1} is assigned to the amorphous cellulose. A small peak at 1640 cm^{-1} is usually assigned to water bound to cellulose. However, amide I peak, the strongest absorption of proteins is roughly at the same frequency so in this case presence of mycobiota might have contributed. No difference in cellulose absorptions was observed between *inoculated* and *referent* samples. No signs of oxidation were observed in any sample.

The only difference between the two types of spectra in Fig. 5a and b is in the range between 1500 and 1300 cm^{-1} and at 874 cm^{-1}. The 874 cm^{-1} absorption arises of C=O bonds in carbonate anion of $CaCO_3$ filler added to paper (Hospodarova et al. 2018). The corresponding C-O bond vibrations should appear at about 1475 cm^{-1} but in this case it produced a wide absorption that overlaps with several cellulose absorptions. The fact that two types of spectra could be distinguished and that they differ in $CaCO_3$ absorption intensities indicate non-homogeneous microdistribution of the filler in paper production.

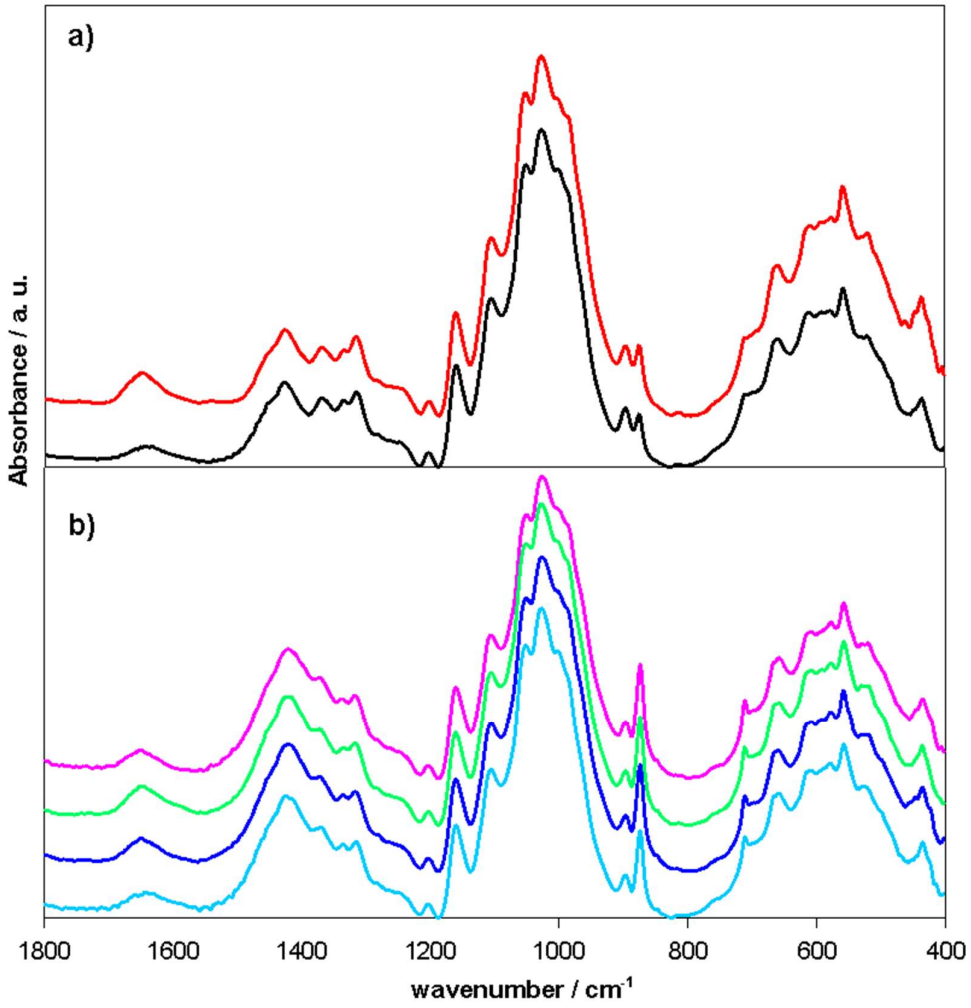

Fig 5. Two types of paper ATR-FTIR spectra (fingerprint region) recorded in studied paper samples:
a) The first type of spectra that contains only cellulose absorptions (lower curve - non-irradiated *referent* sample, higher curve - *referent* sample irradiated to D = 20 kGy at *low-DR*);
b) The second type of paper spectra containing $CaCO_3$ absorptions. From top to bottom: 1st curve - *referent* sample irradiated to D = 20 kGy at *high-DR*; 2nd curve - *inoculated* sample irradiated to D = 20 kGy, *low-DR*; 3rd curve - *inoculated* sample irradiated to D = 20 kGy, *high-DR*; 4th curve - non-irradiated *referent* sample.

In the spectra that contained $CaCO_3$ absorption the dependence of the intensity ratio of 874 cm^{-1} absorption to pyranose ring absorption at 1160 cm^{-1} on the absorbed dose was monitored. Similarly, to colorimetric parameters the ratio increased at lower doses up to 20 kGy (Fig. 6) and then decreased at D = 50 kGy. The increase and decrease of 874 cm^{-1}/1160 cm^{-1} ratio roughly corresponds to changes of the L^* colorimetric parameter. This confirms the role of the carbonate radical anion in observed colorimetric and spectroscopic changes.

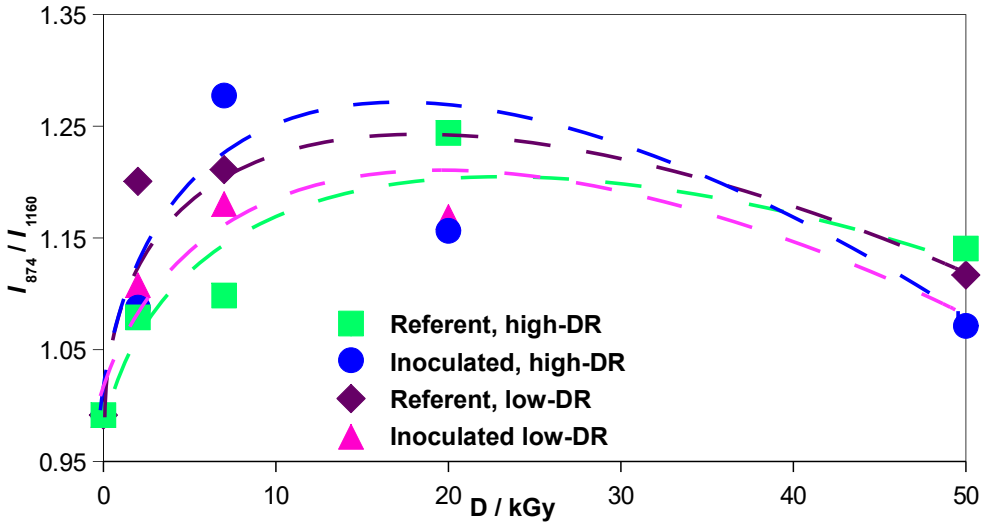

Fig. 6. Absorbed dose dependence of FTIR intensity ratio of carbonate anion absorption at 874 cm^{-1} to pyranose ring absorption at 1160 cm^{-1} for irradiated *inoculated* and *referent* paper samples.

The reduction of colorimetric parameter values in samples irradiated to D=50kGy and appearance of yellowish and reddish hues in *inoculated* samples irradiated at *low-DR* may be a result of the Maillard reaction. Maillard reaction is known to occur upon irradiation (Tegota and Bachman, 1998) particularly in presence of water and mycobiota. It is known that the reaction produces radical scavengers (Morales and Jimenez-Perez, 2001). While the concentration of products of such a reaction may be too low to be observed in FTIR spectra it is high enough to induce slight color changes that were measured in studied samples irradiated to lower doses.

4. Conclusions

The dose rate plays an important yet generally ignored role in the efficacy of the radiation treatment particularly of paper objects and in the likelihood of side-effects appearance. In order to reduce fungal burden to an acceptable level the initial contamination needs to be taken into account since antifungal effectiveness of radiation treatment depends on radiation resistance of fungal species and size of contaminant inoculum. If the contamination is high the applied dose needs to be adjusted taking into account that the dose needed to reduce the molds to an acceptable level significantly increased for the dose rate that was two orders of magnitude lower. However, a threshold dose for that effect cannot be excluded and is a subject of further research. The dependence of obtained D_{10} value on the dose rate is very high, in orders of magnitude thus additional investigations to determine the exact dependence and to reevaluate of the recommended dose of 8 ± 2 kGy are also needed.

Upon irradiation of both *referent* and *inoculated* samples the color changes remained in the *hardly perceptible* range and the effects were visible only in samples irradiated to 20 kGy at the *low-DR*. The shift was mostly to a bluish tone with slight darkening. Such changes are caused by appearance of the carbonate radical-anion. No oxidation or change in crystallinity of cellulose was detected. Overall changes were to insignificant to make any conclusion on the contribution of mycobiota to the irradiation side-effects on paper.

Since high dose rates were shown to be preferable and penetration of electrons into paper is high enough in some cases electron beams treatment of books and paper-based cultural heritage could be applied. Thus further investigations on electron beams treatment may be encouraged.

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