

# Mold Fungi *Syncephalastrum contaminatum* STG-160 and *Cladosporium sphaerospermum* STG-161 from 16 th century icon—Risks of Tempera Pigments Degradation

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## Article

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## Abstract

Many art works, especially tempera paintings on wood, like a "layer cake"—contain a rich organic (animal glues, binders, plasticizers, resins, plant-based pigments) and inorganic components (mineral and synthetic pigments)—all of which can serve as an excellent nutrient medium for mold fungi. As mycelial hyphae grow into the organic "framework," pigment particles become trapped within the growth zone. As it turns out, not all the pigments are equally vulnerable to fungal damage. In the current study, the  $IC_{50}$  for  $Fe^{3+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$ , and  $Cd^{2+}$  was identified for two mold fungi isolated on the 16th century Russian icon "Descent into Hell": *Syncephalastrum contaminatum* STG-160 (4.26, 10.8, 0.22, and 0 mmol/L, correspondingly), and for *Cladosporium sphaerospermum* STG-161 (0.81, 2.16, 0.11, and 0.017 mmol/L, correspondingly). Filamentous fungus *S. contaminatum* STG-160 demonstrated robust tolerance to  $Fe^{3+}$ ,  $Zn^{2+}$ , and high sensitivity to  $Cd^{2+}$ , while melanotic fungus *C. sphaerospermum* STG-161 exhibited resistance to  $Cd^{2+}$ . Whole-genome sequencing of the two fungi revealed two gene clusters in *S. contaminatum* STG-160 in regions 30.1 and 67.1, responsible for the synthesis of siderophores capable of supplying the cell with vital  $Fe^{2+}$  by chelating relatively inert  $Fe^{3+}$  from ochres, siennas, red, brown, and green earth pigments. No siderophores were detected in *C. sphaerospermum* STG-161. The combined results of both *in situ* experiment on mock layers and *in vitro* tolerance testing to certain heavy metal ions allow to identify pigment areas within tempera paintings that require more thorough treatment with biocides for enhancing their aesthetic value and ensuring appropriate conditions for display and storage.

## Introduction

Many restoration workshops, during the restoration process of paintings, whether it is tempera on wood, canvas, or fresco on a wall—have encountered the question: why some areas of pigments show no biodegradation or just trace amounts, while other areas may be significantly affected. Paintings are composed of a support (canvas, wood, paper, or parchment), a ground layer (gesso), and a paint layer, the chemical composition of which varies according to the painting medium (oil, watercolor, acrylic, guash, or tempera) [1]. If the question is icons, then the basis is a wood (in ancient Rus', linden, pine, maple, cypress, and spruce wood were used) [2]. Next, the fabric is glued onto the canvas using fish glue (often sturgeon) or hide or bone glue, which is first soaked in cold and then boiling water to remove adhesive materials that increase the strength of the fabric - starch, polyvinyl alcohol, polyacrylamide, etc.); then a primer is applied, prepared from chalk or slaked gypsum in animal glue. Afterwards the main depiction is painted with ochre paint, sometimes scratching with a sharp needle, creating a graphic effect [2]. After these stages, the background surfaces (halos, clothing elements) are gilded, and mordant (natural resins and oils) is used for gluing to adhere the gold leaf to the surface [3]. Next, the drawing is recorded with mineral, organic, and/or synthetic pigments mixed in egg yolk or egg white. First of all, these are orange-brown earth pigments containing iron oxide (ocher, umber, sienna) and green pigments (glauconite—hydrous iron aluminosilicate, volkonskoite—kaolinite of the group of hydrous aluminum silicates, malachite—hydrous copper carbonate); red pigments, for example, cinnabar, the red color of which is due to the predominant content of mercury sulfide; yellow pigments (strontium yellow, cadmium lemon, etc.), blue and blue pigments ... (azurite - natural copper carbonate, under the influence of atmospheric moisture and binders can slowly turn into green copper carbonate - malachite, lazurite - a sulfate-containing opaque mineral); white (lead, titanium, zinc)[1, 4]. Mold microscopic fungi may attack tempera paintings by binder degradation [5], by weaken mechanical strength of the support or chalk-rich gesso and loss of flexibility of paint layers [6, 7], by pigment chromatic alterations, such as masking, discoloration, pitting and cracking[8][9, 10] causing aesthetic damage.

A numerous investigations illustrating the biodeterioration process of cultural heritage sites have been published [11, 12]. The most common recognized fungi that degrade heritage objects belong to the genera: *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria*, *Trichoderma*, and *Aureobasidium* [13]. As it turns out, not all the pigments are equally vulnerable to fungal damage. The investigation[14] has shown that original materials with iron-oxide and ultramarine pigments on 17th century ceiling in Slovenia are primarily degraded by *Aspergillus* species. *Cladosporium*, *Penicillium* and *Aspergillus* were able to grow in the presence of yellow ochre and ivory black during 18th century canvas painting restoration in Italy [15].

The ability of mold fungi to degrade artistic mediums on heritage sites is explained by their smart metabolic adaptation system determined by their enzymatic diversity and intensity (caseinases, amylases, gelatinases, acid phosphatase,  $\beta$ -glucosidase, as well as esterases, lipases, lecithinases, etc.) allowing to solubilize binders, adhesives and pigments [16] excretion of secondary metabolites such as organic acids (gluconic, citric, oxalic, malic, succinic, itaconic, etc.) which results in the formation of organic salts and complex compounds and which further facilitates the biodeterioration process[17]; the ability to utilize heavy metal ions from mineral pigments for their own benefits [18].

The main goal of this investigation was to evaluate the risks of biodegradation of art pigments used for the contemporary restoration with preliminary determination of their chemical composition and following strategy prediction for preventive protection of tempera paintings. The microscopic mold fungi *S. contaminatum* STG-160 (PRJNA1431907) and *C. sphaerospermum* STG-161 (PRJNA1431979) isolated on the 16th century icon "Descent into Hell" from the State Tretyakov Gallery and identified in current study were the main objects of the investigation.

## Materials and methods

### PCR Amplification and Whole genome sequencing

Ten samples (Fig. 1) were collected from the 16th century icon “Descent into Hell” (78 cm ×64 cm ×3 cm) from the State Tretyakov Gallery and cultivated as described previously [19]. PCR Amplification was performed with fungal isolates no. 5 (STG-161 (PV636606.1)) and no. 6 (STG-160 (PV645139.1)) using primer pairs for hypervariable regions of rDNA: Internal Transcribed Spacer–ITS1 and ITS4 [20]; for locus Calmodulin–CMD5 and CMD6 [21]; for locus Actin–ACT-512 and ACT-783 [22].

Illumina technology was used for the whole-genome sequencing. The NEBNext ultra II library was prepared, and sequencing was performed on a FastaSeq300 (GeneMind) using a 2×300 paired end reads (bp). Adapter sequences in the resulting reads were removed using Cutadapt, and low-quality reads (Q < 33) were removed using Sickle. The metagenomic sequences obtained on the platform were assembled into contigs using SPAdes v.4.0.0. Open reading frames (ORFs) in the contigs were identified using Prodigal v2.6.3. Genome annotation was performed using the RAST server, with subsequent annotation correction by comparing the predicted protein sequences with the National Center for Biotechnology Information (NCBI) databases. Genome for gene clusters has been performed by using the online antiSMASH tool 8.0.4. <https://antismash.secondarymetabolites.org/#!/about>, accessed on 05 March 2026).

### Mock Layer Design and Inoculation

To determine the degree of biodegradation, 2x2 cm wooden mock layers with various art materials were manufactured as a prototype of the miniature icon. The list of materials applied onto the top of the wood panel was classified into three groups: i) linen pavoloka, gypsum gesso with sturgeon glue (30 g/l), as well as gesso with sturgeon glue and with antiseptic benzalkonium chloride (BAC, 1%), and adhesives and binders (egg yolk emulsion consisting of egg yolk, water, and dry white wine [1:2:1]); ii) the colour palette comprising dry pigments in egg yolk emulsion: cadmium yellow, english red, grey-green, zinc white, zinc white and burnt sienna, zinc white and golden ochre, burnt sienna and shellac, cobalt green and burnt sienna; and iii) varnishes: acrylic varnish and linseed oil, and mordant as an adhesive for gilding. The inoculation of each fragment was provided as described before [19]. Fungal growth on mock layers was measured on 3, 6, 9, 12, 15 d, etc. till 30 d. The dynamics of the biodeterioration was estimated by measuring the diameter of the colonies. Measurements were performed in triplicate and repeated at least twice.

### Phenotypic Characterization of Fungal Strains

The morphology of conidiospores and hyphae in PDA medium was studied using the Carl Zeiss Jena microscope (Jena, Germany) at magnification ×1000. The detection of the fluorescent signal of the stain Calcofluor White (CFW, Sigma-Aldrich, St. Louis, MO, USA) was achieved through Celena X microscope (Logos Biosystems, Anyang-si, Korea), equipped with a specific filter set comprising an excitation filter 375/28, an emission filter 460/50, a lens plan apo fluor oil coverslip corrected 100x, with a numerical aperture of 1.25 and a working distance of 0.19.

### Energy-dispersive X-ray spectroscopy of pigments

During scanning electron microscopy (SEM) analysis of the pigments' microstructure, their elemental composition was studied. Spectra of the samples were recorded using Energy-dispersive X-ray spectroscopy (EDX) on a next-generation Ultim Max 100 with an active crystal area of 100 mm<sup>2</sup> (Oxford Instruments Group, Great Britain). Processing these spectra allowed to determine the list and concentration of the chemical elements. This method involves exposing the sample to an electron beam at a high accelerating voltage, causing it to emit characteristic X-rays. Analysis of the resulting spectrum profile allows to quantify the atomic or mass content of elements (from beryllium to californium). The thickness of the analyzed layer, depending on their atomic number, ranges from hundreds of nanometers to 5 micrometers. In addition to determining composition, the method allows to create maps of the elemental distribution across the surface, enabling to assess and visualize the homogeneity of the material and determine the composition of individual components.

### Fifty percent growth inhibitory concentration (IC) determination and growth measurement in metal presence

Conidiospores of *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 with optic density of OD<sub>600</sub> = 0.35 were inoculated onto 6-well culture plates with Chapek-Dox agar medium (CDA, g/L: sucrose – 30; NaNO<sub>3</sub> – 2; K<sub>2</sub>HPO<sub>4</sub> – 1; MgSO<sub>4</sub>·7H<sub>2</sub>O – 0.5; KCl – 0.5; FeSO<sub>4</sub>·7H<sub>2</sub>O –

0.01; agar – 20; pH 7.1) containing metal ions of ferrum (Fe<sup>3+</sup>), zinc (Zn<sup>2+</sup>), cobalt (Co<sup>2+</sup>) and cadmium (Cd<sup>2+</sup>) in following concentrations: Fe<sup>3+</sup> and Zn<sup>2+</sup> – 0.1, 0.25, 0.5, 1.0, 1.5, 3, 6, 9, 11 mmol/L; Co<sup>2+</sup> and Cd<sup>2+</sup>–0.1, 0.25, 0.5, 1.0, 1.5, 3 mmol/L and incubated at 26°C for 3 days. Due to the permissible high toxicity of cadmium, an additional concentration panel was used for this metal 0.01, 0.025, 0.05, 0.075 mmol/L. The 1 M stock solutions of Fe<sup>3+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup> using FeCl<sub>3</sub>·6H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, CoCl<sub>2</sub>·6H<sub>2</sub>O and 10 mM stock solution of Cd<sup>2+</sup> using CdCl<sub>2</sub>·5H<sub>2</sub>O were made in double distilled water.

Colony diameter was measured in mm at 24 h, intervals for 24 h, duration 3 d. Measurements were performed in triplicate and repeated at least twice. Fungi growth inhibition (FGI) was determined by the following formula (1):

$$FGI (\%) = \frac{D_c - D_t}{D_c} \times 100\% \# (1)$$

Wherein, D<sub>c</sub> indicates the colony control diameter (mm) on CDA media without metal addition, D<sub>t</sub> indicates the experimental colony diameter during growth on CDA with metals. Measurements were performed in triplicate and repeated at least twice. IC<sub>50</sub> value for metal ions was derived from graphical plots of growth inhibition versus metal ion concentration.

## Results

### Whole genome sequencing

The whole genome sequences for fungi isolated in samples no. 5 and no. 6 (Fig. 1) have been deposited in the NCBI database as *Cladosporium sphaerospermum* STG-161 PRJNA1431979 (BioSample ID: SAMN56348019) and as *Syncephalastrum contaminatum* STG-160 PRJNA1431907 (BioSample ID: SAMN56344082), correspondingly. The total number of contigs obtained for *Cladosporium sphaerospermum* STG-161 is 2004 with total 2472087716 bp, GC of which are 52.9081791112006; and for *Syncephalastrum contaminatum* STG-160 is 924 contigs with total 3801115290 bp, GC of which are 47.36319318.

In total, 31 secondary metabolites regions were predicted for *Syncephalastrum contaminatum* STG-160, covering 4 NRPS (nonribosomal peptide synthetases), 9 TPC (two-pore channels), 2 NI-siderophores (NRPS-independent)–in the region 30.1 with 44,188 bp, and in the region 67.1 with 33,521 bp, and 16 other regions. (Fig. 2).

In total, 53 regions were predicted for *Cladosporium sphaerospermum* STG-161, covering 15 NRPS, 7 PKS (polyketide synthases), 12 TPC, 2 Fungal-RiPP (fungal ribosomally synthesised and post-translationally modified peptides), 4 Hybrids, 13 other regions, and no NI-siderophores regions (Fig. 2).

### Morphological characterization of fungal strains on mock layers

Mock layers with art materials were subjected to biodegradation by *S. contaminatum* STG-160 (Fig. S1) and *C. sphaerospermum* STG-161 (Fig. S2) with different rates. The active growth of *S. contaminatum* STG-160 began within 2–3 d on the egg yolk emulsion, on pavoloka with the sturgeon glue, and on the gesso with sturgeon glue (with and without BAC). The fungal mycelium quickly entered the sporulation stage, indicating the most favorable growth conditions on these mock layers. Next in destruction score came the english red, grey-green, cadmium yellow, burnt sienna with shellac, and cobalt green with burnt sienna. It can be seen that zinc white and mixed pigments with zinc white have undergone almost no degradation. Mordant degraded faster than all other varnishes; acrylic varnish and linseed oil caught up with it only within 10 d.

Herein the STG-160 mycelium turned gray entering the deep sporulation stage within 10 d on egg yolk (Fig. 3A). In transmitted light, sporangium and sporangiophores with vesicles can be clearly distinguished. The mock layer with english red (Fig. 3B) showed a bit slower rate of degradation than the egg yolk, but on the 10 d it showed almost complete damage to the surface by STG-160. It is possible to distinguish a larger quantity of aerial mycelium with young merosporangia, clearly glowing with CFW stain which is explained by the presence of large amounts of polysaccharides in the young cell walls. Compared to the active damage of the egg yolk and english red, the mixed pigments of the cobalt green with burnt sienna showed significantly low surface damage by STG-160 (Fig. 3C). Sporangiophores are noticed without merosporangia, unlike the well-developed aerial mycelium; rhizoids are clearly visible. Zinc white (Fig. 3D) and other mixed pigments with zinc white showed the lowest biodegradation compared to other pigments, adhesives, binders, and protective varnishes (Fig. S1). Zinc white revealed a dried film of fungal substrate mycelium, and it was very dwarfed as if dried out.

Mock layers with inoculated *C. sphaerospermum* STG-161 are distinguished by the appearance of a characteristic black melanotic pigmentation on the top (Fig. S2). Basis and adhesives with binders were degraded first (the same case as with *S. contaminatum* STG-160)

but all the mock layers with the earth pigments as well as with zinc white are practically unaffected by *C. sphaerospermum* STG-161. The characteristic fluorescence of *C. sphaerospermum* STG-161 distinguish the segmented and more branched hyphae on egg yolk in contrast to cobalt green with burnt sienna (Fig. 4A, B).

The results of this experiment revealed that some art materials biodegrade faster than others (Fig. 5). The most rapidly degraded materials for *S. contaminatum* STG-160 which showed 100–70% of the damage on 3–6 d of inoculation is the egg yolk emulsion, the pavoloka as a basis glued with the sturgeon glue; the ochre, the english red and grey-green pigments; the gilding composition mordant; the gypsum gesso with sturgeon glue and with sturgeon glue with BAC.

The mock layers with less than 50% of biodegradation classified as moderately affected are: the linseed oi, cadmium yellow, cobalt green with burned sienna. The art materials with 1% of the degradation and less are classified as slowly degradable: zinc white and combinations of zinc white with golden ochre, zinc white with burnt sienna, burnt sienna and shellac and the acrylic varnish. Among the rapidly degradable materials for *C. sphaerospermum* STG-161 are the pavoloka, gipsium gesso with sturgeon glue and with sturgeon glue with BAC, the egg yolk emulsion, and the mordant. Other pigments and varnishes did not show any interesting results; compositions with zinc white and its combinations were similarly to *S. contaminatum* STG-160 and practically were not affected by the fungus.

## EDX- analysis of the pigments

SEM and EDX analysis of the pigments' microstructure is applied to assess and visualize the homogeneity of the material and determine the composition of individual pigments (Fig. 6). The flakes in dry pigments are most evenly distributed in english red and zinc white with 0.1–1  $\mu\text{m}$ . Other pigments have the flakes range 0.6–50  $\mu\text{m}$ . Golden ochre, burnt sienna, grey-green and english red pigments—all of them contain Fe in concentrations (%): 5.4, 13.4, 1.4, and 37.45, correspondingly. Also, the trace amounts of metals Na, K, alkali earth metals Ca, Mg, transition elements Zn, Al, Si, Mn, Ti, and metalloids S, P are noticed in approximate quantities. Zinc white pigment is a zinc oxide, the presence of which is noted in cobalt green and cadmium yellow. Cobalt green besides the trace amounts of mentioned elements contains key atoms that determine its color—Zn 41.14% and Co 0.65%. Cadmium yellow contains key atoms of Cd 26.65%, S 26.38% and Zn 10.77%.

## Fifty percent growth inhibitory concentration ( $IC_{50}$ ) and growth measurement

Growth intensity of *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 (Fig. 8) was estimated by measurement of colony diameters on CDA medium with introduced metal ions. The results obtained clearly distinguish between two different classes of *Mucoromycetes* and *Dothideomycetes*, correspondingly, in the rate and nature of  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Co}^{2+}$  absorption (Fig. S3, Fig. S4, Fig. S5). The  $\text{Cd}^{2+}$  sensitivity test for both fungi showed very high toxicity—no colony growth of *S. contaminatum* STG-160 was observed in the specified range of concentrations, while colony growth of *C. sphaerospermum* STG-161 was slightly observed at extremely low concentrations of the metal, descending to 0.01, 0.025, 0.05, 0.075, and 0.1 mmol/L, which indicates the high toxicity of  $\text{Cd}^{2+}$  in relation to the studied microorganisms. Since these results are not included in the main panel of tested metal concentrations, they are not reflected in the diagrams. Plates with  $\text{Cd}^{2+}$  are provided in the supplementary (Fig. S6). The growth of *S. contaminatum* STG-160 on CDA medium with  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$  on 2 d showed an interesting paraboloidal dependence like both metals, with a burst of fungal growth observed in the range from 0.5 to 3 mmol/L. With  $\text{Zn}^{2+}$  the fungus grew even somewhat more actively in this range; further increase in metal concentrations above 3 mmol/L led to fungus growth inhibition (Fig. 7). Within  $\text{Co}^{2+}$  presence, a standard exponential dependence was observed for this fungus—an increasing in the metal concentration led to a decrease in the fungus growth rate. On 3 d, fungal development to a concentration of 3 mmol/L  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$  leveled off and moved from the logarithmic growth stage to a stationary one.

The growth of *C. sphaerospermum* STG-161 on CDA with  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  showed a standard exponential dependence of fungal growth inhibition with increasing metal concentration, in contrast to the increased growth of *S. contaminatum* STG-160 on CDA with  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$  in the range from 0.5 to 3 mmol/L.

The calculation of  $IC_{50}$  which is required to inhibit 50% of a biological process (the fungus growth) has shown varying degrees of *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 tolerance to different metal ions (Fig. 8). To calculate the  $IC_{50}$ , an averaged trend line was constructed. This required the Reliable Approximation Value ( $R^2$ ), the coefficient of determination, which serves as a numerical indicator of quality. For acceptable models, the coefficient of determination should be at least 50%. Models with a coefficient of determination above 80% can be considered sufficiently good. In this regard the logarithmic trend lines were chosen for *C. sphaerospermum* STG-161 on  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ , and for *S. contaminatum* STG-160 on  $\text{Co}^{2+}$ ; polynomial trend lines for *S. contaminatum* STG-160 on  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$ . For  $\text{Cd}^{2+}$  a graph of  $IS_{50}$  was provided only for *C. sphaerospermum* STG-161, since it was not possible to obtain *S. contaminatum* STG-160 growth on  $\text{Cd}^{2+}$  even within a low concentration of (0.01–0.1 mmol/L).

Assessing the IC<sub>50</sub> value in a metal assay allows to estimate the concentration of an inhibitory substance (metals) required to suppress the growth of the studied fungi by 50%. The lower the IC<sub>50</sub> value, the more effective the inhibitor. The IC<sub>50</sub> value depends on the experimental conditions, including the time allowed for the effect to develop; temperature; substrate concentration; and the composition and pH of the reaction medium.

Table 1  
IC<sub>50</sub> estimation

| Fungus                                                                          | IC <sub>50</sub> Fe <sup>3+</sup> , mmol/L | IC <sub>50</sub> Zn <sup>2+</sup> , mmol/L | IC <sub>50</sub> Co <sup>2+</sup> , mmol/L | IC <sub>50</sub> Cd <sup>2+</sup> , mmol/L |
|---------------------------------------------------------------------------------|--------------------------------------------|--------------------------------------------|--------------------------------------------|--------------------------------------------|
| <i>S. contaminatum</i> STG-160                                                  | 4.26                                       | 10.80                                      | 0.22                                       | 0.00*                                      |
| <i>C. sphaerospermum</i> STG-161                                                | 0.81                                       | 2.16                                       | 0.11                                       | 0.017                                      |
| *– inability to achieve 50% inhibition due to high toxicity of Cd <sup>2+</sup> |                                            |                                            |                                            |                                            |

Discussion

Herein we have identified two taxa of mold fungi from the 16th century icon “Descent into Hell” from the State Tretyakov Gallery as *Syncephalastrum contaminatum* STG-160 (PRJNA1431907) and *Cladosporium sphaerospermum* STG-161 (PRJNA1431979). The filamentous fungus *Syncephalastrum* sp. is usually characterized as tropical and subtropical soil-borne with heterothallic system of sexual and asexual reproduction with spores. A new species–*S. contaminatum* was firstly identified not long ago, in 2020 in Australia, as a laboratory contaminant [23]. It forms very fast-growing light colonies that become dark grey over the time. *C. sphaerospermum* STG-161 is a dematiaceous (dark-colored) mold fungus due to the melanin, characterized by slow growth and predominantly asexual reproduction. It remarkable, that one of the *C. sphaerospermum* strain was discovered among other melanized fungi such as *Penicillium lanosum*, *Cladosporium cladosporodites* and others in radioactively contaminated regions of the Chernobyl Exclusion Zone and Nuclear Power Plant where radiation levels are 3–5 orders of magnitude above normal background levels, show a phenomenon called “positive radiotropism” as ionizing radiation becomes an orienting factor for these molds [24, 25]. Moreover, the cultivation of *C. sphaerospermum* aboard the International Space Station was conducted, and the reduction of radiation compared to the negative control was discernable, which is potentially attributable to the fungal biomass [26].

*S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 have a varying degree of sensitivity to different art materials (Fig. 5). To understand and predict the risks of biodegradation of tempera pigments, an EDX analysis was conducted, which allowed to determine the atomic composition of the pigments used the experiment.

Cobalt green used on the top of the mock layer is considered to be similar in color to cobalt blue (CoO·Al<sub>2</sub>O<sub>3</sub>), except that ZnO partially or completely replaces the aluminum oxide—it was noted that cobalt green is made by adding a solution of cobalt salt to a paste of zinc oxide and water with drying and calcinating the mass [27]. The composition of cobalt green varies considerably: 71.5–88% zinc oxide and 11.5–19% cobalt oxide, and the amount of phosphoric acid, iron oxide, etc. also varies, depending on the production process [27]. The pigment used in our work would be more correctly called zinc containing low-cobalt green pigment, and a method for synthesizing such a pigment is known, since cobalt by itself is scarce and expensive [28].

Cadmium yellow pigment [29], from the time of its introduction as an artists’ pigment in the earlier nineteenth century has been generally known as cadmium sulfide (CdS) [27]. Its other historical names include aurora yellow, jaune brilliant as well as mutrie yellow and orient yellow, radiant yellow (trade name of the British colourmen Reeves, late nineteenth century), neutral mineral is hematite (iron oxide, Fe<sub>2</sub>O<sub>3</sub>) or/and goethite (an iron oxyhydroxide, α-FeO(OH)), also, calcite is commonly identified orange and even daffodil yellow [27]. A method for producing cadmium yellow pigment has been known since 1967 comprising a reaction an aqueous mixture of water-soluble salts of zinc and cadmium, a water-soluble sulfide selected from the group of alkali metal and alkali earth metal sulfides, a water-soluble nonsulfide material from the group consisting of aluminates, silicates, hydroxides and carbonates of alkali metals, alkali earth metals and ammonia, separating the solids from the liquids, calcining said solids under non oxidizing conditions at high temperatures [30]. The description of this pigment is largely consistent with its X EDX (Fig. 6), here the content of Cd and S predominates, each slightly more than 26%, besides which we registered significant quantities of Zn 10.77 %.

English red pigment—there is debate about the origin of the term "English red". Recent theories suggest that the pigment is synthetically based—iron oxide, produced by calcining ferrous sulfate, a byproduct of sulfuric acid production: (2FeSO<sub>4</sub>=(t°) Fe<sub>2</sub>O<sub>3</sub>+SO<sub>2</sub>+SO<sub>3</sub>) [27]. The prevalence of Fe (37.45%) and O (49.10%) atoms, as well as trace amounts of S, are confirmed by EDX (Fig. 6).

Zinc white pigment is the zinc oxide (Zn 55.28%, O 44.72%, Fig. 6).

Ochre pigments and siennas belong to earth pigments. Natural ochre pigments are complex materials which may have contributions from different sources. EDX have identified golden ochre and burnt sienna as pigments similar in chemical components (Fig. 6). The main chromophore mineral is hematite (iron oxide,  $\text{Fe}_2\text{O}_3$ ) or/and goethite (an iron oxyhydroxide,  $\alpha\text{-FeO(OH)}$ ), also, calcite is commonly identified as well as variable concentrations of Ti [31]. Siennas, however, differ in chemical composition in that they contain a small percentage of manganese oxides (less than 10%) [27] which was shown in (Fig. 8). They occupy an intermediate position between ochre and umber.

Gray-green pigment is most likely the mineral wollastonite  $\text{Ca}_3(\text{Si}_3\text{O}_9)$  in our experiment, Ca 6,24%, Si 7,14%, O 53,89% (Fig. 8). The mineral may also contain iron, aluminum, magnesium, sodium or potassium, and manganese substituting for calcium giving it a yellow, grey, red or brown color, in our case—the color of the pigment is brownish ochre.

Shellac included in the composition of the top layer with burnt sienna in our experiment is a sesquiterpenic resin secreted by an insect (*Kerria lacca*) and composed mainly of polyesters of oxyacid [32].

After identifying the prevailing metal ions ( $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ) in pigments the IC<sub>50</sub> for *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 was detected by introducing these

metals into a minimal nutrient medium. It turned out to be that the IC<sub>50</sub> is in 5.25 times higher for  $\text{Fe}^{3+}$ , in 5 times higher for  $\text{Zn}^{2+}$  and in 2 times higher for  $\text{Co}^{2+}$  for *S. contaminatum* STG-160 compared to *C. sphaerospermum* STG-161 (Fig. 8, Table 1). It would seem, that a fast-growing and aggressive filamentous fungus of the *Mucormycetes* class should behave similarly towards cadmium, but the picture for this toxic metal is different—it was impossible to achieve 50% inhibition since it was basically impossible to grow *S. contaminatum* STG-160 on a medium with cadmium even with a reduced metal concentration of 0.01, 0.025, 0.05, 0.075 and 0.1 mmol/L. And dark melanotic mold *C. sphaerospermum* STG-161 showed a timid, but still successful, tolerance to cadmium.

Many metals are required in trace amounts by the cells of microscopic fungi. Some of the heavy metals such as iron, zinc, copper and cobalt are required for normal functions of living organisms, they participate in various biochemical processes, acting as cofactors of enzymes or essential elements for maintaining life, but they can be toxic if excess amounts are accumulated. And the higher concentrations of cadmium, chromium, manganese, and lead are studied to be very toxic [31, 32]. They can damage cell membranes, inhibit respiration, alter enzyme activity, disrupt transcription and translation processes, cell division, and damage DNA and proteins. Mold fungi are found to be adaptable to high concentrations of heavy metals through different strategies which include extracellular metal sequestration and precipitation, suppressed influx, enhanced metal efflux, production of intracellular/extracellular enzymes, metal binding to cell walls, intracellular sequestration and complexation [33]. Iron is essential for growth of most living organisms. One of the ways of vital iron utilization by fungi is the production of siderophores—a low molecular weight molecules, which are secreted into the surrounding environment by the cell where they chelate relatively inert  $\text{Fe}^{3+}$  [34, 35] and supply it back to the cell in the form of  $\text{Fe}^{2+}$  [36, 37]. Whereas, in bacteria, the metal-binding receptor is catecholate functional group [38]. Mechanisms of siderophore-bound iron return to the fungi cell has been well studied. The process is regulated by four pathways [37]: (1) in the shuttle mechanism, the  $\text{Fe}^{3+}$ -siderophore complex is transported across the cytoplasmic membrane by siderophore specific transport proteins and the iron is reductively removed from the siderophore complex inside the cell [39]; (2) in the taxicab mechanism,  $\text{Fe}^{3+}$  is transferred from extracellular siderophores to intracellular ligands across the cytoplasmic membrane [40]; (3) in the hydrolytic mechanism, the  $\text{Fe}^{3+}$ -siderophore is transferred inside the cell by specific transport proteins and the metal is removed by simultaneous reduction and degradation of the siderophore by esterases [41]; and (4) in the reductive mechanism, the iron is reduced by membrane bound ferrireductases and  $\text{Fe}^{2+}$  is transported into the cell [34], the siderophore remains outside the cell. The mechanism of iron utilization from the environment has been well studied in the class of *Mucormycetes* in the family *Syncephalastraceae* [37]. As a result of whole-genome sequencing it was established that *S. contaminatum* STG-160 has two genes' clusters mapping in the regions 30.1 and 67.1 (Fig. 2) which are responsible for the synthesis of siderophores. Thus, it can be assumed that the high affinity of *S. contaminatum* STG-160 for iron-containing pigments, especially natural ochres, siennas, red, brown and green earth pigments, is explained by the presence of siderophores. *C. sphaerospermum* STG-161 showed lower tolerance to  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$ , perhaps this is partly explained by the absence of siderophores.

The growth pattern of *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 with  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$  is largely identical (Fig. 7). In eukaryotes, Zn homeostasis is attributed to the coordinated action of two transporter families: zinc-iron permease or ZRT-IRT-like Protein and the Cation Diffusion Facilitator families [42], and the last one has been recently divided into three groups: the first one was identified in *Rhizoglyphus irregularis*, the second in *Hebeloma cylindrosporum* and the third is in *Oidiodendron maius* [18]. Little is known about Zn, Co, and Cd transporters in *S. contaminatum* (as it is a relatively new species) and *C. sphaerospermum*, and this probably be the focus of our next

experiment. However, it was shown with *Saccharomyces cerevisiae* cells that Co and Cd have similar coordination chemistries to Zn and bind to protein

ligands in a similar fashion but just mimic zinc as a functional enzymatic cofactor [31].

Cadmium is the seventh most toxic heavy metal as per Agency for Toxic Substance and Disease Registry ranking [43]. A human may encounter cadmium during his professional activities including the preparation of painting pigments [44]. The primary impact of Cd's toxicity is mainly on fungi's growth and replication. Some fungal species may be eradicated following cadmium exposure in soil, however, in this case of low-level exposure, the selection may occur in favor of the emergence of a more resistant strain [45]. Current experiment demonstrated weak, but still some resistance to cadmium of *C. sphaerospermum* STG-161 with  $IC_{50}$  0.017 mmol/L. Several fungi that were highly tolerant to  $Cd^{2+}$  were found [46], and the fungus *Cladosporium* sp. was among them. *S. contaminatum* STG-160 turned out to be sensitive to cadmium possibly for the following reason: zinc and cadmium share similar chemical properties—the zinc:cadmium ratio of most minerals and soils ranges from 100:1 to 12.000:1 [47]. Unlike zinc that is important for the activity of many metalloenzymes and metal-dependent enzymes, and is complexed with nucleoproteins and other nonenzyme proteins and influences their tertiary and/or quaternary structure [48], cadmium can enter the fungal cell through zinc-transporting proteins and replace zinc by complexing to metallothionein (a family of ubiquitous cysteine-rich low molecular weight proteins) resulting in toxic effect [49]. Most likely, the cadmium-tolerant fungus *C. sphaerospermum* STG-161 uses the ability to form metallothionein complexes with toxic metals to its advantage—it prevents heavy metals from interfering with cellular functions, thereby protecting itself (Table 1, Fig. S6).

*In situ* experiment on mock layers and *in vitro* analysis with metals on 6-well culture plates with CDA show slightly different results. Thus, on mock layers with zinc white and other mixed pigments with zinc, neither *S. contaminatum* STG-160 nor *C. sphaerospermum* STG-161 practically did not develop and were in a depressed state. Meanwhile, on the agar medium containing zinc ions, growth of both fungi was observed. Moreover, a parabolic dependence was observed on the second day of *S. contaminatum* 160 inoculations, with maximum at the range from 0.5 to 3 mmol/L, which transitioned to an exponential dependence on the third day. *C. sphaerospermum* 161 also showed sufficient growth on CDA containing zinc, with an exponential dependence. These results may indicate that the concentration of the leading metals in mock layers significantly exceeds the  $IC_{50}$  in the plate test. The growth of both fungi was noted on the mock layer with cadmium yellow (Fig. S1, Fig. S2), while within the plate test the growth of *S. contaminatum* STG-160 is completely absent, and *C. sphaerospermum* STG-161, on the contrary, present, although with low intensity. This fact can quite possibly be explained by the environmental conditions of the *in situ* experiment—rich organic medium under the top pigment, pH,  $t^{\circ}C$  and humidity—filamentous fungi can grow in cracks—hotspots for mycelial development. All of this contributed to the penetration of fungal spores under the paint layer, accelerating the process of biodegradation. There is a correlation between our results and investigation [50], which demonstrates that yellow CdS pigment does not act separately, but as a part of the composition of egg tempera and egg—linseed oil emulsion subjected to degradation by different fungi *Acremonium chrysogenum*, *Aspergillus niger*, *Mucor rouxii*, *Penicillium chrysogenum* and *Trichoderma pseudokoningii* indicating that microbial attack results in the increase of the content of free fatty acids released from the catalyzed hydrolysis of triglycerides from oil and egg as well as in the breaking down of proteins accompanied by an increase in the irregular and open conformations adopted by the protein molecules.

## Conclusions

Thus, the filamentous fungus *S. contaminatum* STG-160 and the melanotic fungus *C. sphaerospermum* found on the 16th century icon “Descent into Hell” from the State Tretyakov Gallery demonstrated their “food” preferences in relation to tempera art materials, thereby revealing the risks of biodegradation. The data obtained on mock layers and with  $IC_{50}$  regarding metal ions show the ability of fungi to grow and develop in media primarily containing natural ochres, siennas, red, brown and green earth pigment—rich with iron oxides and hydroxides. This is especially appeals to *S. contaminatum* STG-160, which, according to the results of whole genome sequencing, was found to be able to produce siderophores for iron transport into the cell. Also, the ability to manage  $Fe^{3+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$  is most likely associated with the presence of special membrane-bound transporters both in *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161. It was noticed that in areas of the paint layer where the  $IC_{50}$  is exceeded, especially with respect to zinc white and cobalt green pigments, there is slight growth or growth absence occurred for the studied fungi due to the high toxicity of heavy metals. Additionally, *C. sphaerospermum* 161 can utilize  $Cd^{2+}$  in small quantities, and as a feature, mutagenesis methods can be used to produce a more robust fungus, such as for soil bioremediation to remove heavy metals. The State Tretyakov Gallery maintains excellent museum conditions in its exhibition halls and repositories, but it's important to remember that fungal spores remain dormant until the time comes: an emergency, transportation to another exhibit, violation of temperature and humidity conditions.

The information reported herein will be useful for restoration artists which allow to plan the methods of preventive and interventive conservation art works, to prolong life expectancy. This concerns certain pigment areas on tempera paintings, which require more thorough treatment with antiseptics, as well as enhancing their aesthetic value and ensuring appropriate conditions for display and storage.

Furthermore, the high tolerance and degradative capacity of *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 to degrade several pigments suggest their applicability and potential in transversal areas of knowledge.

## Declarations

### Data availability

All data or results mentioned in this review are available and retrieved from online available literature.

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### Author contributions

DA—conceptualization, methodology, resources, writing—review and editing. AE— visualization, methodology, ET— resources. All authors reviewed the manuscript.

### Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Figures



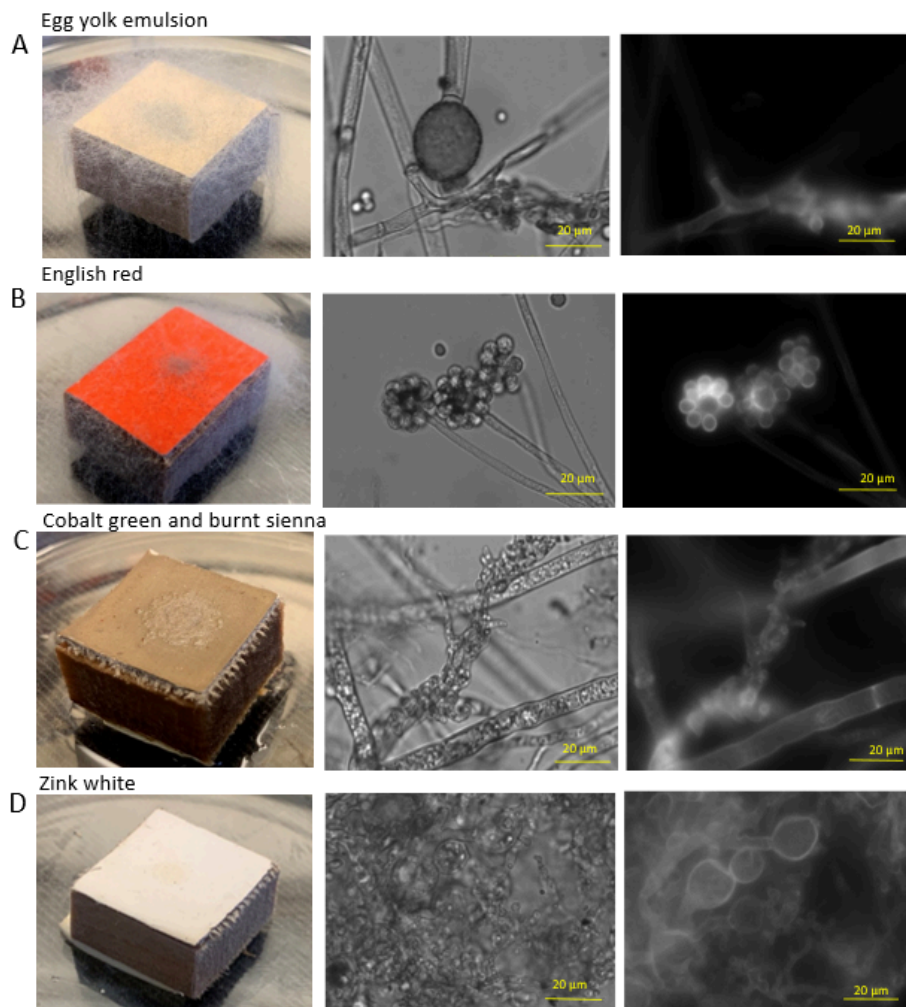


**Figure 3**

Mock layers—degradation by *S. contaminatum* STG-160, 10 d, 26°C, transmission light and fluorescent microscopy, 20 μm

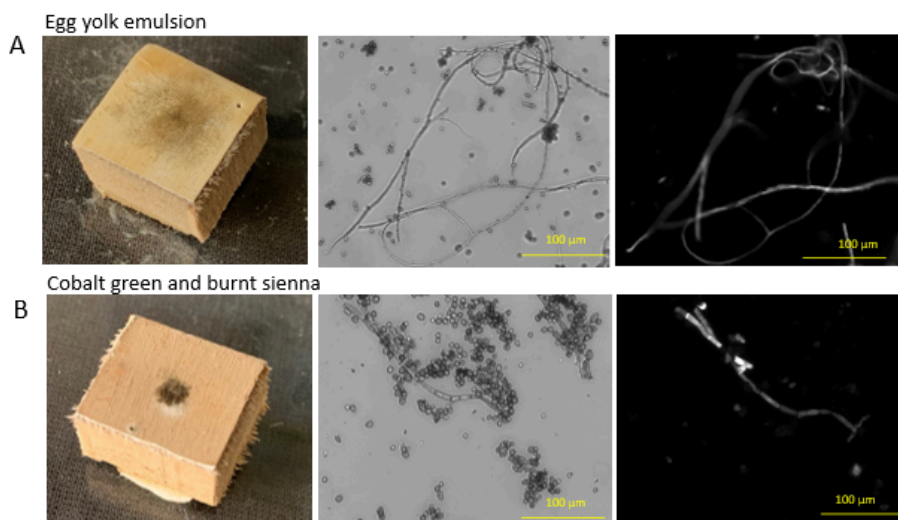


**Figure 4**

Mock layers—degradation by *C. sphaerospermum* STG-161, 10 d, 26°C, transmission light and fluorescent microscopy, 100 μm

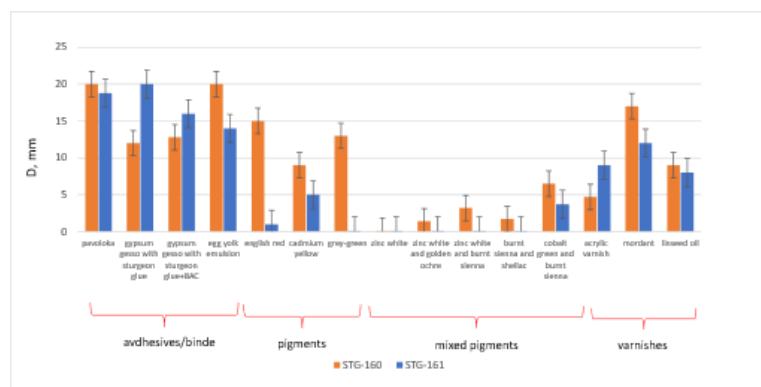


Figure 5

Dynamics of mock layers biodegradation, 12 d

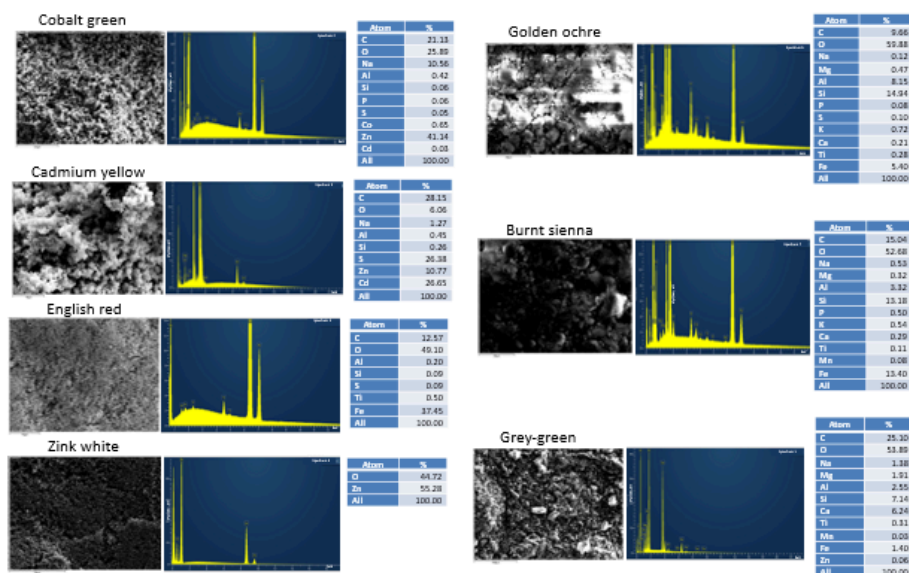


Figure 6

SEM and EDX-analysis: electronic micrographs of the crystalline surface of dry pigments and their atomic composition (%)

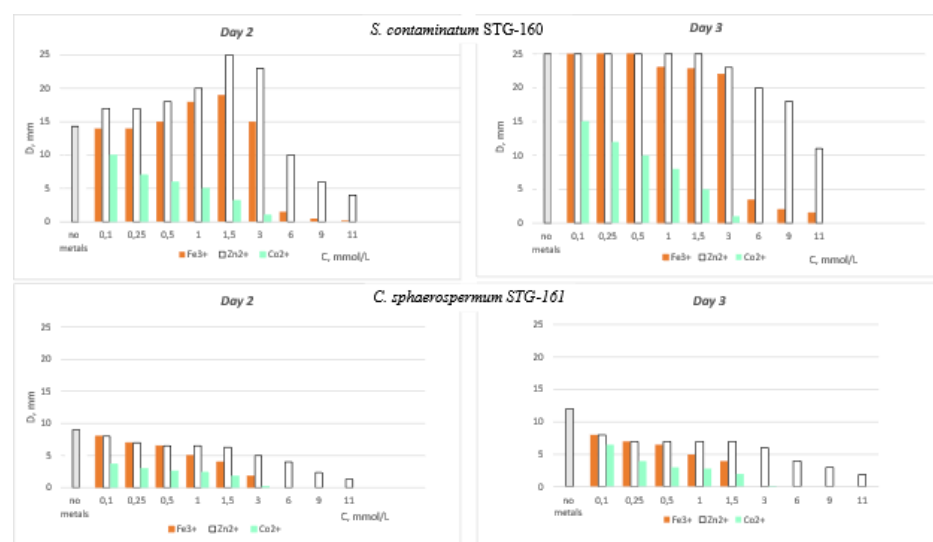


Figure 7

Growth measurement (mm) for 2 d and 3 d for *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 inoculated on CDA medium with metals

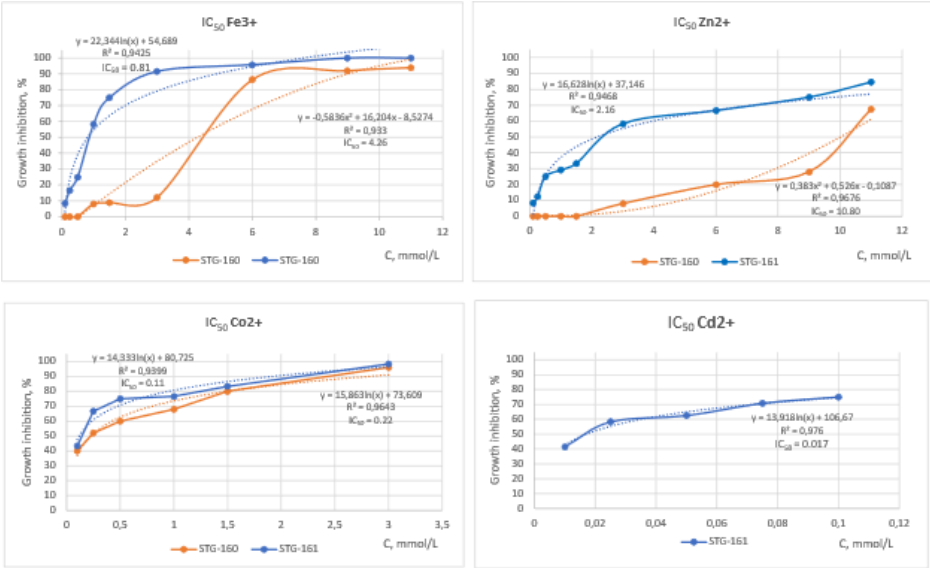


**Figure 8**

Fifty percent growth inhibitory concentration (IC<sub>50</sub>) determination for *S. contaminatum* STG-160 and *C. sphaerospermum* STG-161 on Fe<sup>3+</sup>, Zn<sup>2+</sup> and Co<sup>2+</sup>, 3 d of growth

## Supplementary Files

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