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Abstract

Rosy discoloration has affected the stone steps in the main spiral staircase of the Sully-sur-Loire castle donjon for many years now. This study monitors the development of the coloration and the environmental climatic conditions in the staircase to understand the conditions favoring the presence of this rosy discoloration. High-throughput sequencing was performed on healthy stone and on pink pigmented stone to identify the agent responsible for this discoloration. The results suggest that the rosy discoloration is the mark of a former degradation process which is now inactive, and that bacteria were the main agent of the pink patina formation. *Nitiliruptor* was the main genus identified in the pink parts of the staircase. This is the first time that this bacterial genus has been linked to a cultural heritage biodeterioration process. There was no evidence that this bacterial genus was responsible for the development of the pink patina, but it could have replaced it as a subsequent evolution of the process.

Keywords

Cultural heritage preservation, stone biodeterioration, rosy discoloration, high-throughput sequencing, *Nitriliruptor sp.*, climatic monitoring

1. Introduction

Stone is one of the main and oldest materials used for artistic expression in the field of cultural heritage. It was already used during the upper Paleolithic (between 50 000 and 10 000 years ago) as a support for engraving and sculpting in shelters and caves (Abadía and Morales, 2013). The use of stone ranges from monumental constructions to the sculpture of small statues.

The different types of stone used in the field of cultural heritage include marble, limestone, sandstone and granite, and even man-made stones such as brick, mortar or concrete. Whatever the type of stone used, they are all subject to some degree of biodeterioration (Warscheid and Braams, 2000). This process is defined as an undesirable change in the properties of material caused by the activity of biological agents (Hueck, 1965). The intensity of the biodeterioration process on stone monuments depends on the intrinsic properties that define the bioreceptivity of each stone (Guillitte, 1995; Miller et al., 2012), combined with the effects of climatic changes and air pollution (Brimblecombe and Grossi, 2009; Caneva et al., 1995; Thornbush and Viles, 2006).

The detrimental effects of organisms on stone monuments may be aesthetic, chemical and/or physical (Scheerer et al., 2009). For example, the secretion of inorganic and organic acids by organisms on stones leads to a process known as biocorrosion. This results in the dissolution of the mineral matrix of the stone and the precipitation of salts, possibly causing physical stress in the stone (McNamara and Mitchell, 2005; Warscheid and Braams, 2000). Organisms can also enter the stone via pores in the material, causing mechanical damage and changing the physical properties of the structural material (Scheerer et al., 2009; Sterflinger and Piñar, 2013).

Different types of organisms can be involved in biodeterioration, with both macro and microorganisms observed. Phototrophic microorganisms such as algae and cyanobacteria are generally considered as the initial colonizers (pioneer organisms) and produce colored patinas and incrustations on stone monuments (Crispim and Gaylarde, 2005; Macedo et al., 2009), whilst lichens grow much more slowly (Hoppert and König, 2006). The presence of these first colonizers facilitates colonization by chemo-organotrophic bacteria and fungi (Gorbushina et al., 2005; Li et al., 2016), which are strongly involved in the physical and chemical deterioration process of stone monuments (Pinheiro et al., 2018; Salvadori and Municchia, 2016). Some chemo-organotrophic organisms have also been found on stone monuments without the presence of pioneer organisms, and use organic matter derived from rock mineral, airborne particles or products used in preceding restorations (Scheerer et al., 2009; Warscheid and Braams, 2000). Higher plants are macro-organisms, and may thus play a role in the biodeterioration of stone monuments by an initial colonization of monuments by pioneer plants (herbaceous annuals and perennials) that cause minor damage and are then replaced by shrubs or trees, which can cause substantial physical damage (Korkanç and Savran, 2015; Lisci et al., 2003).

Within the biodeterioration process, the phenomenon of rosy discoloration of stones and wall paintings has attracted increasing amounts of interest over recent years (De Felice et al., 2010; Ettenauer et al., 2014; Gurtner et al., 2000; Imperi et al., 2007; Laiz et al., 2009; Piñar et al., 2014b; Tescari et al., 2018b). This type of discoloration has been described in subterranean and non-subterranean environments and has been widely reported in central and southern Europe (Piñar et al., 2014a). Actinobacteria belonging to *Rubrobacter* genus were found to be involved in discoloration in most of these cases (Jurado et al., 2012; Laiz et al., 2009; Nugari et al., 2009; Schabereiter-Gurtner et al., 2001). In some other cases, other pink pigmented bacteria such as *Arthrobacter*, *Rhodococcus* or *Methylobacterium* were identified (Piñar et al., 2014b; Tescari et al., 2018a; Tescari et al., 2018b). The common link between the different monuments affected by the development of pink biofilms seems to be water infiltration (Piñar et al., 2014a). The crystallization of salts that occurs with these infiltrations may offer optimal growth conditions for halophilic microorganisms in addition to their own destructive effect on the stone.

In France, rosy discolorations are observable in Sully-sur-Loire castle. It is far from being the only monument affected by this phenomenon in France, but it is one of the first to have been intensively studied (Berdoulay and Salvado, 2009). The castle is one of the monuments listed by UNESCO in the Loire Valley in 2000. It is located in the far eastern sector of the area listed by UNESCO in the Loiret region. There has been a castle at this location on the left bank of the Loire since the 12th century, but the construction of the current castle began at the end of the 14th century. Additions and transformations were carried out until the 19th century. Large reconstructions occurred in the 20th century after a great fire in 1918 and damage from bombings during World War II. The castle is made of white tufa, which was the stone typically used as a building material in the Loire Valley during the Renaissance period. Tufa is a yellowish-white, porous and very fine-grained siliceous limestone with a small amount of clayey minerals (Janvier-Badosa et al., 2015). The geometrical and symmetrical distribution of the rosy discoloration on the stones of Sully-sur-Loire castle made it a remarkable study case to compare and determine the conditions affecting its development.

The aim of this study was to confirm or exclude a biological origin for rosy discoloration, and, if applicable, identify the organisms responsible for the biofilm development. In a second step, we sought to compare the environmental conditions in areas of stone affected by rosy discoloration with those observed in healthy areas to widen our knowledge of the factors that lead to this phenomenon.

2. Materials and Methods

2.1 Study area

The rosy discoloration has only been observed on indoor stones and mainly in the "escalier d'honneur": the main spiral staircase of the donjon (Fig.1a). Not all the steps are affected to the same extent by pink coloration, which is mainly located between the first and the second floor, on the underside of the steps and exclusively on the outer part of the steps (Fig. 1b). The steps range from those with no pink coloration to those with almost 75 % of their surface affected by pink coloration.

Two of the most severely affected steps between the first and the second floor were chosen for this study. This area had the further advantage of being closed to the public. The first step was used for colorimetric and climatic monitoring (Fig. 2), while the second was sampled for metagenomic microbial analysis.

2.2 Colorimetric monitoring

Colorimetric monitoring was used to characterize the initial differences between the white area (unaffected) and the pink area (affected) of a given stone step and then to monitor the progression of the coloration within and between these two sections through time according to different factors. The chosen step was first divided into two parts: white and pink (Supplementary fig. 1). Four measurement sections were then delineated within each of these two sections: *i)* section scratched with a scalpel to remove the outside layer of the stone then covered to hide it from direct light; *ii)* section scratched but not covered; *iii)* section covered but not scratched; *iv)* control section left unscratched and not covered. Scratching removed the coloration in the pink section and would thus enable us to observe any return of this coloration. Covering was carried out to check for a possible role of light in the development of the pink coloration. The first colorimetric measurements were made in September 2014 before and after scratching, then colorimetric progression was regularly checked over a three-year period: in May 2015, January 2016, July 2017 and January 2018.

The measurements were made in each defined section with a Chroma meter CR-410 (Konica Minolta, Tokyo, Japan), which uses the L*a*b* CIE 1976 color space under D65 illuminant, and 10° observer. Each measurement returns three values assessed on a sample with a 5 cm diameter: the L* value for lightness (0=black, 100=white); the a* value for the green-red component (-120=green, 120=red); and the b* value for the blue-yellow component (-120=blue, 120=yellow). Each color value was obtained using the mean of two replicated measurements.

2.3 Raman spectroscopy

Raman spectroscopy was used on the pink powder obtained after scratching the stone to characterize the pigment involved. Analysis was performed using an InVia confocal Raman microscope (Renishaw, Wotton-under-Edge, UK) equipped with a 785-nm laser diode and a

50x lens at a power of 10 % (5.05 mW). Raman spectra were obtained by accumulating 50 acquisitions of 10 s.

2.4 Climatic monitoring

The climatic conditions were monitored on the same step as the colorimetric measurements (Supplementary fig. 1). The temperature inside the stone was monitored by drilling a hole in the pink and white sections and installing a Telog R-3300 probe (Trimble Inc., Sunnyvale, USA). This probe records temperatures between -20 and 60 °C with a resolution and an accuracy of 0.01 °C. Environmental conditions were monitored by hanging a Telog R-2162 probe (Trimble Inc., Sunnyvale, USA) between the two areas to measure air temperature and relative humidity. This probe records temperatures between -30 and 80 °C with a resolution of 0.12 °C and an accuracy of 0.4 %, and records relative humidity between 5 and 95 % with a resolution of 0.1 % and an accuracy of 2 %. Each probe recorded a measurement once every two hours. The environmental data collected in this study was then compared to reference data for outdoor temperature and relative humidity were collected from a website (www.historique-meteo.net).

2.5 Microbiological analysis

2.5.1 Sampling

Samples were taken from the white and pink areas of the same step for high-throughput sequencing analysis of microbiological populations. Three samples were taken from the white area (W1, W2 and W3) and from the pink area (P1, P2, P3). Samples were collected by scratching a 25 cm^2 surface with a scalpel, providing a fine powder.

2.5.2 Total genomic DNA extraction and high-throughput sequencing

Bacterial, fungal and eukaryotic communities were identified for each sample. Total genomic DNA was extracted from the samples and from an outgroup (O) composed of non-filtered autoclaved water ("dead DNA" remained in the sample) using FastDNA™ Spin Kit for soil (MP Biomedicals, LLC, Santa Ana, USA), according to the manufacturer's instructions. High-throughput sequencing library preparation and Illumina Miseq (Illumina, San Diego, USA) sequencing were conducted by Genoscreen (Lille, France). The Metabiote protocol (Genoscreen, Lille, France) was used to prepare and barcode the twenty-one libraries (6 samples + 1 outgroup x 3 microbial communities), and targeted the 16S ribosomal V3 and V4 DNA hypervariable regions, the ITS1/ITS2 DNA region and the 18S ribosomal DNA region for bacteria, fungi and eukaryotes, respectively. The twenty-seven libraries were then pooled for single-run sequencing. Sequencing was performed with 4 pM of the pooled library using a 300-bp paired-end sequencing protocol on the Illumina MiSeq platform.

2.5.3 Processing of high-throughput sequencing data

The data were demultiplexed using CASAVA software (Illumina, San Diego, USA). After primer removal, the sequences with a low Phred quality score (Q<30) were removed and the overlapping paired-end reads were assembled using FLASH software (Magoč and Salzberg, 2011). Finally a QIIME 2 2018.11 pipeline was used to remove chimeric sequences, cluster the sequences in operational taxonomic units (OTUs; quality-filter and deblur plugins; q-score and denoise-16S/denoise-other functions) and taxonomically classify the obtained OTUs (plugin feature-classifier; function classify-consensus-vsearch; (Bolyen et al., 2018)). The

taxonomic classification of bacterial, fungal and eukaryotic OTUs was achieved using Greengenes database (McDonald et al., 2012), the UNITE database (Nilsson et al., 2018) and the SILVA database (Quast et al., 2013), respectively.

2.6 Statistics

2.6.1 Colorimetric monitoring

Statistical analyses were carried out using R 3.2.5 with α = 5 % (R Core Team, 2014). The results of colorimetric measurements were analyzed by a generalized linear mixed model (GLMM). The GLMM is a suitable analytical tool for non-normal data collected from complicated designs involving random and fixed effects.

Four qualitative variables were used to design the GLMM, namely the *Color area* (two modalities corresponding to the health status of the sampling area: *White* for the healthy part of stones and *Pink* for the pink part of stones); *Covering* (two modalities: *Yes* for samples that had been covered during the monitoring and *No* for all others); *Scratching* (two modalities: *Yes* for samples which had been scratched prior to monitoring and *No* for all others); and *Measurement date* (six modalities corresponding to the six measurement dates, i.e. *Sept. 2014 before scratching*, *Sept. 2014 after scratching*, *May 2015*, *Jan. 2016*, *July 2017*, and *Jan. 2018*).

The GLMM for this study was:

$$Link(p_{ijklm}) = \eta_{ijklm} = \theta + \alpha_i + \beta_j + \gamma_k + \delta_l + \alpha_i{:}\gamma_k + \alpha_i{:}\delta_l + \gamma_k{:}\delta_l + \alpha_i{:}\gamma_k{:}\delta_l + \mu_{ijkl} + \varepsilon_{ijklm}$$

where θ is the general effect, α_i is the effect of *Color area,* β_j is the effect of *Covering*, γ_k is the effect of *Scratching*, δ_l is the effect of *Measurement date*, $\alpha_i{:}\gamma_k$ is the effect of the interaction between *Color area* and *Scratching*, $\alpha_i{:}\delta_l$ is the effect of the interaction between *Color area* and *Measurement date,* $\gamma_k{:}\delta_l$ is the effect of the interaction between *Scratching* and *Measurement date*, $\alpha_i{:}\gamma_k{:}\delta_l$ is the effect of the triple interaction between *Color area*, *Scratching* and *Measurement date*, μ_{ijkl} is the experimental error associated to each measurement and ε_{ijklm} is the residual error. The effects of other interactions between variables were not included in the model because they do not provide supplementary information. α, β, γ, δ, $\alpha{:}\gamma$, $\alpha{:}\delta$, $\gamma{:}\delta$ and $\alpha{:}\gamma{:}\delta$ were considered as fixed effects, while μ was considered as a random effect. The term η_{ijklm} is known as the linear predictor. This term is obtained by applying the variable p_{ijklm} to the link function of the model. The choice of the link function depends on the p_{ijklm} distribution law. The identity link function is suitable for values provided by colorimetric measurements, i.e. continuous data, either positive or negative (Wolfinger and O'connell, 1993). GLMM parameters were estimated using the penalized quasi-likelihood (PQL) technique (MASS package; glmmPQL function; Wolfinger and O'connell, 1993). A Wald chi-square test was performed to determine the significance degree of each explanatory variable in the GLMM (car package; Anova function; Fox, 2015). The GLMM was completed with a Tukey's post-hoc test in order to check the significance of differences between means (lsmeans package; lsmeans function; Bretz et al., 2016).

2.6.2 Climatic monitoring

The temperatures recorded in the white and pink areas of stone and in the air were compared through a one-way analysis of variance (ANOVA; stats package; aov function; Fisher, 2006) after checking the homogeneity of distribution variances with a Bartlett test (stats package;

bartlett.test function; Bartlett, 1937). This statistical test checks if the means of several samples are equal, and is useful to compare three or more means. The ANOVA test was completed by post-hoc analysis to rank the different means. The significance of differences between means was assessed using Tukey's range test (multcomp package; glht function; Faraway, 2002).

The relative air humidity values were used to calculate dew points to check for potential condensation on the surface of the stone (weathermetrics package; function humidity.to.dewpoint).

2.6.3 Alpha and beta diversity of microbial communities

Alpha diversity, i.e. the diversity of microbial species in each sample, and beta diversity, i.e. the difference in microbial communities between samples, were identified using QIIME 2 2018.11. After generating a phylogenetic tree (phylogeny plugin; align-to-tree-mafft-fasttree function), the Shannon's diversity index and the Pielou's evenness index were calculated to characterize alpha diversity (diversity plugin; core-metrics-phylogenetic function; Hill, 1973), while beta diversity was assessed through principle coordinates analysis (PCoA) based on the determination of the Bray-Curtis distance (diversity plugin; core-metrics-phylogenetic function; Legendre and Gallagher, 2001). The significance of the groups obtained was then assessed with a Kruskal-Wallis non-parametric test for alpha diversity indexes (diversity plugin; alpha-group-significance function; Hollander and Wolfe, 1999), and with PERMANOVA for beta diversity (999 permutations; diversity plugin; beta-group-significance function; Anderson, 2001).

3. Results

3.1 Colorimetric monitoring

Scratching the surface of the stone sufficed to completely remove any visible evidence of pink coloration. The colors in both the white and pink areas showed no sign of change over the three and a half years of monitoring: the scratched sections did not become pink again, and no changes were observed in the colors of the white and pink areas on each of the two steps.

The results of the general linear mixed models designs were the same for the three dimensions of the L*a*b* color space (Table 1): *Color area*, *Scratching* and *Measurement date* and interactions between these factors all had a significant effect on the model, whereas *Covering* did not. The effect of factors was more pronounced for L* and b* variables than for a* variable. The measurements recorded in the unscratched pink area and the first measurement made in September 2014 (i.e. before scratching) in the scratched pink area showed that the pink area was characterized by a lower L* value and higher a* and b* values than those recorded in the white area (Fig. 3 and supplementary fig. 2). The difference was statistically supported for L* and b*, but not for a*. The difference recorded between sections that had not been scratched remained the same throughout the study. Measurements recorded after scratching in September 2014 showed that the scratching led to an increase in the L* value and a decrease in a* and b* values in the pink and white areas. This difference in the evolution of scratched areas was statistically supported by L* and b* values for the pink area and only by the L* value for the white area. Finally, these differences remained statistically stable in terms of L* and a* values for all studied modalities from after scratching in September 2014 until the end of the colorimetric study in January 2018. The differences remained statistically stable in terms of b* value from the second date of assessment, May 2015, until the end of the study. In other words, the significant effect of the *Date* factor was

almost entirely supported by the differences observed at the first date of assessment before and after scratching and the color did not evolve until the end of the study, confirming visual observations.

3.2 Raman spectroscopy

After baseline correction and removal of stone-related noise, Raman spectroscopy revealed the presence of a pigment belonging to the family of carotenoids in the pink powder scratched from the step (Supplementary fig. 3). Carotenoids are usually characterized by three peaks corresponding to C–CH bending, C–C stretching and C=C stretching. C–C and C=C stretching were clearly identifiable at 1151 and 1506 cm^{-1}, and C=CH3 bending was also present at 1003 cm^{-1}.

3.3 Climatic monitoring

The room temperature in the staircase ranged from 3.22 to 27.33 °C during the study (Table 2). Climatic monitoring showed that the room temperature was linked to the outside temperature (Supplementary fig. 4a): the seasonal variations in temperature had a clear impact on room air temperature, with low temperatures recorded during winter and warm temperatures during summer. Daily room temperature variations due to day/night alternation were less marked than those observed outdoors. Moreover, the room temperature was warmer during winter and cooler during summer than the outside temperatures. The relative humidity of room air ranged from 30.01 - 89.2 %. As observed for room temperature, variations in relative humidity of room air followed those recorded for outside relative humidity, although these values are lower than those recorded for outdoor relative humidity. Indoor relative humidity remained between 45 and 65 % for most of the year and rarely exceeded 80 % (Supplementary table 1).

The surface temperature in the white area and in the pink area followed room temperature variations (Supplementary fig. 4b), but the temperature was significantly warmer in the pink area than in both the white area and the room air (Supplementary table 2). The ANOVA study also showed that the temperature in the white area was significantly lower than the room temperature. However, this result should be considered with caution as the interval value was lower than the accuracy of the ambient probe. Finally, the calculation of dew points from ambient relative humidity and surface temperatures showed that no condensation could have been formed at the surface of the step during the year of climatic monitoring.

3.4 Alpha and beta diversity of microbial communities

The only perceptible alpha diversity difference between the white and the pink areas was related to bacterial communities (Supplementary table 3). Bacterial richness (Shannon index) and equitability (Pielou's index) were higher in the pink area than in the white area, and this difference is almost significantly supported (Supplementary table 4). No alpha diversity difference was observed between sampling areas for fungal and eukaryote communities.

The study of beta diversity among bacterial communities clearly separated samples into two groups on the first PCoA axis (Supplementary fig. 5): communities sampled in the pink area shift to positive values on this axis as compared to communities sampled in the white area. The community assessed in the outgroup sample was separated from the two other groups by shifting to positive values on the second PCoA axis. The PERMANOVA test associated to the study of beta diversity confirmed that the sampling area had a significant effect on bacterial

community composition, even if the post-hoc test failed to separate the white and pink areas (Supplementary table 5). Conversely, the PCoA expressed no difference between sampling areas in fungal and eukaryotes communities, with the communities of some samples located closer to the outgroup sample than to other samples in the same area.

The assignment of OTUs revealed that the majority of the bacteria found in the white and in the pink area were Actinobacteria, while the outgroup sample was dominated by Proteobacteria. Among Actinobacteria, the communities of white area samples were largely dominated by the genus *Rubrobacter*, while the communities of pink area samples were largely dominated by the genus *Nitriliruptor* (Fig. 4). Low proportions of *Rubrobacter* were also detected in pink area and low proportions of *Nitriliruptor* were also detected in white area. All other bacterial taxa detected in white and pink areas were in low proportions.

The phylogenetic analysis of fungal and eukaryotes communities did not produce significant differences between samples, especially regarding pink-pigmented yeasts or algae.

4. Discussion

4.1 Bacterial origin of rosy discoloration

The most important result of this study is the confirmation of the biological origin of the rosy discoloration affecting the stone staircase in Sully-sur-Loire castle. Raman spectroscopy assessment of the pink pigment produced on the stone revealed the characteristic bands of C=C and C–C stretching and C–CH bending of carotenoids (De Oliveira et al., 2010). Next-generation sequencing did not reveal any differences between fungal communities and more generally between eukaryote communities on pink and white areas. Conversely, the composition of bacterial communities was strongly related to the type of sampling area. This result is in accordance with past studies that assigned such discoloration to bacterial biofilms (Piñar et al., 2014a). Actinobacteria was the most abundant phylum identified in both pink and white areas in our study. This phylum has already been widely reported in studies that focused on the deterioration of stone in general (Gorbushina, 2007; McNamara and Mitchell, 2005; Zanardini et al., 2016), and on rosy discoloration in particular (Ettenauer et al., 2014; Laiz et al., 2009; Tescari et al., 2018b). *Nitriliruptor* was the main bacterial genus identified in the pink area, while *Rubrobacter* was the main genus identified in the white area. This result differs from those obtained in past studies in which *Rubrobacter* was generally the main genus associated with rosy discoloration (Jurado et al., 2012; Laiz et al., 2009; Nugari et al., 2009). The unusual nature of this result led us to duplicate the study with new samples, but the second batch of samples gave the same result (data not shown). *Nitriliruptor* is an haloalkaliphilic actinobacterium first identified in soda lakes (Sorokin et al., 2009). This single species genus has also been reported in saline-alkali soil (Peng et al., 2017) and in arid soil (Neilson et al., 2012). The only reference of *Nitriliruptor* in the field of cultural heritage was in mineral crystals found on the skin of an 18th century mummy (Kraková et al., 2018). Consequently, this first evidence of the role played by *Nitriliruptor* in stone deterioration is of interest. This bacterial genus has been poorly studied to date, but numerous highly similar environmental clones of this genus have been identified, suggesting that this organism is quite abundant in nature despite being rare in culture collections (Goodfellow et al., 2012a).

4.2 Possible replacement of the main agent of rosy discoloration by Nitriliruptor sp.

The main data about *Nitriliruptor* in relation with this study is that this species is not known to produce pink pigment, with available strains described as colorless (Goodfellow et al., 2012b). Moreover, the colorimetric assessment of the white and the pink areas showed no evolution of the color in each area during the period of monitoring from the day the stone was scratched in September 2014 and January 2018. The only significant change in color was recorded at the beginning of the experiment between the measurements before scratching and the measurements after scratching. This suggests that the process leading to the formation of the rosy discoloration of stones was already finished at the beginning of the experiment and that the presence of *Nitriliruptor* may indicate a subsequent evolution of this process. Bacterioruberin-related carotenoids are frequently associated to *Rubrobacter sp.* and to the rosy discoloration phenomenon on stones (Imperi et al., 2007). In our study, the position of the bands in the Raman spectrum obtained from the pink powder corresponds to that expected for bacterioruberin-related carotenoids (Jehlička et al., 2013; Marshall et al., 2007). A plausible hypothesis is that the rosy discoloration may have been produced under the action of *Rubrobacter* strains, which may have then been replaced by *Nitriliruptor* strains; the pigments usually remain very stable on the materials, even if the causative microorganisms are already dead (Piñar et al., 2014a). The development of pink biofilms on stone has often been linked to water infiltrations and the formation of salt crystals (Piñar et al., 2014a). Carotenoids are known to protect the cells against photo-oxidative damage and to act as membrane stabilizers against salt stress (Köcher and Müller, 2011). The development of *Rubrobacter* would therefore be favoured when desiccation occurs through temperature increase and salt formation (Imperi et al., 2007; Laiz et al., 2009; Schabereiter-Gurtner et al., 2001). Interestingly, the ecological niche of *Nitriliruptor* seems to be very close to that of *Rubrobacter;* we have already mentioned that *Nitriliruptor* has been found in environments with high salinity. The studies that highlighted the presence of *Nitriliruptor* strains in a habitat also detected *Rubrobacter* strains (Ma and Gong, 2013; Neilson et al., 2012). The *Nitriliruptor* development on the staircase of Sully-sur-Loire could therefore be a natural progression of the *Rubrobacter* development under such environmental conditions. Further studies about physicochemical properties of the stone should be performed to determine whether particular conditions had favoured the development of *Nitriliruptor*. This is particularly important in the light of this bacterial genus' ability to use short-chain organic acids, amides and aliphatic nitriles as energy and carbon sources (Goodfellow et al., 2012b). On the other hand, *Rubrobacter* was also detected on stones that did not suffer from rosy discoloration (Chimienti et al., 2016; Li et al., 2016; Zanardini et al., 2019). It is not therefore impossible that *Rubrobacter* DNA was detected in the white area of the staircase, especially since the production of carotenoids by microorganism can vary largely according to environmental conditions (Calegari-Santos et al., 2016; Fong et al., 2001).

The genus *Nitriliruptor* so far includes only one species (Sorokin et al., 2009). Considering that the study of V3-V4 16s rRNA region is not accurate enough to provide species definition (Bukin et al., 2019), and that Actinobacteria are frequently carotenoid producers (Klassen, 2010), the possibility that *Nitriliruptor sp.* detected on the staircase is a new undescribed carotenoid producer species cannot either be dismissed. The production of carotenoid by this species could also vary largely according to environmental conditions. A shotgun metagenomics approach would help to answer this question in future studies by reconstructing the partial genome of the most prevalent species in the samples thus testing the presence of carotenoid synthesis genes.

4.3 Environmental factors involved in the development of rosy discoloration

Previous studies have noted that the development of pink biofilms has often been linked to problems of water infiltration and salt crystallization, and in some cases could be associated with the combined effect of an increase in temperature and the sudden decrease in water availability when the infiltration problem is solved (Imperi et al., 2007; Laiz et al., 2009). The climatic monitoring of the staircase showed that no condensation theoretically occurred at the surface of the stone during the year of study: indeed, the temperature recorded at this surface stayed above the dew point. Moreover, the relative humidity in the staircase air appeared relatively adequate in terms of conservation conditions. It released between 45 and 65 % of humidity more than 60 % of the time, which corresponds to the relative humidity values generally advised for conservation (D'Agostino et al., 2014; Merello et al., 2012). Nevertheless, this humidity range could be problematic if nitrates are present: sodium nitrate and magnesium nitrate crystallize when relative humidity drops below 60 % (Arnold and Zehnder, 1991). However, no efflorescence had been noted in the staircase during the observations and the presence of soluble salts was not assessed during this study. The origin of the water and of possible salt crystallization is most likely the result of external rainfall seeping through the external wall of the staircase. The location of the biofilm at the surface of the staircase steps supports this hypothesis: only the external part of the steps is affected, and the upper part of the staircase seemed to be more affected than the lower part. The administrators of the castle confirmed that external infiltration occurred through the roof of the stairwell, and that the problem had since been resolved. This reinforces the hypothesis that the rosy discoloration observed at the stone surface of the staircase corresponds to a past phenomenon which no longer occurs. Finally, the climatic monitoring showed that the temperature at the stone surface in the pink area was slightly higher than the temperature in the white area. This could have emphasized the desiccation phenomenon and favoured the development of *Rubrobacter* in the pink area (Imperi et al., 2007; Schabereiter-Gurtner et al., 2001).

These observations may explain the distribution of the pink area on the staircase surface and could indicate the action of several factors: the infiltration of external water through the wall, the subsequent formation of salts through crystallization and dissolution process, and the microclimatic differences of temperature occurring at the surface of the staircase.

5. Conclusions

The study confirms that the rosy discoloration occurring at the surface of the spiral staircase was due to bacterial growth. Bacteria belonging to *Rubrobacter* genus may have been the main producer of pink pigment, before being replaced by bacteria from the *Nitriliruptor* genus. Further studies about the physicochemical properties of the stone should be performed to determine if specific conditions favored the development of *Nitriliruptor*.

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References

Abadía, O.M., Morales, M.R.G., 2013. Paleolithic art: a cultural history. J. Archeol. Res. 21, 269-306, https://dx.doi.org/10.1007/s10814-012-9063-8.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26, 32-46, https://dx.doi.org/doi:10.1111/j.1442-9993.2001.01070.pp.x.

Arnold, A., Zehnder, K., 1991. Monitoring wall paintings affected by soluble salts, In: Cather, S. (Ed.), The conservation of wall paintings. Oxford University Press, Oxford, pp. 103-135.

Bartlett, M.S., 1937. Properties of sufficiency and statistical tests. Proc. R. Soc. Lond. A 160, 268-282, https://dx.doi.org/10.1098/rspa.1937.0109.

Berdoulay, M., Salvado, J., 2009. Genetic characterization of microbial communities living at the surface of building stones. Lett. Appl. Microbiol. 49, 311-316, https://dx.doi.org/10.1111/j.1472-765X.2009.02660.x.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., 2018. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. PeerJ PrePrints, https://dx.doi.org/10.7287/peerj.preprints.27295v2.

Bretz, F., Hothorn, T., Westfall, P., 2016. Multiple comparisons using R. CRC Press, Boca Raton.

Brimblecombe, P., Grossi, C.M., 2009. Millennium-long damage to building materials in London. Sci. Total Environ. 407, 1354-1361, https://dx.doi.org/10.1016/j.scitotenv.2008.09.037.

Bukin, Yu.S., Galachyants, Yu.P., Morozov, I.V., Bukin, S.V., Zakharenko, A.S., Zemskaya, T.I., 2019. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci. Data. 6, https://dx.doi.org/10.1038/sdata.2019.7.

Calegari-Santos, R., Diogo, R.A., Fontana, J.D., Bonfim, T.M.B., 2016. Carotenoid production by halophilic archaea under different culture conditions. Curr. Microbiol. 72, 641-651, https://dx.doi.org/ 10.1007/s00284-015-0974-8.

Caneva, G., Gori, E., Montefinale, T., 1995. Biodeterioration of monuments in relation to climatic changes in Rome between 19–20th centuries. Sci. Total Environ. 167, 205-214, https://dx.doi.org/10.1016/0048-9697(95)04581-K.

Chimienti, G., Piredda, R., Pepe, G., van der Werf, I.D., Sabbatini, L., Crecchio, C., Ricciuti, P., D'Erchia, A.M., Manzari, C., Pesole, G., 2016. Profile of microbial communities on carbonate stones of the medieval church of San Leonardo di Siponto (Italy) by Illumina-based deep sequencing. Appl. Microbiol. Biot. 100, 8537-8548, https://dx.doi.org/10.1007/s00253-016-7656-8.

Crispim, C., Gaylarde, C., 2005. Cyanobacteria and biodeterioration of cultural heritage: a review. Microb. Ecol. 49, 1-9, https://dx.doi.org/10.1007/s00248-003-1052-5.

D'Agostino, D., Congedo, P.M., Cataldo, R., 2014. Computational fluid dynamics (CFD) modeling of microclimate for salts crystallization control and artworks conservation. J. Cult. Herit. 15, 448-457, https://dx.doi.org/https://doi.org/10.1016/j.culher.2013.10.002.

De Felice, B., Pasquale, V., Tancredi, N., Scherillo, S., Guida, M., 2010. Genetic fingerprint of microorganisms associated with the deterioration of an historical tuff monument in Italy. J. Genet. 89, 253-257, https://dx.doi.org/10.1007/s12041-010-0035-9.

De Oliveira, V.E., Castro, H.V., Edwards, H.G., De Oliveira, L.F.C., 2010. Carotenes and carotenoids in natural biological samples: a Raman spectroscopic analysis. J. Raman. Spectrosc. 41, 642-650, https://dx.doi.org/10.1002/jrs.2493.

Ettenauer, J.D., Jurado, V., Piñar, G., Miller, A.Z., Santner, M., Saiz-Jimenez, C., Sterflinger, K., 2014. Halophilic microorganisms are responsible for the rosy discolouration of saline environments in three historical buildings with mural paintings. PLoS One 9, 0103844, https://dx.doi.org/10.1371/journal.pone.0103844.

Faraway, J.J., 2002. Practical regression and ANOVA using R. University of Bath, Bath.

Fisher, R.A., 2006. Statistical methods for research workers. Genesis Publishing Pvt Ltd, Guildford.

Fong, N.I., Burgess, M., Barrow, K., Glenn, D., 2001. Carotenoid accumulation in the psychrotrophic bacterium Arthrobacter agilis in response to thermal and salt stress. Appl. Microbiol. Biot. 56, 750-756, https//dx.doi.org/ 10.1007/s002530100739.

Fox, J., 2015. Applied regression analysis and generalized linear models. Sage Publications, Thousand Oaks.

Goodfellow, M., Kämpfer, P., Busse, H.-J., Trujillo, M.E., Suzuki, K.-i., Ludwig, W., Whitman, W.B., 2012a. Bergey's manual® of systematic bacteriology: volume five the actinobacteria, part A. Springer, New York.

Goodfellow, M., Kämpfer, P., Busse, H.-J., Trujillo, M.E., Suzuki, K.-i., Ludwig, W., Whitman, W.B., 2012b. Bergey's manual® of systematic bacteriology: volume five the actinobacteria, part B. Springer, New York.

Gorbushina, A.A., 2007. Life on the rocks. Environ. Microbiol. 9, 1613-1631, https://dx.doi.org/10.1111/j.1462-2920.2007.01301.x.

Gorbushina, A.A., Andreas, B., Schulte, A., 2005. Microcolonial rock inhabiting fungi and lichen photobionts: evidence for mutualistic interactions. Mycol. Res. 109, 1288-1296, https://dx.doi.org/10.1017/S0953756205003631.

Guillitte, O., 1995. Bioreceptivity: a new concept for building ecology studies. Sci. Total Environ. 167, 215-220, https://dx.doi.org/10.1016/0048-9697(95)04582-L.

Gurtner, C., Heyrman, J., Piñar, G., Lubitz, W., Swings, J., Rölleke, S., 2000. Comparative analyses of the bacterial diversity on two different biodeteriorated wall paintings by DGGE and 16S rDNA sequence analysis. Int. Biodeter. Biodegr. 46, 229-239, https://dx.doi.org/10.1016/S0964-8305(00)00079-2.

Hill, M.O., 1973. Diversity and evenness: a unifying notation and its consequences. Ecology, 427-432, https://dx.doi.org/10.2307/1934352.

Hollander, M., Wolfe, D.A., 1999. Nonparametric statistical methods. Wiley, Chichester.

Hoppert, M., König, S., 2006. The succession of biofilms on building stone and its possible impact on biogenic weathering, In: Fort, R., Alvarez de Buergo, M., Gomez-Heras, M., Vazquez-Clvo, C. (Eds.), Heritage, weathering and conservation. Taylor and Francis Group, London, pp. 311-315.

Hueck, H., 1965. The biodeterioration of materials as a part of hylobiology. Mater. Organismen 1, 5-34.

Imperi, F., Caneva, G., Cancellieri, L., Ricci, M.A., Sodo, A., Visca, P., 2007. The bacterial aetiology of rosy discoloration of ancient wall paintings. Environ. Microbiol. 9, 2894-2902, https://dx.doi.org/10.1111/j.1462-2920.2007.01393.x.

Janvier-Badosa, S., Beck, K., Brunetaud, X., Guirimand-Dufour, A., Al-Mukhtar, M., 2015. Gypsum and spalling decay mechanism of tuffeau limestone. Environ. Earth Sci. 74, 2209-2221, https://dx.doi.org/10.1007/s12665-015-4212-2.

Jehlička, J., Edwards, H.G.M., Oren, A., 2013. Bacterioruberin and salinixanthin carotenoids of extremely halophilic Archaea and Bacteria: A Raman spectroscopic study. Spectrochim. Acta A 106, 99-103, https://dx.doi.org/https://doi.org/10.1016/j.saa.2012.12.081.

Jurado, V., Miller, A.Z., Alias-Villegas, C., Laiz, L., Saiz-Jimenez, C., 2012. *Rubrobacter bracarensis* sp. nov., a novel member of the genus Rubrobacter isolated from a biodeteriorated monument. Syst. Appl. Microbiol. 35, 306-309, https://dx.doi.org/10.1016/j.syapm.2012.04.007.

Klassen, J.L., 2010. Phylogenetic and evolutionary patterns in microbial carotenoid biosynthesis are revealed by comparative genomics. PLoS One 5, 11257, https://dx.doi.org/10.1371/journal.pone.0011257.

Köcher, S., Müller, V., 2011. The nature and function of carotenoids in the moderately halophilic bacterium Halobacillus halophilus, In: Ventosa, A., Oren, A., Ma, Y. (Eds.), Halophiles and Hypersaline Environments. Springer, New York, pp. 303-317.

Korkanç, M., Savran, A., 2015. Impact of the surface roughness of stones used in historical buildings on biodeterioration. Constr. Build Mater. 80, 279-294, https://dx.doi.org/10.1016/j.conbuildmat.2015.01.073.

Kraková, L., Šoltys, K., Puškárová, A., Bučková, M., Jeszeová, L., Kucharík, M., Budiš, J., Orovčík, L.u., Szemes, T., Pangallo, D., 2018. The microbiomes of a XVIII century mummy from the castle of Krásna Hôrka (Slovakia) and its surrounding environment. Environ. Microbiol. 20, 3294-3308, https://dx.doi.org/10.1111/1462-2920.14312.

Laiz, L., Miller, A.Z., Jurado, V., Akatova, E., Sanchez-Moral, S., Gonzalez, J.M., DionÃsio, A., Macedo, M.F., Saiz-Jimenez, C., 2009. Isolation of five *Rubrobacter* strains from biodeteriorated monuments. Naturwissenschaften 96, 71-79, https://dx.doi.org/10.1007/s00114-008-0452-2.

Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129, 271-280, https://dx.doi.org/10.1007/s004420100716.

Li, Q., Zhang, B., He, Z., Yang, X., 2016. Distribution and diversity of bacteria and fungi colonization in stone monuments analyzed by high-throughput sequencing. PLoS One 11, 0163287, https://dx.doi.org/10.1371/journal.pone.0163287.

Lisci, M., Monte, M., Pacini, E., 2003. Lichens and higher plants on stone: a review. Int. Biodeter. Biodegr. 51, 1-17, https://dx.doi.org/10.1016/S0964-8305(02)00071-9.

Ma, B., Gong, J., 2013. A meta-analysis of the publicly available bacterial and archaeal sequence diversity in saline soils. World J. Microb. Biot. 29, 2325-2334, https://dx.doi.org/10.1007/s11274-013-1399-9

Macedo, M.F., Miller, A.Z., Dionísio, A., Saiz-Jimenez, C., 2009. Biodiversity of cyanobacteria and green algae on monuments in the Mediterranean Basin: an overview. Microbiology 155, 3476-3490, https://dx.doi.org/10.1099/mic.0.032508-0.

Magoč, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957-2963, https://dx.doi.org/10.1093/bioinformatics/btr507.

Marshall, C.P., Leuko, S., Coyle, C.M., Walter, M.R., Burns, B.P., Neilan, B.A., 2007. Carotenoid analysis of halophilic archaea by resonance Raman spectroscopy. Astrobiology 7, 631-643, https://dx.doi.org/10.1089/ast.2006.0097.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6, 610, https://dx.doi.org/10.1038/ismej.2011.139.

McNamara, C.J., Mitchell, R., 2005. Microbial deterioration of historic stone. Front. Ecol. Environ. 3, 445-451, https://dx.doi.org/10.1890/1540-9295(2005)003[0445:MDOHS]2.0.CO;2.

Merello, P., García-Diego, F.-J., Zarzo, M., 2012. Microclimate monitoring of Ariadne's house (Pompeii, Italy) for preventive conservation of fresco paintings. Chem. Cent. J. 6, 145, https://dx.doi.org/10.1186/1752-153X-6-145.

Miller, A.Z., Sanmartín, P., Pereira-Pardo, L., Dionísio, A., Saiz-Jimenez, C., Macedo, M.F., Prieto, B., 2012. Bioreceptivity of building stones: A review. Sci. Total Environ. 426, 1-12, https://dx.doi.org/10.1016/j.scitotenv.2012.03.026.

Neilson, J.W., Quade, J., Ortiz, M., Nelson, W.M., Legatzki, A., Tian, F., LaComb, M., Betancourt, J.L., Wing, R.A., Soderlund, C.A., 2012. Life at the hyperarid margin: novel bacterial diversity in arid soils of the Atacama Desert, Chile. Extremophiles 16, 553-566, https://dx.doi.org/10.1007/s00792-012-0454-z.

Nilsson, R.H., Larsson, K.-H., Taylor, A.F S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., Saar, I., Kõljalg, U., Abarenkov, K., 2018. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. 47, D259-D264, https://dx.doi.org/10.1093/nar/gky1022.

Nugari, M.P., Pietrini, A.M., Caneva, G., Imperi, F., Visca, P., 2009. Biodeterioration of mural paintings in a rocky habitat: The Crypt of the Original Sin (Matera, Italy). Int. Biodeter. Biodegr. 63, 705-711, https://dx.doi.org/10.1016/j.ibiod.2009.03.013.

Peng, M., Jia, H., Wang, Q., 2017. The effect of land use on bacterial communities in saline–alkali soil. Curr. Microbiol. 74, 325-333, https://dx.doi.org/10.1007/s00284-017-1195-0.

Piñar, G., Ettenauer, J., Sterflinger, K., 2014a. La vie en rose': a review of rosy discoloration of subsurface monuments, In: Saiz-Jimenez, C. (Ed.), The conservation of subterranean cultural heritage. CRC Press, Boca Raton, pp. 113-123.

Piñar, G., Kraková, L., Pangallo, D., Piombino-Mascali, D., Maixner, F., Zink, A., Sterflinger, K., 2014b. Halophilic bacteria are colonizing the exhibition areas of the Capuchin Catacombs in Palermo, Italy. Extremophiles 18, 677-691, https://dx.doi.org/10.1007/s00792-014-0649-6.

Pinheiro, A.C., Mesquita, N., Trovão, J., Soares, F., Tiago, I., Coelho, C., de Carvalho, H.P., Gil, F., Catarino, L., Piñar, G., Portugal, A., 2018. Limestone biodeterioration: A review on the Portuguese cultural heritage scenario. J. Cult. Herit., https://dx.doi.org/10.1016/j.culher.2018.07.008.

669 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner,
670 F.O., 2013. The SILVA ribosomal RNA gene database project: improved data
671 processing and web-based tools. Nucleic Acids Res. 41, D590-D596,
672 https://dx.doi.org/10.1093/nar/gks1219.

673 R Core Team, 2014. R: A language and environment for statistical computing. Vienna,
674 Austria: R Foundation for Statistical Computing; 2014.

675 Salvadori, O., Municchia, A.C., 2016. The role of fungi and lichens in the biodeterioration of
676 stone monuments. Open Conf. Proc. J. 7,
677 https://dx.doi.org/10.2174/2210289201607020039.

678 Schabereiter-Gurtner, C., Piñar, G., Vybiral, D., Lubitz, W., Rölleke, S., 2001. *Rubrobacter*-
679 related bacteria associated with rosy discolouration of masonry and lime wall
680 paintings. Arch. Microbiol. 176, 347-354, https://dx.doi.org/10.1007/s002030100333.

681 Scheerer, S., Ortega-Morales, O., Gaylarde, C., 2009. Microbial deterioration of stone
682 monuments—an updated overview. Adv. Appl. Microbiol. 66, 97-139,
683 https://dx.doi.org/10.1016/S0065-2164(08)00805-8.

684 Sorokin, D.Y., van Pelt, S., Tourova, T.P., Evtushenko, L.I., 2009. *Nitriliruptor alkaliphilus*
685 gen. nov., sp. nov., a deep-lineage haloalkaliphilic actinobacterium from soda lakes
686 capable of growth on aliphatic nitriles, and proposal of Nitriliruptoraceae fam. nov.
687 and Nitriliruptorales ord. nov. Int. J. Syst. Evol. Micr. 59, 248-253,
688 https://dx.doi.org/10.1099/ijs.0.002204-0.

689 Sterflinger, K., Piñar, G., 2013. Microbial deterioration of cultural heritage and works of art -
690 tilting at windmills? Appl. Microbiol. Biot. 97, 9637-9646,
691 https://dx.doi.org/10.1007/s00253-013-5283-1.

692 Tescari, M., Frangipani, E., Caneva, G., Casanova Municchia, A., Sodo, A., Visca, P., 2018a.
693 *Arthrobacter agilis* and rosy discoloration in "Terme del Foro" (Pompeii, Italy). Int.
694 Biodeter. Biodegr., https://dx.doi.org/10.1016/j.ibiod.2018.03.015.

695 Tescari, M., Visca, P., Frangipani, E., Bartoli, F., Rainer, L., Caneva, G., 2018b. Celebrating
696 centuries: Pink-pigmented bacteria from rosy patinas in the House of Bicentenary
697 (Herculaneum, Italy). J. Cult. Herit., https://dx.doi.org/10.1016/j.culher.2018.02.015.

698 Thornbush, M., Viles, H., 2006. Changing patterns of soiling and microbial growth on
699 building stone in Oxford, England after implementation of a major traffic scheme. Sci.
700 Total. Environ. 367, 203-211, https://dx.doi.org/10.1016/j.scitotenv.2005.11.022.

701 Warscheid, T., Braams, J., 2000. Biodeterioration of stone: a review. Int. Biodeter. Biodegr.
702 46, 343-368, https://dx.doi.org/10.1016/S0964-8305(00)00109-8.

703 Wolfinger, R., O'connell, M., 1993. Generalized linear mixed models a pseudo-likelihood
704 approach. J. Stat. Comput. Sim. 48, 233-243,
705 https://dx.doi.org/10.1080/00949659308811554.

706 Zanardini, E., May, E., Inkpen, R., Cappitelli, F., Murrell, J.C., Purdy, K.J., 2016. Diversity
707 of archaeal and bacterial communities on exfoliated sandstone from Portchester Castle
708 (UK). Int. Biodeter. Biodegr. 109, 78-87,
709 https://dx.doi.org/10.1016/j.ibiod.2015.12.021.

710 Zanardini, E., May, E., Purdy, K.J., Murrell, J.C., 2019. Nutrient cycling potential within
711 microbial communities on culturally important stoneworks. Env. Micobiol. Rep. 11,
712 147-154, https://dx.doi.org/10.1111/1758-2229.12707.

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Table 1: Effect of the qualitative variables on L*a*b* color space values, assessed with a Wald X^2 test associated with GLMMs.

	L		***a***		***b***	
Source of variation	X^2-value	p-value	X^2-value	p-value	X^2-value	p-value
Color area (Co)	802	<0.001***	71	<0.001***	366	<0.001***
Covering (C)	1.2	0.26	2.1	0.15	0.2	0.65
Scratching (S)	1052	<0.001***	44	<0.001***	768	<0.001***
Date (D)	1062	<0.001***	57	<0.001***	1163	<0.001***
Co:S	255	<0.001***	19	<0.001***	291	<0.001***
Co:D	27	<0.001***	22	<0.001***	78	<0.001***
S:D	423	<0.001***	21	<0.001***	264	<0.001***
Co:S:D	74	<0.001***	13	0.02*	74	<0.001***

* p < 0.05, ** p < 0.01, *** p < 0.001

Table 2: Summary of climatic data assessed on the stone steps between September 2014 and January 2018.

	Temperature (°C)			Relative humidity (%)		
Color area	Minimum	Mean	Maximum	Minimum	Mean	Maximum
White	3.22	14.35	25.78	(-)	(-)	(-)
Pink	4.44	15.39	26.67	(-)	(-)	(-)
Room air	3.22	14.66	27.33	30.01	58.95	89.2
Outdoor	-3	12.53	35	43	80.65	98

(-): parameter not assessed

Captions for Figures

Figure 1: Location of the study area in Sully-sur-Loire castle.

The study focused on the stones of the "escalier d'honneur" of the donjon (a). The cross-section of the "escalier d'honneur" shows the location of the pink discoloration (b).

3D model realized with SketchUp 2016 from plans, video recordings and pictures.

Figure 2: Half-pink step chosen for colorimetric and climatic monitoring.

The picture shows the distribution of the rosy discoloration on the outer part of the stairs.

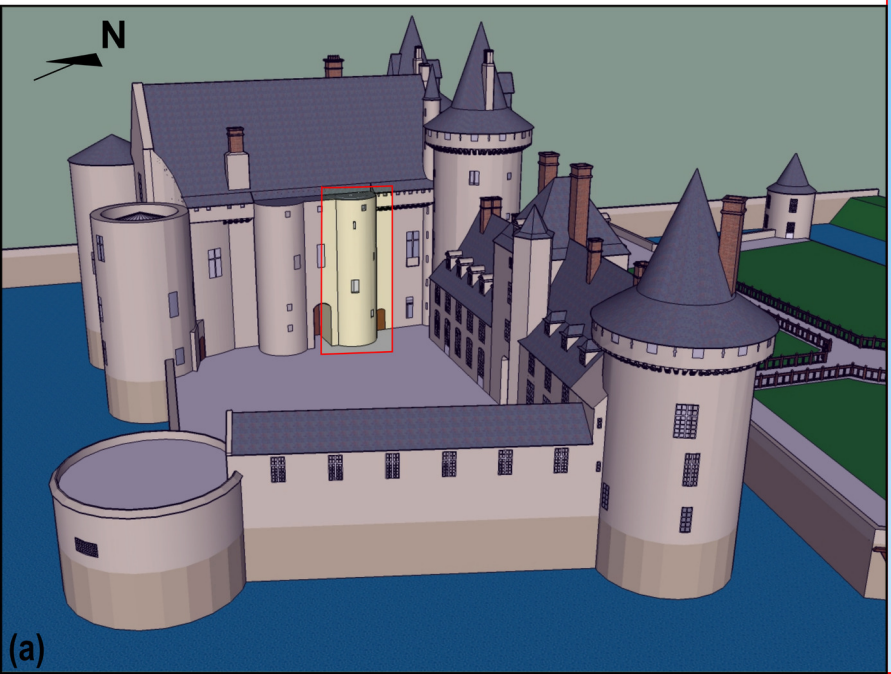
Figure 3: 3D representation of the colorimetric monitoring of the stone step in the L*a*b* colour space.

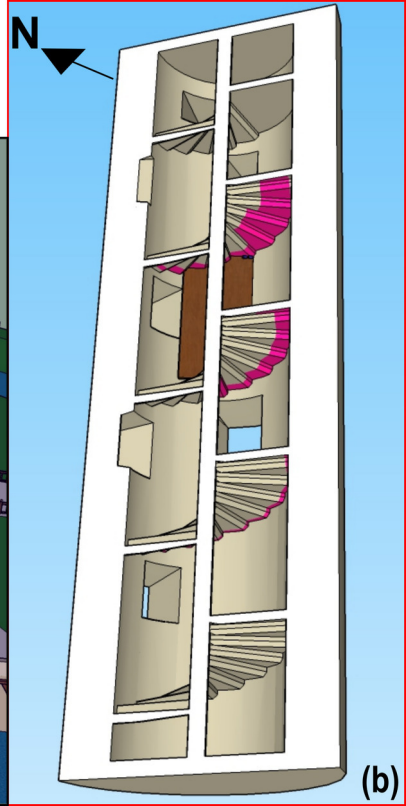
Values are depicted for the pink and in the white areas, scratched (crosses) or not (circles), between September 2014 and January 2018.

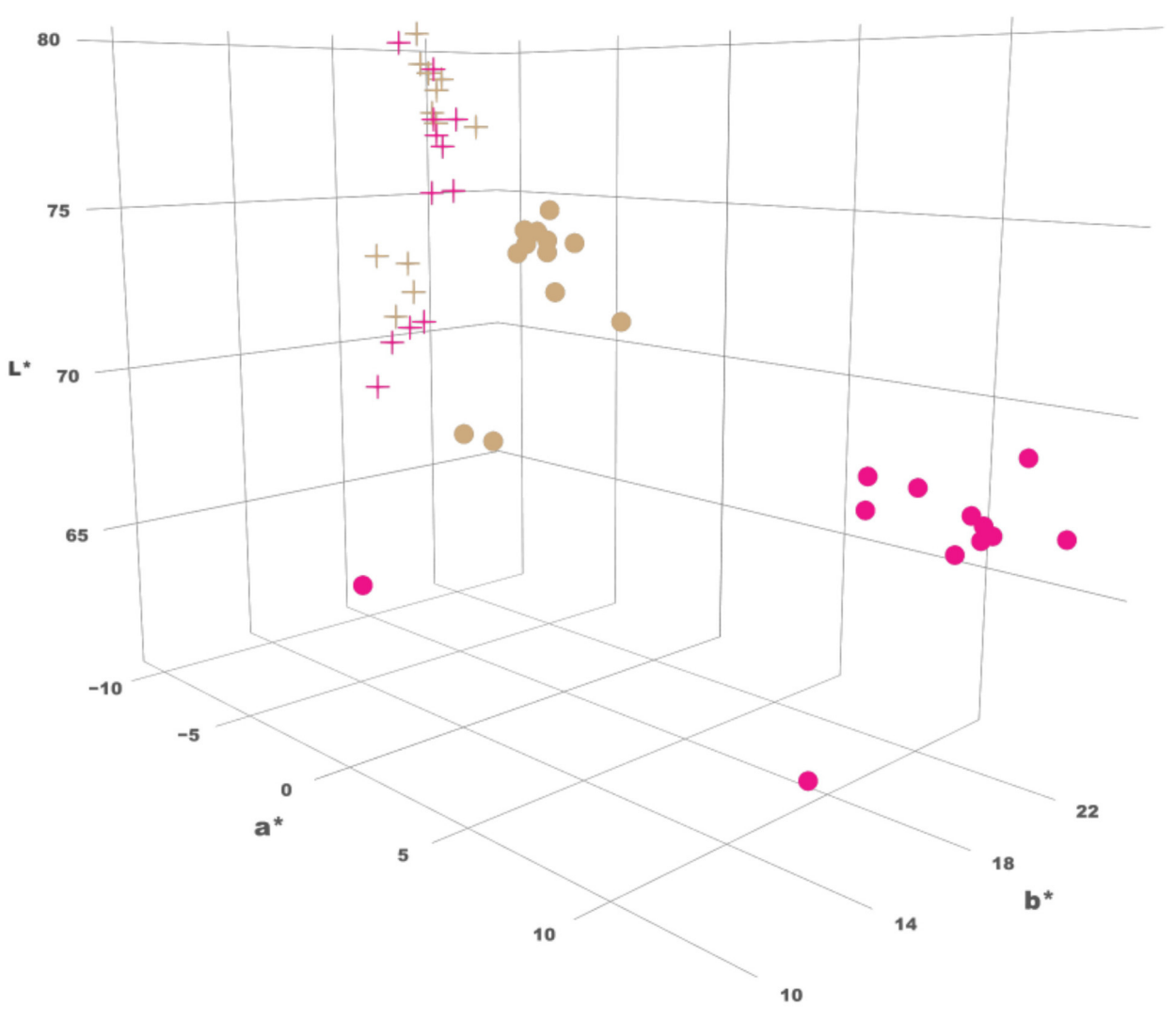
Figure 4: Heatmap of bacterial communities according to each sample at the genus level.

W: white sample; P: pink sample; O: outgroup sample.

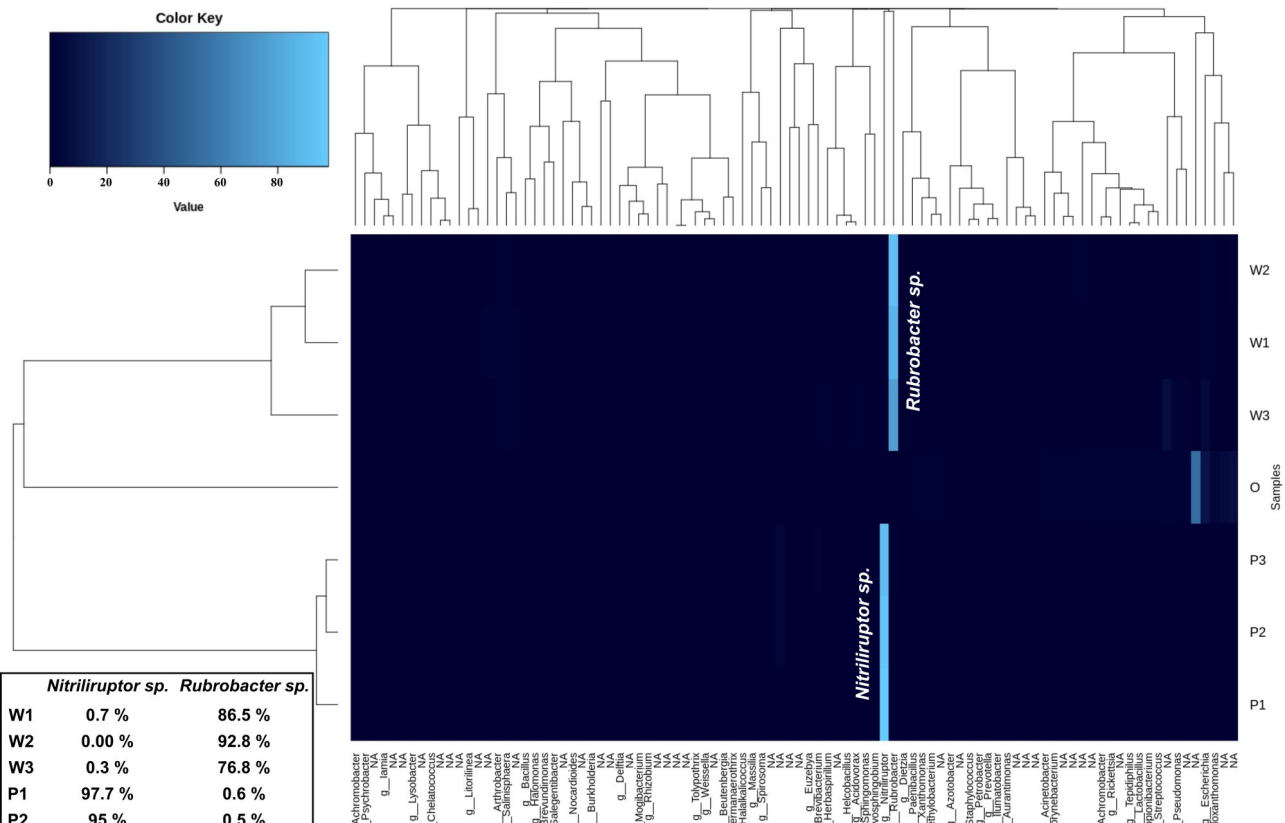
The most abundant genera are labelled in pale blue, while the less abundant genera are labelled in dark blue. The dendrograms show the proximity between the different samples and the proximity between identified bacterial genera.


N
(a)


N
(b)



- Pink zone / Not scratched
- Pink zone / Scratched
- White zone / Not scratched
- White zone / Scratched



	Nitriliruptor sp.	*Rubrobacter sp.*
W1	0.7 %	86.5 %
W2	0.00 %	92.8 %
W3	0.3 %	76.8 %
P1	97.7 %	0.6 %
P2	95 %	0.5 %
P3	91.8 %	1.00 %
O	0.00 %	0.00 %