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► **To cite this version:**

Valentina Valbi, Johann Leplat, Alexandre François, Anne Perez, Barbara Trichereau, et al.. Bacterial diversity on stained glass windows. *International Biodeterioration & Biodegradation*, 2023, 177, pp.105529. 10.1016/j.ibiod.2022.105529 . hal-04012600

HAL Id: hal-04012600

<https://hal.science/hal-04012600>

Submitted on 6 Feb 2024

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1 ***Bacterial diversity on stained glass windows.***

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13

14 **Abstract**

15 In order to explore the role of bacteria in the alteration of stained glass windows, and especially
16 on the browning phenomenon, it is necessary to have an overview of the microbial inhabitants
17 likely to be found on this kind of material. In this study, biological samples were collected on
18 stained glass of different colors, both on site and stored in a conservation workshop. Cultivable
19 bacterial communities were identified by 16S rDNA sequencing. Similarity values ranged from
20 99 to 100% to known bacteria. Sequence analysis showed different bacterial communities
21 depending on the sampling site. The more represented genera were *Arthrobacter*, *Micrococcus*,
22 *Paenibacillus* and *Bacillus*. Some of the identified bacteria are known for their weathering
23 potential.

24 **Keywords**

25 Stained glass, bacteria, 16S rDNA, alteration, manganese, browning, weathering

26 **1. INTRODUCTION**

27 The conservation of stained glass windows exposed over centuries to episodes of rain and high
28 humidity implies the understanding of the weathering phenomena that undermine their stability
29 over time. The main mechanisms underlying glass alteration involve i) the interdiffusion, or ion
30 exchange, between modifier ions (mainly alkalis) and hydrogenated species (H^+ , H_3O^+ , H_2O)
31 from the environment and ii) hydrolysis of the silicate network ionic-covalent bonds (Si–O–Si
32 or Si–O–Al). These mechanisms lead to the formation, at the surface, of an altered layer of glass
33 that is hydrated, porous, and alkali-depleted (Sterpenich and Libourel, 2001; Verney-Carron et
34 al., 2015, 2017; Sessegolo et al., 2017, 2018). The alkali leached from the glass further react
35 with atmospheric gasses and might form salts at the surface of the glass, such as carbonates
36 (calcite) and sulphates (gypsum, syngenite) (Lombardo et al., 2010; Gentaz et al., 2016). The
37 alteration kinetics depend on the environmental conditions such as hygroscopic conditions, pH,
38 temperature, rainfall intensity and composition, as well as on the glass surface original state
39 (Wolff-Boenisch et al., 2004; Alloteau et al., 2017, 2019; Sessegolo et al., 2019, 2020; Majérus
40 et al., 2020). The composition of glass is also a key-feature (Wolff-Boenisch et al., 2004;
41 Cailleateau et al., 2008; Alloteau et al., 2020).

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as: <https://doi.org/10.1016/j.ibiod.2022.105529>

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42 Another factor that should be taken into account is the effect of microorganisms. They can
43 indeed cause physico-chemical damages to the surfaces of the materials they use as a support
44 for living (Perez y Jorba et al., 1980; Krumbein et al., 1991; Drewello and Weissmann, 1997;
45 Gorbushina and Palinska, 1999; Carmona et al., 2006; Rodrigues et al., 2014; Weaver et al.,
46 2021). Microorganisms are also suspected to play a significant role in the widespread pathology
47 called “browning phenomenon”. This latter appears as brown spots at the surface of the glass
48 (Schalm et al., 2011; Ferrand et al., 2015), is caused by the oxidation of manganese (originally
49 introduced in the composition in order to obtain colorless or murrey glass (Gliozzo, 2016;
50 Bidegaray et al., 2019; Rossano et al., 2022)) and might be linked to microbiological activity
51 (Perez y Jorba et al., 1980; Oriol et al., 2007).

52 In order to further explore the role of microorganisms in glass weathering and in the browning
53 phenomenon, a first step is to get an overview of the inhabiting microbial populations on stained
54 glass surfaces. A few studies previously identified several species of fungi and bacteria on
55 stained glass windows in Germany (Rölleke et al., 1999; Schabereiter-Gurtner et al., 2001),
56 Spain (Carmona et al., 2006; Piñar et al., 2013) and Italy (Marvasi et al., 2009). Piñar et al.,
57 2013 showed that the bacterial communities on stained glass were far more diverse than the
58 fungal communities, and these communities included bacteria known to possess metabolic
59 activities able to cause biodeterioration phenomena. Thus, they concluded that the role of
60 bacteria has probably long been underestimated in the alteration of glass, compared to the easier
61 to identify mechanical effect of fungi with their hyphae leaving marks on the glass surface.
62 Although of importance for the study of the effect of microorganisms, these previous works
63 often employed sampling techniques destructive for the glass substrate that discourage the
64 development of more extensive experimental sampling campaigns. Thus, no study of this kind
65 has been realized in France to date, despite the considerable stained glass heritage. Moreover,
66 in order to have a more realistic representation of the microorganisms living on stained glass,
67 the samples should be collected on site, still in their original places in historical buildings, which
68 is rarely possible due to the unreachable positions of the bays.

69 In the present work, sampling campaigns were realized mainly on colourless or murrey stained
70 glass, both deposited and *in situ*, affected by the browning phenomenon or not, in order to
71 identify the bacteria present and especially those likely to participate in the alteration of glass.
72 The aim of the present study is to i) analyze the bacterial communities present on historical
73 glass coming from four different French sites without recurring to destructive sampling, ii)
74 identify possible bacteria involved in glass weathering and iii) collect bacteria possibly suitable
75 for further laboratory-based studies on bioalteration of glass.

76 2. MATERIALS AND METHODS

77 2.1 Sampling sites

78 Samples were collected *in situ* from the Royal Chapel of Versailles Castle (Île-de-France
79 region) and from the church of Saint-Etienne in Bar-sur-Seine (Grand-Est region) during their
80 restoration campaigns giving an access to the windows with scaffoldings. A few panels of
81 stained glass coming from Lachalade Abbey church (Grand-Est region) and Wissembourg
82 Abbey church of Saint-Pierre-et-Saint-Paul (Grand-Est region) were also sampled in a
83 conservation workshop (Atelier Parot, Bourgogne Franche-Comté region) shortly after their
84 removal from the buildings and before proceeding to their conservation treatments (Figure 1).

85 Concerning the *in situ* samplings, both Versailles and Bar-sur-Seine have a warm temperate
86 climate, with an annual medium temperature around 12 °C, with respectively 682 and 661 mm
87 of rainfall and 1902 and 1894 hours of sunlight (data MétéoFrance). Different locations were

88 sampled in order to eventually observe differences in the microbial population by changing the
89 exposure conditions. The factors taken into account were the color of the glass pieces
90 (colourless and murrey glasses were preferentially sampled), the internal or external
91 localization of the sampled windows and the geographical exposure. Special attention was
92 given to realize the sampling on original not restored glass pieces when the information was
93 available. These strategies were obviously subjected to the limitations given by the access to
94 the stained glass and were variable depending on the sampling site.

95 The Royal Chapel in Versailles Castle was constructed between 1700 and 1710 at the end of
96 the reign of Louis XIV. Five samples were collected on the external face of the site coming
97 from different expositions: three samples at North-East (B11-2p.68, B11-2 p.11, B11-1 p.15)
98 and two samples at South (B4-2 p.68, B4-1 p.15). Three additional samples were collected on
99 the internal face: two from the north (B91-2 p.17, B91-2p.17 blue) and one from the south (B4-
100 2 p.17). The sampling was carried out on wide unpainted colorless sheet of glass except for one
101 blue stained glass panel located in the borders of the bays (Figure 2 A), B), C)).

102 The Church of Saint-Etienne of Bar-sur-Seine and its stained-glass windows were realized
103 during the 16th century. An equal number of samples were collected both on external and
104 internal faces (seven samples each) with different expositions : nine samples from the north
105 (B101_LD_P7_FI, B101_LC_P7_FI, B101_LD_P7_FE, B101_LC_P7_FE, B103_R16_FE,
106 B103_LC_R9_FI, B103_LD_P4_FE, B103_R16_FI, B103_LD_P3_FI), two samples from the
107 east (B100_LD_P7_FI and B100_LD_P7_FE) and three from the south (B104_R17-FI,
108 B104_R17_FE, B104_LB_P4_FE). The glass pieces in this site are mainly painted and
109 samplings were realized on unpainted surface whenever it was possible (Figure 2 D), E), F)).

110 Concerning the stained glass panels in the conservation workshop, one panel was from
111 Wissembourg and three others from Lachalade. Storage conditions in the workshop were
112 around 18-20 °C and 50 % relative humidity (RH), in a dark sheltered space with occasional
113 artificial and natural lightening. The stained glasses from these two sites both suffer from the
114 browning phenomenon, thus the samples were collected on the brown spots.

115 The Abbey church of Wissembourg is a gothic church and its stained glass windows date from
116 the 13th century. Four samples were collected, two on the internal face and two on the external
117 face of the glass (tracery BOR3, originally with East exposition).

118 The construction of the Cistercian Abbey of Lachalade lasted from the 12th to the 14th century
119 and its stained glass date from the 14th century. Two samples, both on the internal face, for each
120 of the three panels chosen for this study (B5P4, BOR4, B6P3, all of them originally with East
121 exposition) were collected (Figure 2 I), J), K)).

122 The complete list of the collected samples is available in Table 1.

123 **2.2 Sampling methodology, cultivation and isolation of bacteria**

124 Each sample was taken with a sterile cotton swab (sampled surface approximatively 1 cm²),
125 then inoculated in Petri dishes (diameter 8 cm) containing Lisogen Broth supplemented with
126 agar (5 g/l NaCl, 5 g/l yeast extract, 10 g/l peptone). The samples were kept at environmental
127 temperature following sampling, and only a few hours elapsed from swabbing to cultivation.
128 The inoculated Petri dishes were incubated at 30 °C for 3 days. Then, bacterial strains that
129 developed on Petri dishes were separated through sub-cultures (Leplat et al., 2017, 2020). The
130 isolated bacterial strains were identified to the species level through molecular identification.

131 **2.3 DNA extraction, PCR amplification and sequencing**

132 DNA (deoxyribonucleic acid) was extracted as described by (Edel et al., 2001). fD1 and rP2
133 primers were used to amplify the 16S ribosomal DNA (16S rDNA) region (Weisburg et al.,
134 1991). PCR was performed in 25 μ l reactions, with 1.5 μ l of each 10 mM primer (Eurogentec,
135 Seraing, Belgium), 1 μ l of template DNA, 0.2 μ l of Taq DNA polymerase 5 U/ μ l (Invitrogen,
136 Carlsbad, USA), 2.5 μ l of 10X Taq DNA polymerase buffer, 0.75 μ l of MgCl₂ 50 mM, 2.5 μ l
137 of 2 mM dNTPs (Thermo scientific, Waltham, USA) and 15.05 μ l of distilled sterile water.
138 Amplifications were performed on a 5 PrimeG thermocycler (Antylia Scientific, Vernon Hills,
139 USA) with the following parameters: a 5 min step at 94 °C, followed by 35 cycles at 94 °C for
140 30 s, at 56 °C for 45 s, and at 72 °C for 90 s, and a final 10 min extension at 72 °C. PCR
141 products were sequenced by Genoscreen (Lille, France) using the same primer set. Isolates were
142 identified by BLASTn comparison to the NCBI GenBank database (Altschul et al., 1990; Clark
143 et al., 2016). The generated sequences were deposited in the Genbank database under the
144 numbers OP574250 to OP574343 (Tables S1 to S6 in Supplementary Materials).

145 **2.4 Data treatment**

146 *2.4.1 Sequence alignment and phylogenetic analysis*

147 A phylogenetic tree was built to compare the isolated strains to reference strains known to
148 perform manganese oxidation. The sequences of a too poor quality were not included in the
149 tree. The sequences related to reference strains (Yang et al., 2013) were downloaded from the
150 NCBI GenBank database. Multiple sequence alignment was performed using the FFT-NS-2
151 alignment strategy from the MAFFT v. 7 web server (Katoh et al., 2019). The uninformative
152 gaps and ambiguous regions were removed using the Gblocks program v. 0.91.1 implemented
153 in the NGPhylogeny.fr service (Talavera and Castresana, 2007; Lemoine et al., 2019; Leplat et
154 al., 2021). The maximum likelihood (ML) analysis was performed using the PhyML software
155 with a GTR model of nucleotides substitution, with 100 bootstraps to verify the branches
156 robustness (Guindon et al., 2010).

157 *2.4.1 Beta diversity of bacterial communities*

158 The β -diversity, i.e. the diversity of the bacterial communities between samples, was assessed
159 through Principal Coordinate Analysis (PCoA, package ape; function pcoa; (Legendre and
160 Gallagher, 2001)) based on the determination of the Bray-Curtis distance (package vegan;
161 function vegdist; method bray; (Legendre and Legendre, 2012)) using R 4.0.1 (R core team,
162 2014). The significance of differences obtained between sampling groups was assessed with
163 PERMANOVA (999 permutations; package vegan; function adonis; (Anderson, 2001)). The
164 proximity between both sampling sites and between identified species was assessed through
165 hierarchical clustering based on the Bray-Curtis distance (package stats; function hclust,
166 method complete; (Legendre and Legendre, 2012)) and the results were presented through an
167 heatmap representation.

168 **3. RESULTS**

169 102 bacterial strains were isolated and identified by sequencing: 23 strains were isolated from
170 Versailles samples, 47 from Bar-sur-Seine samples, 17 from Wissembourg samples and 15
171 from Lachalade samples (Tables S1 to S6 in Supplementary Materials).

172 The *in situ* sampling sites were characterized by different comparative abundances of recovered
173 bacteria depending on internal face (IF) and external face (EF). For the site of Bar-sur-Seine,
174 seven samples were made on the internal face (IF) and seven samples on the external face (EF).
175 The majority of bacteria that developed on culture media came from the external face of the
176 stained glass (31 from EF and 16 from IF). For the site of Versailles only 3 samples out of 8

177 were collected in IF and 5 bacterial strains developed from them, while 18 bacterial strains
178 developed from the 5 samples collected on EF.

179 Most of the sequences showed at least 99 % of similarity with the sequences given in GenBank,
180 and were clearly identified at the species level. Other sequences also showed a high similarity,
181 but were only identified at the genus level because of the phylogenetic proximity between
182 several species regarding the 16S rDNA. Thus, these bacteria were grouped in “clusters” for
183 the statistical data treatment. For example, the *Paenibacillus amyloleticus* cluster grouped
184 bacteria for which the species was impossible to differentiate among 6 phylogenetic close
185 species. Finally, some sequences presented lower similarity percentages because of a poor
186 quality of these sequences, but they allow to identify the kind of bacteria without a certain
187 identification (<97 % of similarity).

188 The bacterial communities detected on the sites of Bar-sur-Seine and Lachalade were similar
189 based on bacterial Phyla, with a majority of Actinobacteria and lower percentages of
190 Proteobacteria and Firmicutes, but with inversed proportions (Fig. S1 in Supplementary
191 Materials). In Bar-sur-Seine, 66 % of the bacteria were Actinobacteria, 19 % were Firmicutes
192 and 15 % were Proteobacteria. In Lachalade, 67 % of the bacteria were Actinobacteria, 20 %
193 were Proteobacteria and 13 % were Firmicutes. The abundance of recovered bacteria was
194 completely different in Versailles and Wissembourg sampling sites. In Versailles, 87 % of the
195 bacteria belonged to the phylum of Firmicutes, 9 % to Actinobacteria and 4 % to Proteobacteria.
196 Concerning the site of Wissembourg, 100 % of the identified bacteria were Actinobacteria.

197 The most frequently isolated bacterial species belonging to Actinobacteria encountered in this
198 study were *Arthrobacter* spp. and *Micrococcus* spp., while *Paenibacillus* spp. and *Bacillus* spp.
199 were the most frequently isolated species belonging to Firmicutes. Isolated α -Proteobacteria
200 were *Sphingomonas* spp. and *Agrobacterium* spp. for example, while γ -Proteobacteria were
201 *Pantoea* spp., and *Psychrobacter* spp. (Tables S1-S6 in Supplementary Materials). The
202 phylogenetic tree displaying the identified bacteria together with some Mn-oxidizing bacteria
203 from the literature showed that *Arthrobacter crystallopoietes*, which was only isolated from the
204 stained glass of Wissembourg and represented all the strains isolated from this site, was the
205 only recovered species related to known Mn-oxidizing bacteria (Fig. S2 in Supplementary
206 Materials). In particular, *Bacillus* spp. isolated on stained glass in this study seemed
207 phylogenetically different from the reference *Bacillus* spp. known to be Mn-oxidizing.

208 The Principal Coordinates Analysis (PCoA) showed that the samples were generally grouped
209 depending on the origin of the stained glass (Fig. 3). The PERMANOVA test associated to this
210 PCoA confirmed that the sampling site had a significant effect on bacterial community
211 composition. Conversely, the PCoA expressed no difference between sampling areas in external
212 or internal face.

213 Bar-sur-Seine and Lachalade represent two distinct groups in the PCoA analysis (Fig. 3).
214 However, their proximity highlights similarities that can be explained in terms of Phyla
215 distribution.

216 The samples from the Versailles site and the samples from Wissembourg formed two different
217 and distant groups isolated from the other two sites. However, one sample from Versailles was
218 positioned next to the group of Lachalade and Bar-sur-Seine and two external samples from
219 Versailles (13 and 14) were also distant from the other Versailles samples.

220 The hierarchical clustering study also underlined the importance of the original building
221 location factor in the composition of bacterial communities (Fig. 4). The communities from

222 Versailles and Wissembourg being clearly separated from the ones coming from Lachalade and
223 Bar-sur-Seine and from each other. Bacterial communities in Versailles samples were strongly
224 affected by the predominance of the *Paenibacillus amyloleticus* cluster, while samples from
225 Wissembourg site were strongly affected by the presence of *Arthrobacter crystallopoietes*, this
226 species being the only one identified from this site, and the heatmap showed a low bacterial
227 diversity for these two sites (Fig. 4). On the contrary, the sites of Bar-sur-Seine and Lachalade
228 had more diversified bacterial communities. The dendrogram on the columns gives the
229 proximity among the different bacterial clusters. Some clusters were specific to certain sites
230 like *A. crystallopoietes* for Wissembourg and *P. amyloleticus* for Versailles. Other clusters, like
231 the *Micrococcus luteus* cluster, were more transversally distributed and were recovered in
232 samples from Versailles, Lachalade and Bar-sur-Seine. This result explained why one sample
233 from Versailles superimposed with the group of samples from Lachalade and Bar-sur-Seine in
234 the PCoA study (Fig. 3).

235 4. DISCUSSION

236 4.1 Bacterial diversity on the sampled stained glass

237 The results showed that the bacterial communities on stained glass were different depending on
238 the geographical localization of the monuments. The differences between the sites of Bar-sur-
239 Seine and Versailles can mainly be explained by different surrounding environments, as the
240 two sites have almost identical weather conditions through the year, with an annual average
241 temperature of 12°C, ≈700 mm of rainfall, and ≈1900 hours of sunlight.

242 The site of Versailles showed a predominance of *Paenibacillus* species, with a few
243 *Micrococcus*, *Pantoea*, and *Bacillus*. *Paenibacillus* species are often associated to soil and plant
244 roots (Grady et al., 2016; Cho et al., 2017; Jeong et al., 2019) and their presence might be
245 explained by the proximity to the Versailles gardens surrounding the Chapel.

246 The site of Bar-sur-Seine shows more variability among the identified species. This site was
247 particularly chosen to investigate the difference between bacterial communities depending on
248 the internal or external exposure of the sampled surface. Thus, the same number of samples
249 were collected in internal and external exposition. The results showed that more
250 microorganisms developed on the Petri dishes when sampled on external faces (31 isolates out
251 of 7 samplings in external face versus 16 isolates out of 7 samplings in internal face). This result
252 is however to be taken cautiously because a bias can be introduced by the cultivation step: more
253 non-cultivable bacteria could be present in internal face and would therefore not have been
254 detected in this study. Even though this observation is not statistically supported, this finding
255 could suggest that the external face of *in situ* stained-glass windows is more prone to
256 microbiological contamination caused by the transport and deposition of microorganisms
257 through the atmospheric agents. This could be supported by the presence on the external face
258 of bacteria associated to soil and plants, like for example the genera *Paenibacillus*,
259 *Agrobacterium*, *Agreia*, *Plantibacter* and *Kocuria* (Leyval and Berthelin, 1989; Takarada et al.,
260 2008; Tuo et al., 2019), probably coming from the surrounding vegetation, the St. Etienne
261 church being located in a rural area. On the contrary, species on the internal face, bacteria
262 mainly belonged to the *Sphingomonas*, *Micrococcus*, *Staphylococcus* and *Arthrobacter*. The
263 only work to our knowledge that attempted to differentiate the bacterial communities from
264 internal and external faces of stained glass was performed by Marvasi et al., 2009. They also
265 isolated cultivable bacteria and they only identified bacteria belonging to Firmicutes phylum
266 (*Bacillus*, *Paenibacillus*) from the external faces of the windows, while no Firmicutes were
267 found on the internal face of the windows, suggesting a different microbial presence in the
268 confined environment of the Church. Our study did not display such contrasted results.

269 Firmicutes were identified on both external and internal faces. However, in Bar-sur-Seine, no
270 *Paenibacillus* nor *Bacillus* were found in the internal face, although they were detected on the
271 external face. The only Firmicutes recovered from internal face were *Staphylococcus* spp.
272 which are generally linked to the presence of humans in the Church (Gorbushina et al., 2004;
273 Otto, 2009; Rakotonirainy et al., 2016). Moreover, in Versailles, *Paenibacillus* spp. were found
274 both in external and in internal face, but this might be explained, as stated before, by the close
275 proximity to the gardens and the frequent air exchange with the exterior related to the masses
276 tourism that make Versailles's site a special one, allowing the penetration of plant-associated
277 bacteria also in the interior of the building.

278 The bacteria from the genus *Frigobacterium*, *Psychrobacter glacincola* and *Brachybacterium*
279 *arcticum* isolated from the site of Bar-sur-Seine both in internal and external face (preferentially
280 in the northern exposition) are associated to tempered and cold environments such as marine
281 environments and hypogaeum (Bowman et al., 1997; Agogu e et al., 2005; Sprocati et al., 2008;
282 Ettoumi et al., 2016), which suggests that they can resist to ample temperature variations. This
283 characteristic is compatible with microorganisms living on stained-glass windows, exposed for
284 example to low temperature and atmospheric agents during winter. Some of the identified
285 bacteria are halotolerant (i.e. resistant to the presence of salts) and they were previously found
286 on archaeological walls and hypogaeum (Saiz-Jimenez and Laiz, 2000; Carrillo-Gonz alez et al.,
287 2016; Grottoli et al., 2020). These characteristics are also compatible with the "system stained-
288 glass window" where salts (secondary phases) can be formed during the glass alteration process
289 by reaction of leached glass elements with atmospheric components (Lombardo et al., 2010;
290 Gentaz et al., 2016; Maj erus et al., 2020).

291 The panels coming from the site of Lachalade were only sampled on the internal face. The
292 species identified for this site correspond mainly to the genera *Micrococcus*, *Agrobacterium*,
293 *Bacillus* (the three of them are considered plant-related species) and *Arthrobacter*. The genus
294 *Arthrobacter* is composed of heterotrophic species ecologically diverse and with metabolic
295 versatility and adaptability (Cacciari and Lippi, 1987; van Waasbergen et al., 2000). At a first
296 observation, the sites of Lachalade and Bar-sur-Seine share some similarities concerning the
297 identified Phyla, but the PCoA that takes into account the information of the species, allowed
298 discriminating at a finer level the communities from Lachalade and Bar-sur-Seine. The
299 similarity between these two sites was given by the presence of bacteria from the genus
300 *Arthrobacter* and *Micrococcus* present in both sites and representing the majority of the
301 identified genera on Lachalade panels.

302 Wissembourg panel was sampled on the two faces (internal/external). The only bacterial species
303 identified on this panel was *Arthrobacter crystallopoietes*, belonging to the Actinobacteria
304 Phylum. Numerous isolates of this species were recovered from both faces of the panel.
305 *Arthrobacter crystallopoietes* is known for its ability to survive to extreme desiccation and long
306 starvation (Ensign, 1970; Boylen, 1973), which are conditions compatible to an "extreme
307 environment" such as stained-glass windows.

308 Independently from the site, and as already noticed in the case of Bar-sur-Seine, some bacteria
309 found on stained glass are typically associated to the natural flora of human skin such as
310 *Staphylococcus hominis*, *Staphylococcus epidermidis*, *Acinetobacter lwoffii* (Gorbushina et al.,
311 2004; Regalado et al., 2009; Rakotonirainy et al., 2016). Their presence was probably caused
312 by the manipulation of the stained glass (for example during the transportation of the object for
313 the ones in the workshop), or simply by the presence of the public in their direct environment
314 as they were mainly found on internal face samplings.

315 Several species identified in this study were previously identified on other cultural heritage
316 sites. *Bacillus muralis* was first described on mural paintings (Heyrman and Swings, 2001) and
317 it has since been found on other mural paintings but also on organic materials (Pepe et al., 2010;
318 Pangallo et al., 2013). In the same way, *Arthrobacter parietis*, *Arthrobacter tumbae*,
319 *Arthrobacter crystallopoietes*, *Bacillus simplex*, *Micrococcus luteus*, *Arthrobacter agilis* and
320 different species of *Paenibacillus* were previously found on mural paintings, stones and stained
321 glass (Heyrman et al., 1999, 2000, 2005; Rölleke et al., 1999; Heyrman and Swings, 2001;
322 Gorbushina et al., 2004; Marvasi et al., 2009; Pangallo et al., 2009; Urzi et al., 2010; Piñar et
323 al., 2013; Mihajlovski et al., 2017; Tescari et al., 2018). Concerning stained-glass windows,
324 Rölleke et al., 1999 identified bacteria on German sites belonging to the *Flexibacter*,
325 *Nitrosospira*, *Arthrobacter*, *Micrococcus*, *Streptomyces*, *Frankia* and *Geodermatophilus*. The
326 bacteria identified by Marvasi et al. 2009 belong to *Bacillus*, *Paenibacillus*, *Arthrobacter* and
327 *Leucobacter* genera. Pinar et al. 2013 identified complex bacterial communities belonging to
328 *Methylobacterium*, *Ralstonia*, *Acinetobacter*, *Micrococcus*, *Prevotella*, *Kocuria*, *Delftia* and
329 *Arthrobacter*.

330 The comparison of the results from these previous works and the present work allow to identify
331 a subgroup of bacterial genera recurrently found on stained-glass windows, such as the
332 *Arthrobacter*, *Micrococcus*, *Bacillus* and *Paenibacillus*.

333 It is important to remind that glass composition changes through the ages due to the raw
334 materials used for glass production (Tite et al., 2002; Shortland et al., 2006; Verità, 2006;
335 Adlington et al., 2019). In this study, it was not possible to associate the microbiological
336 sampling with a systematic study of the composition of the glass because sampling of the glass
337 material was not allowed. This factor then could not be fully considered but it definitely
338 deserves to be investigated because i) the substrate composition can cause a selection of the
339 bacterial communities' development (Uroz et al., 2015; Kelly et al., 2016; Colin et al., 2017;
340 Plymale et al., 2021), and ii) reciprocally, different glass compositions are affected diversely
341 by the presence of microorganisms and their exudates (Koestler et al., 1987; Drewello and
342 Weissmann, 1997; Rodrigues et al., 2014; Valbi et al., 2022). However, some considerations
343 can be done on the basis of what is known about the evolution in the use of glass composition
344 in France through the ages, and on the basis of the color of the sampled glass pieces. In Medieval
345 times, stained glass windows in northern France/central Europe are mostly made with potash-
346 lime glass because of the use of plant ashes as a flux, even if some regional differences can be
347 observed (Douglas and Frank, 1972; Dungworth, 2012; Adlington et al., 2019). In the 12th–13th
348 centuries, stained-glass windows like the ones from Wissembourg were made with glass with
349 low silica (around 50 wt% SiO₂) and high amounts of calcium and potassium (around 15 wt%
350 of K₂O and CaO each) (Sterpenich and Libourel, 2001). In the 14th century, stained-glass
351 windows like the ones from Lachalade and Bar-sur-Seine generally have a similar composition
352 to the previous centuries, but with silica content slightly increasing, and with higher amounts
353 of calcium and lower amounts of potassium (Adlington et al., 2019). In the late medieval time,
354 beginning from the 15th century, the silica rate increases, as well as lime content, while
355 potassium and sodium concentrations decrease to obtain High Lime Low Alkali glass (HLLA)
356 (Schalm et al., 2007; Dungworth, 2012; Trichereau et al., 2022). In the 18th century, glass
357 production is completely changing with the introduction of glass casting. In stained-glass
358 windows like the ones from Versailles, glass sheets may have been produced by blowing or by
359 casting as both techniques coexisted (Hamon, 2005). In their composition, the amounts of silica
360 rise up to 65-70 wt%, with varying amounts of CaO, and K₂O (Schalm et al., 2007; Dungworth,
361 2012).

362 The similar bacterial communities between Lachalade and Bar-sur-Seine might then be
363 explained by a similar substrate, since both these sites have stained glass dating from the 14th
364 century. Nevertheless, this kind of comparison should be supported with more data in future
365 works.

366 If the composition of the studied stained glass is not known, the different colours of the glass
367 on which bacteria were preferentially sampled are also reflecting chemical variations in the
368 composition of the glass substrates. Colourless and murrey glasses were preferentially targeted
369 in this study, in order to improve the understanding of the browning phenomenon occurring on
370 colourless and murrey glass pieces, which are likely to contain Mn. Nevertheless, two samples
371 were collected for comparison on a blue glass at Bar-sur-Seine, while one sample was collected
372 on a painted glass at Versailles (see Table 1). It is worth noticing that the only sample collected
373 on a painted glass (blue paint, in internal face of the Versailles windows) did not induce the
374 development of cultivable bacteria. This could be attributed to the already discussed factor of
375 “internal face”, but another possible explanation could be the presence of the paint and its rich-
376 lead-chemical composition (Schalm et al., 2009) that could have an inhibiting effect on
377 microbial colonization. Similarly, no cultivable bacteria were detected in the sample collected
378 on blue glass (unpainted, but colored in the mass) in internal face in Bar-sur-Seine, while the
379 sample collected on external face on the same blue glass only showed the presence of
380 *Arthrobacter parietis*, a strain that was previously identified on comparable environments like
381 mural paintings.

382 By contrast, the identification of only one species, *Arthrobacter crystallopoietes*, a bacterial
383 strain known for its Mn-oxidizing ability (Tebo et al., 2005; Yang et al., 2013) on the murrey
384 glass from the Wissembourg panel (Douglas and Frank, 1972; Trichereau et al., 2022) could be
385 explained by the presence of Mn in the glass, introduced as a chromophore in the glass
386 composition (Gliozzo, 2016; Bidegaray et al., 2019; Rossano et al., 2022).

387 The composition of bacterial communities could also change with time (the bacterial
388 communities living on the stained-glass windows a hundred or two hundred years ago may
389 differ from the ones living on stained glass today) and with environmental conditions. This
390 could especially be a concern for the sampling realized on the stained glass removed and stored
391 in the conservation workshop, which represents a change of environmental conditions as
392 compared with stained glass *in situ*.

393 **4.2 On the sampling and identification methodology**

394 Only a few studies in the past were able to collect microbiological samples on stained-glass
395 windows, using different methodologies. Rölleke et al., 1999 identified bacteria on German
396 stained glass by sequencing excised bands of 16S rDNA fragments after Denaturing Gradient
397 Gel Electrophoresis (DGGE). For that study, 5-10 mg of glass were dissolved in water and
398 subjected to freeze-thawing cycles in order to lyse the bacterial cells for DNA extraction. Piñar
399 et al., 2013 grinded 0.2-0.25 g of glass from Spanish stained glass to obtain a powder that was
400 added to the lyse matrix for DNA extraction, the 16S rRNA fragments were then identified by
401 DGGE. Marvasi et al., 2009 used a non-destructive sampling method with contact plates filled
402 with Nutrient Agar with 1 % glucose for the sampling of microorganisms on an Italian stained
403 glass, then they obtained isolated strains by subculture and identified the bacteria through
404 DGGE of 16S rRNA fragments and sequencing. In the present work, a non-destructive
405 sampling method was chosen as well, allowing to collect a limited amount of biological sample,
406 but compatible with the difficulties of accessibility to the bays. The protocol applied here is
407 simple and is standardly used on other kinds of monuments (Leplat et al., 2019) and can be
408 easily used in the future in a wider project in order to extend the knowledge of microorganisms

409 living on French stained-glass windows. However, the culture of the microorganisms results in
410 a loss of information since only 1% of microorganisms are cultivable (Laiz et al., 2003). This
411 limit has to be considered and the protocol could be completed by direct DNA extraction from
412 the cotton-swab followed by metagenomic analyses. However, the results obtained in this study
413 were in agreement with the existing literature, and the most frequently genera found on stained-
414 glass windows on this basis could be identified as *Bacillus*, *Paenibacillus*, *Arthrobacter* and
415 *Micrococcus*.

416 4.3 Bacteria and weathering potential

417 The microorganisms identified on stained glass windows may not all be involved in alteration
418 phenomena. In the environment, bacteria, fungi, lichen and plants all produce organic acids and
419 chelating molecules, such as siderophores, which can strongly affect minerals and glass
420 weathering by increasing the solubility and mobility of elements (Staudigel et al., 1995; Ahmed
421 and Holmström, 2014; Perez et al., 2015, 2016, 2019; Valbi et al., 2022). Most microorganisms
422 are able to release organic acids such as oxalic, citric, acetic, fumaric and malic acid that can
423 cause bioalteration by changing the pH of the environment or by chelating ions from the
424 environment (Gadd, 1999; Neaman et al., 2005; Hausrath et al., 2009; Calvaruso et al., 2013;
425 Salek et al., 2013). Several kinds of bacteria are reported in literature to be able to produce
426 siderophores, such as the genus *Pseudomonas*, *Streptomyces*, *Nocardia*, *Arthrobacter*, *Erwinia*,
427 *Vibrio*, *Altermona*, *Micrococcus*, *Paenibacillus* and *Bacillus*, especially the siderophores
428 belonging to the families of hydroxamates and catecholates (Liermann et al., 2000; Peña et al.,
429 2007; Challaraj-Emmanuel et al., 2012; Perez et al., 2015; Saha et al., 2015; Grady et al., 2016).
430 Some of these genera were identified on stained glass (*Arthrobacter*, *Bacillus*, *Micrococcus*,
431 *Paenibacillus*) and could then have a weathering potential linked to the production of these
432 chelating molecules. Moreover, the weathering ability of some of the identified species was
433 already documented on mineral substrates. *Arthrobacter* spp. were able to promote the
434 dissolution of minerals such as hornblende, feldspar, quartz, and Fe-bearing glass through the
435 production of organic acids and siderophores (Ullman et al., 1996; Barker et al., 1998; Liermann
436 et al., 2000; Kalinowski et al., 2000; Frey et al., 2010). Other studies tested the weathering
437 potential of *Bacillus* spp. on granite and serpentine (Song et al., 2007; Yao et al., 2013) and of
438 *Paenibacillus* spp. on biotite, bauxite, and other rocks (Modak et al., 1999; Vasan et al., 2001;
439 Zhou et al., 2010; Liu et al., 2012). *Bacillus* spp. and other bacterial species like *Agobacterium*
440 spp. and *Shpingomonas* spp., were studied for their ability to promote the dissolution of
441 phosphates, phlogopite and biotite in the soil to improve the bioavailability of mineral nutrients
442 for plant roots (Leyval and Berthelin, 1989; Vassilev et al., 2006; Uroz et al., 2007).

443 A complete review of the glass-colonizing organisms is lacking in the literature, as stated in the
444 recent review on microbial interactions with silicate glass by (Weaver et al., 2021). Crossing
445 the results obtained in the present work and the literature previously cited, *Arthrobacter*,
446 *Bacillus* and *Paenibacillus* could be considered as the bacterial genera most likely to be able to
447 contribute to the alteration of stained glass windows as for today's state of knowledge. Notably,
448 the finding of the Mn-oxidizing species *Arthrobacter crystallopoietes*, which was isolated from
449 the murrey glass of Wissembourg suffering from the browning phenomenon could be crucial
450 for the understanding of this pathology, especially since this species has already been identified
451 on stained glass once before (Marvasi et al., 2009). The microbiological samples on the
452 Wissembourg panel were specifically sampled on the dark spots caused by the browning
453 phenomenon. Thus, the selection of *A. crystallopoietes* is an encouraging lead for the
454 investigation of the occurrence of this pathology, that has to be further investigated. In the frame
455 of a more targeted study of the browning phenomenon, future work should involve a larger *in*
456 *situ* sampling of medieval stained glass windows affected by this pathology. The study of the

457 effect of *A. crystallopoieties* on glass alteration also deserves further investigation; this
458 cultivable strain could thus be suitable for future laboratory-based glass bioalteration
459 experiments.

460 5. CONCLUSIONS

461 This work focused on the identification of bacteria at the surface of French stained-glass
462 windows and aimed to be a first non-exhaustive screening of the microorganisms likely to be
463 present on stained glass. The bacterial diversity was investigated by DNA extraction from
464 cultivated bacteria, and by amplification and sequencing targeting the 16S rDNA region. The
465 differences observed among the sampling sites showed that the sampling method used allowed
466 studying the microbial diversity without invasive sampling of the glass. Several genera known
467 for their weathering potential on mineral substrates were identified: *Agrobacterium*,
468 *Sphingomonas*, *Acinetobacter*, *Arthrobacter*, *Bacillus* and *Paenibacillus*. In particular, the Mn-
469 oxidizing species *Arthrobacter crystallopoietes* and its interaction with a glass substrate or its
470 involvement in the browning phenomenon need further investigations.

471 Acknowledgements

472 This work was supported by the Paris Ile-de-France Region – DIM “*Matériaux anciens et*
473 *patrimoniaux* » and by the Doctoral school “*Science Ingénierie et Environnement*” of
474 Université Paris-Est. The authors are grateful to the curators from the “*Directions régionales*
475 *des affaires culturelles, CRMH*” of both the region Ile-de-France and Grand-Est for their
476 collaboration. Frédéric Didier and Pierre Bortolussi, chief architects of respectively the Royal
477 Chapel of Versailles and St. Etienne church in Bar-sur-Seine are acknowledged for the access
478 to the monuments during their restorations. Pierre-Alain Parot is also acknowledged for the
479 access to the panels from Lachalade and Wissembourg in his workshop. The authors wish to
480 thank Céline Rommevaux for the advice and kind recommendations for the improvement of the
481 work.

482

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853 Table 1 List of all the samples collected, with information about the internal/external exposure
854 (Int./Ext.), the geographical exposure (E=East, NE=North-East, S=South), the colour and the
855 origin of the piece. The exposure information is also reported for Wissembourg and Lachalade,
856 but it is not applicable(*) in these sites because the panels were stored in the conservation

857 workshop. All the samplings were effectuated at least 3 cm away from any metal
 858 casings/bindings and on unpainted surfaces, except when indicated.

Site	Sample	Exposure	Glass colour	Original/restored
Versailles	VER_B11-2_P68	Ext., NE	Colourless	unknown
	VER_B11-2_P11	Ext., NE	Colourless	unknown
	VER_B11-1_P15	Ext., NE	Colourless	unknown
	VER_B91-2_P17_FI	Int., N	Colourless	unknown
	VER_B91-2_P17_FI_blue**	Int., N	Colourless, blue painted	unknown
	VER_B4-2_P68	Ext., N	Colourless	unknown
	VER_B4-2_P17_FI	Int., S	Colourless	unknown
	VER_B4-1_P15	Ext., S	Colourless	unknown
Bar-sur-Seine	BAR_B100_LD_P7_FI	Int., E	Blue	original
	BAR_B100_LD_P7_FE	Ext., E	Blue	original
	BAR_B101_LD_P7_FI	Int., NE	Colourless	original
	BAR_B101_LC_P7_FI	Int., NE	Colourless	original
	BAR_B101_LD_P7_FE	Ext., NE	Colourless	original
	BAR_B101_LC_P7_FE	Ext., NE	Colourless	original
	BAR_B103_R16_FE	Ext., N	Colourless	original
	BAR_B103_R16_FI	Int., N	Colourless	original
	BAR_B103_LC_R9_FI	Int., N	Colourless	original
	BAR_B103_LD_P4_FE	Ext., N	Colourless	original
	BAR_B103_LD_P3_FI	Int., N	Colourless	original
	BAR_B104_LB_P4_FE	Ext., S	Colourless	original
	BAR_B104_R17_FE	Ext., S	Colourless	original
	BAR_B104_R17_FI	Int., S	Colourless	original
Wissembourg	WIS_B0R3_1_FI	Int., E*	Murrey	original
	WIS_B0R3_1_FE	Ext., E*	Murrey	original
	WIS_B0R3_2_FI	Int., E*	Murrey	original
	WIS_B0R3_2_FE	Ext., E*	Murrey	original
Lachalade	LAC_B5P4_1	Int., E*	Colourless	original
	LAC_B6P3_2	Int., E*	Colourless	original
	LAC_B0R4_1	Int., E*	Colourless	original
	LAC_B0R4_2	Int., E*	Colourless	original

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863 List of figures

864

865 Figure 1 Map of France with the position of the four sites sampled *in situ* (red) or after
866 deposition (green), and the restoration workshop (blue) included in this study.

867

868 Figure 2 A) Plan of the Versailles Chapel (Didier, 2017) with location of the sampling points
869 and pictures of two sampled glass pieces on B) internal face of panel B91-2 p.17 in transmitted
870 light, and C) external face of panel B4-2 p.68 in reflected light. D) Plan of the church of Bar-
871 sur-Seine (Prache et al., 1992) with location of the sampling points, and pictures of E) panel
872 B101_LD_P7 on internal face, transmitted light and F) panel B101_LC_P7 on external face,
873 reflected light. G) Plan of Wissembourg Abbey (Héroid and Gatouillat, 1994) with original
874 location of the sampled glass, and picture taken at the restauration workshop (credits Atelier
875 Parot) of the tracery B0R3 on H) internal face and I) external face. J) Plan of Lachalade Abbey
876 (Vincent-Petit, 2016) with original location of the sampled glass, and pictures taken at the
877 restauration workshop of K) tracery B0R4 and L) panel B5P4 both in transmitted light. The
878 sample names include information about the sampling site (VER-BAR-WIS-LAC), the type of
879 structure (B=bay, P = panel, R = tracery).

880

881 Figure 3 Principal Coordinates Analysis (PCoA) results showing the groups of samples formed
882 by bacterial communities similarities. The factors taken into account are the “site” provenance
883 and the “external/internal” exposure of the sampled panel.

884

885 Figure 4 Heatmap of bacterial communities according to each sample at the genus level. The
886 most abundant genera are labelled in pale blue, while the less abundant genera are labelled in
887 dark blue. The dendrograms show the proximity between the different samples and the
888 proximity between identified bacterial genera.

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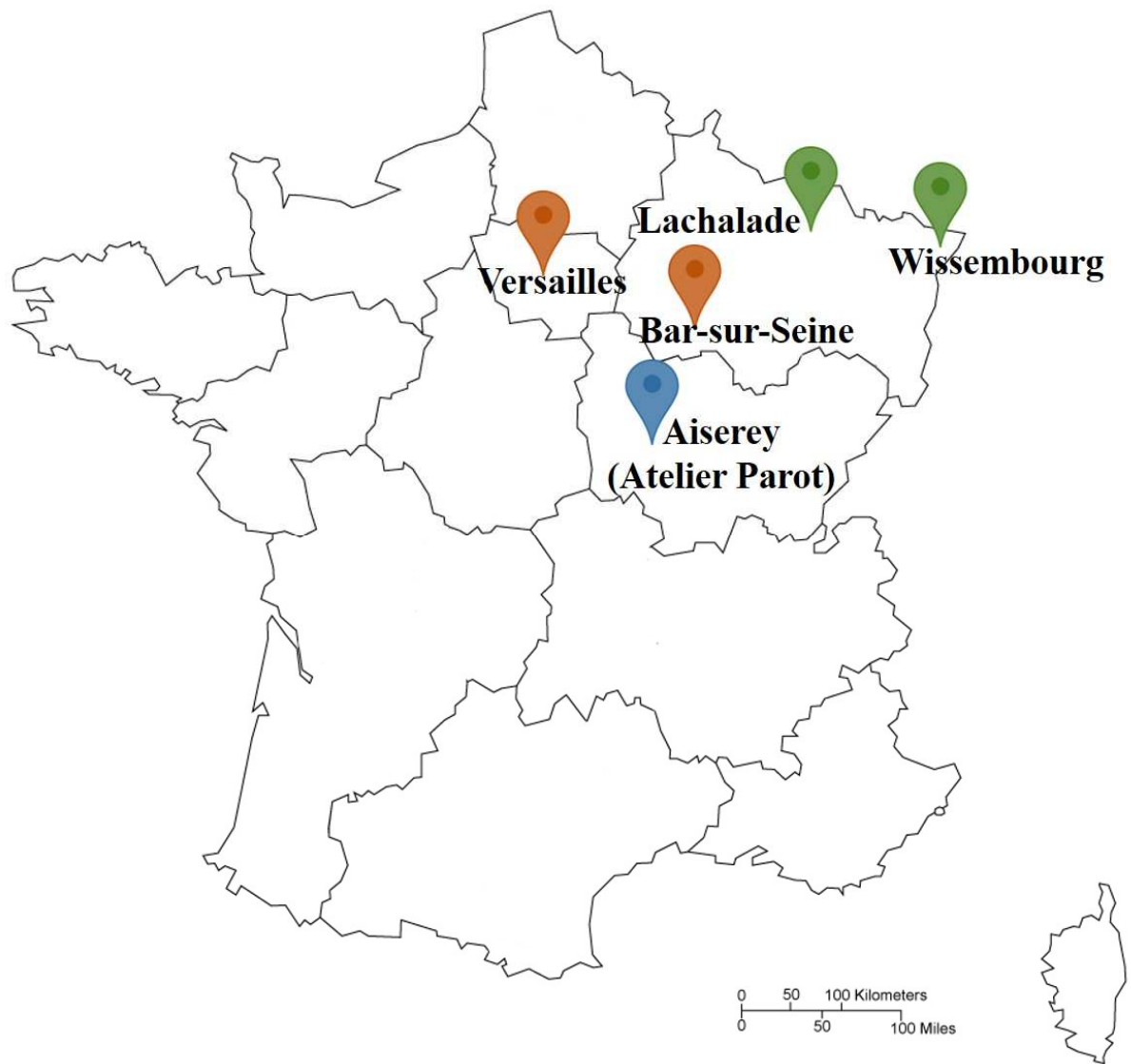
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899 Figure 1



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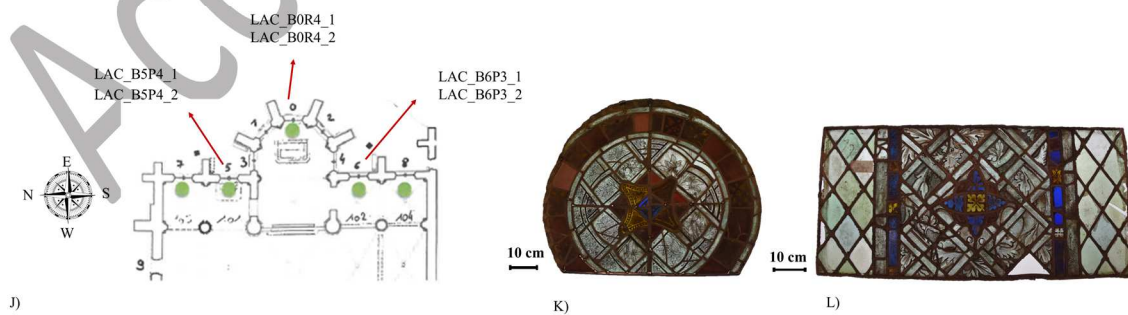
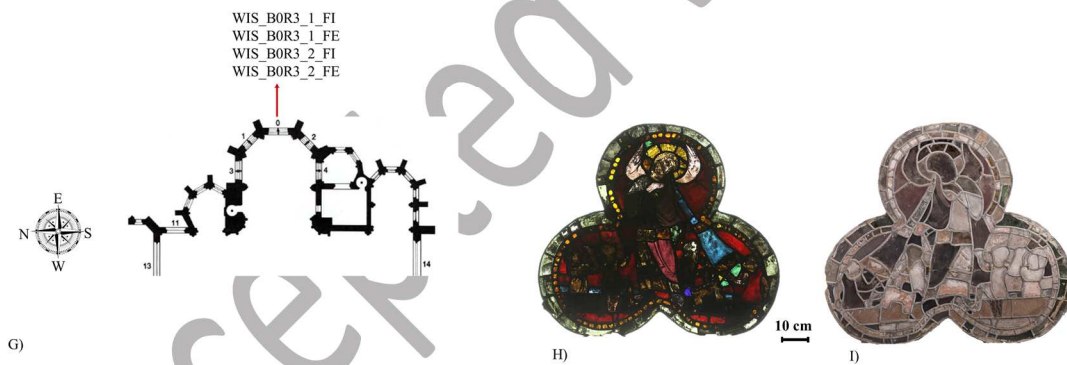
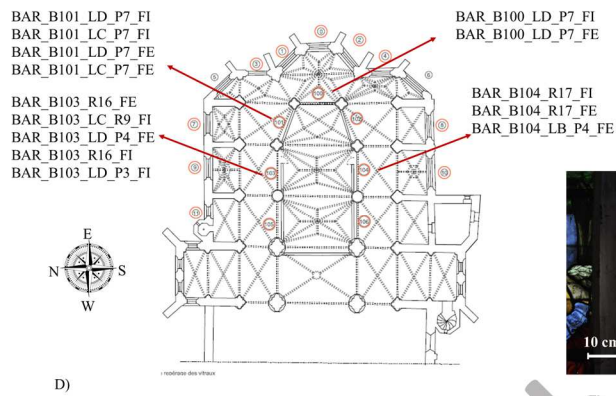
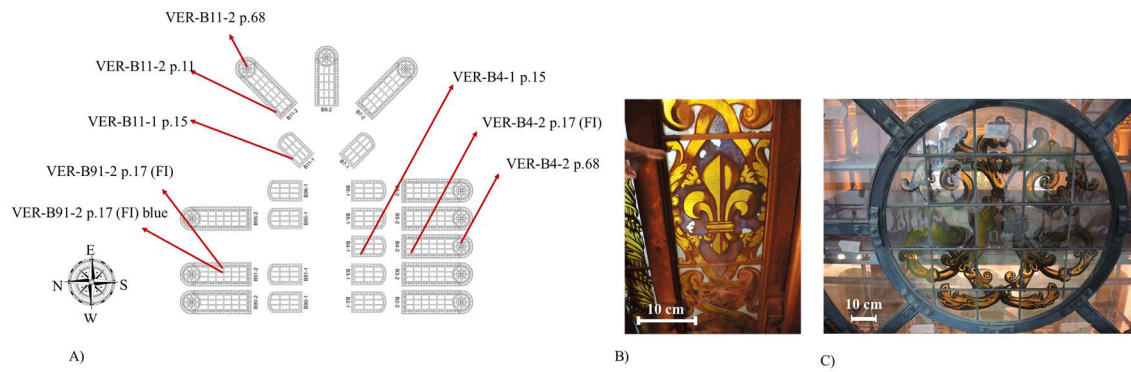
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911 Figure 2



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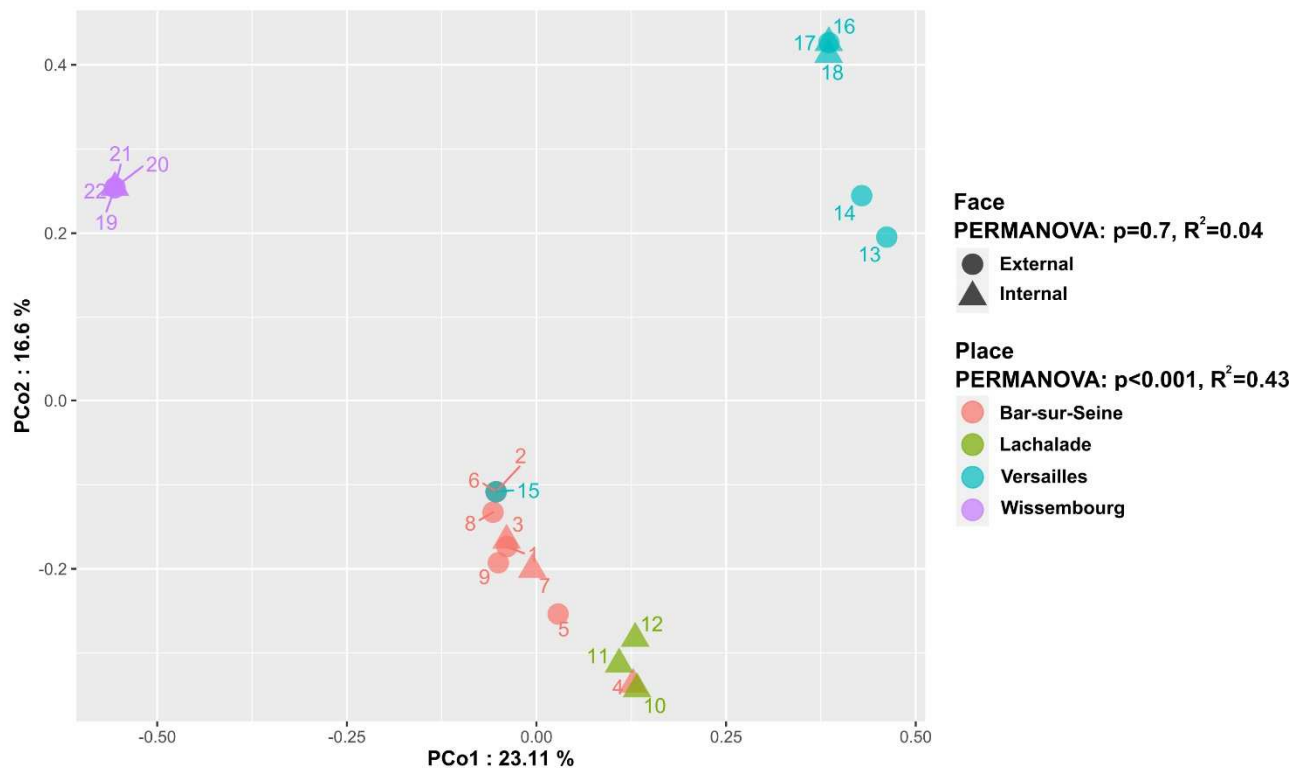
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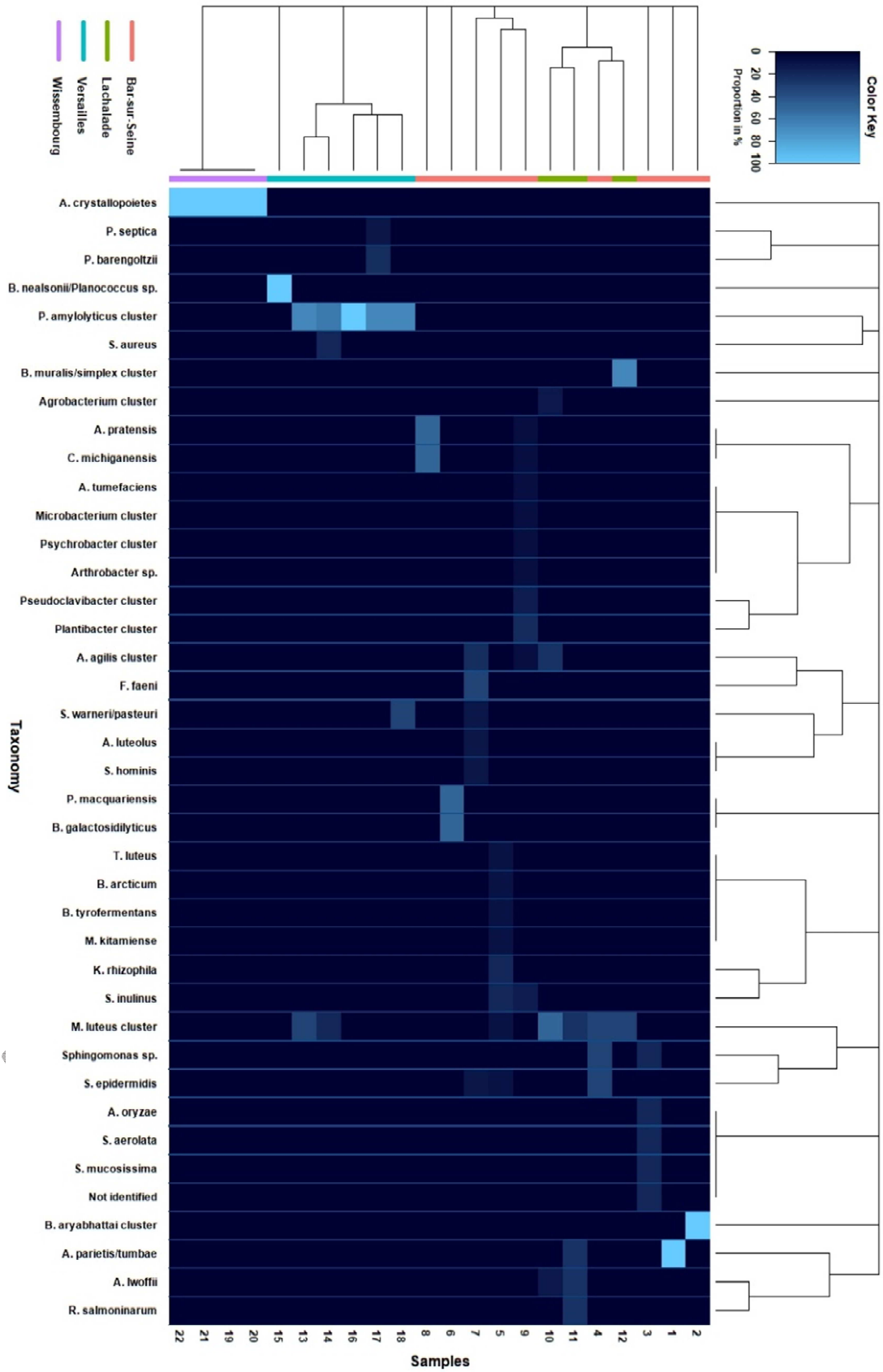
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934 Figure 4



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