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Biodeterioration of wall painting at the UNESCO site of Varallo: pink patina.

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Abstract **– Chapel 11 at the Sacro Monte di Varallo, a UNESCO World Heritage site, is decorated with wall paintings and life-size statues. The wall paintings date from 1591 but were heavily repainted at the end of 19th century and again in 1955. Visual examination of the surface revealed the signs of possible microbiological growth (MBG). *In situ* investigations with portable microscopy (using visible and ultraviolet radiations) and with Adenosine Triphosphates (ATP) measurements were carried out to determine the presence of MBG and the level of their activities. Several types of MBG were identified, but this work discusses the main and more extended discoloration found: the pink patina. In laboratory, isolation and identification by 16S ribosomal RNA (rRNA) and internal transcribed spacer (ITS) sequencing were carried out to determine the species. The results obtained will be important for planning the remedial intervention, the post treatment monitoring and the maintenance processes.**

Key words: Varallo, wall painting, biodegradation, ATP, 16S rRNA, ITS, pink patina

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I. INTRODUCTION

The Sacro Monte di Varallo is a UNESCO World Heritage site located in Val Sesia in the North of Italy. It includes 45 chapels representing episodes of the life of Christ (De Filippis 2006). Since 2015 the University of Applied Sciences and Arts of Southern Switzerland (SUPSI) is working on a project to conserve one of the largest chapel representing the 'Massacre of the Innocents' (Fig. 1). This chapel (Chapel 11), completed in 1591, is decorated with wall paintings covering an area of approximately 300 m^2 and contains 71 realistic life-size polychrome terracotta sculptures. The walls and the vault were painted by the Della Rovere brothers, also known as "the Fiamminghini". The wall paintings were extensively repainted in at least two phases, first in the late 19th century and again in 1955 (Piqué et al. 2016).

Fig. 1. Chapel 11, view of 'The massacre of the innocents' before conservation (Picture by Centro Conservazione Restauro "La Venaria Reale", 2012)

Chapel 11 is located in a depression behind the Basilica and is only partially hit by sunlight. Throughout the year, the air temperature within the chapel varies between -3 and 27°C. It falls below zero for several consecutive days during winter periods. The climate of the chapel is

characterized by high relative humidity values, ranging from 20% to 100%, often reaching favorable conditions for condensation events (particularly on the East wall, but also on the North and South walls in the high parts) and for biological colonization.
In these environmental conditions of light and humidity, the microbial activity can play an important role in the majority of the transformations, discolorations, patinas and color changes.
Among the various types of deterioration visible in Chapel 11, we focused on the pink patina spread on the surface of the wall paintings to determine its nature and its association with biodeterioration. The microorganisms isolated from this patina were identified and characterized using optical microscopy (OM) and microbiology techniques involving cultivation, isolation, 16S rRNA and ITS sequencing. This work summarize some of the researches carried out for a Bachelor thesis which focused in developing an investigation methodology to tackle the study of biodeterioration.

MATERIAL AND METHODS

Environmental data

Environmental parameters were recorded at 15 min intervals for 14 months using a Testo 6621 sensor (for air temperature and relative humidity) and a thermocouple type K (for surface temperature). Data were collected in 6 locations within the Chapel.

Microscopy

To carry out in situ microscopy analysis, damaged areas were directly analyzed with portable microscopes, Dino-Lite (non-invasive observations directly on the surface) and Cyscope HP (invasive observation immediately after sample collection) (Fig.2). Powder samples, obtained by scraping off surface material with a scalpel, from the selected areas were rehydrated directly on a microscope slide with a drop of the following solutions: distillated water, 0.01% Calcofluor white and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) from a 1 mg/mL stock solution. Samples were then covered with a cover-glass and immediately analyzed under the microscope (Cyscope HP) with visible and UV light.

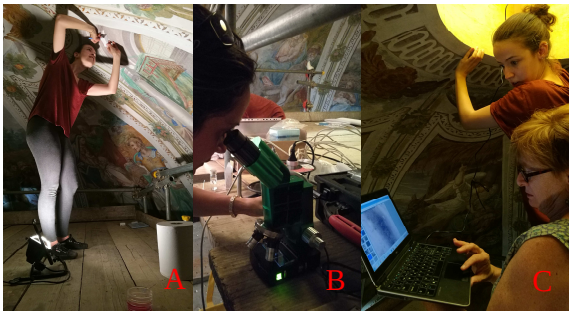

Fig. 2 Site investigation on the wall paintings in Chapel 11 at the Sacro Monte di Varallo. A: sampling by scraping off surface material with a scalpel. B: *in situ* microscopic analysis with portable microscope (Cyscope HP). C: *in situ* microscopic analysis with portable microscope (Dino-Lite).

ATP measurements

Defined areas of 1 cm^2 were sampled with sterile swabs moistened with sterile 0.9% NaCl solution in spring and summer seasons 2017 (autumn and winter season analysis are in progress). After sampling, swabs were soaked in 0.5 ml of sterile water and 0.1 ml were used to determine ATP bioluminescence using CellTitre-Glo (Promega). The assay was carried out immediately after swabbing, according to the manufacturer´s instructions). Blanks were obtained using sterile swabs moistened with sterile 0.9% NaCl solution (control 1) and with sample were no visible alterations were detected (control 2). Bioluminescence readings were expressed as relative light units (RLU).

Sampling and isolation of microorganisms

Samples representative of the main visive alteration (pink patina) from the wall paintings were collected from two distinct areas (nearby the points selected for ATP measurements and microscopic analysis) with a sterile cotton swabs immersed in sterile 0.9% NaCl solution and by scraping off surface material with a scalpel only on spring samplings. Sample material from scraping from the pink patina was suspended in 0.9% NaCl solution and shaken for 1 h. 100 µl of appropriate dilutions were transferred to Plate Count Agar (PCA), PCA supplemented with 3% sodium chloride, Reasoner`s 2A Agar (R2A), R2A supplemented with 3% sodium chloride, Trypticase Soy Agar (TSA), TSA supplemented with 3% sodium chloride and Sabouraud Agar.
Sample swabs were kept in transport medium until the transfer to the growing media described above.
Plates were incubated at 22.5 °C for at least 2 weeks

under aerobic conditions. Colonies were counted at different time interval. Well-defined single colonies, selected according to their morphology (color and shape) were isolated on a fresh agar plate and identified by 16S rDNA/ITS sequencing.

DNA extraction and sequencing

Bacterial genomic DNA was extracted using InstaGene Matrix (BIO-RAD) according to the manufacturer's instructions. The primers used for the 16S rDNA PCR were UniR 5' ATG GTA CCG TGT GAC GGG CGG TGT GTA 3' and UniL 5' ATT CTA GAG TTT GAT CAT GGC TCA 3'. The PCR reaction was performed with 5 µL of genomic DNA as a template in a 25 µL reaction mixture by using HotStar-Taq (Qiagen) for the following cycles: activation of Taq polymerase at 95℃ for 15 minutes, followed by 35 cycles at 95℃ for 30 seconds, 52℃ and 72℃ for 1 minute each, finishing with a 7-minute step at 72℃. Fungal DNA was extracted by the phenol-chloroform method. The ITS regions were amplified using primers ITS1 5`TCC GTA GGT GAA CCT GCG G 3`and ITS4 5`TCC TCC GCT TAT TGA TAT GC 3`. The PCR reaction was performed with 5 µL of genomic DNA as a template in a 25 µL reaction mixture by using HotStar-Taq (Qiagen) for the following cycles: activation of Taq polymerase at 95℃ for 15 minutes, followed by 40 cycles at 95℃ for 30 seconds, 55℃ and 72℃ for 1 minute each, finishing with a 10-minute step at 72℃.
The amplification products were purified and sequenced using primers UniL or ITS4. Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems), incubated at 95℃ for 5 minutes, put on ice for 5 minutes, and analyzed by an ABI Prism 3730XL DNA analyzer (Applied Biosystems). Sequences were compared to publicly available databases.

Methodology

The sequence of investigations followed is illustrated in Fig. 3. It started from in situ visual examination and investigations, which allowed to identify areas of interest which were then sampled for laboratory analysis.

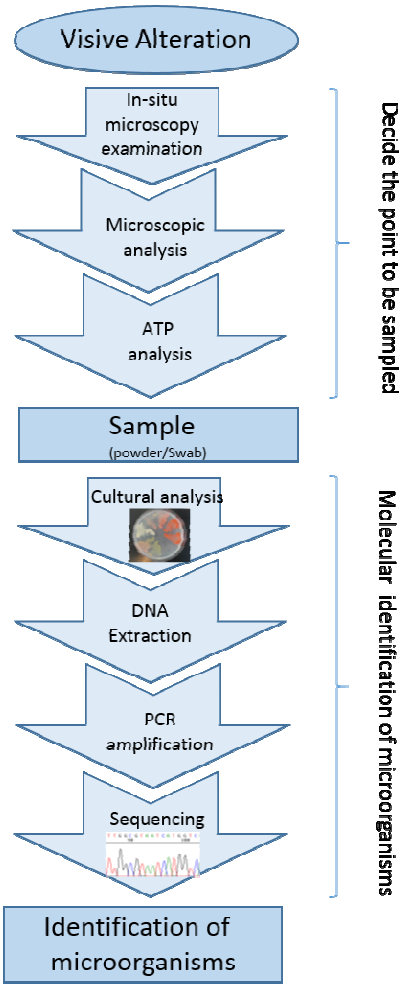

Fig.3 General scheme of the approach used in this work to analyze the microbiological growth.

Results and Discussion

Visual examination by Macroscopic Analysis

Visual examination of the wall paintings showed discolorations varying from white to pink (Fig.4) and brown patinas in some areas; dark spots were also detected. Some of these alterations were well delimited, while others, such as the pink patina, were visible on larger areas of the chapel, covered or not with paintings, suggesting the possible presence of microbial growth and/or microbial colonization.

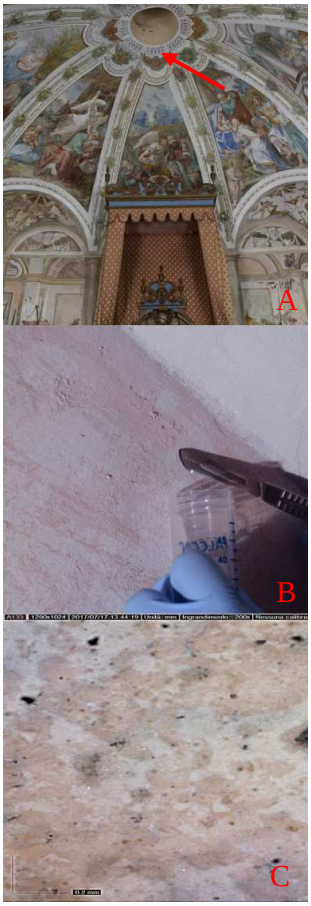

Fig. 4. Pink patina. A: Area where the pink patina was visible, indicated with an arrow. B: point of sampling, C: image of the pink patina taken by Dino-lite, 200x.

Mapping of the discolorations, patinas and color changes showed that the most significant and diffused phenomenon was a pink patina, on which we focused our work.

Microscopic Analysis

Chlorophylls are the dominant pigments in green algae, while phycobilins are the main light harvesting pigments in cyanobacteria and red algae. Observing the fluorescence due to the presence of these pigments by using epifluorescence microscopy, it is possible to recognize these two groups of microorganisms, The absence of autofluorescent cells (data not shown), suggested that algae and cyanobacteria were not present in our samples. The low light condition inside the Chapel can explain the absence of these phototrophic microorganisms on the surfaces analyzed
DAPI (Fig.5) staining reveal the presence of bacterial cells while fungal cells were not detected by microscopy after Calcofluor white stain (data not shown)

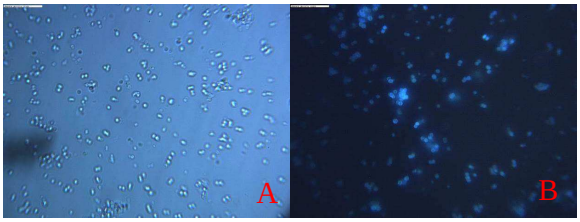

Fig.5 Sample of Pink patina in water dispersion A: visible light, B: DAPI staining. 1000x magnification

ATP Analysis

ATP is the principal energy carrier for all living organisms, and can be used as an indicator of microbial presence and biological activity.
Results of ATP measurements are presented in Table 1.

Table 1. Seasonal measurements of temperature and bioluminescence of the selected areas. Bioluminescence readings are expressed as relative light units (RLU).

Visive Alteration	Spring measurement Temp. = 12-15 °C	Summer measurement Temp. = 22-25 °C
Control 1 (NaCl)	0.005	0.012
Control 2 (no visible alteration)	5.13	6.08
Pink patina 1	10.22	104.6
Pink patina 2	11.18	40.23

ATP values increased during the summer months, proving an increased microbial activity at the site, due probably to better temperature conditions for bacterial growth. We do not have the results of the autumn and winter seasons yet, but we can speculate that the ATP values will decrease again in the cold season.

Isolation of microorganisms

The samples collected with the two sampling methods, swabbing and scraping off the surface material to obtain powder sample, gave consistent. Quantitative analysis of the powder samples and qualitative analysis (no growth, low, medium and high) of the swabs are shown in Table 2.

Table 2. Total microbial counts of aerobic microorganisms

	PCA	PCA+ NaCl	R2A	R2A+ NaCl	TSA	TSA+ NaCl	Sab
Swab	low	high	high	high	low	high	0
Powder	2	89	90	110	15	120	0

After 5-7 days, samples taken from the pink patina developed numerous dark pink and light pink bacterial colonies. Some white and yellowish bacterial colonies with diverse size and shape were also observed (Fig. 6). The growth of the pinkish colonies was higher in agar media containing NaCl, indicating the presence of halotolerant and/or halophilic bacteria.

Fig.6 Inoculated plate with powder sample of pink patina on R2A

We could also observe that the pink colonies were more abundant on R2A medium than on TSA or PCA without NaCl. This can be explained by the fact that R2A has a higher salt concentration than the other media. Moreover, R2A is recommended for the detection of slow growing microorganisms adapted to environments with low concentration of nutrients, as the conditions of the wall paintings.

Fungal growth was also observed in some plates, not specific for their growth, after two weeks incubation.

DNA extraction and sequencing

The 16S rRNA and ITS sequencing based analyses are fundamental methods in microbiology to identify microorganisms and to explore the microbial diversity. Genomic DNA was extracted from isolated bacterial or fungal colonies, amplified and sequenced. Sequences obtained were compared to the 16S rRNA or ITS sequences in Genbank collection using the Basic Local Alignment Search Tool (BLAST), which finds regions of local similarity between sequences.

The pink colonies (dark and light) most present in culture media were identified by 16S rRNA as *Arthrobacter agilis*. The association between this kind of alteration (pink patina) and the presence of *Arthrobacter agilis* has already been observed in other studies [F.Imperi 2007, M.M Lopes-Miras 2013] The genus *Arthrobacter* contains more than 70 species, some described as halotolerant bacteria [H.J Busse 2016], that can be either pigmented or non-pigmented. Pinkish pigmentation has been identified in several species of the genus: *Arthrobacter roseus sp. Nov*., *Arthrobacter agilis* and *Arthrobacter hyalinus*. In the case of *Arthrobacter agilis*, the pink coloration is due to the production of carotenoids. The nature of these compounds is still partially unknown, although some studies have reported that carotenoids are involved in the survival to oxidative damage and in UV radiation resistance [Sutthiwong, 2014].

Besides *Arthrobacter agilis*, we could also identify other microorganisms, such as *Rhodococcus* sp, *Chaetomium* sp., *Cryptococcus* sp., *Actinobacteria* sp., *Penicillium* sp., *Bacillus* sp., and *Cladosporium sphaeropermum* that have already been associated with biodeterioeration of several substrates. [G. Caneva 2007, M. Falkiewicz-Dulik 2015], as well as *Staphylococcus warnerii* and *Staphylococcus epidermidis*, common commensal organisms found as part of the human skin flora

The partial sequences we obtained for some bacteria and fungi strains did not allowed an identification beyond the genus level.

It is important to analyze the microbiological results critically to distinguish between the microorganisms responsible for the observed damage from those that do not contribute to it. Clearly, the *Staphylococcus* spp. identified are contaminants, since frequent human skin colonizers, and are not involved in the biodeterioration process. On the other hand, the proliferation of *Arthrobacter agilis*, which contains carotenoid pigments such as β-carotene in their cell membranes [Oren 2009], can significantly modify the optical appearance of the wall surfaces. To confirm that this microorganism is responsible for the pink color alteration, we plan to compare the pigments absorption and emission spectra after extraction from *Arthrobacter agilis* cultures and pink powder samples

OUTLOOK

The next phases of this project will focus on completing the microbiological analysis of all samples in laboratory. Furthermore, we plan to construct a calibration curve to correlate ATP measurements with the number of bacterial cells present *in situ*, in particular for the main microorganism (*Arthrobacter agilis*) detected in the pink discolorations. This will allow a rapid evaluation of the discoloration and the monitoring of its evolution. Species identification will be implemented through mass spectrometry by using protein analysis with Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS).
Methods that allow the study of microbiological populations, such as automated ribosomal intergenic spacer analysis (ARISA) and next generation sequencing (NGS), will be applied, to detect viable but not culturable (VBNC) microorganisms and to identify species that are present at very low number.

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