An integrated approach for assessing the bioreceptivity of glazed tiles to phototrophic microorganisms

M.L. Coutinho^{a,b,c}, A.Z. Miller^d, M.A. Rogerio-Candelera^d, J. Mirão^e, L. Cerqueira Alves^f, J.P. Veiga^g, H. Águas^g, S. Pereira^h, A. Lyubchik^g, M.F. Macedo^{b,c}

^aLAQV – Requimte - Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Monte de Caparica, Portugal.

^bDepartamento de Conservação e Restauro, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Monte de Caparica, Portugal.

^cVICARTE, Research Unit Vidro e Cerâmica para as Artes, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus Caparica, 2829-516 Caparica, Portugal.

^dInstituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, Av. Reina Mercedes 10, 41012 Sevilla, Spain.

^eLaboratório HÉRCULES, Universidade de Évora, Largo Marquês de Marialva, 8, 7000-554 Évora, Portugal.

^fC2TN/Instituto Superior Técnico, Universidade de Lisboa, Estrada Nacional 10, 2686-066 Bobadela, Loures, Portugal.

^gCENIMAT/I3N, Departamento de Ciência dos Materiais, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal.

^hLNEC, Laboratório Nacional de Engenharia Civil, Av. do Brasil no. 101, 1700-066 Lisbon, Portugal.

* Corresponding author

Tel./Fax: +34 954 62 47 11

E-mail address: anamiller@irnas.csic.es (A.Z. Miller)

Running title: Bioreceptivity of glazed tiles

A laboratory-based methodology was designed to assess the bioreceptivity of glazed tiles. The experimental set-up consisted of multiple steps: manufacturing of pristine and artificially aged glazed tiles, enrichment of phototrophic microorganisms, inoculation of phototrophs on glazed tiles, incubation under optimal conditions and quantification of biomass. In addition, tile intrinsic properties were assessed to determine which material properties contributed to tile bioreceptivity. Biofilm growth and biomass were appraised by digital image analysis, colorimetry and chlorophyll *a* analysis. SEM, micro-Raman and micro-particle induced X-ray emission were carried out to investigate the biodeteriorating potential of phototrophic microorganisms on the glazed tiles. This practical and multidisciplinary approach showed that the accelerated colonization conditions allowed distinguishing different types of tile bioreceptivity and to relate them to precise characteristics of the material. Aged tiles showed higher bioreceptivity than pristine tiles due to their higher capillarity and permeability. Moreover, biophysical deterioration caused by chasmoendolithic growth was observed on colonized tile surfaces.

Keywords: glazed tiles, bioreceptivity, biodeterioration, phototrophic microorganisms, ceramic

Introduction

Microbial communities colonizing historical glazed tiles have recently been assessed by several authors, providing a first insight on the diversity of bacteria, cyanobacteria, microalgae and fungi dwelling on ceramic tiles (Oliveira et al. 2001; Pedi et al. 2009; Giacomucci et al. 2011; Coutinho et al. 2013; Silva et al. 2014). Phototrophic microorganisms (cyanobacteria and microalgae) were identified as major components of the biomass developing on glazed wall tiles (e.g. Oliveira et al. 2001; Coutinho et al. 2013, 2015). These microorganisms are known to be pioneer in the colonization of monuments and historical buildings due to their photosynthetic ability and adaptation to growth under extreme conditions through the formation of biofilms (Ortega-Calvo et al. 1995; Tiano et al. 1995; Cecchi et al. 2000; Coutinho et al. 2013; Guimet et al. 2013). It has been reported that the growth of photosynthetic-based biofilms on inorganic building materials promote biofouling and contribute to biogeophysical-chemical deterioration (Brehm et al. 2005; Crispim & Gaylarde 2005; Macedo et al. 2009; Noack-Schönmann et al. 2014; Nowicka-Krawczyk et al. 2014; Shirakawa et al. 2015). The development of biofilms can consequently cause irretrievable material loss, a particular concern in the case of cultural heritage assets.

Glazed ceramic tiles have been used as building materials for centuries, being particularly important on the cultural heritage of south European countries. In most of these countries, glazed tiles have been produced according to the majolica technique (Coentro et al. 2012; Van de Voorde et al. 2014). This tile production technique consists of applying a white opaque glaze (lead- tin opacified coating) over a porous ceramic body; subsequently, this glaze is

76 painted with colorants, and the tile is fired at high temperatures, which makes
77 the glaze vitrify.

78 In order to preserve a material from biodeterioration, it is essential to
79 understand degradation mechanisms, as well as the susceptibility of the
80 material to colonization by living organisms (Guillitte 1995). The influence of
81 intrinsic properties of a material on the development of biological colonization
82 was defined by Guillitte (1995) as bioreceptivity. It can be divided into three
83 types: 1) primary bioreceptivity, which is related to the potential of a pristine
84 material to be colonized; 2) secondary bioreceptivity, which refers to the
85 potential of a weathered material to be colonized; and 3) tertiary bioreceptivity
86 which is the colonization potential of a material after being subjected to
87 conservation treatments (Guillitte 1995). Several studies have been devoted to
88 stone bioreceptivity (e.g. Miller et al. 2012; Guillitte & Dreesen 1995; Tiano et al.
89 1995) and only a few have been focused on modern ceramic materials, such as
90 roofing tiles and bricks (Gazulla et al. 2011; Gladis & Schumann, 2011; Portillo
91 et al. 2011; D'Orazio et al. 2014). Most of these studies relied on the artificial
92 inoculation of organisms on a substrate under controlled environmental
93 conditions (e.g. Tiano et al. 1995; Miller et al. 2008, 2009a,b; Marques et al.
94 2015). These approaches were developed for stone, glass, unglazed ceramics
95 and concrete materials, providing important data on their bioreceptivity and
96 mechanisms of biodeterioration (Ranogajec et al. 2008; Miller et al. 2009b;
97 Herisson et al. 2013; Rodrigues et al. 2014). No laboratory studies have been
98 performed to assess bioreceptivity and biodeterioration of majolica glazed tiles.
99 The aim of this study was to develop an integrated laboratory-based
100 methodology to assess the primary and secondary bioreceptivity of majolica

glazed tiles to phototrophic microorganisms and additionally analyze their biodeteriorating potential.

Materials and methods

Experimental design

The experimental set-up of this work followed five specific steps: i) manufacture of majolica glazed tile models based on the composition of historical glazed wall tiles from Pena National Palace (Sintra, Portugal); ii) artificial aging of the manufactured model tiles to simulate the natural aging process; iii) chemical and physical characterization of the tiles reproduced in this work; iv) inoculation and incubation of the majolica glazed tile models with a selected mixture of phototrophic microorganisms, and finally v) assessment of microbial growth and biodeteriorating potential of the inoculated microorganisms on the majolica glazed tile models.

Manufacture of majolica glazed tile models

Majolica glazed tile models were manufactured in laboratory according to the traditional tile production technique. The ceramic body was prepared with a commercial ceramic paste (PF, SIO-2 CERÁMICA COLLET S.A., Spain), which was kneaded manually, rolled-out and cut into squares (approximately 2.5 x 2.5 cm). A slight circular depression was made on the upper surface to contain the inoculum. After air-drying during two weeks, the ceramic body was fired at 950°C (100°C per hour and 1 hour dwell time at 950°C). The lead–tin glaze was prepared with 100 g of the commercial frit TR29 (Ferro, USA). For adjusting the composition, pure raw materials were added: carbonates (3.4 g of Na₂CO₃, 1.5

g of MgCO_3 , 13.23 g of K_2CO_3 , 3.6 g of CaCO_3) and oxides (60 g of SiO_2 , 15 g of SnO_2 , 0.1 g of TiO_2 and 0.3 g of Fe_2O_3). The powders were ground and mixed in an agate mortar for 15 minutes and suspended in deionized water. Following the traditional manufacturing of glazed tiles, the glaze suspension was then poured over the upper surface of the dry ceramic body inclined at 45° to allow the runoff of excess glaze suspension. After 24h air-drying, the tiles were fired at 980°C (100°C h^{-1} heating rate with 1h dwell time at 980°C).

Artificial aging

For studying the secondary bioreceptivity of glazed tiles (Guillitte 1995; Miller et al. 2012), 19 out of 38 tile models were subjected to artificial aging for mimicking weathered tiles. For this purpose, the reproduced tile models were chemically corroded by immersion into alkaline NaOH solution (pH 10) for 10 days. An alkaline medium was selected because it is well known that silicate glasses are susceptible to alkaline corrosion (White 1992; Cannillo et al. 2009). Moreover, tiles applied on buildings can be subjected to alkaline corrosion due to water-mortar interactions (Makhloufi et al. 2012). Inert reaction containers (polyethylene) were selected to avoid any reaction between the electrolyte and the container walls, and sealed to prevent interactions with the atmosphere. Afterwards, the samples were rinsed with distilled water and air-dried. To simulate mechanical decay, the samples were subjected to thermal shock through cycles ($n = 3$) of heating (at 150°C during 20 minutes) and cooling (immersion in ice-cold water). This is a common procedure for testing ceramic durability due to the decrease of mechanical resistance caused by thermal shock (Lewis 1983). A set of 38 glazed tiles from both typologies (19 pristine

and 19 aged surfaces) was used for the subsequent tile colonization experiment.

Laboratory-based tile colonization experiment

Tested microorganisms and tile inoculation

The photoautotrophic microorganisms used in this study were selected according to the occurrence and type of phototrophs dwelling on the glazed wall tiles from Pena National Palace, Portugal (Coutinho et al. 2013). The Chlorophyta *Trentepohlia laginefera* (strain ACOI 1067), *Chlorella ellipsoidea* and *Apatococcus lobatus* (strain ACOI 193), and the cyanobacterium *Nostoc microscopicum* (strain ACOI 578) from the microalgae collection at the University of Coimbra, Portugal (*Algoteca de Coimbra*, <http://acoi.ci.uc.pt/>), were enriched separately in BG11 liquid medium (Fluka Biochemika, Switzerland) for three weeks. At the inoculation day, a volume of 5 ml of each liquid culture were mixed and used as inoculum (approximately 1.25×10^4 cell ml^{-1}).

Pristine and aged tiles (16 of each) were placed on a grid inside glass petri dishes with distilled water at the bottom (Figure S1a). As control, non-inoculated pristine and aged tile samples (three of each) were used (Figure S1b). Before inoculation, these set-ups were autoclaved at 121°C for 20 min. After slow cooling at room temperature, 150 μl of the mixed phototrophic culture were inoculated on the depression of the upper surface of each glazed tile. All tile samples (inoculated and control) were maintained under the same conditions (22-23°C and 75-95% relative humidity), close to a window, but sheltered from direct sunlight to provide optimal growth conditions during the time span of the

experiment (12 months). Tile samples were re-inoculated with 150 µl of the mixed phototrophic culture every 3 months to simulate constant deposition of cells that occurs in outdoor environments.

Characterization and quantification of phototrophic growth on the glazed tiles

Digital image analysis. Photographic records were made with an Olympus C-5060 digital camera every 3 months. A Kodak color scale was included for light and color adjustment of the images. The extent of the tile surface area covered by the green biofilms was estimated by digital image analysis at the end of the incubation period (12 months). Digital images of the colonized tile surfaces were individually recorded. Images were then submitted to principal component analysis (PCA) for the enhancement of the colonized area according to the protocols described by Miller et al. (2010a, 2010b) and Rogerio-Candelera et al. (2011). Photographic analyses relied on: a) geometry rectification of the digital images to minimize the different distortions introduced by the kind and structure of the lenses, the focal distance or the relative position of the camera; b) digital decorrelation of red, green, and blue light (RGB) images by means of PCA, allowing contrast enhancement of minority elements (of different nature and composition) apparently absent in the initial RGB digital image, and avoiding the loss of information implicit in the methods which redefine the histograms; c) image classification by the application of a thresholding algorithm (Sezgin & Sankur 2004) to segment the images into binary, allowing the selection of the colonized areas to be quantified; d) area estimations by scaling the images and counting of the selected pixels (Rogerio-Candelera et al. 2011). HyperCube

(Army Geospatial Centre, USA) and ImageJ (National Institutes of Health, USA) were the software packages used for digital image analysis. These methodological approaches have been widely used for monitoring biofilm growth on stone surfaces (Miller et al. 2010a, b, 2012).

The surface areas covered by the green biofilms were calculated from the segmentation of the first and third bands obtained by PCA (PC1 and PC3, respectively). After measuring the colonized areas in every band, the outlines < 0.001 mm² (one pixel) were ignored and the averages of PC1 and PC3 were calculated.

Color measurements. The color of the biofilms on the pristine ($n = 3$) and aged ($n = 3$) tile samples was measured directly over the tile surface by portable spectrophotometry (Minolta CM-508i). The CIELab color space was chosen for characterizing the biofilm color. Color is described by three parameters: lightness (L^*) redness-greenness (a^*) and chromatic coordinates (b^*), defined by the Commission Internationale de l'Éclairage (2004). In the color space, L^* varies from 0 black to 100 white; a^* , from -60 (green) to +60 (red) and b^* , from -60 (blue) to +60 (yellow).

Chlorophyll a (chl_a) fluorescence. Chl_a fluorescence of the green biofilms was measured by fluorescence spectrometry. The biofilms on the inoculated pristine ($n = 3$) and aged ($n = 3$) samples were scraped with a sterile scalpel from each tile directly into microcentrifuge tubes. Chl_a was extracted with dimethyl sulfoxide (Shoaf & Lium 1976) and subsequently measured by fluorescence spectroscopy. Emission spectra of the Chl_a solution were measured using a spectrofluorometer SPEX Fluorolog-3 FL3-22 with an excitation wavelength of

430 nm, optimum for Chla molecules (APHA/AWWA/WEF 2005), slits of 4.5 nm, an integration time of 0.3 s and an increment of 1.0 nm.

Characterization of pristine and aged majolica tile models before and after the colonization experiment

Prior to the laboratory-based colonization experiment ($t = 0$ months), pristine and aged tiles were chemically and mineralogically characterized by wavelength dispersive X-ray fluorescence (WD-XRF) and X-ray diffraction (XRD). The glaze layer was also characterized by micro-particle induced X-ray emission (micro-PIXE) and micro-Raman. The morphology and microstructure of the glaze were characterized by scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS). In addition, intrinsic physical characteristics of the model tiles, specifically water absorption by capillarity, water vapor permeability and surface roughness, were assessed.

After the colonization experiment ($t = 12$ m), all glazed tile models (control and inoculated pristine and aged samples) were further characterized by micro-PIXE, micro-Raman and SEM-EDS. In addition, the water absorption by capillarity of the glazed tile surfaces was assessed after 12 months-incubation.

Wavelength dispersive X-ray fluorescence (WD-XRF). Elemental analyses were conducted by WD-XRF spectrometry on finely grinded samples. A PANalytical XRF-WDS 4 kW AXIOS (PANalytical B.V., Almelo, The Netherlands) sequential spectrometer was used with an Rh X-ray tube; samples were measured under a

He flow. Spectra deconvolution by the iterative least squares method and standard less semi-quantitative analysis based on the fundamental parameter approach were performed with the SuperQ IQ+ software package (PANalytical B.V., Almelo, The Netherlands).

X-ray diffraction (XRD). XRD analyses were performed for the identification of crystalline phases (mineralogical characterization) on the powdered ceramic body before and after artificial aging. The XRD patterns were recorded on a Rigaku Dmax III-C 3 kW diffractometer (Rigaku Corporation, Tokyo, Japan), using Cu K α radiation at 40 kV and 30 mA settings in the 2 θ range from 20° to 80°, and an acquisition time of 1s and 2 θ increment of 0.08°. The resulting spectra were deconvoluted using a suitable software (EVA, Bruker AXS GmbH, Karlsruhe, Germany) and crystalline phases were identified by comparing the peak positions and intensities with those listed in the software standard files (ICDD, Newtown Square, PA, USA).

Micro-particle induced X-ray emission (micro-PIXE). Micro-PIXE analyses were performed on five glaze fragments of the historical tiles from Pena National Palace (Sintra, Portugal) to determine their chemical composition. This composition was used as reference for the manufacture of the glazed tile models.

Post-experiment analyses of glazed tile models were carried out after repeated cleaning of the glazed tile surfaces with a cotton swab soaked in a 1:1 water ethanol solution to remove biofilm traces. An Oxford Microbeams OM150 type scanning microprobe was used for focusing down to 3 x 4 μm^2 at 1 MeV proton

beam. The glaze samples were irradiated in vacuum; X-rays were collected by a 8 μm thick Be windowed Si(Li) detector with a crystal active area of 80 mm^2 and 145 eV resolution. The system configuration allowed efficient detection of low energy X-rays. Operation and basic data manipulation, including elemental distribution mapping, were achieved through the OMDAQ software code, and quantitative analysis was done with the GUPIX software. The quantification was performed in a representative area of the glaze cross-sections with an area of analysis of ca. 500 x 500 μm . Consistency and comparability of micro-PIXE data were assessed by measuring the glass standard CMOG-C (The Corning Museum of Glass, New York, USA).

Micro-Raman spectroscopy (micro-Raman). Micro-Raman analyses were carried out on the glaze surface for characterizing the mineralogy of the glaze crystalline inclusions and crystals deposited on the glazed surface before biofilm removal. A Labram 300 Jobin Yvon spectrometer was used, equipped with a He-Ne laser of 17mW power operating at 632.8 nm and a solid state external laser of 50 mW power operating at 514.5 nm. Spectra were recorded as an extended scan. The laser beam was focused either with a 10x, 50x or a 100x Olympus objective lens. The laser power at the surface of the samples varied with the aid of a set of neutral density filters (optical densities 0.3, 0.6, 1 and 2).

Scanning electron microscopy - energy dispersive X-ray spectroscopy (SEM-EDS). Colonized pristine and aged model tiles, as well as control samples, were analyzed by SEM to assess microbe-substrate interactions. In addition,

biodeterioration patterns were assessed after repeated cleaning of the surface with a cotton swab soaked in a 1:1 water ethanol solution to remove biofilm traces. Tile samples were directly mounted on sample stubs, sputter-coated with gold/palladium, and examined on a HITACHI 3700N scanning electron microscope interfaced with a Quantax EDS microanalysis system. The Quantax system was equipped with a Bruker AXS XFlash® Silicon Drift Detector (129 eV Spectral Resolution at FWHM - Mn Ka). The operating conditions were: backscattered electron mode, 20 kV accelerating voltage, 10 mm working distance and 120 mA emission current.

Intrinsic physical characteristics of the glazed tile models. The intrinsic physical characteristics of the pristine ($n = 3$) and aged ($n = 3$) tile models were assessed through hydric properties: water absorption by capillarity and water vapor permeability. Water absorption by capillarity via the glaze layer was quantified by weighting the water uptake of the samples at increasing time intervals of 3, 10, 15 and 30 min and hourly according to Pereira and Mimoso (2012). The capillary coefficient (Q) is defined as the coefficient between water suction mass and the square root of time, thus adopting the unit $\text{g m}^{-2}\text{s}^{-1/2}$. This material property refers to the water suction by the glazed tile surface through the crack network on the glazed surface and the open capillary pores of the ceramic body.

Water vapor permeability of the glazed tiles was estimated by the dry method according to Pereira and Mimoso (2012). A tile shaped hole was made on the lid of a polypropylene box containing dehydrated CaCl_2 at the bottom. A

tile sample was then mounted on the top of the polypropylene container, which was sealed with honey wax and placed inside a chamber with controlled environmental conditions (20 °C and 75% relative humidity); the weight uptake of water passing through the tiles was then registered.

Surface roughness of the pristine ($n = 3$) and aged ($n = 3$) glazed tiles was measured using an Ambios XP-Plus 200 Stylus profilometer; three scan lines on each sample surface with 2 mm length were performed at a speed of 0.1 mm and a stylus tracking force of 0.1 mg. The values of roughness parameters, namely average roughness (R_a), root-mean-square roughness (R_q) and total roughness height (R_y), were calculated using XP-Plus Software.

Statistical analysis

In order to find significant differences between pristine and aged tile models regarding chemical composition, Chla concentration and physical parameters (capillarity coefficient, water vapor permeability coefficient and surface roughness), experimental data were subjected to analysis of variance using the Microsoft Excel 2010 for Windows. The averages were compared by the Tukey's honest significant difference (HSD) test at 5% level of significance.

Results

Majolica tile models characterization

Micro-PIXE analyses performed on the white glaze fragments collected from the nineteenth century Pena National Palace tiles (Sintra, Portugal) indicated that they were composed of: 1.5% of Na_2O , 0.6% of MgO , 5% of Al_2O_3 , 50% of

SiO₂, 4% of K₂O, 1.2% of CaO, 0.1% of TiO₂, 0.5% of Fe₂O₃, 9% of SnO₂ and 30% of PbO. The relative standard deviation for SiO₂, K₂O, CaO, and PbO of the glass standard CMOG-C was below 11% and for the remaining elements or oxides (Na₂O, Al₂O₃, Fe₂O₃ and Cl) was below 35%, except for Cl. The relative differences between the measured value and the certified composition were below 20% for Na₂O, below 15% for MgO, Al₂O₃, K₂O and CaO, and below 5% for SiO₂, Fe₂O₃ and PbO (Table S1). Values close to the detection limit, such as Cl showed high relative difference. The content of SnO of the reference glass was below detection limit, thus it was not quantified.

Table 1 shows the chemical composition of the glaze produced in laboratory obtained by micro-PIXE. SiO₂ and PbO were the main oxide components of the pristine glaze; SnO₂ was the opacifier used, which was found in a considerable amount, followed by Al₂O₃ which was also a major component of the glaze (Table 1). The proportions of alkalis (Na₂O + K₂O) were < 2 wt % of the total concentration of the glaze. Regarding the aged glazed tile samples, their composition was similar to the pristine model tile samples as confirmed by the analysis of variance. Statistical analysis revealed that the average composition of the pristine and aged tiles was not significantly different (Table 1).

According to WD-XRF analyses, the manufactured model tiles showed a ceramic body composed of: 60% of SiO₂, 20% of Al₂O₃, 13% of CaO, 3.4% of K₂O, 1.3% of TiO₂ and 2.4% of Fe₂O₃, as major oxide components. After firing, the main mineralogical phases of the ceramic body were quartz (SiO₂), gehlenite (Ca₂Al[AlSiO₇]) and anorthite (CaAl₂Si₂O₈), as revealed by X-ray diffraction (Figure S2).

Regarding the mineralogical composition of the crystalline inclusions of the glaze, quartz and cassiterite (SnO_2) were identified by micro-Raman (Figure S3). Micro-Raman spectra showed the characteristic bands of quartz at 125, 200 and 460 cm^{-1} (Coentro et al. 2012). Cassiterite was identified by its most intense characteristic band at 633 cm^{-1} (Coentro et al. 2012).

The surface morphology and microstructure of the pristine and aged glazes, before inoculation, were examined by SEM-EDS (Figure 1). Differences were identified between pristine and aged tile models. The pristine glaze surfaces showed irregularities due to the mineral inclusions of cassiterite and quartz dispersed on a smooth glassy phase matrix (Figure 1a,b). Moreover, a network of microcracks could be observed by SEM (Figure 1a,b). This microcrack network was enhanced significantly on the aged tiles (Figure 1c,d); however no well-defined hydrated corrosion layer was visible on the aged tile surfaces, but the fissure edges showed corrosion and irregularities (Figure 1c,d).

Intrinsic physical characteristics of the pristine and aged tiles

Capillary coefficient of the aged samples showed a higher and more rapid water uptake (Q mean: $10.8\text{ g.m}^{-2}.\text{s}^{-1/2}$) than pristine samples (Q mean: $1.3\text{ g.m}^{-2}.\text{s}^{-1/2}$), as shown in Table 2 and Figure 2. The pristine tiles displayed similar values of capillary coefficient among the three replicates. In contrast, the capillary coefficient values of the aged tiles varied among samples (Table 2).

Concerning the water vapor permeability, a similar pattern was obtained. The pristine tiles before inoculation showed lower permeability values than the aged tiles (Table 2 and Figure 3). R_a , R_q and R_y of the aged samples were

greater than for the pristine samples, but they were not significantly different (Table 2).

Characterization of the biofilm after 12 months of incubation

After the incubation period, pristine and aged samples showed differences in terms of color and extent of tile surface covered areas. Judging by visual examination, the inoculated tile samples developed green biofilms similar in appearance to those occurring on outdoor wall tiles (Figure 4). The biofilm areas on the tile surfaces were greater on the aged tiles than on the pristine tile samples (Figure S4). In addition, a thicker and darker green layer was observed on the aged tiles (Figure 4). Table 3 summarizes the values of tile surface coverage area, colorimetry and photosynthetic biomass obtained for the pristine and aged tiles.

The tile surface areas covered by biofilms on the pristine samples ranged from 47.9 to 7.9 mm², whereas on the aged samples the values ranged between 69.4 and 20.4 mm², of a total surface area per tile of approximately 625 mm². Mean covered area was higher on the aged samples (43 mm²) than on the pristine model tiles (24 mm²) (Table 3). Statistical analysis ($p < 0.05$) revealed that the coverage areas on the pristine and aged samples were significantly different (Table 3).

Concerning color measurements, the L* parameter was significantly different for the pristine and aged samples (Table 3). Lower L* values of the biofilm on the aged samples resulted in a darker green color. Regarding the a* parameter (redness-greenness), the surfaces of the aged samples were significantly greener than on the pristine samples. In terms of yellowness-

blueness b^* parameter, pristine and aged samples were not significantly different (Table 3).

Chla fluorescence analyses showed significant differences between pristine and aged tiles (Table 3). The aged samples showed higher fluorescence and hence higher Chla concentration, which indicated higher photosynthetic biomass on the aged tiles. This is in accordance with the CIELab color measurements.

SEM observations

SEM images showed dense microbial colonization growing over the glazed surfaces on both pristine and aged tile samples (Figure 5). Chasmoendolithic growth by phototrophic microorganisms in glaze fissures was also observed on both types of tile samples (Figure 5a,b). Comparing with control samples, the inoculated tiles, particularly those with chasmoendolithic growth, showed rougher fissure edges (Figure 5a,d,e). The adhesion of microorganisms by extracellular polymeric substances (EPS) to the glaze and their penetration into fissures were noteworthy (Figure 5b). After cleaning the tile surfaces with cotton swabs soaked in water-ethanol solution, superficial deposits with imprints of rod-shaped cells (Figure 5d) and biofilm remains (Figure 5e) were observed.

Characterization of the corrosion products

After the incubation experiment, both control and inoculated pristine and aged tile samples had corrosion products on their surface (Figure S3). Micro-Raman analyses revealed crystalline compounds identified as calcium carbonate (probably calcite) and calcium sulfate (probably gypsum) on both control and

inoculated tile samples. Raman spectra of the calcium compounds showed bands at 156, 280, 712 and 1088 cm^{-1} for carbonates (Maguregui et al. 2009) and bands at 412, 494 and 1008 cm^{-1} for sulfates (Maguregui et al. 2009).

Characterization of the glaze surface after the colonization experiment

The uptake and/or depletion of elements from the glaze by microorganisms during the tile colonization experiment were analyzed by micro-PIXE (Tables 4 and 5). Control samples showed no significant changes regarding the major and minor components of the glaze before and after the colonization experiment, with the exception of CaO, which increased from 1 wt. % to 2-3 wt. % (Table 4). Concerning the inoculated samples, although mean values seemed to indicate an increase of PbO in relation to the control samples, these differences were not significant (Table 4).

For detecting biogeochemical damage on the glazed tile surfaces, elemental distribution maps of Si and Pb were performed by means of micro-PIXE on colonized and non-colonized areas of the same sample. This mapping together with chemical analyses confirmed that there were no significant chemical alterations on the glazed tile surfaces. The observed heterogeneity was associated with the glaze microstructure (Figure 6 and Table 5).

Post-experimental capillary water absorption

Pristine control samples showed lower capillary coefficients (1.5 - 2.1 $\text{g m}^{-2}\text{s}^{-1/2}$) than aged control samples (7.9 - 13.4 $\text{g m}^{-2}\text{s}^{-1/2}$) (Figure 7). Aged inoculated tiles showed lower water uptake and thus lower capillary coefficient (5.6 – 6.3 g

$\text{m}^{-2} \text{s}^{-1/2}$) than control samples (Figure 7). Capillary coefficient values of control and inoculated samples were not significantly different ($p < 0.05$).

Discussion

Glazed tile models

The characterization of historical tiles from Pena National Palace (Sintra, Portugal) showed an alkali-lead silicate glaze, common in majolica tiles (Tite 2009). The composition of these 19th century tiles was similar to earlier majolica glazes retrieved on Portuguese tiles from the 17th and 18th centuries (Coentro et al. 2012; Silva et al. 2014).

The production of representative tile models was complex due to the wide variety of ceramic raw materials and other technological aspects that influence the final characteristics of the ceramic body, such as granulometry and firing conditions (temperature, cycle and atmosphere). Therefore, a commercial ceramic paste was used for manufacturing the tile models. As revealed by micro-PIXE, the chemical composition of the produced glaze was similar to the historical glazes from Pena National Palace tiles (Table 1). However, the concentration of alkalis in the glazed tile models was lower than in the historical glazes (Table 1). This was probably due to the addition of carbonates instead of feldspar minerals. In fact, other crystalline phases different from quartz and cassiterite can be found in historical glazes, such as potassium feldspars (Coentro et al. 2012).

Traditional firing is often accompanied by production faults. Differential thermal properties of the glaze and ceramic body, and tensions of the glassy matrix containing crystalline inclusions, produce microfissures during cooling

(Eggert 2006; Carter & Norton 2013). The presence of microfissures has been commonly described on historical glazed tiles (Mimoso et al. 2011; personal observation). Therefore, microcracks were intentionally produced on the tiles manufactured under laboratory conditions (Figure 1). Although the glaze is a non-porous layer, surface flaws, such as pitting or microfissures, make it permeable to water, as observed for the aged tile models; the more fissures, the higher the uptake of water (Figure 2). Zhou et al. (2012) reported a strong correlation between crack network parameters and water transport properties. These properties have been described as determinant on the primary bioreceptivity of a substrate, because water availability is the limiting factor for microbial growth (Miller et al. 2009b, 2012; Gazulla et al. 2011; Portillo et al. 2011).

The presence of quartz and cassiterite inclusions on the glaze of the tile models was also an important factor to mimic historical glazes. According to Fröberg and Hupa (2008), crystals, raw materials and surface flaws determine not only the surface roughness of a glaze, but also the glaze corrosion process. Cannillo et al. (2009) reported that the corrosion in an alkaline environment of glazes containing mineral inclusions is accompanied by an increase of surface roughness due to the differential corrosion of the glassy matrix and the crystalline inclusions. Surface roughness has also been regarded as a favorable feature for the settlement and development of microbial communities on a substrate (Koestler et al. 1997; Miller et al. 2009b). However, no significant differences in surface roughness were detected between pristine and aged samples using a contact surface profilometer. According to Santos and Júlio (2013), bidimensional measurements of surface texture by mechanical stylus

profilometers might have some drawbacks in relation to the microstructure characterization. The surface roughness on the aged tiles at the microscale by SEM (Figure 1) might not have been assessed using a stylus profilometer. This could explain the absence of significant differences between the pristine and aged tile models.

Based on the chemical and mineralogical composition of the manufactured glazed tile models and on their physical features (hydraulic properties), the historical majolica glazed tiles from Pena National Palace were reproduced under laboratory conditions. The aging process caused alterations similar to those observed on naturally weathered tiles. The physical deterioration of glazed wall tiles used on buildings occurs due to several factors, such as water movements, freeze-thaw cycles and salt crystallization (Borges et al. 1997). These factors lead to volume fluctuations resulting in crack propagation and scaling of the glaze (Eggert 2006). These alterations increase water permeability and decrease the physical resistance of glazed wall tiles (Borges et al. 1997; Larbi 2004; Figueiredo et al. 2009). Hence, the chemical and physical properties of the tile models manufactured in this work (pristine and aged) were suitable for conducting the subsequent laboratory-based tile colonization experiment.

Bioreceptivity of glazed tile models

Considering that the bioreceptivity assessment of a given material involves the quantification of microbial growth (Guillitte 1995; Miller et al. 2012), a set of techniques (digital image analysis, colorimetry and fluorescence spectroscopy)

was applied for characterizing the photosynthetic-based biofilms developed on the glazed tile surfaces. These methodologies have been extensively used in the characterization of photosynthetic biofilms on inorganic surfaces (Miller et al. 2009b; Sanmartín et al. 2010; Rogerio-Candelera et al. 2011). Miller et al. (2012) in a literature review concerning the bioreceptivity of stone, claimed that these techniques were the most reliable for estimating epilithic biomass on stone surfaces. Therefore, digital image analysis, colorimetry and fluorescence spectroscopy were considered as the most suitable for studying biofilm development on glazed tiles.

Digital image analysis was used to determine the extent of growth of the green biofilms on tile surfaces. Data indicated that the growth of photoautotrophic microorganisms was larger on the aged tiles than on the pristine tile models (Figures 4 and 4S). These data were also supported by Chla fluorescence measurements, which revealed larger amounts of Chla on the aged tile models than on pristine tiles (Table 3).

In addition, CIELab color parameters were used to quantify the differences noticed by visual examination (Table 3). Lower values of lightness coordinate L^* (darker color) were observed for the aged samples, which suggested higher concentration of Chla and thus greater photosynthetic biomass (Sanmartín et al. 2010). Also, the a^* parameter (redness-greenness) showed that the biofilm on the aged samples was significantly greener than on the pristine samples (Table 3). This color parameter, despite being less significant than the L^* parameter, has been linked to the concentration of photosynthetic pigments, such as Chla, carotenoids and phycocyanins (Sanmartín et al. 2010). The data indicated that the biofilm development was

greater on the aged tiles than on pristine surfaces. Consequently, the bioreceptivity of aged tile surfaces was larger than that of pristine model tiles.

Differences in bioreceptivity between pristine and aged tiles were due to the hydric properties of the substrate (water absorption by capillarity and water vapor permeability). Aged model tiles showed higher permeability than pristine models, which could justify their bioreceptivity. In fact, studies on the bioreceptivity of clay roofing tiles to phototrophic microorganisms also concluded that higher porosity led to increased bioreceptivity (Gazulla et al. 2011; Portillo et al. 2011). Water, anchoring sites and micro-refuges on surfaces, support the attachment and settlement of microorganisms (Scardino et al. 2008). Thus, aged glazed surfaces with surface irregularities were more susceptible to colonization by phototrophic microorganisms than pristine glazed tiles. Aging induced changes of the properties of the ceramic tiles, which promoted increased bioreceptivity. Thus, the conservation state of glazed ceramics is an important issue regarding tile bioreceptivity and biodeterioration, as more deteriorated glazed tiles might be more rapidly colonized than sound glazed surfaces.

Biodeterioration of glazed tile models

Biodeterioration, particularly biogeophysical alterations caused by the inoculated phototrophic microorganisms, were observed on the tile models after 12 months of incubation under laboratory conditions. As reported by several authors, microbial growth within fissures of the glaze, as in other building materials, caused chemical and physical changes on the substrate and may

lead to the flaking of the glaze (Palmer & Hirsch 1991; Mandal & Rath 2013). This damage has been reported in the literature as a serious problem which compromises the durability of glazed wall tiles (Palmer & Hirsch 1991; Coutinho et al. 2013). In fact, the values of water uptake by capillarity were lower for the inoculated aged samples ($5.6 - 6.3 \text{ g m}^{-2} \text{ s}^{-1/2}$) compared with control aged samples ($7.9 - 13.4 \text{ g m}^{-2} \text{ s}^{-1/2}$) (Figure 7). The presence of EPS and other substances in the fissures of the glaze could explain these changes in the permeability of the aged samples. However, there was no significant change in the hydric properties of the glazed tile models due to microbial activity as revealed by statistical analysis (Figure 7).

Regarding the biochemical attack on the glazed surface, corrosion products such as carbonates, common corrosion products of silicate glass (Rodrigues et al. 2014) and ceramic body (Maguregui et al. 2009), were identified after the colonization experiment. However, carbonates were also present on the control samples. Carbonates may be a natural product of the tile aging when exposed to moisture conditions. Nevertheless, the precipitation of calcium carbonate might also be promoted by the phototrophic microorganisms inoculated on the tile models, as cell imprints on a bed of calcium carbonate deposits were observed on the aged tile samples (Figure 5d). The chelating action of EPS and other macromolecules produced by phototrophic microorganisms to capture divalent ions and other metal ions have been well described in the literature (eg Rossi et al. 2012; Suresh Kumar et al. 2014). Some cyanobacterial strains can bind calcium ions, which is indicative of chemical and physical deterioration on the substrate (Rossi et al. 2012). However, the amount and distribution of Ca-rich crystals on the controls and

inoculated samples could not be estimated, partly because the dense biofilm formed on the colonized surfaces hindered the visualization of corrosion products.

Regarding alterations on the chemical composition of the glazed surfaces, there was a slight increase in lead concentration after 12 months-incubation (Tables 4 and 5). The corrosion of historical lead-based glazes involves lead lixiviation and the formation of a lead-rich layer which detaches when it reaches a certain thickness (Bonnet 2003). Some phototrophic microorganisms can excrete organic acids (Rossi et al. 2012), and the corrosion of lead-glazed tiles in acid media results in increased lead lixiviation rate (Wood & Blachere 1978). However, the chemical composition obtained for colonized and non-colonized tile areas was not significantly different. Thus, no conclusion could be drawn regarding their influence on glaze chemical corrosion or on the formation of biogenic compounds. Colonization of the glazed tile models by phototrophic microorganisms under laboratory conditions showed that the presence of fissures and other surface defects enabled chasmoendolithic growth, which promoted biogeophysical deterioration on the glazed substrate.

Conclusions

The methodological approach designed in this study, involving the production of tile models, their characterization, inoculation with phototrophic microorganisms, quantification of biomass and analysis of damage, was suitable to assess the bioreceptivity of glazed wall tiles under laboratory conditions.

Data showed that the bioreceptivity of pristine tile samples was lower than aged samples indicating that the susceptibility to phototrophic colonization is related to the intrinsic properties of the substrate.

The laboratory-based colonization experiment demonstrated the biodeteriorating potential of phototrophic microorganisms on glazed wall tiles, through chasmoendolithic growth. In this experiment, it was possible to observe that this damage can occur without any other tile deterioration form, i.e., on non-weathered glazed materials.

An accurate assessment of the biogeochemical deterioration on glazed ceramic tiles is crucial, particularly for complex composite materials. Therefore, further research will be necessary to quantify the effect of the microorganisms on the chemical corrosion of glazed surfaces.

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TABLES

Table 1. Chemical composition (wt. %) of the glaze analyzed by micro-PIXE on pristine ($n = 3$) and aged ($n = 3$) tile model samples. Mean values are presented together with the standard deviation (SD) and ANOVA results.

Oxides (wt. %)	Pristine (mean $\pm$ SD)	Aged (mean $\pm$ SD)
Na ₂ O	0.3 $\pm$ 0.04 (a)	0.3 $\pm$ 0.06 (a)
MgO	0.4 $\pm$ 0.03 (a)	0.4 $\pm$ 0.01 (a)
Al ₂ O ₃	3.4 $\pm$ 0.30 (a)	3.2 $\pm$ 0.10 (a)
SiO ₂	47.0 $\pm$ 2.70 (a)	45.2 $\pm$ 5.20 (a)
Cl	0.1 $\pm$ 0.05 (a)	0.1 $\pm$ 0.10 (a)
K ₂ O	1.5 $\pm$ 0.10 (a)	1.3 $\pm$ 0.01 (a)
CaO	1.0 $\pm$ 0.08 (a)	1.1 $\pm$ 0.10 (a)
TiO ₂	0.1 $\pm$ 0.02 (a)	0.1 $\pm$ 0.01 (a)
Fe ₂ O ₃	0.2 $\pm$ 0.01 (a)	0.2 $\pm$ 0.03 (a)
SnO ₂	10.2 $\pm$ 0.10 (a)	10.0 $\pm$ 1.00 (a)
PbO	35.7 $\pm$ 2.50 (a)	38.0 $\pm$ 4.50 (a)

Values followed by the same letters in brackets in the same line are not significantly different by the Tukey HDS test at $p < 0.05$.

Table 2. Physical parameters (capillary water absorption, water vapor permeability and surface roughness) measured for pristine ($n = 3$) and aged ($n = 3$) tile model samples. Mean values of each parameter are presented together with the standard deviation (SD) and ANOVA results.

Model tile	Capillary	water vapor	Surface roughness		
	coefficient	permeability			
	Q (g m ⁻² s ^{-1/2})	δ (Kg m ⁻¹ h ⁻¹ Pa ⁻¹)	Ra (Å)	Rq (Å)	Ry (Å)
Pristine	1.3 $\pm$ 1.4 (a)	1.5 $\times 10^{-10}$ $\pm$ 4.6 $\times 10^{-11}$ (a)	12.6 $\pm$ 3.3 (a)	14.9 $\pm$ 3.3 (a)	57.9 $\pm$ 14.0 (a)
Aged	10.8 $\pm$ 6.1 (a)	3.9 $\times 10^{-10}$ $\pm$ 1.3 $\times 10^{-10}$ (b)	13.0 $\pm$ 1.4 (a)	15.6 $\pm$ 1.2 (a)	60.0 $\pm$ 3.5 (a)

Ra, average roughness; Rq, root-mean-square roughness; Ry, total roughness height.

Values followed by the same letters in brackets in the same column are not significantly different by the Tukey HDS test at $p < 0.05$.

Table 3. Characteristics of the photosynthetic-based biofilms on the pristine and aged samples after 12 months of incubation: tile covered area obtained by digital image analysis ($n = 16$); colorimetric parameters (L^* , a^* and b^*) ($n = 16$) and intensity of Chla fluorescence measured at 684 nm ($n = 3$). Mean values are presented together with the standard deviation (SD) and ANOVA results.

Sample	Covered area (mm ²)	Colorimetry			Intensity of Chla fluorescence at 684 nm (cps)
		L^*	a^*	b^*	
Pristine	24.0 ± 9.7 (a)	64.3 ± 7.9 (a)	-0.6 ± 2.5 (a)	22.1 ± 7.4 (a)	2.5 × 10 ⁶ ± 4.8 × 10 ⁵ (a)
Aged	43.0 ± 13.4 (b)	55.0 ± 4.8 (b)	-4.4 ± 1.1 (b)	18.3 ± 2.1 (a)	7.2 × 10 ⁵ ± 6.4 × 10 ⁵ (b)

Values followed by the same letters in brackets in the same column are not significantly different by the Tukey HDS test at $p < 0.05$.

Table 4. Chemical composition (wt. %) of the glaze analyzed by micro-PIXE on control and inoculated samples after 12 months of incubation.

	Control		Inoculated	
	Pristine	Aged	Pristine	Aged
Na ₂ O	0.3 ± 0.10	0.3 ± 0.10	0.3 ± 0.10	0.3 ± 0.10
MgO	0.4 ± 0.04	0.4 ± 0.02	0.4 ± 0.01	0.4 ± 0.10
Al ₂ O ₃	3.1 ± 0.15	3.0 ± 0.07	3.0 ± 0.20	3.1 ± 0.40
SiO ₂	45.5 ± 4.00	42.7 ± 1.00	45.4 ± 4.40	41.7 ± 4.80
P ₂ O ₅	n.d. ¹	0.1 ± 0.20	n.d. ¹	0.2 ± 0.30
Cl	0.1 ± 0.09	n.d. ¹	0.1 ± 0.10	0.2 ± 0.01
K ₂ O	1.5 ± 0.20	1.4 ± 0.10	1.4 ± 0.10	1.4 ± 0.20
CaO	2.1 ± 1.80	2.9 ± 3.20	1.3 ± 0.20	1.5 ± 0.80
TiO ₂	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.10
Fe ₂ O ₃	0.2 ± 0.03	0.3 ± 0.02	0.2 ± 0.01	0.2 ± 0.10
SnO ₂	9.9 ± 0.48	10.0 ± 0.72	10.3 ± 1.60	10.8 ± 1.00
PbO	36.7 ± 3.90	38.9 ± 3.40	37.5 ± 2.90	40.1 ± 3.40

¹n.d. – not detected

927 **Table 5.** Surface composition of the colonized samples (1AP, 2AP and 9AP)
928 analyzed on the same areas mapped by micro-PIXE (Figure 6). Analyses were
929 performed on non-colonized and colonized areas after biofilm removal.

Sample	1AP		2AP		9AP	
Area	NC ¹	C ²	NC ¹	C ²	NC ¹	C ²
Na ₂ O	0.36 ± 0.02	0.30 ± 0.03	0.65 ± 0.03	0.31 ± 0.02	0.32 ± 0.02	0.34 ± 0.03
MgO	0.35 ± 0.01	0.37 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.38 ± 0.02
Al ₂ O ₃	3.08 ± 0.02	2.99 ± 0.02	3.03 ± 0.02	3.07 ± 0.02	3.03 ± 0.02	3.17 ± 0.02
SiO ₂	50.21 ± 0.05	46.08 ± 0.05	49.6 ± 0.05	48.96 ± 0.05	47.90 ± 0.05	48.23 ± 0.05
Cl	0.15 ± 0.01	0.18 ± 0.01	0.24 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.17 ± 0.01
K ₂ O	1.65 ± 0.02	1.47 ± 0.02	1.79 ± 0.02	1.52 ± 0.02	1.62 ± 0.02	1.42 ± 0.02
CaO	1.06 ± 0.02	1.71 ± 0.03	1.33 ± 0.02	1.15 ± 0.02	1.21 ± 0.02	0.97 ± 0.02
TiO ₂	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.11 ± 0.01	0.07 ± 0.01	0.10 ± 0.01
Cr ₂ O ₃ ²	n.d.	0.08 ± 0.01	n.d.	n.d.	n.d.	0.01 ± 0.01
Fe ₂ O ₃	0.23 ± 0.01	0.22 ± 0.02	0.16 ± 0.01	0.23 ± 0.01	0.22 ± 0.01	0.20 ± 0.02
SnO ₂	9.28 ± 0.09	10.14 ± 0.09	9.33 ± 0.09	9.16 ± 0.09	9.78 ± 0.09	9.39 ± 0.09
PbO	33.54 ± 0.50	36.39 ± 0.05	33.43 ± 0.5	34.99 ± 0.54	35.34 ± 0.51	35.63 ± 0.53

930 ¹NC, non-colonized area; ²C, colonized area; ³n.d.,not detected

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FIGURES

Figure 1. SEM-BSE images of the microstructure and surface morphology of representative pristine and aged glazed tile samples before inoculation. (a) General view of a pristine tile, and (b) detailed view of a fissure indicated in (a) with an arrow. (c) General view of an aged tile, and (d) detail of a fissure indicated in (c) with an arrow.

Figure 2. Water uptake by capillarity of the pristine (Prist 1-3) and aged (Aged 1-3) tile models before the colonization experiment. The corresponding linear trendlines and capillary coefficient equation values are shown.

Figure 3. Water vapor permeability of pristine (Prist 1-3) and aged (Aged 1-3) tile samples before inoculation. The corresponding linear trendlines and capillary coefficient equation values are shown.

Figure 4. Photograph of the inoculated pristine (left) and aged (right) tile samples after 12 months of incubation.

Figure 5. SEM images of majolica tile models after the colonization experiment. (a) Microbial cells within a fissure of a pristine tile. Arrows indicate EPS. (b) Colonization near a fracture of an aged tile with EPS into the fracture. (c) Biofilm of phototrophic microorganisms on a pristine glaze sample. (d) Imprints of rod-shaped cells after removal of the biofilm on the same pristine sample. (e) Growth of microorganisms within a surface fracture of a pristine tile. (f) Surface of a control pristine glazed tile after the colonization experiment.

Figure 6. Elemental maps obtained by micro-PIXE of three pristine inoculated samples (1AP, 2AP and 9AP) on non-colonized and colonized surface areas after biofilm removal. Mapped area of ca. 500 x 500 μm .

Figure 7. Water uptake by capillarity of the pristine and aged inoculated samples (APrist and AAged) and control samples (CPrist and CAged). The corresponding linear trendlines and capillary coefficient equation values are shown.

Supplemental Material

Table S1. Reference composition and analytical results (wt.%) for CMOG C reference glass ($n = 5$).

Oxides	Na ₂ O	MgO	Al ₂ O ₃	SiO ₂	Cl	K ₂ O	CaO	TiO ₂	Fe ₂ O ₃	SnO ₂	PbO
CMOGC Certified (wt.%)	1.1	2.8	0.9	34.9	0.1	2.8	5.1	0.8	0.3	0.2	36.7
CMOGC Measured (wt.%)	1.3	2.4	0.8	33.3	0.3	2.5	4.5	0.5	0.3	0.0	36.0
RSD ¹ (%)	25.0	14.0	20.0	6.0	32.0	4.0	4.0	69.0	16.0	-	11.0
LOD ² (ppm)	236	105	101	81.0	411	403	708	88.0	88.0	797	1620

¹RSD – Relative Standard deviation; ²LOD – Average Limit of detection.

Figure S1. Illustration of the experimental set-up. (a) Glass Petri dish ($\varnothing = 9$ cm) with distilled water at the bottom and a grid to separate the water from the glazed tile samples; (b) experimental scheme.

Figure S2. Diffractogram obtained by XRD for the fired ceramic body with signalization of the main mineral phases of the tile models.

Figure S3. Raman spectra of crystalline compounds of the produced white glaze and corrosion products over the glaze. (i) Quartz and (ii) cassiterite with the Raman signatures of the glass structure identified *in situ* on the white glaze; (iii) calcium sulfate (gypsum) and (iv) calcium carbonate (calcite) detected over the glazes after the biodeterioration experiment.

Figure S4. Example of digital image analysis of a pristine and an aged tile model covered by the green biofilm. (a) Original image of a pristine glazed tile. (b) Segmented image (binary) of the first Principal Component. (c) Outlines of the areas selected for quantifying the surface covered area of the pristine glazed tile. (d) Original image of an aged glazed tile. (e) Segmented image (binary) of the first Principal Component. (f) Outlines of the areas selected for quantifying the surface covered area of the aged glazed tile.