Highlights

- An improved method is proposed, shortening to 14 days the bacterium-paint interaction

- Microtopography and digital image for the first time assessed graffiti biodegradation

- Microtopography is more effective than Rz in assessing biodegradation effects

- Sequencing results identified ATCC 700440 as *C. testosteroni*

- FTIR spectra and colour measurements proved better biodegradation by *C. testosteroni*

Graphical Abstract
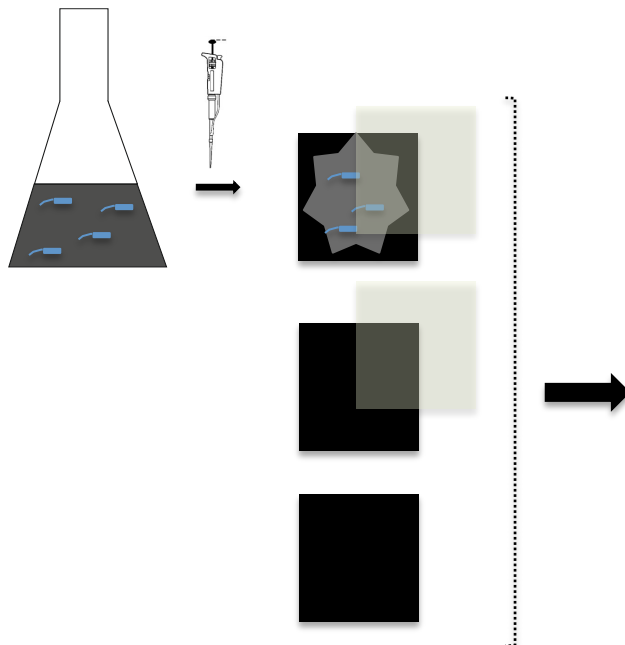
Klebsiella aerogenes or Comamonas testosteroni

Solution supplemented with powdered graffiti paint

Graffiti-coated granite and graffiti-coated concrete

Agar carrier
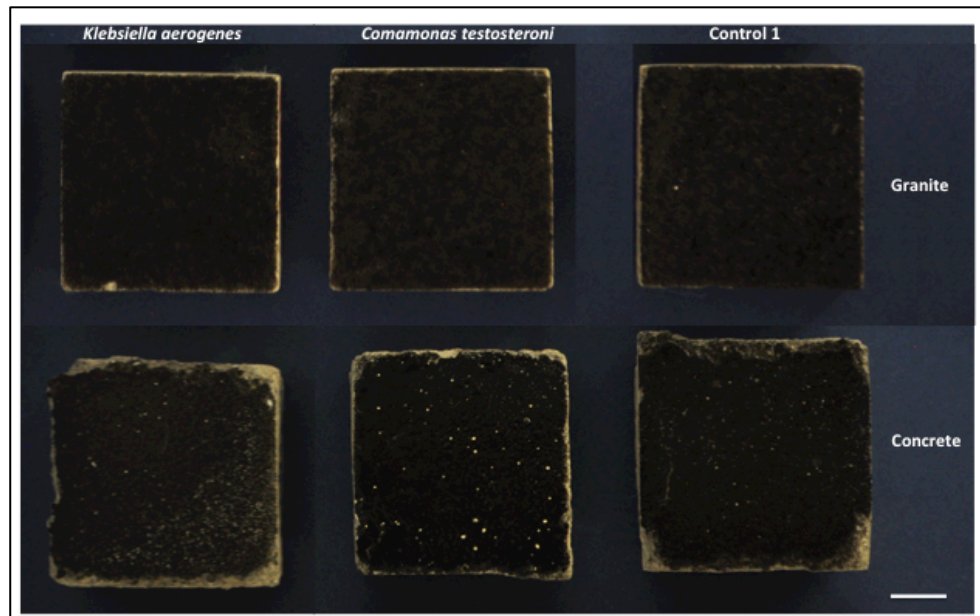
- Bacterial presence
- Naked-eye observation
- Digital image analysis
- Surface microtopography
- Wetting, gloss and roughness characterization
- Colour assessment
- Infrared (ATR-FTIR) spectroscopy

1   *Klebsiella aerogenes* **(ATCC 13048) and** *Comamonas testosteroni* **(ATCC 700440) as**
2   **biodegradation agents on graffiti coated concrete and granite**
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27   **Abstract**

28   *Klebsiella aerogenes* ATCC 13048 and *Comamonas* sp. ATCC 700440 (here identified
29   as *Comamonas testosteroni*) have been previously shown as good candidates for graffiti
30   bioremoval, demonstrating in both immersion and subaerial strategies high levels of
31   tolerance to the presence of the graffiti paint and some ability to degrade the graffiti
32   material. To explore further the graffiti biodegradative capacity of these two newfound
33   suitable bacteria, an experiment has been performed encompassing an improved assay
34   protocol (protocol time is reduced from 20 to 14 days). Pinholes formation -noticeably
35   higher on concrete than on granite- was already observable by naked eye and further
36   proved by digital image analysis, novel to this experiment, which showed holes greater in
37   number due to *K. aerogenes* and greater in size due to *C. testosteroni*. Complementarily,
38   surface microtopography -also novel for biodegradation studies with bacteria- offered
39   detailed information on surface irregularities that allows better understanding of the
40   behaviour of bacteria. In contrast non-mapping techniques, such as wetting by drop,
41   specular gloss and roughness measured in line transects provided lesser information for
42   the study. Infrared (ATR-FTIR) spectroscopy and colour change assessment – mainly in
43   the achromatic parameter L* - proved a better performance by *Comamonas testosteroni.*

44   **Keywords:** Bacteria; biocleaning; graffiti; microbial degradation; stone; cultural
45   heritage.

1. Introduction

In the field of built heritage preservation, cleaning is still a challenging operation, mainly due to its irreversible nature and potential side-effects. Safe, low risk and relatively cost-effective cleaning approach for built heritage materials is offered by biocleaning procedures (Batabyal, 2020). Gauri et al. (1992) published the first work on biocleaning of cultural heritage stone surfaces, where sulphate-reducing bacterium *Desulfovibrio desulfuricans* was used for removal of black sulphated crust from a marble statue. That first study had many shortcomings, including that the work of art was immersed in a growth medium for the test, the analytical data of sulphate removal from the statue were not reported (the bioremoval was evaluated only by visual observation), and control experiments were not conducted. The advancements in biocleaning in recent years allowed for its effective application on real case-studies, for the removal of inorganic and organic substances. Further research is needed for a broader and sustainable technological transfer to the building conservation practice (Gioventù et al., 2011; Ranalli et al., 2018, 2019). According to Pinna (2017) and Upadhyay et al. (2020), bacteria belonging to the genera *Desulfovibrio* and *Pseudomonas* are the most used in biocleaning processes against sulphates, nitrates and organic substances that are typical components of soiling and deposits covering the cultural heritage surfaces. However, extrapolating the findings on bioremoval of salts and organic matter from xenobiotic (synthetic) compounds, such as graffiti, is not an easy or direct task, and it is necessary to first identify microorganisms as potential candidates, and then carry out biodegradation tests to assess the degree of disturbance of target substances (Bosch-Roig and Sanmartín, 2021).

Less than ten years ago, graffiti paint was included as a novel potential bioremovable material, leading Giacomucci et al. (2012) to conduct the first studies with *Desulfovibrio desulfuricans* ATCC 13541 applied to nitrocellulose-based red graffiti paints. Later, a feasibility study involving the isolation of natural strains of microorganisms capable of degrading graffiti showed bacteria belonging to the genera *Arthrobacter, Bacillus, Gordonia, Microbacterium, Pantoea* and *Pseudomonas* and fungi belonging to the genus *Alternaria* as potential bioremediation agents in black graffiti paint (Sanmartín et al., 2015). So far, the use of fungi and bacteria to remove graffiti has been investigated in a limited way. Nevertheless, it provides an effective and ecologically safe method particularly useful for cleaning porous materials due to its high-level controllability. Some shortcomings given by traditional chemical and physical (including laser) methods of graffiti removal are well known (Sanmartín et al., 2014 and therein references), especially on porous substrates (such as mortars, concretes, natural stones, bricks) where graffiti can penetrate, thereby leading to limited cleaning results (Fig. 1).

Recently, in immersion (Cattò and Sanmartín et al., 2021) and subaerial (Sanmartín and Bosch-Roig, 2019) strategies, *Enterobacter aerogenes* ATCC 13048 and *Comamonas* sp.

ATCC 700440 have shown a promising profile for biocleaning of black and silver graffiti paints. Previously, different strains of *Enterobacter aerogenes*, a common flora found in the human gastrointestinal tract (Sanders and Sanders, 1997) have been reported to degrade xenobiotic compounds, such as pyrethriodspesticide residues (Liao et al., 2009), anionic surfactants like alkylbenzene sulfonate compounds (Fathi et al., 2016), and acrylamides (Buranasilp and Charoenpanich, 2011; Jangkorn et al., 2018; Madmanang et al., 2019). An *E. aerogenes* strain isolated from domestic wastewater in Thailand proved to be an acrylamide-degrading bacterium, degrading acrylamide to acrylic acid and further to lower polarity compounds. The bacterium showed hydrolysis potential of acrylamide to acrylic acid and ammonia up to 5000 ppm at the mesophilic temperatures and could degrade other aliphatic amides especially short to medium-chain length but not amide derivates (Buranasilp and Charoenpanich, 2011). Similarly, *Comamonas* sp. strains are well-known degraders of the phenol, viz. *C. testosteroni* ZD4-1 (Chen et al., 2003), the organochlorine compound chloroaniline, viz. *C. testosteroni* I2 (Boon et al., 2001), and the chromone, quinolone or quinoline, viz. *Comamonas acidovorans* DSM 6426 (Miethling et al., 1993), *C. testosteroni* sp. bdq06 (Geng et al., 2015). In Changchun (China), high concentrations of TOC (total organic carbon), quinoline, phenol and other refractory compounds from an accidentally released dye wastewater were successfully removed by adding *Comamonas testosteroni* bdq06 (Liu et al., 2016).

In the present work, a further attempt to enhance graffiti biodegradation by applying single strains of *E. aerogenes* ATCC 13048 or *Comamonas* sp. ATCC 700440, has been made by: (i) using an advanced onsite biocleaning system with a short application contact time of fourteen days between bacterium and graffiti paint, and (ii) using a multi-analytical approach to assess the bacterial-induced alteration of black non-metallic paint, chosen among other colours because it is the most commonly used graffiti spray paint (Sanmartín et al., 2014), on granite and concrete substrates.

2. Materials and methods

2.1 Selected bacteria and growth conditions

Klebsiella aerogenes ATCC 13048 (at the time of the purchase sold as *Enterobacter aerogenes*) and *Comamonas* sp. ATCC 700440 were selected for the assays. DNA analysis by sequencing of 16S rDNA gene of both bacteria was realized following the protocol described by Sanmartín and Carballeira (2021). Bacterial sequences were deposited in the NCBI database under GenBank accession numbers MW322923 (ATCC 700440) and MW322925 (ATCC 13048).

For biodegradation, bacteria are initially grown in a medium enriched with the same material they are expected to remove during the treatment. A culture medium enriched with powdered graffiti was used for their growth, following the protocol described in

121 Sanmartín and Bosch-Roig (2019), to facilitate and accelerate their adaptation to the
122 graffiti painted surface. Thus, strains of bacteria were grown separately at 28ºC for 20
123 days in 400ml Erlenmeyer flask with the M9 mineral medium (M9 broth (75.2g/L
124 $Na_2HPO_4–2H_2O$, 30g/L KH_2PO_4, 5g/L NaCl, 5g/L NH_4Cl) + $MgSO_4$ (1M) + $CaCl_2$
125 (1M)) supplemented with powdered black graffiti as a selective agent. Bacteria viability
126 was analysed over time (5, 10 and 20 days incubation) by serial dilutions and colony
127 forming units (CFU)/ml counts.

2.2 Graffiti spray paint samples preparation

129 Black graffiti paint (R-9011) from Montana Colors (Barcelona, Spain) was applied to
130 granite and concrete substrates according to Sanmartín and Cappitelli (2017). Blocks of
131 ca. 4x4x1.5 cm^3 of a *Silvestre* granite and a concrete produced from Portland cement,
132 both described in Sanmartín and Bosch-Roig (2019) were used. Once painted and dried,
133 the graffiti-coated samples were sterilized by leaving them for 24 h in a sealed box
134 containing 37% formaldehyde (Sanmartín et al., 2015).

2.3 Biodegradation assays

136 The assays were carried out using an agar carrier to apply the bacteria (oriented towards
137 an onsite application) on the graffiti coated blocks for a total of 14 days, following the
138 steps detailed in Figure 2. This is an improved version of the protocol described in
139 Sanmartín and Bosch-Roig (2019). The main advancement being the reduced interaction
140 time between the bacterium and the graffiti paint to be removed from 20 days to 14 days.

141 Suspensions of bacteria, first adapted to the powdered graffiti (section 2.1), were
142 centrifuged (4900 rpm for 10min) and pellet was resuspended in 20 ml of sterile water.
143 One ml of the final cell suspension (10^6 cells/ml) was applied on the painted surface of
144 each block with the aid of a micropipette and sterile brush. Six ml of 2% warm agar was
145 immediately added as a delivery system (Sanmartín and Bosch-Roig, 2019). Samples
146 were then placed on a plastic tray and covered with plastic film to prevent agar
147 desiccation. To minimize ambient contamination, treatments were done inside a Laminar
148 flow hood. Experiments were performed in triplicate. Five controls without treatment,
149 only blocks painted with graffiti (hereinafter, Control 0), and five controls prepared by
150 adding sterile water instead of bacterial suspension (hereinafter, Control 1) were included
151 to check the results obtained. After 14 days, agar was removed and the painted surfaces
152 were cleaned with sterile water and a sterile sponge, and then allowed to dry.

153 The comparison between Control 0 (no treatment, graffiti coated sample) and Control 1
154 (agar carrier without bacteria) allows for measuring variability in the graffiti paint
155 ascribable to the 14-day contact with agar.

156 *2.4 Graffiti biodegradation assessment*

157 *2.4.1 Bacterial presence at the end of treatment*

158 At the end of the experiment, agar was removed and the presence of microorganisms on
159 the treated surfaces was checked before and after the final sterile water-cleaning step.
160 RODAC plates with Nutrient Agar (NA, supplier Merck Millipore) were applied and then
161 incubated for 24h-48h at 28ºC.

162 *2.4.2 Digital analysis*

163 To quantify the effects visually detectable of the biological treatment, which were
164 observable also by naked eye, digital images of the Control 0, Control 1, treatment with
165 ATCC 13048 and treatment with ATCC 700440 on granite and concrete substrates coated
166 with black graffiti paint were taken. In order to standardize the images, a digital camera
167 (Canon EOS 50 D) mounted on a tripod with fixed conditions (ISO100, f/8.0, 1/250 s,
168 focal length 19 mm) was used, according to Bosch-Roig et al. (2019). For digital image
169 analysis, a specific software scheme and method was developed in MatLab matrix
170 programming environment (Bosch-Roig et al., 2019).

171 Image analysis involved organizing the results of the photographic data and selecting the
172 images for each of treatment condition. Subsequently, the pixels of each image were
173 determined and stored, and then converted from RGB values to grey scale. Next, the grey
174 levels of each pixel area were analyzed and quantified by selecting an experimental 0.3
175 threshold (calculated ad hoc based on the images and requirements of the study) on the
176 group-averaged images. The purpose of this grey level thresholding is to extract from the
177 image those pixels which represent the damage caused on the graffiti paint (related to
178 clearer areas), to obtain binary detection masks where the areas with the highest light
179 values (white dots, pixels) of each image can be easily seen against the dark values of
180 graffiti paint. The results obtained are shown as the percentage of white pixels over the
181 total number of pixels in each image. In addition, the size of clearer areas was calculated
182 as a relative percentage of area, obtaining the number of white dots on the black paint
183 surface according to their size.

184 *2.4.3 Roughness measurement*

185 The microtopography (surface roughness) of three 5×5mm ($25mm^2$) surface zones (one
186 in the center and the other two at 1 centimeter from the vertices) on each surface sample
187 (i.e. the two controls and two bacterial treatments on both substrates, including also in
188 this case, unpainted granite and concrete substrates) was obtained with a resolution of 2.5
189 µm using a 3D optical roughness (INNOWEP TRACE-iT®). From each measured plane,
190 the roughness parameter Rz that best discriminates the differences between the profiles

191 (Vázquez-Calvo et al., 2012; Dąbski and Tittenbrun, 2013) was determined from the
192 average of 5 peak-valley measurements across 5 mm transects in both X- and Y-axes.

193 *2.4.4 Surface wetting characterization by contact angle analysis and water drop*
194 *penetration time*

195 A Drop Shape Analyzer DSA 100 (Krüss GmbH, Hamburg, Germany) was used for
196 water repellency measurements: a droplet of distilled water (10 µL) was deposited onto
197 the graffiti painted surfaces and the water surface contact angle was immediately
198 measured. At least 5 measurements were made on different zones of the target surface at
199 25 °C. The contact angle was measured on digital microphotographs with Image J2
200 software (Rueden et al., 2017). The water drop penetration time (WDPT) test was
201 conducted according to Leelamanie et al. (2008)'s repellency category, as follows: ≤ 1 s:
202 Non-repellent; 1–60 s: Slightly repellent; 1–10 min: Strongly repellent; 10–60 min:
203 Severely repellent; and, $\geq$ 1h: Extremely repellent.

204 *2.4.5 Colour and gloss measurements*

205 A total of 10 colour readings were taken at different randomly selected zones on each
206 graffiti painted surface, according to Sanmartín and Cappitelli (2017), by use of a Konica
207 Minolta colorimeter with a CR–300 measuring head (8-mm-diameter viewing area). The
208 working conditions were: CIE standard daylight illuminant D65 and observer 10°. Colour
209 measurements were analysed using the CIELAB colour system (CIE S014-4/E:2007),
210 which represents each colour by means of three scalar parameters or Cartesian
211 coordinates: L*, lightness, which varies from 0 (absolute black) to 100 (absolute white);
212 a*, associated with changes in redness-greenness (positive a* is red and negative a* is
213 green); and b*, associated with changes in yellowness-blueness (positive b* is yellow and
214 negative b* is blue). Partial colour differences (ΔL*, Δa* and Δb*) and the total or global
215 colour difference (ΔE*$_{ab}$) were determined according to UNE-EN 15886:2011 by using
216 the following equations:
217

$$\Delta L^* = L^*_i - L^*_0, \tag{1}$$
$$\Delta a^* = a^*_i - a^*_0, \tag{2}$$
$$\Delta b^* = b^*_i - b^*_0, \tag{3}$$
$$\Delta E^*_{ab} = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}, \tag{4}$$

218

219 The changes (Δ) in each parameter (L*, a* and b*) were calculated between the values at
220 the end of the treatments with ATCC 13048 or ATCC 700440 (subscript i) and the
221 control 0 (subscript 0). Control without bacterial cells (control 1) was also included and
222 compared with control 0 for reference purposes.

223 Similarly, 3 specular gloss measurements were made per sample surface, obtaining the
224 mean value, at an angle on incidence of 60° - because the surfaces are not very shiny -
225 with a Konica Minolta GM-268 glossmeter. The changes in gloss were calculated in the
226 same way as the colour changes.

227 *2.4.6 Infrared (ATR-FTIR) spectroscopy*

228 FTIR analyses were conducted in Attenuated Total Reflectance mode (ATR-FTIR,
229 VARIAN FTIR 670, Palo Alto, CA, USA) in the 4000–400 cm^{-1} spectral range. Average
230 spectra from 150 scans were acquired with a resolution of 4 cm^{-1}. At least three spectra
231 were recorded for each condition.

232 **3. Results**

233 Sequencing results identified the strain ATCC 13048 as *Klebsiella aerogenes* (100%
234 similarity in nucleotide identity) and the strain ATCC 700440 as *Comamonas testosteroni*
235 (99.93% similarity in nucleotide identity). From now on the strains are named as
236 identified as in the case of *Comamonas testosteroni* we have identified the species.

237 Results of the adaptation to the powdered graffiti of the two bacteria are shown in Table
238 1. They indicate that both bacteria showed an initial viability decrease (higher in *C.
239 testosteroni*) followed by an increase and stabilization after 10 days of incubation.
240 Despite the initial decline, the growth of *C. testosteroni* was then higher and faster, and
241 after 20 days the growth of *K. aerogenes* was an order of magnitude less than *C.
242 testosteroni*, i.e. 10^6 CFU mL^{-1} versus 10^7 CFU mL^{-1}. The adapted bacteria were then
243 used for the direct application on the graffiti coated blocks as described on Section 2.3.
244 At the end of treatment (14 days), the presence of bacteria on the treated surfaces was
245 analysed by NA contact plates (Fig. S1).

246 Figure 2 shows a schematic diagram of the protocol followed in this research. Bacterial
247 growth was detected on all samples treated with *K. aerogenes* or *C. testosteroni* after agar
248 removal and before the final water cleaning with microbial counts media values over 300
249 CFU per 25cm^2. After the final sterile water cleaning step bacterial load was drastically
250 reduced (up to 40±10 CFU per 25cm^2).

251 Figure 3 shows the visible effects, in number and size, caused by the treatments on the
252 graffiti paint and the results of the digital image analysis. On pristine concrete, only
253 0.0417% of the surface showed pinholes (white dots or pixels), while after agar
254 application (Control 1) this value increased almost sevenfold to 0.2796 %. The treatment
255 with bacteria provoked an additional increase in pinholes concentration, with average
256 values of 0.8970 % after *C. testosteroni.* This was around three times higher than agar
257 alone (Control 1), and average values of 1.4862% after *K. aerogenes* bacterial treatment,

258   around five times higher than agar alone (Control 1). In granite, the differences between
259   the treatments (including Control 1) and Control 0 are negligible, with values of 0.0250%
260   for Control 0, 0.0429% for Control 1, 0.0092% for treatment with *C. testosteroni* and
261   0.0190% for treatment with *K. aerogenes.*

262   Regarding the size of the pinholes, the histogram of concrete shows how, despite a higher
263   number of dots resulting from *K. aerogenes*, their size was notably smaller than the one
264   observed after treatment with *C. testosteroni*, which provoked pinholes even five times
265   larger in size (0.05%area/point) than *K. aerogenes* (Fig. 3). Control 1 on concrete
266   provoked only a limited number of small pinholes. In the granite samples, few numbers
267   of pinholes of small size were detected, not clearly linked to any treatment condition.

268   Regarding roughness measured in line transects (Fig. 4a), although the initial Rz of
269   granite was twice of the average value of concrete, once the graffiti spray paint was
270   applied (Control 0), roughnesses were leveled up to Rz values around 30 μm. In the same
271   way, the treatment without bacteria (Control 1) produced a very similar roughness in both
272   substrates, with Rz=43 mm approx. The roughness (Rz) values achieved after applying
273   the bacteria were different depending on the substrate. For granite, the roughness
274   increased with both bacterial treatments compared to Control 1, while in the case of
275   concrete it decreased (Fig. 4a).

276   With regard to surface microtopography (Fig. 4b), the unevenness on the surface of
277   unpainted granite, unlike concrete with a quite homogeneous surface in height
278   distribution, was maintained after the samples were painted. Surface microtopography of
279   coated concrete showed few irregularities in the surface, while in granite there was an
280   accumulation of the highest levels - marked in red - in the center of sample (Fig. 4b).

281   In concrete the treatment with bacteria produced surface irregularities with the highest
282   levels (peaks) close to 300 μm in the case of *K. aerogenes* and peaks of around 230 μm
283   in the case of *C. testosteroni* (Fig. 4b). In addition, *C. testosteroni* seemed to have caused
284   more areas of lower height - less than 100 microns, marked in blue in the image - when
285   compared to the microtopography generated in Control 1 (Fig. 4b).

286   In granite, with *K. aerogenes* higher height values of the surface were observed, around
287   290 μm, marked in red in the center of the sample (Fig. 4b). In *C. testosteroni*, few high
288   levels appeared in height distribution, the mean value being around 180 μm, with areas of
289   lower values of 100 μm - valleys marked in blue - which that are not observed in the
290   corresponding Control 1 (Fig. 4b).

291   In general, a slightly more heterogeneous surface in height distribution with peaks and
292   valleys was observed after treatment with *C. testosteroni* compared to that with *K.*
293   *aerogenes* (Fig. 4b).

294 As seen in Table 2, an increase in the hydrophilic behaviour (associated to a reduction of
295 the contact angle values) of graffiti paints was obtained after the treatment with agar
296 (Control 0 versus Control 1) and also after the application of bacterial cells (Control 1
297 versus treatments with *K. aerogenes* or *C. testosteroni*). Enhancement of hydrophilicity
298 was particularly important with both bacteria on concrete, comparing the results with
299 those of the treatment only with agar (Control 1), by reaching an average reduction of the
300 contact angle of almost 50%.

301 The results of water drop penetration time and its related water repellency category
302 (Table 2) indicate a shorter time in the samples treated with bacteria compared to Control
303 1. *K. aerogenes* reduced the water absorption time more than its counterpart.

304 In the case of graffiti on concrete, *C. testosteroni* caused colour changes greater than *K.*
305 *aerogenes* (Fig. 5). In contrast, on granite, *K. aerogenes* caused colour variations similar
306 to those produced by Control 1 and greater than those provoked by *C. testosteroni.* For
307 the achromatic parameter (L*), the variations (ΔL*, Fig. 5a) were on concrete higher and
308 positive (indicating a lightening of graffiti paint) and on granite lower and negative
309 (indicating a darkening of graffiti paint). Neither of the two chromatic parameters (a* and
310 b*) varied appreciably, the variations (Δa* and Δb*, Fig. 5b and 5c) were near-zero for
311 all conditions, not exceeding the threshold of 0.73 CIELAB units. Therefore, the total
312 colour changes (ΔE*$_{ab}$, Fig. 5d) were almost entirely due to changes in lightness, being
313 ΔE*$_{ab}$ values practically the same as those of ΔL*.

314 Fig. 5e shows that changes in gloss did not reach 3° and they were very similar on both
315 substrates. With respect to Control 0, the treatment only with agar (Control 1) and the
316 treatment with *C. testosteroni* varied the gloss around 1.7° - 2.5°, while the treatment
317 with *K. aerogenes* barely modified the gloss (less than 0.3°).

318 With regard to infrared spectroscopy, the black graffiti paint was previously identified as
319 an alkyd paint (Sanmartín and Cappitelli, 2017; Cattò and Sanmartín et al., 2021) with
320 main absorption peaks at 1718 cm^{-1} and 1252 cm^{-1} attributed to the carbonyl and C-O
321 stretching mode, respectively (Ploeger et al., 2008). The characteristics absorptions peaks
322 due to asymmetric and symmetric C-H stretching at 2922 cm^{-1} and 2852 cm^{-1} are
323 observed in Control 0. The application of the agar gel without bacteria did not induce
324 significant changes to the paint, as confirmed by the almost unaltered spectral features of
325 Control 1 (data not shown).

326 The effects of the treatment on the compositional features of the paint were evaluated
327 following the same protocol as in Cattò and Sanmartín et al. (2021). In particular,
328 changes were evaluated with respect to the general spectral features and the variation of
329 the relative intensities of the characteristics peaks of the paint. Overall, as a result of both
330 bacterial treatments, the C-H absorption in the 2900-2800 cm^{-1} region is reduced

331   compared to both Controls 0 and 1, and a new peak could be observed around 1020 cm^{-1}.
332   The latter cannot be unambiguously associated to the treatment, as it can derive from the
333   contribution of the inorganic substrates due to the Si-O-Si stretching absorption.
334   Treatment with *C. testosteroni wa*s associated with more intense changes, and the
335   formation of an additional small peak at 1652 cm^{-1}. The 1721/1256 cm^{-1} ratio of the paint
336   was also slightly altered upon treatment with both bacteria, decreasing from 0.91-0.95 of
337   Controls 0 and 1 to 0.83-0.86 of the treated with bacteria surfaces.

338 **4. Discussion**

339   At present, living bacteria are successfully used for biocleaning of undesired substances
340   such as salts and organic matter (see e.g., Chandra et al., 2020 and references therein;
341   Parulekar-Berde et al., 2020 and references therein), but the graffiti removal is a more
342   complicated process which requires more planning, more steps, and more detailed
343   assessment of the degree of disturbance in the unwanted material to know how to move
344   forward into the research.

345   According to Bosch-Roig and Sanmartín (2021) if the bioremoval of graffiti is analysed
346   in the context of current biocleaning research on sulphates, nitrates and organic matter
347   where only a few hours of treatment are necessary (72 hours maximum), one important
348   remaining challenge is to reduce the treatment application times. Following an immersion
349   strategy, graffiti biodegradation application times of up to 49 days for *Desulfovibrio*
350   *desulfuricans* ATCC 13541 applied under anaerobic conditions to red alkyd-
351   nitrocellulose-based binder spray paint have been reported (Giacomucci et al., 2012).
352   Cattò and Sanmartín et al. (2021) reported aerobic treatment requiring an application time
353   of 27 days when ATCC 13048, ATCC 53922 (mixed culture of *Bacillus* sp., *Delfia*
354   *lacustris*, *Sphingobacterium caeni* and *Ochrobacterium anthropi*) and ATCC 700440
355   were used. This was reduced to 25 days when aerobic microorganisms (*Arthrobacter,*
356   *Bacillus, Gordonia, Microbacterium, Pantoea, Pseudomonas* and *Alternaria*) were used
357   to remove black paint (Sanmartín et al., 2015). In a subaerial strategy, placing the strains
358   onto spraying graffiti paint on stone material in an aerial environment, i.e. suitable for
359   real field conditions, the application time of bacteria onto graffiti paint was 20 days
360   (Sanmartín and Bosch-Roig, 2019). In the present study, , also following a subaerial
361   strategy, this time was reduced to 14 days.

362   As at the time of the purchase from the microbial collection ATCC, 13048 was identified
363   as *Enterobacter aerogenes* (now sold as *Klebsiella aerogens*) we performed an accurate
364   identification of the two strains involved here in the biodegradative experiments. Indeed,
365   results from partial 16S rDNA sequencing indicated the need to update the name of the
366   ATCC 13048 strain (according to Tindall et al., 2017), and allowed a better identification

367 as *Comamonas testosteroni* of ATCC 700440 currently sold by the microbial collection
368 as *Comamonas* sp.

369 Microorganisms develop mechanisms of resistance and specific metabolisms once
370 exposed to novel organic compounds (van der Meer and Sentchilo, 2003). Thus, before
371 any biodegradative test was performed, tests of adaptation were conducted. Findings
372 showed that *C. testosteroni* was more able by one order of magnitude than *K. aerogenes*
373 to grow using graffiti as the sole carbon and energy source.

374 After the treatment, bacteria were easily removed using a washing step (Bosch-Roig et
375 al., 2012; 2013). As this step was also performed in other biocidal treatments in cultural
376 heritage assets (Sanmartín and Carballeira, 2021), it has become evident the effectiveness
377 of water in the elimination of bacteria

378 The naked eye observations after treatment give quick and qualitative information to
379 conservators and scientists on the activity on bacteria-exposed specimens (Cappitelli et
380 al., 2007; Bosch-Roig et al., 2015). Visible changes in form of pinholes formation
381 occurred in most of the samples treated with bacteria. These changes, to a much lesser
382 extent were also observed in Control 1. A method of visually monitoring and
383 mathematically modelling changes to graffiti paints was used here based on the
384 technology of digital image processing (Clogg & Diaz-Andreu, 2000; Mirmehdi et al.,
385 2001; Gazzano et al., 2009). Digital image analysis, novel to this experiment, showed that
386 the percentage of white pixels over the total of pixels in each image was noticeably
387 higher on concrete than on granite. Further *K. aerogenes* activity led to a greater number
388 of white dots in relation to *C. testosteroni* but of smaller size. In contrast, *C. testosteroni*
389 activity led to bigger size white dots formation suggesting that both the bacteria species
390 can affect the final type of biocleaning visual results as suggested before by these (Cattò
391 and Sanmartín et al. 2021 and Sanmartín and Bosch-Roig 2019) and other authors
392 (Barbabietola et al., 2016; Romano et al., 2019).

393 The authors believe this is the first time that an optical roughness device (TRACE-iT®)
394 has been used to assess the biodegradative capacity of a treatment using bacteria. This
395 device provides average values of linear roughness, such as Rz and Ra, but also 3D
396 microtopographic maps of the materials surface, accurately detecting the surface
397 irregularities on a micro-scale (μm) with measuring fields of 25 mm^2 and resolution along
398 both the Z and the X/Y axes of 2.5 μm (Aly et al., 2015; Perez-Monserrat et al., 2017).
399 As roughness (measured in line transects) of Control 0 and Control 1 for both concrete
400 and granite was the same, it is suggested here that surface microtopography of graffiti
401 coatings can affect the final biocleaning result. Indeed, graffiti coated concrete was
402 smoother than graffiti coated granite, which showed more irregularities, and this likely
403 prompted a better performance of the biodegradative activity. A smoother surface could

404 promote a better adhesion between the bacteria and the underlying material, which in turn
405 could help bacteria develop their biodegradable activity. However, understanding the
406 actual effect of surface topography on the overall biodegradation activity and the biofilm-
407 surface topography interaction remains challenging (Cheng et al., 2019).

408 The less homogenous topographic surface after the treatment with *C. testosteroni*
409 compared to treatment with *K. aerogenes* may explain the difference in the size of the
410 holes created by the two bacteria. Anyway, differences in microtopography for the two
411 bacteria in respect to Control 1 showed that both were able to degrade the graffiti paints.

412 The results obtained in this paper suggest, therefore, that simple roughness values alone,
413 like those provided by Rz or Ra, are not sufficient for the assessment of the
414 biodegradation activity and should be integrad by surface microtopography information.

415 Liquid–solid contact angle and the WDPT are the most common approaches to
416 characterize the magnitude of water repellency, the former being related to the degree of
417 water repellency and the latter to its persistence. According to Leelamanie and Karube
418 (2009) WDPT or the repellency persistence can be considered the time taken to increase
419 the surface free energy to overcome water repellency, not only on the surface in contact
420 with the droplet, but also in the adjacent layers below the surface. The WDPT is directly
421 related to surface erosion or alteration (Wessel, 1988). In the case of the graffiti layers in
422 this study, their alteration upon biotreatment, especially at the level of microporosity,
423 may be closely related to the time required for the infiltration of water drops.

424 The limited change in colour and gloss of graffiti paint on granite is in line with a less
425 degradative effect on this substrate by bacteria. The biodegradative capacity of bacteria
426 may fade the black graffiti colour, and also may favour the pinholes formation
427 (Sanmartín et al., 2015). Only in the case of *C. testosteroni* in black graffiti on concrete
428 the change in lightness exceeded the threshold of 2 CIELAB units, indicating that an
429 experienced observer could easily notice the difference (Mokrzycki and Tatol, 2011). The
430 same bacterium applied on black graffiti on granite produced an unnoticeable lightness
431 difference, below the threshold of 0.73 CIELAB units, considered the just noticeable
432 difference (jnd) and below which the difference is completely inappreciable to an
433 experienced observer (MacAdam, 1942; Melgosa et al., 1995). It is possible to
434 hypothesize that holes larger in size affected more lightness than those smaller.

435 FTIR spectroscopy is one reference technique for characterizing historical and
436 contemporary paints and following their degradation pathways (Ploeger et al., 2008;
437 Duce et al., 2014). Its application has also been successfully extended to the identification
438 of the main classes of components of modern spray paints (Germinario et al., 2016). The
439 ATR geometry employed here has the additional advantage of being non-destructive and
440 allow for repeated measurements at the various step of the treatments. On the other hand,

441 the ATR crystal-surface interaction is a well-known potentially limiting factor especially
442 in presence of rigid and non-flat substrate like natural and artificial stone. In addition to
443 the crystal contact, the morphology of the layer under study plays a relevant role. Among
444 the tested substrates, the graffiti paint on granite provided the most reliable
445 characterization results. The limited porosity of granite prevents a deep penetration of the
446 paint, possibly resulting in a thicker surface layer that can be better evaluated by FTIR.
447 The absence of significant spectral changes between Control 0 and 1 confirmed that the
448 agar treatment alone is not responsible for any chemical alteration of the paint. As for the
449 bacterial activity, their potential for biodegradation is indicated by the significant
450 reduction of the absorption peak in the C-H region. Considering the overall spectral
451 features, the treatment with *C. testosteroni* was associated with more intense changes,
452 and the formation of additional absorption peaks possibly due to the proteinaceous
453 contribution can be linked to the permanence of residual biofilm on the treated surface.

454 **5. Conclusions**

455 Naked-eye observation, digital analysis and surface microtopography (the last two, novel
456 to this experiment) proved that both *K. aerogenes* and *C. testosteroni* are capable of
457 altering the black graffiti paint. They act as biodegradative agents, but more efficiently
458 when the coating surface is smoother (as in the case of concrete). More intense lightness
459 and FTIR changes provided further information about the best performing of *C.
460 testosteroni* in comparison to its counterpart, which is also in agreement with the results
461 of adaptation by bacteria to graffiti. However, although treatment time was strongly
462 shortened, the biocleaning treatment performance is still relatively low and cannot be
463 straightforwardly applicable to real cases. A possible solution to be developed in future
464 studies might be a combination of more traditional techniques, like laser cleaning, to
465 remove the bulk of the graffiti paint followed by the biocleaning treatment.

478 Coruña, Spain) for his help on the submission process for the sequences in the NCBI
479 database.

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Figure captions:

Figure 1. Left: Graffiti which appeared on a 12th century apostolic marble figure on the Romanesque facade of Platerías, Cathedral of Santiago de Compostela (Spain), in August 2018. Right: Current appearance of the figure, where the remains of graffiti paint are still visible as 'ghosting' or remnant markings in blue, after the use of microlaser techniques. Photos: diariodepontevedra.es.

Figure 2. Schematic diagram of the protocol followed to assess the graffiti biodegradative capacity of both bacteria.

Figure 3. Digital image analysis Top: Images (a) original RGB, (b) grey-scale conversion, (c) applying 0.3 thresholding. Average results of the percentage of white dots/pixels are shown below the images Bottom: Histograms of the number of points (white dots/pixels) quantified according to their size (% area/point).

Figure 4. (a) Roughness parameter Rz measured in line transects. The histograms represent mean values (vertical bars represent the SD). (b) 3D surface roughness (microtopography). In each case a representative 5×5 mm area is shown, with vertical height (Z) deviation represented in colour shading.

Figure 5. (a-d) Colour differences (ΔL^*, Δa^*, Δb^* and ΔE^*_{ab}) and (e) gloss differences of the graffiti coated samples after the treatments with respect to the untreated graffiti coated samples (Control 0). The histograms represent mean values (vertical bars represent the SD). In the colour graphs, orange dashed line marks the threshold of 2 CIELAB units and blue dashed line marks the threshold of 0.73 CIELAB units.

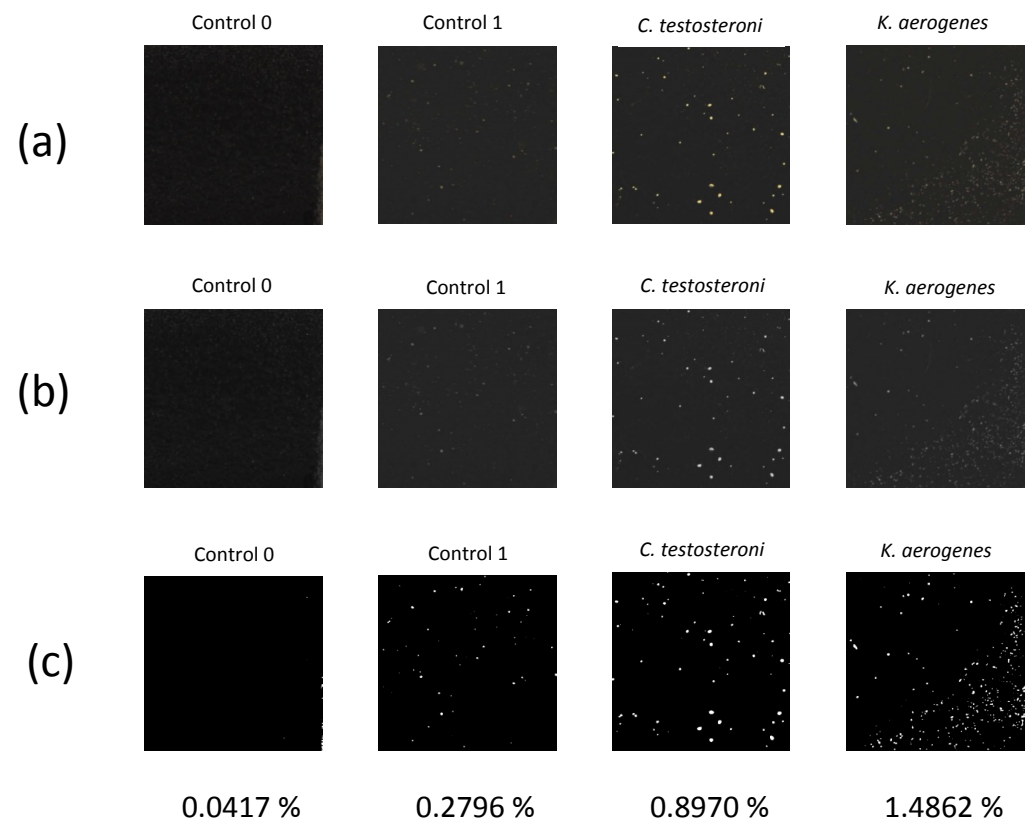**Figure S1.** Bacterial presence analysis by NA contact plates after biocleaning, before (A) and after (B) the final cleaning step with sterile water.

Figure 1

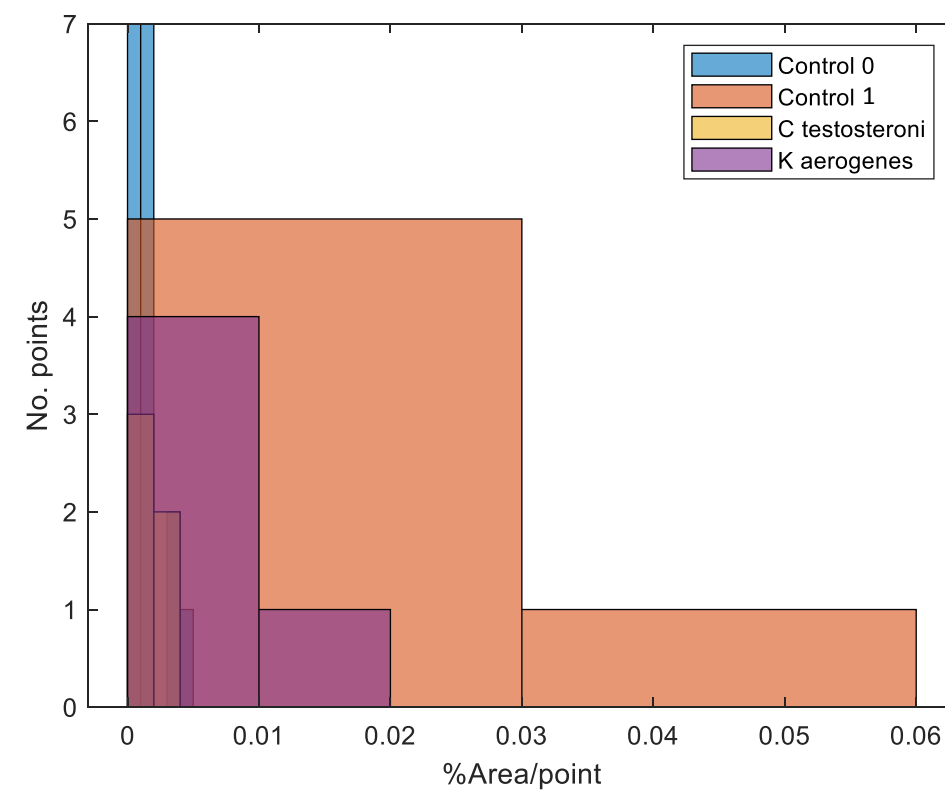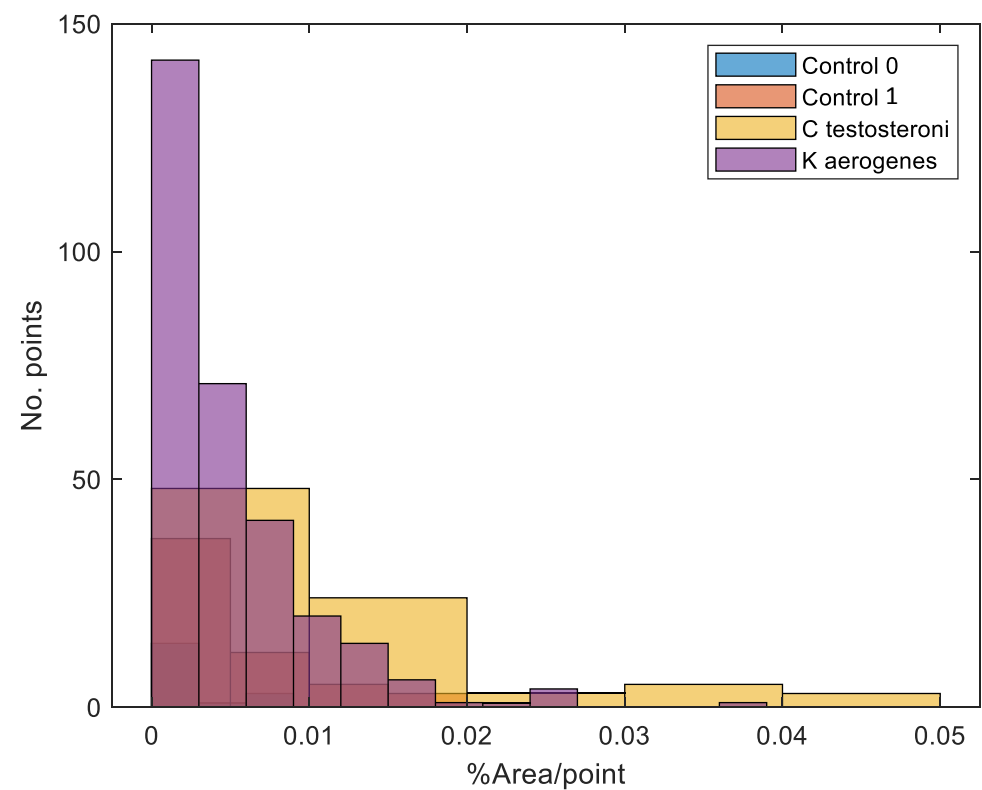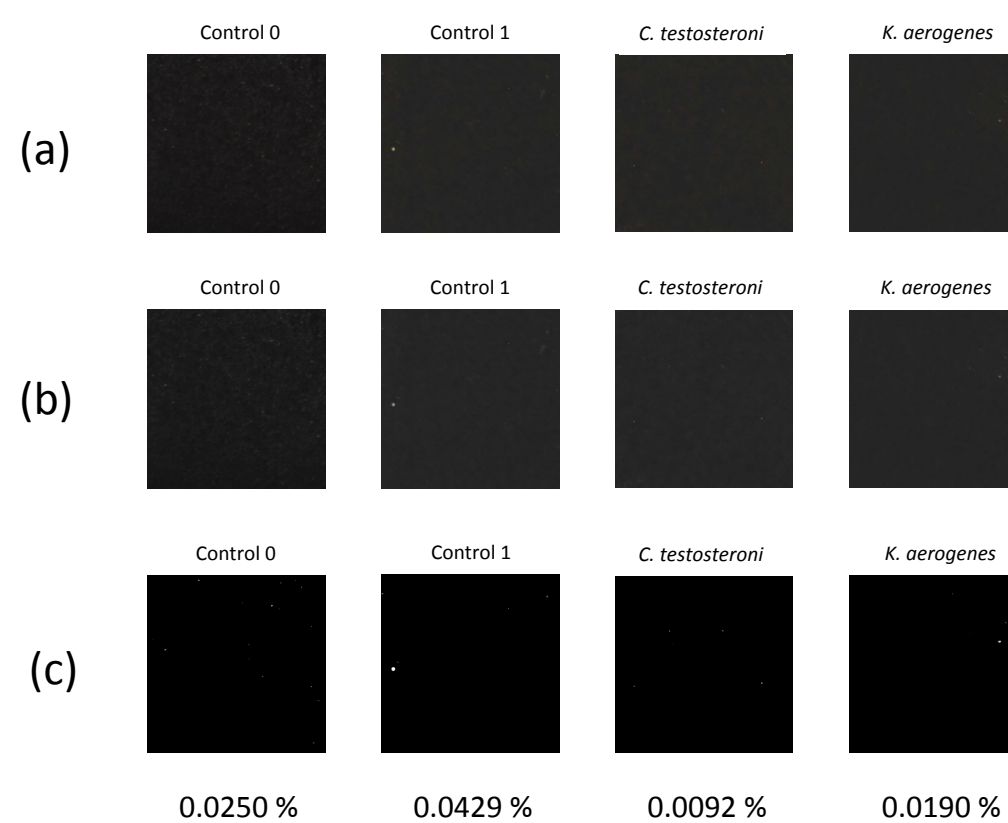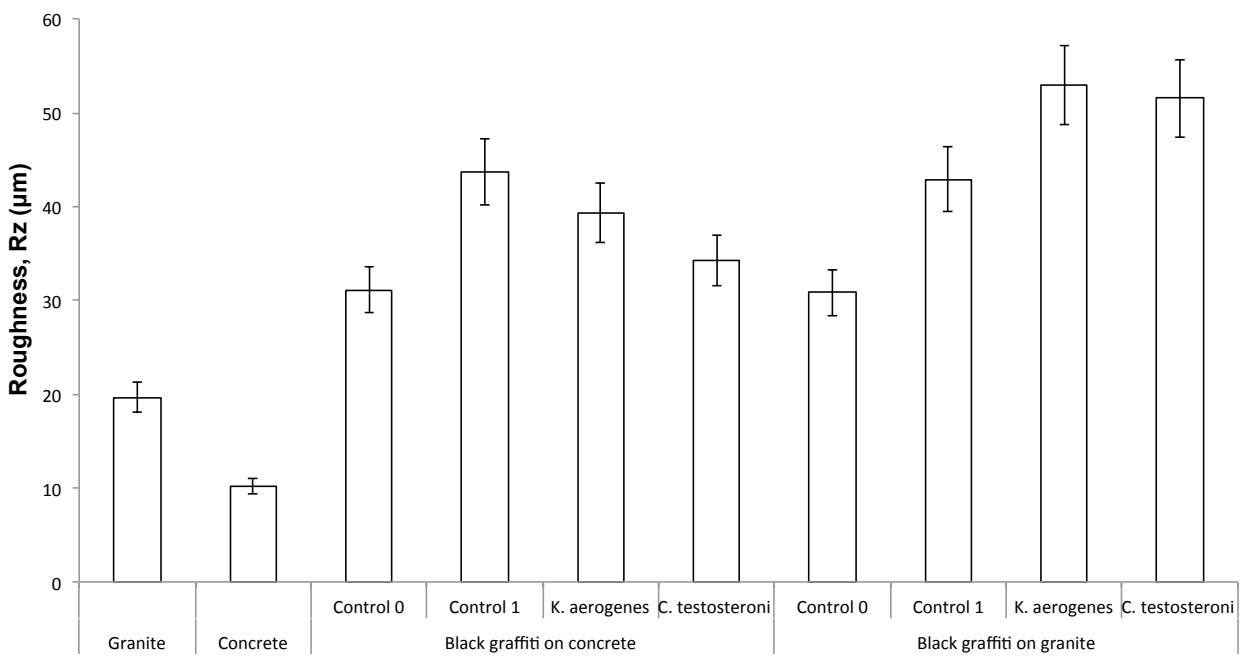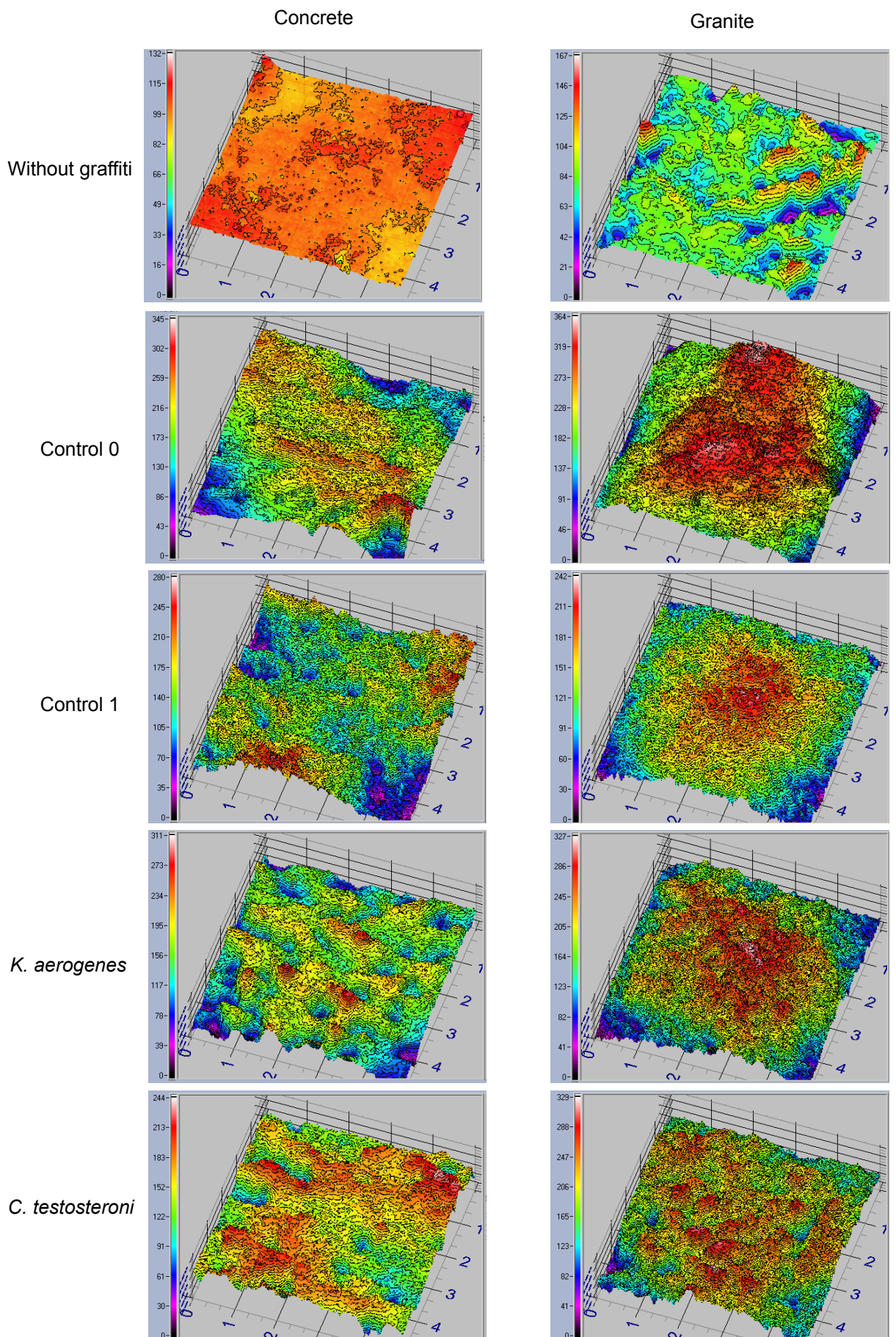[Click here to download Figure FIG 1.pdf](#)

Figure 2
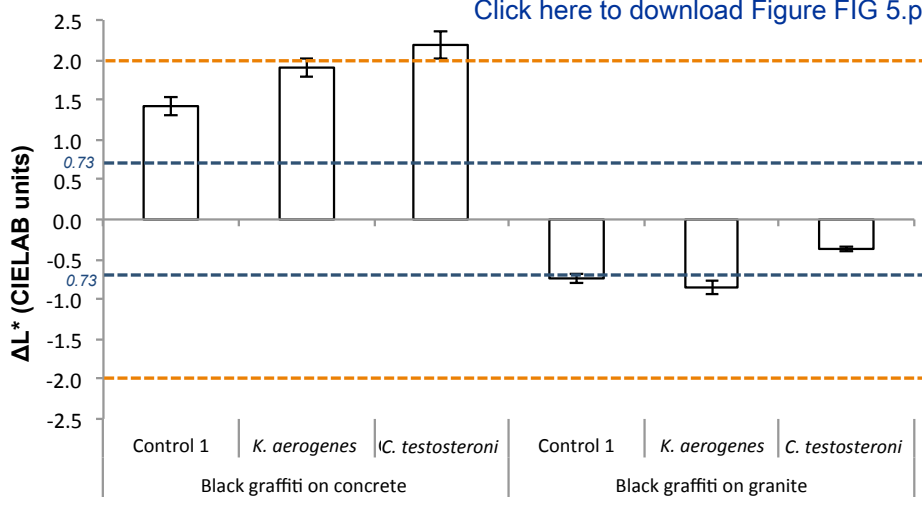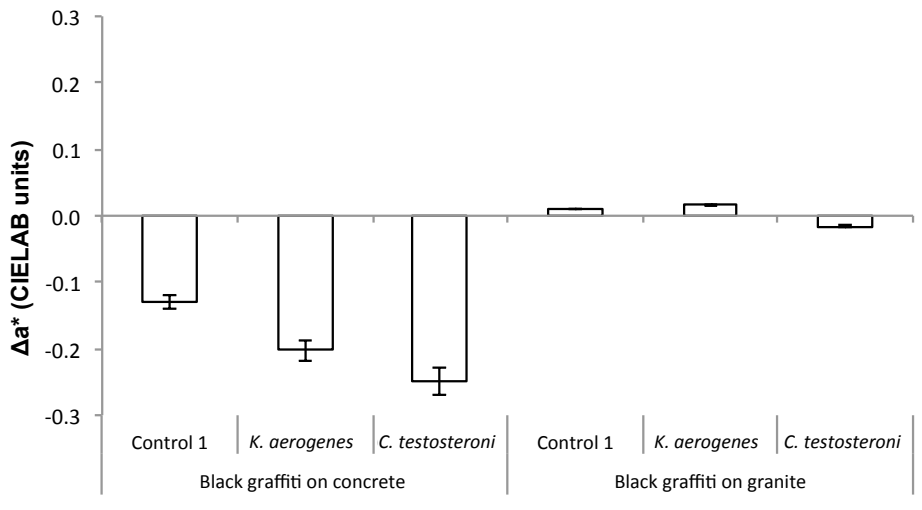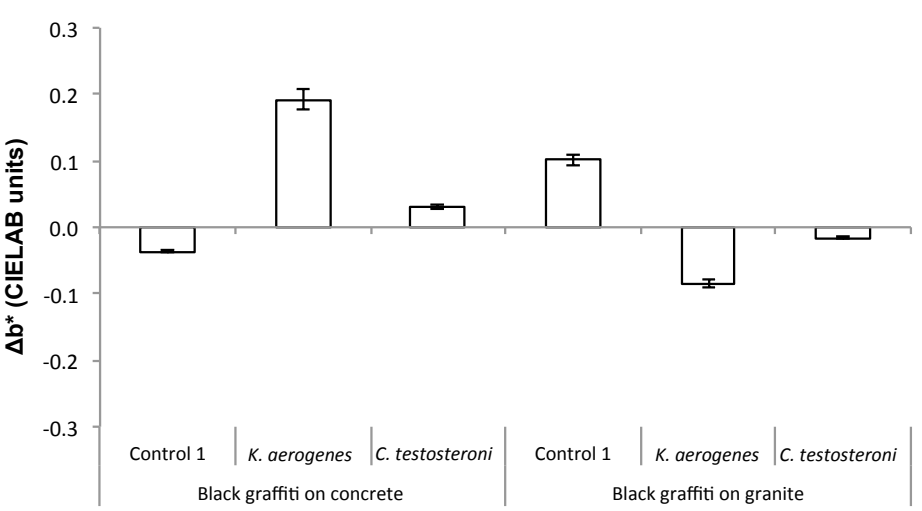
M9 mineral medium supplemented with powdered graffiti paint

+

Klebsiella aerogenes or *Comamonas testosteroni*

Centrifuge at 4500 rpm for 10min and then

Pellet resuspended in sterile water

Treated with agar + bacteria

Control 1 (agar carrier without bacterium)

After 14 days

agar removal and final cleaning with sterile water

Control 0 (no treatment, graffiti coated sample)

- Bacterial presence
- Naked-eye observation
- Digital image analysis
- Surface microtopography
- Wetting, gloss and roughness characterization
- Colour assessment
- Infrared (ATR-FTIR) spectroscopy

Figure 3

CONCRETE

GRANITE

Figure 4

(a)

(b)

Concrete

Granite

Without graffiti
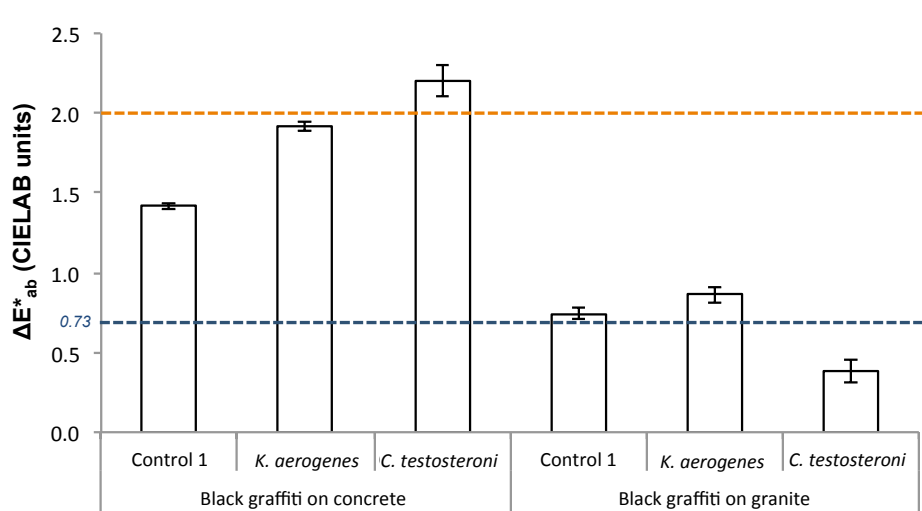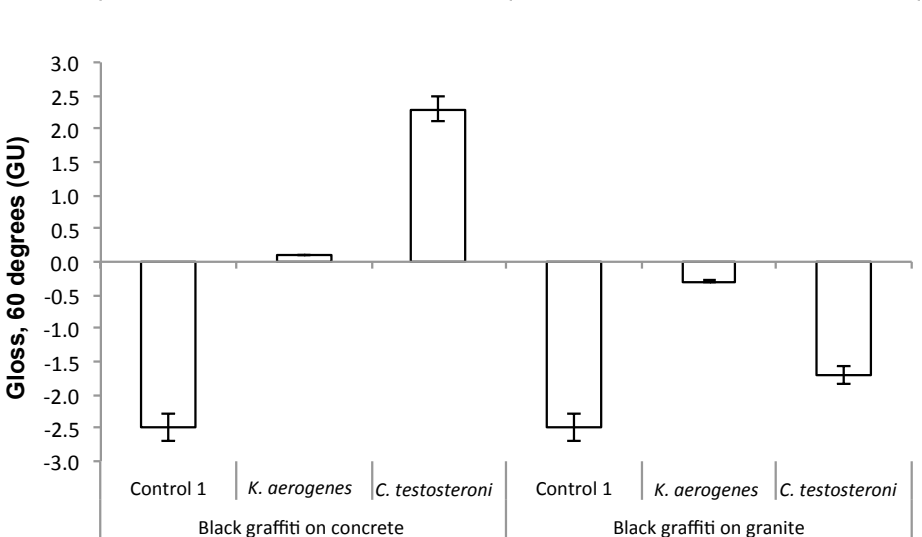
Control 0

Control 1

K. aerogenes

C. testosteroni

Figure 5

Table 1. Viability time evolution (after 5, 10 and 20 days) of both bacteria in M9 mineral medium supplemented with powdered graffiti. CFU mL^{-1}: colony forming units per mL.

Bacterium	Initial inoculum (CFU mL^{-1})	After 5 days (CFU mL^{-1})	After 10 days (CFU mL^{-1})	After 20 days (CFU mL^{-1})
Klebsiella aerogenes	1.0×10^9	5.0×10^7	2.0×10^6	2.9×10^6
Comamonas testosteroni	1.0×10^9	1.0×10^4	1.0×10^7	1.7×10^7

Table 2. Contact angle, water drop penetration time (WDPT) and water repellency categories according to Leelamanie et al. (2008).

Sample	Treatment	Contact angle (°)	WDPT (min)	Repellency category
Black graffiti on concrete	Control 0	75.0 ± 4.2	> 60	Extremely repellent
	Control 1	66.0 ± 1.4	40	Severely repellent
	Klebsiella aerogenes	37.5 ± 0.7	25	Severely repellent
	Comamonas testosteroni	36.5 ± 4.9	30	Severely repellent
Black graffiti on granite	Control 0	73.5 ± 2.1	> 60	Extremely repellent
	Control 1	60.0 ± 2.8	> 60	Extremely repellent
	Klebsiella aerogenes	52.5 ± 2.1	45	Severely repellent
	Comamonas testosteroni	54.5 ± 2.1	55	Severely repellent

Control 0: No treatment, graffiti coated sample. Control 1: Agar carrier without bacteria.

Figure S1