ZINC OXIDE NANOPARTICLES HINDER FUNGAL BIOFILM DEVELOPMENT IN AN ANCIENT EGYPTIAN TOMB.

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ABSTRACT

Nanoparticles (NPs) have been proposed as an innovative strategy to prevent fungal colonization of cultural heritage, but until now their efficacy has mainly been proved against fungi in planktonic conditions. Four fungi from microbial alterations of the royal tomb of Tausert and Setnakht in the Valley of the Kings, Thebes, West Bank, Modern Luxor, Egypt, were isolated and identified as *Alternaria alternata*, *Aspergillus niger*, *Penicillium chrysogenum* and *P. pinophilum*. This work deals with four developing fungal biofilms grown with a colony biofilm approach, and exposed to two concentrations of zinc oxide NPs (0.25 and 0.5 %) for 10 days. A significant reduction in the biofilm growth was observed in presence of 0.5 % NPs for all fungi, except *A. niger*. Moreover, the morphology of the fungal biofilm exposed to NPs differed from that of the control, and there was a different polysaccharide to protein ratio in the matrices, and earlier production of coloured compounds and spores. Despite the success of the zinc oxide NPs, this early metabolite production needs to be monitored as a possible source of substances affecting cultural heritage surfaces.

KEYWORDS: Biodeterioration, mural paintings, biofilm, fungi, zinc oxide nanoparticles.

1. Introduction

Fungi are ubiquitous colonizers and, according to some researchers, the most important agents in the biodeterioration of cultural heritage in outdoor environments (Warsheid and Braams, 2000; Masaphy et al. 2014). Fungal hyphae can penetrate and widen fissures in rocks and building materials and produce a broad range of enzymes, and of other compounds including pigments, causing erosion, discoloration, exfoliation of stone and mural paintings (Griffin et al. 1991; Sterflinger 2010; Unkovic et al. 2016).

Fungi grow on historical and artistic surfaces as sub-aerial biofilms (SABs), i.e. communities of microorganisms embedded in an exopolymeric matrix interacting with the underlying substrate and the atmosphere. The growth as a community and the presence of a matrix allows fungi to tolerate a very broad range of multiple and fluctuating environmental stresses (e.g., low organic nutrient content, low water activity, wide temperature fluctuations, intense solar irradiation) (Gorbushina 2007), but also to withstand exposure to antifungal agents, up to 1000-fold better than planktonic cells (Ramage et al. 2012). Several studies on biofilms of the human pathogens *Candida* spp. and *Aspergillus fumigatus* have highlighted the critical role played by the biofilm matrix in detoxifying antifungal agents, acting as a protective physical and chemical barrier (Mitchell et al. 2013; Manavathu et al. 2010). Except for these studies in the sanitary field, the role of fungal biofilm matrix in protecting fungal cells has not yet been studied.

Metallic nanoparticles were recently explored as an innovative strategy to inhibit fungal colonization of cultural heritage. Indeed, antimicrobial efficacy of various NP materials has been demonstrated against bacteria and fungi, and, among these are zinc oxide NPs (ZnO-NPs), which meet the requirements for application on artworks because they are biocompatible, chemically stable, bioactive and white-coloured, thus not affecting the colour of the substrate (Ditaranto et al. 2015). ZnO-NPs have been shown to significantly decrease the planktonic growth of a number of fungi: *C. albicans*, *Saccharomyces cerevisiae*, *Rhizopus stolonifer* (Sawai and Yoshikawa, 2004; Lipovsky et al., 2011), the plant pathogenic fungi *Pythium debarynum* and *Sclerotium rolfsii* (Sharma et al. 2011), the wood-decay fungi *Trametes versicolor* and *Gloeophyllum trabeum* (Terzi et al. 2016), and the two postharvest pathogenic fungi *Botrytis cinerea* and *Penicillium expansum* (He et al. 2011). In addition to their antimicrobial activity, coatings containing ZnO-NPs are also reported to prevent dust accumulation and UV aging on oil paintings (El-Feky et al. 2014). Thanks to their efficacy and compatibility with the conservation of cultural heritage, ZnO-NPs were recently proposed as a method for preventing the microbial growth of *Aspergillus niger* on stone substrates (van der Werf et al. 2015; Ditaranto et al. 2015) and on oil paintings (El-Feky et al. 2014). Nevertheless, no data are available on ZnO-NPs efficacy in inhibiting the growth of fungi as a biofilm on heritage surfaces.

In this study, we evaluated the preventive action of ZnO-NPs against fungal biofilm that is growing on ancient Egyptian paintings in the Valley of the Kings.

2. Materials and methods

2.1. Description of case site and sampling

The wall paintings of the royal tomb of Tausert and Setnakht in the Valley of the Kings (position KV14 in the Valley, Thebes, West Bank, Modern Luxor, Egypt; Fig.1) show various alterations ranging in color from green to brown and black, including salt efflorescence, and pitting and cracking of the ceiling of the burial chamber, probably due to water seepage that has led to the deterioration and loss of part of the painted surface. Alterations ascribable to fungal colonization are also visible. Despite the extensive decay of some parts of the tomb, the only intervention undertaken to date has been to secure some cracks with lime mortar.
With the authorization of the Egyptian Ministry of Cultural Heritage six samples were taken, using sterile swabs, from wall paintings in the burial chamber and on the ceiling of corridor D, all presenting deterioration and signs of fungal colonization.

2.2. Isolation, storage and identification of fungi

To culture the fungi present on the paint surface, each swab was streaked directly onto Potato Dextrose Agar (PDA) medium and incubated at 26°C for five days, then the fungi were isolated according to their different morphologies. For each fungus, spores were collected from 2-day old plates, adding phosphate buffer solution (PBS) and glass beads to scrape the aerial hyphae. The suspension was collected, centrifuged and filtered by glass beads in a sterile tube. Spores were stored at -80°C in 15 % glycerol.
DNA was extracted from fungal spores as described by Polo et al. (2010). The internal transcribed spacer 1 (ITS1) region, 5.8 S rDNA, and the ITS2 (Chen et al. 2001) were amplified in gene fragments extracted from samples, amplified by PCR using the primers ITS1f and ITS4 with 1X of PCR buffer, 1.8 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.5 µM of each primer, 5 % dimetylsolfoxide and 0.63 U of GoTaq DNA polymerase (Promega, Italy) in 25-µl PCR reaction. The cycling programme consisted in an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 2 min and a final extension at 72°C for 10 min.
The amplified fragments were identified by sequencing (Macrogen, Korea) and the sequences were analysed using BLAST software (www.ncbi.nlm.nih.gov/BLAST) and the Ribosomal Database Project classifier (Wang et al. 2007). The ITS sequences from fungi isolated in this study have been deposited in the GenBank database under accession numbers KY052055, KY052056, KY052057, and KY052058.

2.3. Zinc oxide nanoparticle production and characterization

Zinc oxide nanoparticles (ZnO-NPs) were prepared following the wet chemical method (Yadav et al. 2006). Briefly, 0.1 % soluble starch was prepared in a microwave oven for use as the stabilizing agent, then 0.1 mol of zinc nitrate and 0.2 mol of sodium hydroxide were added to the starch solution under stirring. After complete addition of sodium hydroxide, the supernatant was decanted and the remaining solution centrifuged at 10,000 rpm for 10 min. The ZnO-NPs were washed 3 times with distilled water and dried at 80 °C for 8 h.
The purity and chemical composition of the ZnO-NPs were determined using the enhanced mini-materials analyser (εMMA) D143 X-Ray Diffractometer (GBC Scientific Equipment, USA). Transmission Electronic Microscopy (TEM) was used to determine the size on ZnO-NPs by using the JEOL JEM-1010 TEM, Japan, equipped with Kodak Megaplus Camera, Model 1.6i with image analysis and processing software (AMT, USA).

2.4. Zinc oxide nanoparticles on planktonic fungi

Appropriate quantities of ZnO-NPs were homogenously dispersed in PDA before pouring onto plates to obtain concentrations of 0, 0.125 and 0.25 %. Three plates for each concentration and each fungus were inoculated, dropping on 10 µl of a suspension of 10^6 spores ml^{-1}. Plates were incubated for 48 h at 26°C and growth was observed.

2.5. Colony biofilm formation

A protocol for the growth of fungal colony biofilms was set up by modifying the method reported by Anderl and colleagues (2000). Briefly, 10 µl of cell suspension containing 10^6 spores ml^{-1} were used to inoculate sterile black polycarbonate filter membranes (0.22 mm pore size, Whatman, UK) that were placed on PDA plates, at 26°C, either in the absence or in the presence of ZnO-NPs (0.25 and 0.5%). The membranes were transferred every 48 h to fresh media, and grown for a total of 7 days.

2.6. Colony biofilm quantification with Bradford assay

Total protein amount was determined to assess the relative amounts of biomass in the colony biofilms. For protein determination a membrane was collected every 48 h, resuspended in a tube with 1 ml of 6M urea and small glass beads (G9268, Sigma Aldrich, Italy) and vortexed for 15 min. The tubes were then incubated for 48 h at 4 °C to complete the protein extraction, centrifuged 15 min at 4°C at 11,000 rpm and the supernatant was collected. The protein amount was quantified with Bradford assay (Bradford 1976), using bovine serum albumin as a standard. Experiments were performed in triplicate.

2.7. Extraction and characterization of the extracellular polymeric substances (EPS)

EPS extraction and characterization was conducted by modifying the protocol described for bacterial biofilms by Villa and collaborators (2012), on seven-day old biofilm biomass, grown in contact with 0 and 0.25 and 0.5 % ZnO-NPs. Two biofilms were resuspended in 4 ml of 2% ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, Italy) by vortexing for 5 min and homogenization for 1 min at each speed. Forty ul of Tween 20 were added and biofilm cell suspensions were shaken at 300 rpm for 3h at 4°C. The Bradford method was applied to quantify protein content, whereas the optimized microplate phenol-sulfuric acid assay was applied for carbohydrate determination (Masuko et al. 2005) using glucose as standard. The results obtained were normalized by the cellular protein concentration. Experiments were performed in triplicate.

2.8. Statistical analysis

A t-test or analysis of variance (ANOVA) via Graphpad Software (San Diego California USA) was applied to statistically evaluate any significant differences among the samples. Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Statistically significant results were depicted by p-values 0.05.

3. Results

3.1. Isolation and identification of fungi

Four fungi were isolated from the samples and were identified through molecular analysis as, *Alternaria alternata* (100% identity with closest relative KX074005.1), *Aspergillus niger* (99% identity with closest relative KX010799.1), *Penicillium chrysogenum* (99% identity with closest relative KX151962.1) and *Penicillium pinophilum* (99% identity with closest relative KU945888.1). The four species were retrieved from all the sampling points of the royal tomb, indicating a homogenous presence of fungal contamination on the deteriorated parts of mural paintings.

3.2. Zinc oxide nanoparticles characterization

The diameter and characteristics of NPs can deeply influence their biological reactivity and toxicity (Hsueh et al. 2015). To better compare the results of this research with the literature data, ZnO-NPs used in this study were derived via a wet chemical method (Yadav et al. 2006) and characterized by XRD and TEM. Figure 2a shows XRD patterns of ZnO-NPs. As no characteristic peaks of impurities were detected, it is possible to assess the good quality of the ZnO NPs synthesized. The overall pattern was comparable with XRD spectra previously reported for such ZnO-NPs in other studies (Akhtar et al. 2012). TEM revealed the average particle diameter of the ZnO-NPs to be about 30-70 nm (Fig. 2b).

3.3. Zinc oxide nanoparticles on planktonic fungi

The four fungal species were inoculated on PDA plates with increasing concentrations of ZnO-NPs (0, 0.125 and 0.25 %) in order to evaluate the effects on planktonic growth. *P. pinophylum* was the most sensitive species with a reduction of 57% of growth diameter with respect to the control in the presence of 0.125% of ZnO-NPs. The same ZnO-NPs concentration reduced *P. chrysogenum* growth 36% and *A. alternata* growth 27%. The least sensitive fungus was *A. niger*, its growth diameter was reduced 13% in the presence of 0.125% ZnO-NPs. Exposure to 0.25% ZnO-NPs slightly exacerbated the growth reduction for *P. pinophylum* (68%) and for *P. chrysogenum* (39%), while no additional effect was measurable for diameters of *A. niger* and *A. alternata* with respect to the lowest nanoparticle concentration. Standard deviation was always less than 20% of the measure.

3.4. Colony biofilm formation

The colony biofilm methodology had already been used to grow sub-aerial biofilms of bacteria (Gambino et al. 2015). In this study, the technique was adapted for the cultivation of fungal biofilms, adjusting the number of inoculated spores to 10^6 spores, the time between the media changes to 2 days and the monitoring period extended to 2 weeks.

The quantification of proteins was used to monitor the biofilm growth. Given the high physical resistance and hydrophobicity of fungal biofilm, a specific protocol was set up, one that combines both physical (vortexing with beads) and chemical (high molarity urea for 48 h) steps to effectively and homogenously extract biofilm proteins. *A. niger* sessile growth was the most rapid (2939 ± 673 μg of cell proteins), while the slowest fungal biofilm was that of *A. alternate* (359 ± 11 μg of cell proteins),

3.5. Zinc oxide nanoparticles effect on fungal biofilms

The fungal biofilms were, from the first day of growth, exposed continuously to 0, 0.25 and 0.5 % ZnO-NPs, and their development was monitored by protein quantification (Figure 3). It was seen that the growth rate of the *P. chrysogenum* (days 4, 7 and 9; Fig. 4c) and *P. pinophylum* (days 7 and 9; Fig. 4d) biofilms at both 0.25 and 0.5% concentrations was severely slowed down by the ZnO-NPs. The monitoring of *A. alternata* biofilm revealed that both concentrations of ZnO-NPs affected the morphology and growth of colony biofilms up to day 7 only, possibly because of a phase of adaptation to the stressful condition. The only exception being *A. niger* biofilm where the lower ZnO-NPs concentration (0.25%) was enough to invert the trend by significantly increasing biofilm growth at day 7.

In addition to affecting biofilm growth, ZnO-NPs also affected biofilm morphology. Figure 4 shows the biofilm morphology of the four fungi exposed to increasing ZnO-NPs concentrations after 9 days of growth. For all the fungi, exposure to ZnO-NPs caused a change in the aerial part of the biofilms, stimulating the earlier production of spores in *A. alternata* and *A. niger* and coloured compounds in *P. chrysogenum* and *P. pinophylum*, as revealed by the chromatic change in biofilm exposed to ZnO-NPs, with respect to the control.

3.6. Extraction and characterization of the extracellular polymeric substances (EPS)

Morphological differences were observable in the ZnO-NP treated biofilms. Given that these differences are often due to different structures in the matrix the fungal biofilm matrix was separated out from the cells, and the main components (i.e., proteins and polysaccharides) were quantified. The protocol employed to separate matrix and cells was achieved by modifying the traditional method used for bacterial biofilms (Villa et al. 2012): there was the addition of a homogenization step and the use of Tween20, a non-ionic detergent used as an emulsifying agent to prepare stable oil-in-water; this increases the contact between matrix components and EDTA.

According to our results, the polysaccharide and protein quantities in the fungal biofilm matrix were species-specific. As shown in Fig. 5, *A. alternata* matrix biofilm contains many more polysaccharides (10862.7 ± 834 μg μg^{-1} cell proteins) than *P. chrysogenum* (1890.4 ± 557 μg μg^{-1} cell proteins), *P. pinophylum* (1235.4 ± 448 μg μg^{-1} cell proteins) and *A. niger* (889.7 ± 133 μg μg^{-1} cell proteins).

Also in the case of EPS proteins, *A. alternata* biofilm matrix (0.142 ± 0.010 μg μg^{-1} cell proteins) was richer than *P. pinophylum* (0.043 ± 0.005 μg μg^{-1} cell proteins), *P chrysogenum* (0.021 ± 0.003 μg μg^{-1} cell proteins) and *A. niger* (0.015 ± 0.003 μg μg^{-1} cell proteins).

The effect of NPs in shaping the characteristics of the biofilm matrix was likewise various.

The ZnO-NP treated *P. chrysogenum* and *P. pinophylum* biofilms showed increased exopolysaccharide and exoprotein content, and, whereas in the *A. alternata* biofilm matrix ZnO-NPs increased the matrix proteins there was no quantifiable change in the polysaccharides. With regard to the *A. niger* biofilms no statistically significant difference was detected in the matrix components.

On separating the biofilm matrix from cells, it was evident from the different colour of the suspension of EPS that *P. chrysogenum* and *P. pinophylum* biofilms treated with ZnO-NPs were affected by the production of compounds of different colour - or different quantities - from the control, turning from orange to yellow in the first case and from red to pink in the second case

(Fig. 6). No comparable effect on the production of coloured compounds was observable for *A. alternata* and *A. niger*.

4. Discussion

4.1 Effect of zinc oxide nanoparticles on planktonic fungi

In this study, four fungal species (*A. niger*, *A. alternata*, *P. chrysogenum*, *P. pinophylum*) were isolated from the tomb of Tausert and Setnakht in the Valley of the Kings. Parts of the tomb are in a bad conservation state and show evident microbiological growth, seriously damaging the ancient mural paintings. An intervention to save these paintings is urgent and a strategy to preserve the paintings from future microbiological attack is needed.

In the last ten years, nanoparticles have been proposed in various fields as new antimicrobial compounds, able to eradicate bacterial and fungal colonization (Rai et al. 2012; Sawai and Yoshikawa 2004). Recently, ZnO-NPs were proposed as an antifungal strategy for cultural heritage, thanks to their efficacy against a number of microorganisms (Janaki et al. 2015) and their transparency, thus maintaining the readability of artworks without damaging their aesthetics (Ditaranto et al. 2015; van der Werf et al. 2015). In this study, ZnO-NPs were produced in a commonly equipped laboratory according to the protocol of Yadav et al. (2006), obtaining ZnO-NPs of a size of between 30-70 nm (Fig. 2).

A comparison with literature data is difficult because of the different formulations, targets and experimental strategies used to verify the antifungal efficacy of ZnO-NPs (Lipovsky et al. 2011; He et al. 2011; Ditaranto et al. 2015; Terzi et al. 2016). The ZnO-NPs produced in the present study were effective in delaying the planktonic growth of the four isolated fungi at a concentration of 0.25%. Thus, further experiments on biofilm inhibition activity were carried out considering 0.25% as the lowest ZnO-NP concentration to test. Considering that the resistance of the biofilms to the antimicrobial compounds could nullify treatments previously shown to be very effective against planktonic cells (Polo et al. 2011), the antibiofilm experiments also included 0.5% as the highest concentration.

4.2 Effect of zinc oxide nanoparticles on the growth of fungal biofilms

Fungi grow on cultural heritage surfaces as sub-aerial biofilms, communities embedded in a matrix of self-produced substances growing on a solid surface and exposed to the atmosphere (Dornieden et al. 2000; Chertov et al. 2004; Goburshina 2007). Given that biofilm response to antimicrobial compounds can be dramatically different from planktonic response, it was necessary to set up a protocol to test biofilm inhibition strategies in the laboratory. While such a protocol was developed recently for dual species biofilm of prokaryotes (Villa et al. 2015), there is no protocol for exposing a fungal subaerial biofilm to antibiofilm compounds. Here we propose colony biofilm as an easy method to grow fungi as subaerial biofilm and to expose it to two different ZnO-NP concentrations.

The ZnO-NPs were very effective in slowing down the biofilm growth of the two *Penicillium* spp. (0.25%) and *A. alternata* (0.5%), confirming ZnO-NPs as a promising solution to inhibit fungal biofilm formation. The mechanism of action of ZnO-NPs is still controversial (Zhang et al. 2008; Lipovsky et al. 2011). The *A. niger* biofilm was more resistant to NP exposure and the concentration of 0.25% triggered its growth, probably due to a dose-response phenomenon called hormesis, which is characterized by low-dose stimulation and high-dose inhibition (Southam and Ehrlich 1943; Calabrese et al. 2011; Gambino and Cappitelli 2016). This raises

292 the possibility that ZnO-NPs could elicit a hormetic response in fungal biofilm, with important
293 consequences for the conservation of cultural heritage, i.e. different antimicrobial doses can
294 either kill or promote resistance mechanisms in the sessile community, thus allowing
295 biodeterioration agents to grow even in presence of NPs.
296 *4.3 Effect of zinc oxide nanoparticles on the matrix of fungal biofilms*
297 As demonstrated by the change in biofilm morphology (Fig. 3), and in the composition of the
298 exopolymeric matrix (Fig. 4), ZnO-NPs affect the development of fungal biofilm.
299 In *A. alternata* and *A. niger* biofilms, the exposure to ZnO-NPs led to a more rapid sporulation
300 and, according to the EPS characterization, stimulated the accumulation of proteins in the
301 matrix of *A. alternata* biofilm. The sporulation process in these species leads to mannitol
302 accumulation in the conidiospores to protect the spores from stress (Ruijter et al. 2003; Calmes
303 et al. 2013). *A. niger* is a saprophytic fungus, pathogenic in immune-compromised patients
304 (Kousha et al. 2011), and *A. alternata* is a phytopatogen that causes considerable economic
305 losses worldwide (Hausland and Leiminger 2009). Unfortunately, the quantification of the
306 exopolysaccharides with the sulphuric acid test did not allow the distinguishing of the sugars
307 formed. Further experiments are necessary to verify mannitol accumulation in *A. niger* and *A.*
308 *alternata* biofilm matrixes in response to ZnO-NP exposure. Another strategy adopted by *A.*
309 *niger* to protect cells from stress is, during spore formation, the synthesis of melanins, dark
310 pigments formed by the polymerization of phenolic compounds, for deposition in the cell wall
311 (Calvo et al. 2002). As visible from the chromatic differences in treated and control biofilms
312 (Fig. 4), *A. niger* biofilms treated with ZnO-NPs proceed towards sporulation, and melanin
313 production, faster than the control. This response could provide a selective advantage to *A.*
314 *niger*, conferring a long-term resistance over other fungal species in the presence of ZnO-NPs.
315 The *Penicillium* species are well-known producers of secondary metabolites, e.g., antibiotics
316 and pigments (Peng et al. 2014). According to our results, *Penicillium* spp. biofilm exposure to
317 ZnO-NPs interferes with the production of coloured compounds, possibly triggering the
318 synthesis of either different products or of the same product in different quantities. This was
319 evident from the chromatic changes observable in both the biofilm morphology (Fig. 4) and the
320 solution containing EPS of biofilm exposed to ZnO-NPs (Fig. 5). Environmental conditions
321 strongly affect fungal metabolism, by both directly interfering with metabolite production and
322 accelerating the developmental process (Medina et al. 2015; Schmidt-Hejdt et al. 2015).
323 Although further studies are needed to identify the synthetized coloured compounds in
324 *Penicillium* biofilms, such compounds could have an important role in buffering stress caused
325 by exposure to ZnO-NPs. In addition, EPS characterization of *Penicillium* biofilm matrix also
326 demonstrated that ZnO-NPs affect both the protein and the polysaccharide content. A similar
327 effect has already been observed in sub-aerial biofilms of *B. subtilis* exposed to sub-lethal
328 concentrations of silver nanoparticles (Gambino et al. 2015). EPS in sub-aerial biofilms plays
329 an important role as a barrier against environmental stresses (Sutherland 2011), in gathering
330 toxic compounds, and limiting the diffusion of Reactive Oxygen Species (ROS) released by
331 nanoparticles (Peulen et al. 2011).
332
333 **5. Conclusion**
334 ZnO-NPs have been proposed as an innovative strategy to inhibit fungal colonization of cultural
335 heritage. By testing the ZnO-NP effect on the sub-aerial biofilms of 4 fungi isolated from the

tomb of Tausert and Setnakht in the Valley of the Kings, we proved that ZnO-NPs could be a valid option to prevent fungal growth on mural paintings. Nevertheless, the response of the fungal biofilms after exposure to ZnO-NPs could lead to the acceleration of the sporulation process and an earlier production of metabolites, two events that could be detrimental to cultural heritage conservation. Further studies to identify the metabolites produced and the pathways involved are necessary.

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492

493 **Table and figure capture**

494 Figure 1. Two pictures of the sampling area, a) the burial chamber and b) the corridor of the
495 tomb.

496 Figure 2. Characterization of produced ZnO-NPs by a) XRD and b) TEM.

497 Figure 3. Colony biofilm growth monitored by quantification of cell proteins. Data represent
498 the means + the SD of three independent measurements. The histograms provide the *p*-values
499 obtained by ANOVA analysis. According to *post hoc* comparison of results (Tukey's HSD, p
500 <0.05), means sharing the same letter are not significantly different.

501 Figure 4. Top view of the fungal colony biofilms (from left, *A. alternata*, *A. niger*, *P.*
502 *chrysogenum*, *P. pinophylum*) exposed to increasing concentrations of ZnO-NPs (from top, 0,
503 0.25 and 0.5%).

504 Figure 5. Extracellular proteins and polysaccharides in the matrix of mature biofilms of a) *A.*
505 *alternata*, b) *A. niger*, c) *P. chrysogenum*, d) *P. pinophylum*. Data represent the means ± the SD
506 of three independent measurements. Letters provide the graphical representation for *post hoc*
507 comparisons. The histogram provides the *p*-values obtained by ANOVA analysis. According
508 to *post hoc* analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly
509 different from each other.

510 Figure 6. EPS of fungal biofilms exposed to ZnO-NPs before filtration.