Bioremoval of graffiti using novel commercial strains of bacteria

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Abstract

Previous studies have provided evidence that bioremediation deals a novel approach to graffiti removal, thereby overcoming well-known limitations of current cleaning methods.

In the present study eight bacteria aerobic, mesophilic and culturable from the American ATCC and the German DSMZ collections of microorganisms, some isolated from car paint waste, coloured deposits in a pulp dryer and wastewater from dye works, were tested in the removal of silver and black graffiti spray paints using immersion strategies with glass slides. Absorbance at 600 nm and live/dead assays were performed to estimate bacterial density and activity in all samples. Also, pH and dissolved organic carbon (DOC) and inorganic carbon (DIC) measurements in the liquid media were made, as well as, thickness, colorimetric and infrared (FTIR) spectroscopy measurements in graffiti paint layers were used to evaluate the presence of the selected bacteria in the samples and the graffiti bioremoval capacity of bacteria. Data demonstrated that of the eight bacteria studied, *Enterobacter aerogenes*, *Comamonas* sp. and a mixture of *Bacillus* sp., *Delftia lacustris*, *Sphingobacterium caeni*, and *Ochrobactrum anthropi* were the most promising for bioremoval of graffiti. According to significant changes in FTIR spectra, indicating an alteration of the paint polymeric structure, coupled with the presence of a consistent quantity of live bacteria in the medium as well as a significant increase of DIC (a measure of metabolic activity) and a change in paint color.

Keywords: graffiti; bacteria; bioremoval; spray paint; innovative cleaning method.

1. Introduction

Graffiti vandalism is an unavoidable visual component of the urban fabric, resulting in the defacing of public or private properties accessible to the public (Sanmartín et al., 2014, 2016). According to Careddu and Akkoyun (2016), the current economic losses caused by graffiti are estimated at over a million dollars a year on a world scale. In the city of Glasgow, Scotland, from 2009 to 2014, more than £2 million was spent targeting the damage caused by graffiti vandals (according to the data provided by the Glasgow Times, 2014). Nearly ten years ago, in Santiago de Compostela, Spain, more than €150,000 were spent in one year on removing graffiti across the city (Sanmartín and Cappitelli, 2017). These costs are derived from the use of chemical and physical cleaning techniques, and, on special cases related to cultural heritage, the use of laser (Sanmartín et al., 2014). Compared to these techniques, which do not fully meet the requirements for protecting the substrate or remove substances selectively, the bioremoval of graffiti, although its research is ongoing (Sanmartín and Bosch-Roig, 2019), is a low-cost, powerful (particularly for porous materials), and environmentally friendly solution for graffiti removal, with no risk to human health.

Graffiti spray paints contain xenobiotic compounds that make them resistant to removal and degradation. The effective use of microbes in biodegradation has been already proved in the last few years (Giacomucci et al., 2012; Sanmartín et al., 2015, Sanmartín and Bosch-Roig, 2019). For example, an efficient technique for the biodegradation of nitrocellulose-based spray-painted graffiti was obtained by Giacomucci et al. (2012). Here, ammonification was postulated as a degradation pathway, and it was also demonstrated that the anaerobic sulphate-reducing bacterium *Desulfovibrio desulfuricans* ATCC 13541

is able to transform nitrocellulose-based paint binder and is therefore a strong candidate as a nitrocellulose-degrading bacteria (Giacomucci et al., 2012). The use of anaerobic bacteria requires restrictive treatment conditions, limiting their application in a real field. Therefore, subsequent studies were oriented towards the use of aerobic bacteria. In Sanmartín et al. (2015) a total of 54 different strains of bacteria and fungi were isolated from graffiti paints in two areas in the North-eastern United States, at Cambridge, Massachusetts. Bacteria showing their potential as bioremediation agents, with pinholes in the coating, blister formation, cracking, color fading and loss of gloss, were identified as belonging to the bacterial genera *Arthrobacter, Bacillus, Gordonia, Microbacterium, Pantoea* and *Pseudomonas*, and fungal genus *Alternaria*. Further bioremoval assays were carried out to develop a suitable biological protocol of action for walls and building surfaces. Sanmartín and Bosch-Roig (2019) showed that (i) *Pseudomonas stutzeri* DSMZ 5190, a bacterium previously identified as a good candidate for biocleaning purposes (Bosch-Roig et al., 2016), is a potential candidate for use in the bioremoval of graffiti; (ii) a liquid culture medium enriched with powdered graffiti enhances the selection of appropriate bacteria; (iii) agar and water are suitable carrier agents in the biocleaning treatment; and (iv) some improvements were proposed to the existing methodology, e.g., shortening the time required for adaptation of the microorganisms and application time .

Most of previous research were focused on the isolation of bacteria from graffiti paints suggesting that graffiti surfaces are a good source of putative biodegradative microbial populations, which may aid in the remediation of damaged surfaces (Sanmartín et al., 2015). However, a unique methodology to assess the potential of these bacteria as bioremediation agents has never been proposed so far and only tests limited to the specific

47 experimental condition were done.

48 In this paper, a global methodology to easily assess the potential of bacteria to remove
49 graffiti has been proposed by combing data obtained from Fourier Transform Infrared
50 Spectroscopy (FTIR), color and thickness paint analysis, measurements of absorbance,
51 number of cells, organic and inorganic carbon and pH in the medium surrounding the paint
52 layer. Indeed, the methodology has been used to select the paint bioremoval ability of
53 microorganisms bought from ATCC and DSMZ international collections. In comparison
54 to *P. stutzeri* DSMZ 5190, which had to be first adapted to the powdered graffiti before it
55 was able to degrade them (Sanmartín and Bosch-Roig, 2019), the strains used in this study
56 were firstly isolated in places where synthetic polymers were present.

57 **2. Materials and methods**

58 ***2.1. Bacterial strain selection and growth conditions***

59 Commercial strains of *Enterobacter aerogenes* ATCC 13048 (Bac_1), *Bacillus subtilis*
60 ATCC 27328 (Bac_2), mixed culture ATCC 53922 (i.e., a mixture of *Bacillus* sp., *Delftia*
61 *lacustris*, *Sphingobacterium caeni*, and *Ochrobacterum anthropi* Bac_3), *Comamonas* sp.
62 ATCC 700440 (Bac_4), *Rubellimicrobium thermophilium* DSMZ 16684 (Bac_5),
63 *Chelatoccocus daeguensis* DSMZ 22069 (Bac_6), *Escherichia coli* DSMZ 787 (Bac_7)
64 and *Marinospirillum* sp. DSMZ 9662 (Bac_8) were used for bioremoval assays. Bacterial
65 features are reported in Table 1. The strains were stored at –80 °C in suspensions containing
66 20% glycerol and 2% peptone and were routinely grown overnight in the most appropriate
67 medium at 30 °C. Prior to use, cells were washed with phosphate buffered saline (PBS).

68 Bacterial growth in rich and low culture nutrient media and mineral medium was monitored

to find the optimal culture condition with both high and low availability of nutrients. Nutrient Broth (NB; 3.0 g/l beef extract, 5.0 g/l peptone, Sigma Aldrich, USA), Tryptic Soy Broth (TSB; 17 g/l casein peptone, 2.5 g/l K_2HPO_4, 2.5 g/l glucose, 5.0 g/l NaCl, 3.0 g/l soya peptone, Sigma Aldrich, USA) and R2B (0.5 g/l casein acid hydrolysate, 0.5 g/l dextrose, 0.3 g/l K_2HPO_4, 0.024 g/l $MgSO_4$, 0.5 g/l proteose peptone, 0.3 g/l sodium pyruvate, 0.5 g/l soluble starch, 0.5 g/l yeast extract) were used as rich media; 10% TSB (i.e., TSB at 1/10 strength) and Complete Mineral (CM; 10.5 g/l K_2HPO_4, 1.13 g/l KH_2PO_4, 1.0 g/l $(NH_4)_2SO_4$, 0.5 g/l $Na_3C_6H_5O_7$× $2H_2O$, 0.25 g/l $MgSO_4$ × $7H_2O$, 0.015 g/l CaCl × $2H_2O$) plus 0.5 g/l glucose (CM-glu) were used as low nutrient media; CM was used as a mineral medium.

Planktonic assays were carried out in 96-well microtiter plates according to Cattò et al. (2015). Briefly, bacteria of a washed overnight culture were added to each medium to a final concentration of 10^6 cells/ml and incubated at 30 °C. Absorbance at 600 nm (A_{600}) was measured every 10 min for 30 h (rich media) or 60 h (low nutrient and mineral media) using the Infinite 200 PRO Microplate Reader (Tecan, Switzerland). The A_{600} of suspensions minus the A_{600} of the non-inoculated medium was plotted against the incubation time and absorbance-based growth kinetics were constructed. The polynomial Gompertz model adapted for microbial growth by Zwietering (1990) was used to fit the growth curves, and the length of lag time (λ) and maximum specific growth rate (μm) were calculated using GraphPad Prism software (version 5.0, San Diego, CA, USA). Media were chosen that enabled both a short lag time and a high growth curve.

2.2. Graffiti spray paint sample preparation

A black non-metallic spray paint (R-9011, Montana Colors) and a silver metallic spray

92 paint (Silver Chrome, Montana Colors) were selected for the study. Both the black and
93 silver paints have been characterized in previous studies (Rivas et al., 2012; Sanmartín and
94 Cappitelli, 2017), and were identified as alkyd and polyester-based resin, and polyethylene-
95 based resin, respectively. Thirty painted glass slides (7.6 cm × 2.6 cm) of each of the two
96 graffiti paints were prepared outdoors by spraying a thin layer on one side following the
97 method described by Giacomucci et al. (2012). Samples were allowed to dry at room
98 temperature for 3 months. Before testing, samples were sterilized by leaving them for 18 h
99 in a sealed box containing 37% formaldehyde (Sanmartín et al., 2015).

100 ***2.3. Immersion experimental set-up***

101 Bioremoval assays were carried out by immersion of graffiti-coated slides held static at
102 90° in liquid media for a total of 27 days under aerobic conditions, darkness and gentle
103 agitation at 30 °C. First, slides were immersed in 30 ml of TSB rich nutrient medium
104 containing 10^6 cells/ml of each of the eight strains incubated separately, promoting cell
105 growth and adhesion on the graffiti layer (step 1). After 48 h, slides were transferred to a
106 30 ml fresh 10% TSB low nutrient medium for 5 days (step 2). In this step, the reduction
107 of nutrients promoted adhesion of cells to the paint, which were seeking another food
108 source. Then, slides were transferred to a CM mineral medium for an initial period of 10
109 days (step 3) and were then transferred to a new fresh CM medium for a further 10 days
110 (step 4). In these last two steps, adhered bacteria were forced to use paint samples as the
111 only source of energy and carbon.

112 These assays with each of the eight bacteria (Section 2.1) were performed in triplicate.
113 Additionally, three negative control replicates (painted slides immersed in the liquid
114 medium without bacterial cells, hereafter Control 0) were included in the experiment. For

purposes of comparison, analysis from TSB, 10% TSB and CM media were used to check the results obtained in the surrounding media (Section 2.4) and the analysis of non-immersed graffiti coated slides was used to check the results obtained on the graffiti paint layers (Section 2.5). Both are referred to hereafter as Control 0_0.

At the end of each of the steps (steps 1–4), the culture media slides were analyzed after immersion (Section 2.4), while the graffiti paint layers were analyzed at the beginning and end of the experiment, i.e., steps 0 and 4 (Section 2.5).

2.4. Analysis of the liquid media after immersion of the painted slides

2.4.1 pH medium

In order to exclude any bacterial growth inhibition due to changes in the physiological conditions and verify the accumulation of specific metabolic products from paint degradation, the pH of the media following each experimental step was measured using a Jenway 3510 pH meter (Jenway, Staffordshire, United Kingdom).

2.4.2 Bacterial growth

To monitor bacterial growth, absorbance at 600 nm (A_{600}) was measured after each experimental step using an UV/VIS 7315 Spectrophotometer (Jenway, United Kingdom). Data were reported as A_{600} minus the A_{600} of the corresponding non-inoculated medium.

At the end of the experiment (step 4), the amount of live and dead cells within the media was counted using the Live/Dead BacLight viability kit (L7012, Molecular Probes-Life Technologies). One ml of medium from each sample was incubated with 2 µl of each fluorescent probe at room temperature in the dark for 25 min, according to the manufacturer's instructions. Control 0_0 and Control 0 were also stained. A total of 200 µl

of stained suspension were aliquoted on black-sided plates and fluorescence intensity was measured using the Infinite 200 PRO Microplate Reader (Tecan, Switzerland) with excitation at 480 nm and emission at 516 nm for the green channel and excitation at 581 nm and emission at 644 nm for the red channel. A standard curve of fluorescence intensity was used to quantify the number of live and dead cells. Relative viability was calculated by dividing the percentage of live cells by the percentage of dead cells in each sample.

2.4.3 Dissolved total, inorganic and organic carbon concentration

After measuring the pH and absorbance at each experimental step, samples were filtered through a 0.2 μm filter (Millipore, Italy). Both dissolved total carbon (DTC) and dissolved inorganic carbon (DIC) concentrations were measured in triplicate in the filtrates with a total organic carbon analyser, model TOC-5000 (Shimadzu, Tokyo, Japan), equipped with a platinum catalyst on quartz wool. The dissolved organic carbon (DOC) concentration was estimated by subtracting the DIC from the DTC.

2.5. Analysis of the paint coatings on the slides

2.5.1 Thickness

Fifteen thickness measurements were randomly taken on each graffiti layer (total number = 54) using a coating thickness tester CG204 (Extech Instruments, USA).

2.5.2 Color

On the same samples, color measurements were made according to Giacomucci et al. (2012) by taking five measurements at random positions on the painted glass slides. A Konica Minolta colorimeter with a CR–300 measuring head (8 mm diameter viewing area) was used under the following conditions: illuminant D65 and observer 2º. Color

measurements were analyzed by considering the CIELAB color system (CIE 1986), which represents each color by means of three coordinates: L*, lightness of color, which varies from 0 (absolute black) to 100 (absolute white); a*, associated with changes in redness–greenness (positive a* is red and negative a* is green); and b*, associated with changes in yellowness–blueness (positive b* is yellow and negative b* is blue). The total color difference (ΔE^*_{ab}) after immersion assays was calculated as follows:

$$\Delta E^*_{ab} = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$$

where $\Delta L^* = L^*_i - L^*_0$, $\Delta a^* = a^*_i - a^*_0$, $\Delta b^* = b^*_i - b^*_0$, $_i$ denotes the color parameter in each of the bacteria treatments (Bacs_1–8) at step 4 of the experiment, and $_0$ denotes the color parameter in Control 0 at step 4 of the experiment. Threshold colorimetric values based on the perception of color changes by the human eye (Sanmartín and Pozo-Antonio, 2020, and references therein) were considered to determine whether these changes were perceptible and to what extent.

2.5.3 FTIR

The infrared spectra obtained by FTIR in the spectral range 600–4000 cm^{-1} at a resolution of 4 cm^{-1} were recorded in the Attenuated Total Reflectance mode (ATR-FTIR, Perkin Elmer Spectrum 100, USA). For each condition (Control 0_0, Control 0, and Bacs_1–8), 8 measurements were conducted on different areas of each glass slide. The variation in the main spectral features and the ratio between the main absorption peaks of the paint (1720/1256 cm^{-1}) were used to qualitatively monitor alterations of the paint induced by the

different treatments. The peak at 1650 cm^{-1}, which corresponded to the amide I bond in proteins (Yu and Irudayaraj, 2005), was observed after immersion and, therefore, was related to the presence of bacterial biofilm on the sample. The shoulder at 1550 cm^{-1} was also associated with the amide II contribution of the biofilm. The broad peak at 1080 cm^{-1} was related to the ring vibration of bacterial polysaccharides (Schmitt and Flemming, 1998; Yu and Irudayaraj, 2005).

2.6 Statistical analysis

In each assay, at least three technical replicates were carried out for each of three experimental replicates. Obtained data were subjected to one-way analysis of variance (ANOVA) and Tukey's HSD post hoc tests to compare means among the bacteria and control treatments. Homogeneity of variance was tested using Levene's test, and the normality of residuals was checked using the Shapiro–Wilk test. Statistical significance was set at $p < 0.05$. A principal component analysis (PCA), based on the correlation matrix of the data at the end of the experiment (step 4), was also carried out. Statistical tests were conducted using R software for MacOSX (R Core Team, 2018).

3. Results

3.1. Selection of common growth conditions for the bacteria under study

Bacterial growth was monitored in rich, low nutrient and mineral media to find the optimal culture conditions for immersion strategy experiments. Obtained growth curves for each strain are reported in Figures S1 and S2 and the growth parameters λ and μm are reported in Table S1 in the Supplementary Materials.

Among the rich media tested (Figure S1 and Table S1), TSB led to the highest growth rates

in all bacterial strains, with plateau absorbance values significantly higher than those obtained by their counterparts NB and R2B media. Indeed, the μ_m displayed higher values when all bacteria were cultured in TSB than in NB or R2B media. NB and R2B did not facilitate bacterial growth of Bac_5, whereas Bac_8 did not grow in the presence of NB. In light of these results, TSB was chosen as the best rich medium to be used in experimental step 1 of the immersion strategy.

Both 10% TSB and CM-glu low nutrient media enabled bacterial growth of all strains, except Bac_8, which did not grow in the presence of both these media, and Bac_6, which did not grow in the presence of CM-glu (Figure S2 and Table S1)., Growth was generally higher in the presence of 10% TSB than in the presence of CM-glu, with higher μ_m values. Additionally, λ values were smaller when bacteria were grown with 10% TSB instead of CM-glu, indicating a greater ability of bacteria in adapting to 10% TSB. Therefore, 10% TSB was chosen as the best low nutrient medium to be used in step 2 of the immersion strategy.

Bac_1–Bac_5 were able to grow in CM medium (Figure S2 and Table S1). On the contrary, this mineral medium did not enable the growth of Bac_6, Bac_7 or Bac_8. However, in the former strains, the λ values were large, at over 13 h, suggesting a long period of adaptation before bacterial growth began.

3.2. Analysis of the liquid media after immersion of the painted slides

3.2.1 Comparison between Control 0_0 and Control 0

The comparison between the Control 0_0 (medium) and Control 0 (medium with paint layer submerged and without bacteria) was performed to measure any changes in the media

ascribable to the presence of the submerged paint layer in the media. Both black and silver paints altered neither the pH of the medium (except the silver paint at step 4, which slightly increased the medium's pH), absorbance values or DOC concentrations. On the contrary, a significant increase of DIC was measured in step 1 after the immersion in the medium of both black and silver paints. A release of DIC in the medium was also observed in the case of black paint at step 4.

As the paint layer alone was subjected to changes in the culture medium, to highlight the bacterial behaviour, subsequent data obtained from the analysis of the surrounding liquid media and related to each bacterial strain were compared to Control 0.

3.2.2 pH

The pH of culture media after each experimental step (from 1 to 4) were measured to detect changes in physiological conditions due to the accumulation of specific metabolic products. Obtained data are reported in Figures 1A and S3. For the black paint, pH significantly decreased in Control 0 and Bac_1, Bac_2, Bac_3, Bac_5 and Bac_8 at step 1. No significant changes were detected at step 2, while a significant decrease in pH was recorded for Bac_6 and Bac_8 at step 3, and of Bac_4 at step 4 when compared with Control 0. With regards silver paint, significant reductions of pH values in comparison to Control 0 were observed for Bac_1–5 and Bac_8 at step 1; Bac_2, Bac_4, Bac_5 and Bac_8 at step 2; Bac_6 and Bac_8 at step 3; and Bac_2, Bac_3, Bac_4, Bac_6, Bac_7 and Bac_8 at step 4.

3.2.3 Bacterial growth in liquid media

A_{600} in the liquid media was measured to assess microbial growth. Obtained data are

reported in Figures 1B and S4. At steps 1 and 2, the trend of microbial growth was similar for both black and silver paint. Indeed, at step 1, a significant increase of absorbance in comparison to Control 0 was found for all bacterial strains, except Bac_6 and Bac_7, suggesting microbial growth. At step 2, only culture media with Bac_1, Bac_3, Bac_4 and Bac_8 displayed a significant increase in cell population, whereas the remaining cultures did not show bacterial growth. On the contrary, at steps 3 and 4, different results were obtained for each paint. In the presence of black paint, only media with Bac_1, Bac_3, Bac_4 and Bac_6 showed significant growth at steps 3 and 4. At the same experimental steps, in the presence of silver paint, all culture media, except Bac_7 and Bac_8, displayed a significantly higher absorbance in comparison to Control 0.

At the end of the experiment (step 4), the number of live and dead cells within the media was evaluated (Figure 1C). Control 0_0 and Control 0 did not display fluorescence, indicating a complete absence of cells. In both black and silver paints, Bac_1, Bac_3 and Bac_4 had the highest number of cells, both live and dead. Bac_5 and Bac_6 had the lowest number of cells (live plus dead cells). Relative viability (Figure 1D) was generally higher in the presence of black paint, as a greater proportion of live cells in comparison to dead cells was observed in this paint. In both paints, Bac_4 had the highest viability (black: 9.6; silver: 3.4), followed by Bac_7 (black: 6.2; silver: 2.6) and Bac_8 (black: 5.1; silver: 2.7). In black paint, Bac_3 had a high number of cells but, with a ratio of live/dead cells lower than 1, had the lowest relative viability, indicating a major proportion of dead cells compared to living cells. Regarding the silver paint, all strains displayed values above 1, with the exception of Bac_2 (silver: 0.5) and Bac_6 (silver: 0.7).

3.2.4 DIC and DOC concentration

Figures 1E and S5 show the results of the DOC concentration for the different culture media after each experimental step. For black paint, a reduction of DOC in the medium was measured at step 1 in the presence of Bac_3 only, whereas in the presence of all other strains no significant differences were detected. With the same paint, at step 2, no differences with Control 0 were seen. At steps 3 and 4 the effect was similar in the presence of black paint. Indeed, DOC significantly decreased in the presence of Bac_1, Bac_3, Bac_4 and Bac_6 at step 3; and for these strains as well as Bac_5 at step 4. Regarding the silver paint, media with Bac_1, Bac_3 and Bac_4 significantly decreased in DOC steps 1 and 2. Similar to black paint, the effect of bacteria on silver paint was similar at steps 3 and 4. At these steps, a reduction in the DOC concentration was detected in the presence of all strains except Bac_7 and Bac_8.

The amount of DIC is reported in Figures 1F and S6. At step 1, the only significant change recorded was a reduction of DIC with respect to Control 0, in the culture media with black paint incubated with Bac_7 and with silver paint incubated with Bac_7 and Bac_8. At step 2, there was a significant increase in the concentration of DIC in the culture media of Bac_1, Bac_3 and Bac_4 incubated with black paint and all strains except Bac_7 for silver paint. At step 3, in both paints, DIC increased with Bac_1–5. At step 4, for black paint, the culture media that showed a significant increase in the concentration of DIC were those of Bac_1, Bac_3, Bac_4 and Bac_6. In the presence of silver paint, a significant increase was measured in all culture media, except for Bac_7 and Bac_8.

3.3. Analysis of the paint coatings on the slides

3.3.1 Comparison between Control 0_0 and Control 0

A comparison between Control 0_0 (untreated paint layer without immersion) and Control

293 0 (paint layer after immersion without bacteria) was performed to measure any changes in
294 the paint layers ascribable to the immersion in the respective media. Indeed, after
295 immersion without bacteria, at step 4, black paint presented an increased thickness and
296 changed in color. The color parameter L* was significantly higher in Control 0 compared
297 to Control 0_0. A change in color was also measured in the case of silver paint. In this case,
298 L* was significantly lower in Control 0 than Control 0_0. In black paint, the L* chromatic
299 difference between Control 0_0 and Control 0 did not exceed the threshold of 2 CIELAB
300 units, indicating that only an experienced observer could notice the difference (Mokrzycki
301 and Tatol, 2011). However, in silver paint, this difference ranged between 14.7 and 16.9
302 CIELAB units, a very wide and noticeable color difference. No changes were recorded
303 between Control 0_0 and Control 0 for parameters a* and b*.

304 In terms of FTIR analysis, a comparison between Control 0_0 and Control 0 with the black
305 paint showed no significant changes as a result of immersion in the medium (Figure 3A).
306 On the contrary, significant spectral changes were observed for the silver paint between
307 Control 0 and the untreated paint (Control 0_0). This suggests that immersion in the
308 medium itself, even in the absence of any bacterial strain, can induce an alteration of the
309 paint and will require further investigation.

310 *3.3.2 Thickness*

311 Variations in paint layer thickness with bacteria in comparison with Control 0 were not
312 recorded for both paints (Figure 2A).

313 *3.3.3 Color*

314 Both paints showed perceptible and significant variations in color due to bacterial

315 treatments (Figure 2B–D). The most significant changes occurred in color parameter L*
316 (Figure 2B). In black paint, only Bac_3 and Bac_8 showed significant differences with
317 respect to Control 0, the color of the paint becoming clearer in both cases. In silver paint,
318 Bac_1–4 and Bac_6 showed differences to Control 0, fading in the case of Bac_1–4, and
319 darkening in the case of Bac_6. For color parameter a* (Figure 2C), a significant greening
320 in the black paint was noticed compared to Control 0 for Bac_1, Bac_3, Bac_4, Bac_8, and
321 especially Bac_6. In silver paint, there were no significant changes in this parameter
322 between the bacterial treatment and Control 0. This was also the case for parameter b* in
323 both paints (Figure 2D).

324 In terms of the total color change (ΔE^*_{ab}), in black paint, Bac_1, Bac_3 and Bac_8 showed
325 differences greater than 2 CIELAB units, whereas Bac_2 and Bac_7 exhibited changes of
326 less than 1 CIELAB unit. For silver paint, the greatest global color differences occurred
327 with Bac_1 and Bac_4, with 9.9 and 8.9 CIELAB units of difference, respectively. The rest
328 of the bacteria, except Bac_5 and Bac_7, exhibited differences exceeding 3 CIELAB units.

329 *3.3.4 FTIR*

330 Sessile growth on the painted slides was studied using bacterial proteins and carbohydrates,
331 as well as by observing any significant changes in the paint that suggested degradation by
332 the bacterial strains. Such changes were evaluated in terms of overall spectral features, and
333 by monitoring the variation of the relative ratio of the main absorption peaks of the paint
334 (1720/1256 cm^{-1}). All these changes can be collectively associated to alterations of the
335 paint (Duce et al., 2014; Anghelone et al., 2016). The comparative evaluation of bacterial
336 spectra with Control 0 spectra in terms of the 1720/1256 cm^{-1} peak ratio and overall
337 spectral changes highlighted three distinctive behaviors: Bac_2 and Bac_5 induced no

change; Bac_1, Bac_4, Bac_6 and Bac_8 promoted small changes; and Bac_3 and Bac_7 were associated with major alterations to the spectral features of the paint.

The comparison between Bac_2 and Bac_5 with Control 0 showed that all of the original spectral features were preserved (Figure 3A). A small shoulder at 1550 cm^{-1} and a slight shift of the broad peak at 1650 cm^{-1} towards higher wavenumbers were the only differences detectable, and these were very limited. The latter cannot be definitively associated with bacterial activity as it can be noticed already in the Control 0, albeit to a lesser extent.

The main alteration induced by Bac_1, Bac_4, Bac_6 and Bac_8 was identified in the spectral region around 1650 cm^{-1} (Figure 3B). The absorption peak of the paint was broadened and clearly shifted towards a higher wavenumber, and a small peak at 1650 cm^{-1} was observed after treatment. Additionally, a depletion of the 1720/1256 cm^{-1} peak ratio was detected in all strains (from an average value of 0.8 in the untreated paint to 0.64 after treatment with Bac_4). Such a change suggests an alteration of the paint's polymeric structure. Similar variations were observed in the infrared spectra of black paint with Bac_1, Bac_6 and Bac_8.

Bac_3 and Bac_7 were associated with the most relevant spectral changes, and the main bands of the paint at 1720 and 1256 cm^{-1} disappeared as a result of the biotreatment (Figure 3B). In both cases, spectra were dominated by the signals of the biofilms with some significant differences. Bac_3 showed a clear pattern at 1650–1545 cm^{-1} due to the protein contribution, with a broader peak centred at 1080 cm^{-1}. For Bac_7, the characteristic pattern of amide I-II was not observed and the carbohydrate contribution was the main absorption band.

The generally low-intensity response of the resulting ATR spectra did not allow a

comparative evaluation of the treatments in the case of silver paints (data not shown).

3.4. Relationship between studied bacteria and graffiti bioremoval-related data

The principal component analysis (PCA) shown in Figure 4 was used to assess relationships among variables collected from the liquid media surrounding the painted slides (pH, A_{600}, absolute and percentage data of live and dead cells and relative viability, DOC and DIC) and the paint layers (thickness, total color changes [ΔE^*_{ab}] and 1721/1256 cm^{-1} peaks ratio by FTIR) for all treatments at the end of experiment (step 4). FTIR data were included only for the black paint, because of the limitations related to the ATR analysis of the silver paint (see Section 3.3).

For both paints, the first two components (PC1 and PC2) accounted for over half of the total variance (65.6% and 59.5% for black and silver paints, respectively).

For the black paint, PC1 accounted for 47.4% of the total variance. The variables from the most relevant to the least relevant were FTIR ratio (1720/1256 cm^{-1}); absolute data of living cells, A_{600} nm, and DIC (the three being closely related, as shown by their almost overlapping vectors); total color change (ΔE^*_{ab}); and DOC (showing an inverse relationship to DIC). PC1 linked the alteration of the graffiti with the presence of bacteria and their metabolic activity. PC2 accounted for 18.2% of the total variance, and although it was not clearly associated with any variable, it was mainly related to the cell relative viability. The positions of the vectors for thickness, pH, and absolute and percentage data of dead cells were at about 45° angles to both PC1 and PC2 axes, indicating that these parameters were not as well represented in the PCA as the other variables. In the case of thickness, this was also indicated by the small vector shown for this variable. The two controls were well clustered and separated from bacterial treatments. According to PC1,

Bac_3, Bac_1, and Bac_4, listed in that order, showed the greatest distances to the controls, which translated into the largest difference of results of those bacteria with respect to controls.

For the silver paint, PC1 accounted for 41.4% of the total variance, and was mainly related to the DOC and DIC (again inversely related), and to a lesser extent A_{600}. PC2 (18.1% of the total variance) is explained primarily by data for total color change (ΔE^*_{ab}), and to a lesser extent the relative cell viability. This result may be explained by the significant change in color of the silver graffiti paint produced by the immersion process, as indicated in Section 3.3. Again, the controls appeared well clustered and were clearly separated from the bacteria treatments, especially Bac_3 and Bac_1, and to a lesser extent Bac_4.

4. Discussion

Although biotechnology in cultural heritage conservation (i.e., biocleaning and bioconsolidation) has proved to be very useful (Troiano et al., 2013; Sanmartín et al., 2018; Joseph and Junier, 2020), the bioremoval of organic paint layers of graffiti from artworks has been investigated less often (Sanmartín and Bosch-Roig, 2019). In this work, a clean bio-based method has been proposed to remove graffiti from surfaces of both historic and contemporary buildings. Graffiti samples were prepared and the effect of eight bacteria in the paint removal process was investigated.

In order to introduce a ready-to-use biocleaning product to the restoration market one of the main basic aspects that must be taken into account is the microorganism to choose (Bosch-Roig et al., 2015). In the present manuscript the goal has been addressed and a careful selection of the appropriate microorganisms that perform well the removal of graffiti has been done. Indeed, graffiti samples were prepared and the effect of eight

bacteria in the paint removal process was investigated. Notably, the biological treatment was not always visible to the eye. However, as the research in the field of graffiti removal is at the beginning, and graffiti display a very complex structure, the finding of microorganisms able to alter the paint spray represents the reaching of an important milestone.

As the spraying and brushing cell applications are limited by rapid and excessive drying, with a consequent reduction in bacterial cell viability and activity (Ranalli et al., 2005), here, a submerged strategy with bacteria in a liquid suspension was chosen.

Graffiti is a hostile environment for microorganisms because of the complex composition of the spray paints that include organic and inorganic, natural and synthetic components, i.e., various pigments, binders and additives, and even low concentrations of biocides, fungicides and/or algicides (Sanmartín et al., 2014). Therefore, microorganisms used in the removal procedure must demonstrate a high level of tolerance to graffiti and must have a removal impact on the paint, as well as be active in aerobic conditions (Sanmartín and Bosch-Roig, 2019). In this research, bacteria were chosen from some previously found that were associated to paints and were used both as single strains and in combination (i.e., Bac_3). In the latter case, the basic idea was to take advantage of a pool of metabolic pathways rather than from a single one. Indeed, a mixture of microorganisms can have advantages over a single strain, especially when the substance to be cleaned is complex and encrusted (i.e., for graffiti paint), due to the pool of enzymes produced (Bosch-Roig and Ranalli, 2014).

Microorganisms in a planktonic suspension have been found to efficiently degrade paint (Giacomucci et al., 2012). However, it has been reported that bioremoval is significantly

improved when microorganisms form biofilms (Gilan et al., 2004; Tribedi and Sil, 2013). Here, sequential use of media with a progressively reduced amount of organic nutrients was used to promote the transition from the planktonic to the biofilm mode of life. In a low nutrient medium, bacteria were forced to adhere to the paint slide and in the mineral medium they were forced to use paint samples as the only source of energy and carbon. Consistent with this experimental strategy, at the end of the experiment, FTIR spectra of black paint after immersion with Bac_3 and Bac_7 were dominated by the signals of biofilm, with a clear pattern of the protein contribution for the former and a carbohydrate contribution for the latter.

Black non-metallic paint was chosen among other colors because it is the most commonly used graffiti paint (Sanmartín et al., 2014). Silver metallic paint, with a chemical composition different from non-metallic graffiti paints, was selected for being very difficult to extract, even using laser-based techniques, which is considered the most sophisticated and precise technology for graffiti removal (Pozo-Antonio et al., 2018).

The FTIR results for the black paint confirmed the presence of the characteristic absorption peaks of an alkyd paint, dominated by the sharp and intense absorptions at 1720 and 1256 cm^{-1}, due to the stretching mode of the carbonyl and C–O bonds. The peak at 1600–1580 cm^{-1}, and the sharp peak at 1068 cm^{-1} were attributed to the stretching and in-plane deformation of the aromatic ring. Additionally, the double peak at 707–704 cm^{-1} can be related to the polyester portion of the paint (Ploeger et al., 2008). The small and broad absorption around 1650 cm^{-1} indicated the presence of N–O groups (Germinario et al., 2016). In contrast, the FTIR results for silver graffiti were strongly affected by the particularly high reflectance of this paint, which has been found in previous studies (Rivas

et al., 2012). In silver paint, the generally low-intensity response of the resulting ATR spectra did not allow for a comparative evaluation among the treatments.

The comparison between Control 0_0 and Control 0 highlighted that the immersion strategy alone contributed somewhat to changes in the paint layer properties (Figure 5A,B). Indeed, the black paint increased in thickness, probably due to a hydration effect of the paint layer and consequent volume expansion. A change in color was also measured, although the L* parameter did not exceed the threshold of 2 CIELAB units. Similarly, a noticeable effect on the color after immersion was measured in the silver paint; in this case, the L* parameter following immersion differed by more than 14 CIELAB units to the same paint that had not been immersed. In both paints, an increased amount of DIC was observed in the surrounding medium. DIC may be present as a constituent in the paint layer and may be leached into the surrounding medium after the paint layer is submerged (Cappitelli, 2010; Jesionowski and Ciesielczyk, 2013). Besides this immersion effect, data demonstrated an additional action of bacteria on the paint layer.

Among the parameters investigated, changes in FTIR spectrum coupled with DIC increase and bacterial growth were shown by PCA to be the most indicative of biodegradation activity. Indeed, FTIR is a valid method to assess changes in the paint structure, but as shown by PCA, alone it does not highlight the microbial activity on the paint layer. In addition, as in the case of the silver paint, this information is not always available.

On the other hand, a DIC increase is valid proof to assess the presence of bacteria in an active mode of life and its metabolic activity. Carbon is the main element in graffiti spray paints (above 50% of total composition; Sanmartín et al., 2014). Using an immersion strategy, graffiti layers release DOC into the liquid medium, an action that can be promoted

476 in the presence of bacteria. We hypothesize that the metabolic activity of bacteria, related
477 to their bioremoval action of graffiti, triggers the break down and transformation of DOC
478 into DIC, mainly CO_2 (bacterial respiration can be estimated by this DIC accumulation),
479 together with low molecular weight organic compounds, hydrogen peroxide, and other
480 substances (Amado et al., 2006). However, the presence of bacteria and their metabolism
481 alone is not enough to prove a degradation effect on the paint layer.

482 Previous work has shown how color measurements are a key tool to assess the 'degree of
483 efficiency' in bioremoval processes on paints and inks (Giacomucci et al., 2012;
484 Germinario et al., 2017). Indeed, Giacomucci et al. (2012) on red graffiti paint covering a
485 glass slide support found a noticeable color fading which correlated with the degradation
486 of the paint binder, the removal of nitro groups from the nitrocellulose molecule, and the
487 degradation of other ingredients of the paint formulation; processes that altogether would
488 lead to the observed paint detachment. In the present study, changes in color are considered
489 a good parameter together with FTIR, bacterial growth and DIC data in the evaluation of
490 microbial performance. Among the investigated parameter, L*, associated with lightness,
491 was the most useful parameter with which to measure the bacterial effect on the paint
492 layers.

493 DOC and paint thickness were less indicative of biodegradation. Indeed, no changes in
494 thickness were measured after bacterial treatments. This was also confirmed by the short
495 vector for thickness in the PCA. On the other hand, DOC is highly influenced by organic
496 matter in the medium. In our experiments, DOC appeared to be reduced in the media with
497 both black and silver paint, ascribable to the consumption of organic carbon content in the
498 rich and low nutrient media used in steps 1 and 2, respectively. The mineral medium also

499 contains organic carbon in the form of citrate. Therefore, a reduction of DOC was also
500 often observed in steps 3 and 4.

501 The contribution of FTIR (for black paint only), bacterial growth, DIC and color change
502 indicated a similar effect of bacteria on the bioremoval of both paints. In black paint,
503 Bac_1, Bac_3 and Bac_4 were most promising (Figure 5C). In these samples, significant
504 changes in the FTIR spectra, indicating an alteration of the paint polymeric structure, were
505 coupled with the presence of consistent amounts of live bacteria in the medium and a
506 significant increase of DIC, probably due to bacteria metabolism, and a change in paint
507 color. The same strains showed a good effect in the bioremoval of silver paint (Figure 5D).
508 In silver paint, Bac_1 and Bac_4 exhibited a more than 8 CIELAB units difference in
509 comparison with the control. Such values are almost twice the 5 CIELAB threshold, above
510 which an observer notices two different colors (Mokrzycki and Tatol, 2011), and that is
511 probably the most applied perception limit in the context of cultural heritage conservation
512 (Palazzi, 1995). Consistently, Bac_1, Bac_3 and Bac_4 showed the greatest differences to
513 the controls in the PCA plots.

514 According to the PCA plots, Bac_3 was the most distant from Control 0, indicating it had
515 the best performance in degrading both black and silver paint. Bac_3 was a mixture of
516 different strains previously isolated in soil contaminated by paints (WO1991003327A1,
517 1990). The stronger results of using a pool of bacteria rather than a single strain highlights
518 the idea that the synergy of several metabolic pathways might be highly effective when
519 biotreating a complex material such as spray paint.

520 Notably, Bac_1, Bac_3 and Bac_4 accounted for the highest values of A_{600} at the end of
521 experiment, showing a great ability to live with the paint as its sole source of carbon and

522 energy. This was confirmed by previous literature. Bac_1, consisting of *Enterobacter*
523 *aerogenes*, demonstrated high levels of tolerance to the presence of paint (Macklin et al.,
524 2013) and for this reason is used for testing the resistance of painted surfaces to microbial
525 attacks (ASTM Standard Test Method D4783-89). Similarly, Bac_4 (*Comamonas sp*.) has
526 been found to associate with wall paintings (Pinar et al., 2013) and is able to degrade the
527 precursor of paints in wastewater (Ordaz-Cortes et al., 2014). Bac_3 is a mixture of
528 *Bacillus* sp., *Delftia lacustris*, *Sphingobacterium caeni*, and *Ochrobactrum anthropi*. The
529 microbial community of pre-painted steel commonly used in roofing applications was
530 found to be dominated by *Bacillus* ssp. (Huynh et al., 2017). *Delftia lacustris* can degrade
531 peptidoglycan and probably other polymers (Jørgensen et al., 2009). The genus
532 *Sphingobacterium* has been found to degrade recalcitrant polymers such as lignin (Rashid
533 et al., 2018). Finally, *Ochrobactrum anthropi* can degrade complex molecules like
534 azoxystrobin (Feng et al., 2020).

535 Our data showed that Bac_6 and Bac_8 had an effect on both paint slides (Figure 5C,D).
536 FTIR spectra revealed changes in the paint structure and the color was altered after bacterial
537 treatment. However, bacterial growth and DIC increases were limited to steps 1 and 2 in
538 the case of Bac_8, and to steps 3 and 4 in the case of Bac_6, indicating that the bio-
539 degradation activity occurred only under specific experimental conditions. In the latter
540 case, biodegradation occurred at the end of experiment, probably after a period of
541 adaptation. Notably, bacteria are known to produce not only constitutive but also inducible
542 enzymes that attack and degrade different types of molecules (Ranalli et al., 2005).

543 Bac_2 and Bac_5 did not affect black paint, as none of the most indicative parameters for
544 these treatments differed in comparison with the Control 0 (Figure 5C). Accordingly, in

the black paint PCA, Bac_2 and Bac_5 were the closest to both controls. For the silver paint, Bac_5 exhibited bacterial growth as well as an increase of DIC, but did not cause a change in the color, so we could not confirm a degradation effect of the paint layer by that bacteria. On the contrary, after treatment with Bac_2, the silver paint was discolored and bacterial growth and a DIC increase were observed (Figure 5D).

For both paints, Bac_7 did not show a biodegradation effect (Figure 5C,D). The FTIR spectra of the black paint showed some changes, but no bacterial growth was found. All of the parameters most indicative of biodegradation of the silver paint were not altered in comparison with the controls.

5. Conclusion

The method presented in this research was used to investigate the implementation of a protocol designed to remove graffiti using bacteria. This removal method is not harmful to substrates, is environmentally friendly and is safe for humans.

Data demonstrated that Bac_1, Bac_3 and Bac_4 were the most promising for the bioremoval of graffiti. For future applications, a mixture of bacteria may be preferred over the use of a single strain. Bac_6 and Bac_8 required specific experimental conditions to have an effect, and Bac_2 had a different response to the tested paints and is therefore considered less suitable for outdoor application in the presence of mixed-color paint graffiti. Bac_5 and Bac_7 did not exhibit biodegradation activity.

Besides data showing that black graffiti paint was chemically altered by bacteria, the effects following biological treatment did not seem significant to the eye. However, the selection of bacterial strains able to degrade graffiti paint is a step forward in the field of

graffiti bioremoval that has so far been under-studied. Notably, upon microbial biodegradation, graffiti can be altered in ways that make traditional interventions more effective, e.g., mechanical or solvent-based chemical removal.

Some limitations need to be still resolved and the research is ongoing to address them. Indeed, there is still some work to set up a delivery system for the application of microorganisms, providing them an adequate microenvironment to optimize in situ their activity. The future research will address the critical evaluation of the main properties and characteristics of the systems adopted until now as well as the development of new strategies in order to find the best delivery solution for the presented bioremoval technology. Compatibility with bacteria and graffiti substrates, easy application, lack of health risks for restorers and safety for the environment are, among others, aspect that will be taken into consideration for the choice (Bosch-Roig et al., 2015).

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733 Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Vantriet, K., 1990. Modeling of the
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735 http://doi.org/10.1128/aem.56.6.1875-1881.1990

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738 **Table 1**. Characteristics of the eight strains of bacteria tested.

739

Label	Bacterial strain	Code	Isolation	Known applications
Bac_1	*Enterobacter aerogenes* (formerly *Aerobacter aerogenes*)	ATCC 13048	Sputum	Bacterial resistance testing paint (ASTM D4783-89, 1989)
Bac_2	*Bacillus subtilis*	ATCC 27328	Spoiled polyvinylacetate paint	Bacterial resistance testing paint (ASTM D2574-06, 2006). It causes spoilage of latex paints (US 6902727B2, 2005)
Bac_3	Mixture of mixture of *Bacillus* sp., *Delftia lacustris*, *Sphingobacterium caeni*, and *Ochrobacterum anthropi*	ATCC 53922	Soil containing paint waste	Degrades paint, removes paint and coatings from metallic surfaces (WO1991003327A1, 1990)
Bac_4	*Comamonas* sp.	ATCC 700440	Paint stripping waste	Degrades m-nitrobenzoic acid (Nadeau and Spain, 1995)
Bac_5	*Rubellimicrobium thermophilium*	DSMZ 16684	Colored deposits in a pulp dryer (Denner et al., 2006)	–
Bac_6	*Chelatoccocus daeguensis*	DSMZ 22069	Wastewater of a textile dye works (Yoon et al., 2008)	–
Bac_7	*Escherichia coli*	DSMZ 787	–	Bacterial resistance testing paint (Bogdan et al., 2018). Ink grids on membrane filter testing (ASTM D4200-82, 2019)
Bac_8	*Marinospirillum* sp.	DSMZ 9662	Car paint waste	–

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744

745 **Figure captions**

746 **Figure 1**. Analysis of the liquid media without (Control 0_0) or with black and silver paints (Control 0)
747 and the addition of bacteria (Bac_1–8) at step 4 of the experiment. The histograms represent mean values
748 of pH (Panel A), A_{600} (Panel B), live and dead cells (Panel C), relative viability (Panel D), dissolved
749 organic carbon (DOC) (Panel E) and dissolved inorganic carbon (DIC) (Panel F). Black lines represent
750 the standard deviation. Different letters indicate significant differences based on Tukey's HSD ($p \leq 0.05$)
751 between samples.

752 **Figure 2**. Analysis of black and silver paint coatings before (Control 0_0) and after immersion (Control
753 0) and the addition of bacteria (Bac_1–8) at step 4 of the experiment. Histograms represent the mean
754 values of thickness (Panel A) and the color parameters L* (Panel B), a* (Panel C), and b* (Panel D).
755 Black lines represent the standard deviation. Different letters indicate significant differences based on
756 Tukey's HSD ($p \leq 0.05$) between samples.

757 **Figure 3**. FTIR spectra of the graffiti-coated slides. The dashed vertical lines indicate the main absorption
758 peaks of the paint used to track treatment-induced changes. Panel A: Control 0_0 (reference untreated
759 black paint; below, red line), Control 0 (middle, black line), and Bac_5 treatment (above, green line);
760 Panel B: Bac_4 (below, grey line), Bac_3 (middle, blue line), and Bac_7 (above, orange line) treatments.

761 **Figure 4**. Biplot obtained from PCA of variables measured in the media surrounding the painted slides
762 and the paint coatings (including both Controls 0_0 and 0). Panel A: Black graffiti paint. Panel B: Silver
763 graffiti paint.

764 **Figure 5**. Summary of all experiments related to the media and paint layers without (Control 0_0 and
765 Control 0) and with bacterial strains. A comparison between Control 0_0 and Control 0 is reported in
766 panels A and C. A comparison between paints treated with bacteria and Control 0 is reported in panels

767 B and D. Orange cells highlight significant differences: + indicates a significant increase of the
768 parameter, whereas – indicates a significant decrease. White cells indicate that there was no significant
769 difference. Gray cells indicate experiments not performed in the step. na*: not available, as the 1721 peak
770 is not present.

771

772

Figure 1

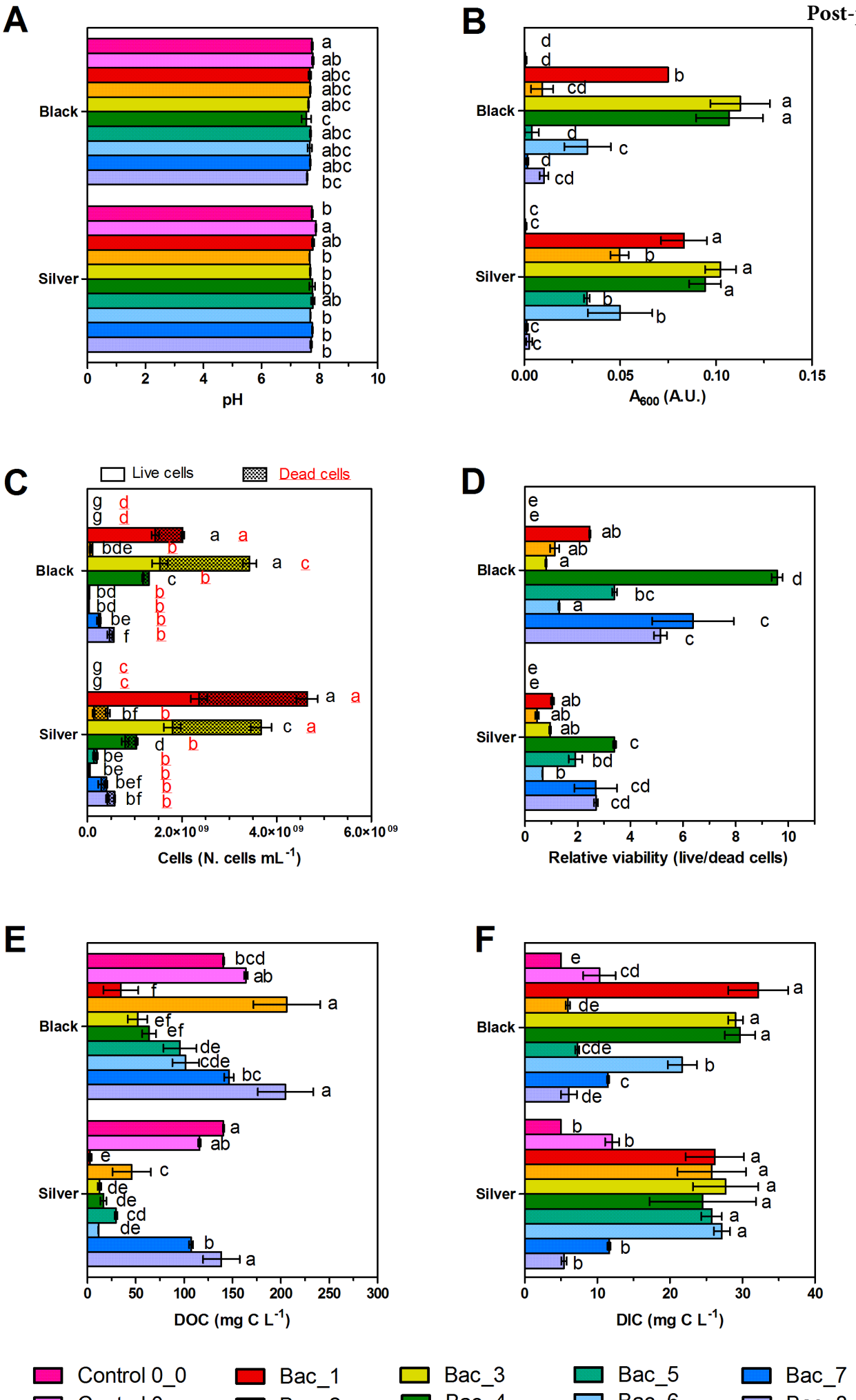

A
Black
Silver
pH
B
A600 (A.U.)
C
Live cells
Dead cells
Cells (N. cells mL-1)
D
Relative viability (live/dead cells)
E
DOC (mg C L-1)
F
DIC (mg C L-1)
Control 0_0
Control 0
Bac_1
Bac_2
Bac_3
Bac_4
Bac_5
Bac_6
Bac_7
Bac_8

Figure 2

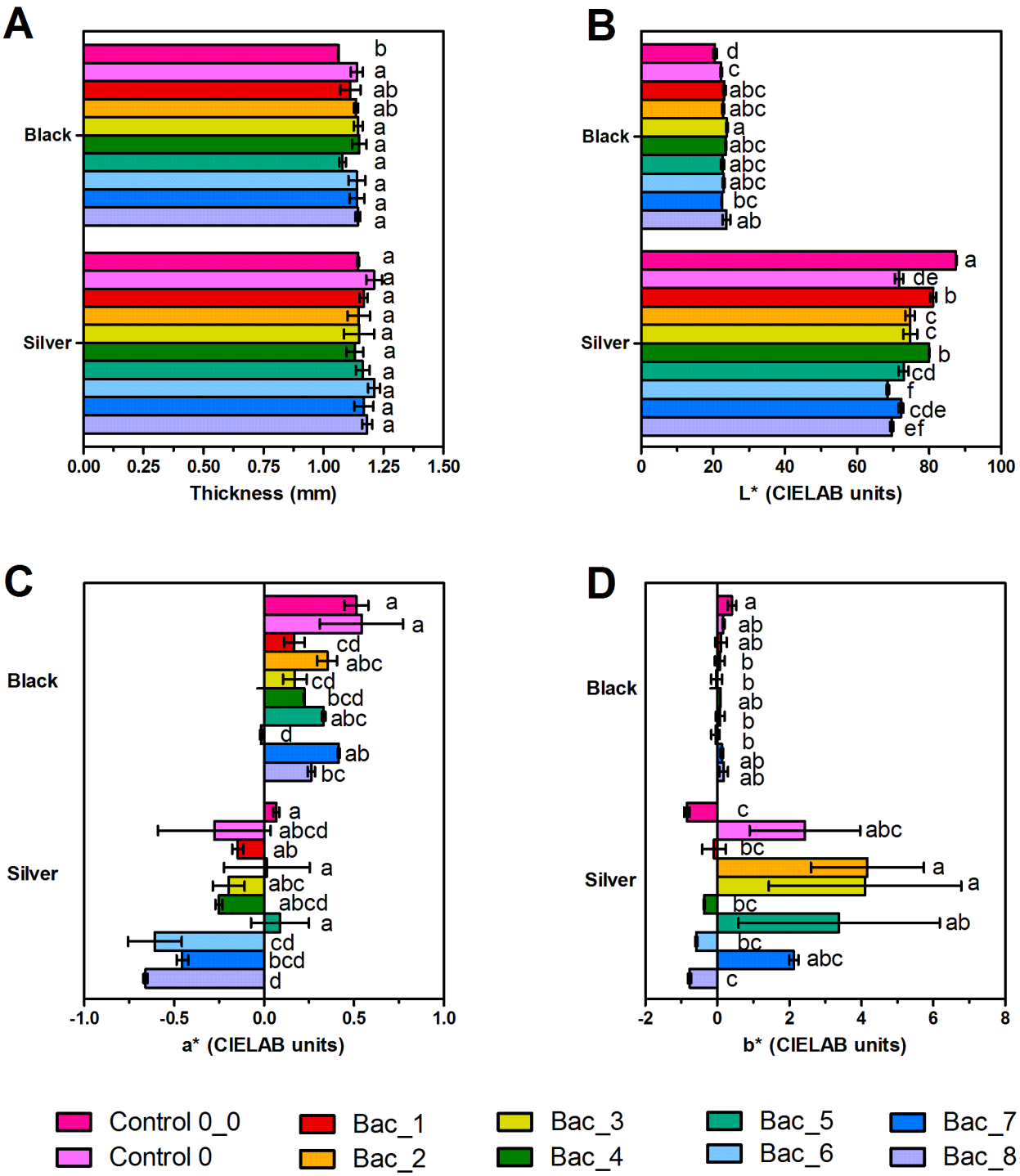

A
Black
Silver
b a ab ab a a a a a a
a a a a a a a a a a
0.00 0.25 0.50 0.75 1.00 1.25 1.50
Thickness (mm)
B
Black
Silver
d c abc abc a abc abc abc bc ab
a de b c c b cd f cde ef
0 20 40 60 80 100
L* (CIELAB units)
C
Black
Silver
a a cd abc cd bcd abc d ab bc
a abcd ab a abc abcd a cd bcd d
-1.0 -0.5 0.0 0.5 1.0
a* (CIELAB units)
D
Black
Silver
a ab ab b b ab b b ab ab
c abc bc a a bc ab bc abc c
-2 0 2 4 6 8
b* (CIELAB units)
Control 0_0
Control 0
Bac_1
Bac_2
Bac_3
Bac_4
Bac_5
Bac_6
Bac_7
Bac_8

Figure 3

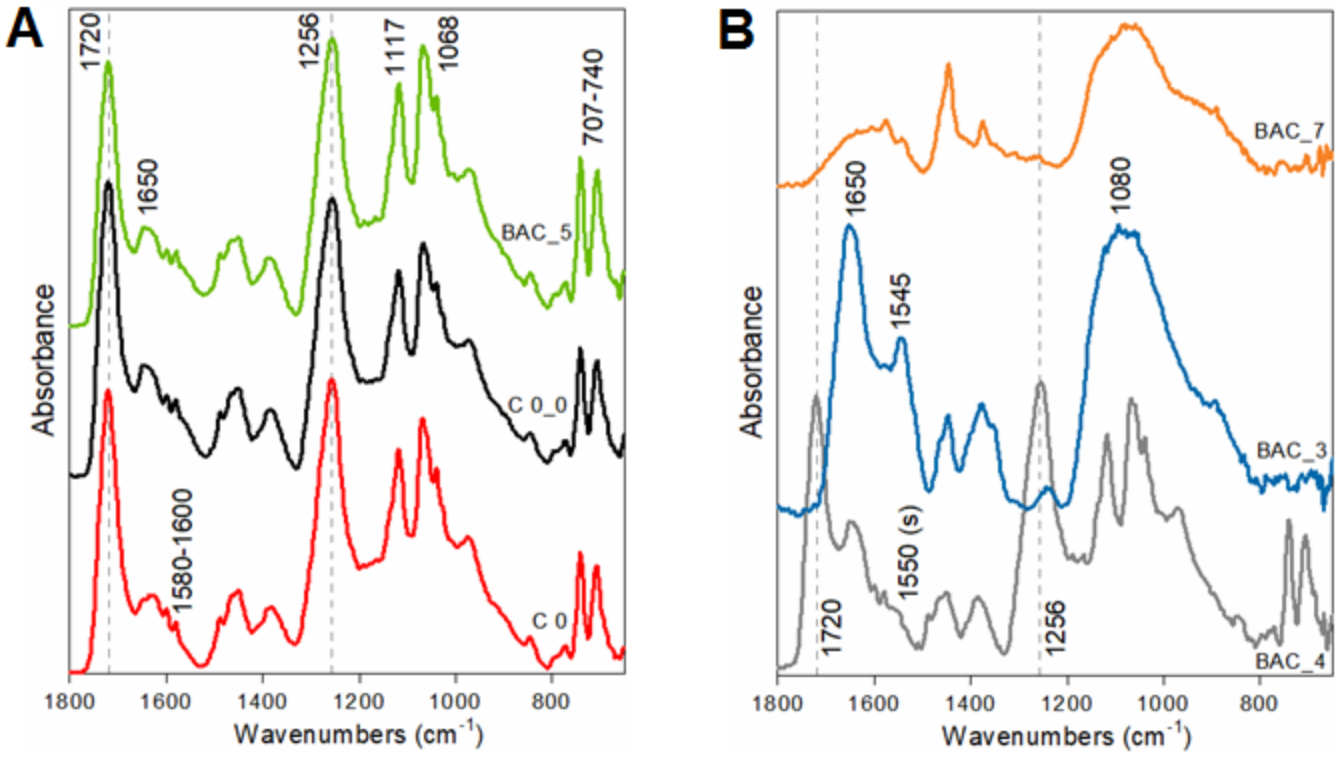
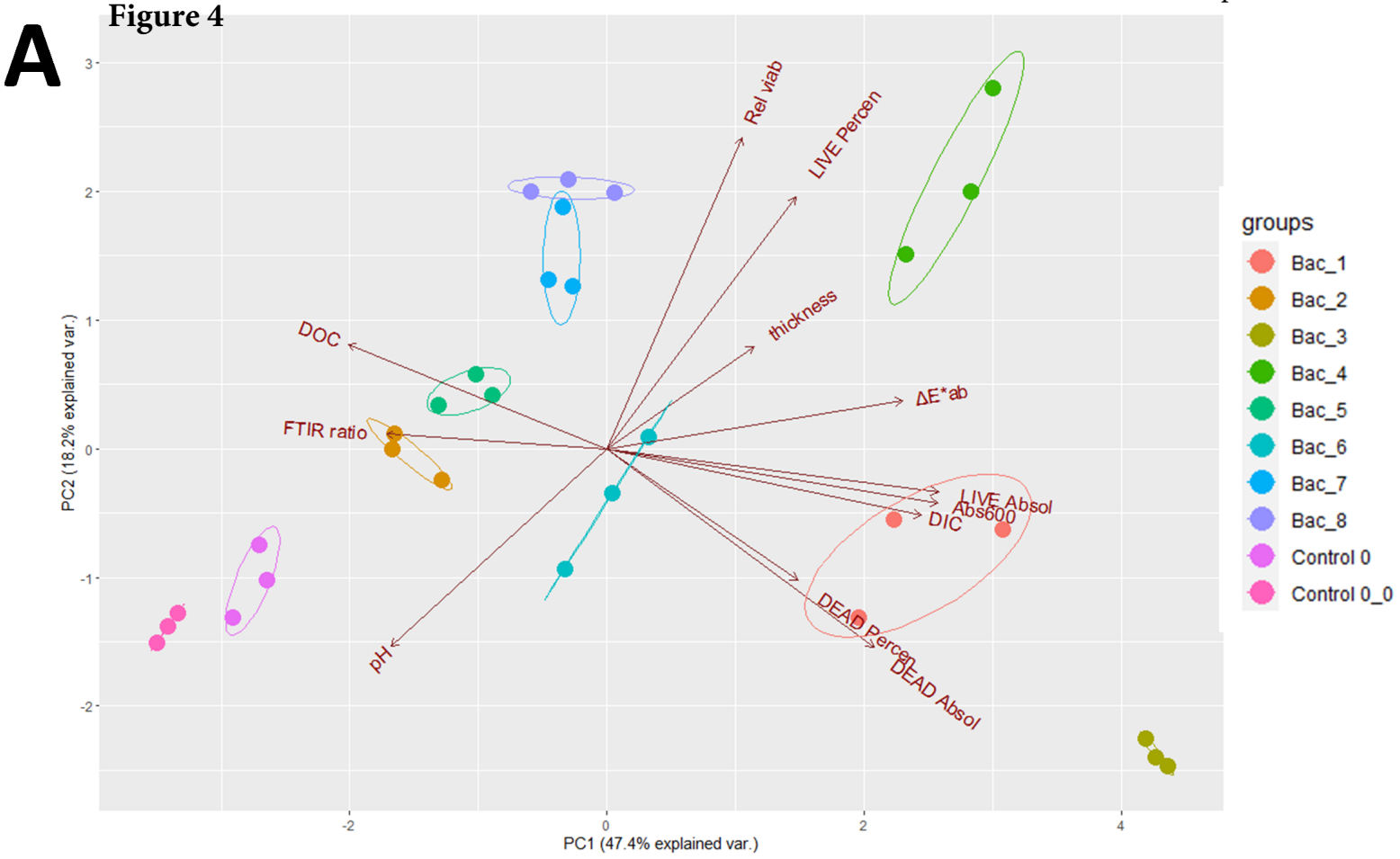
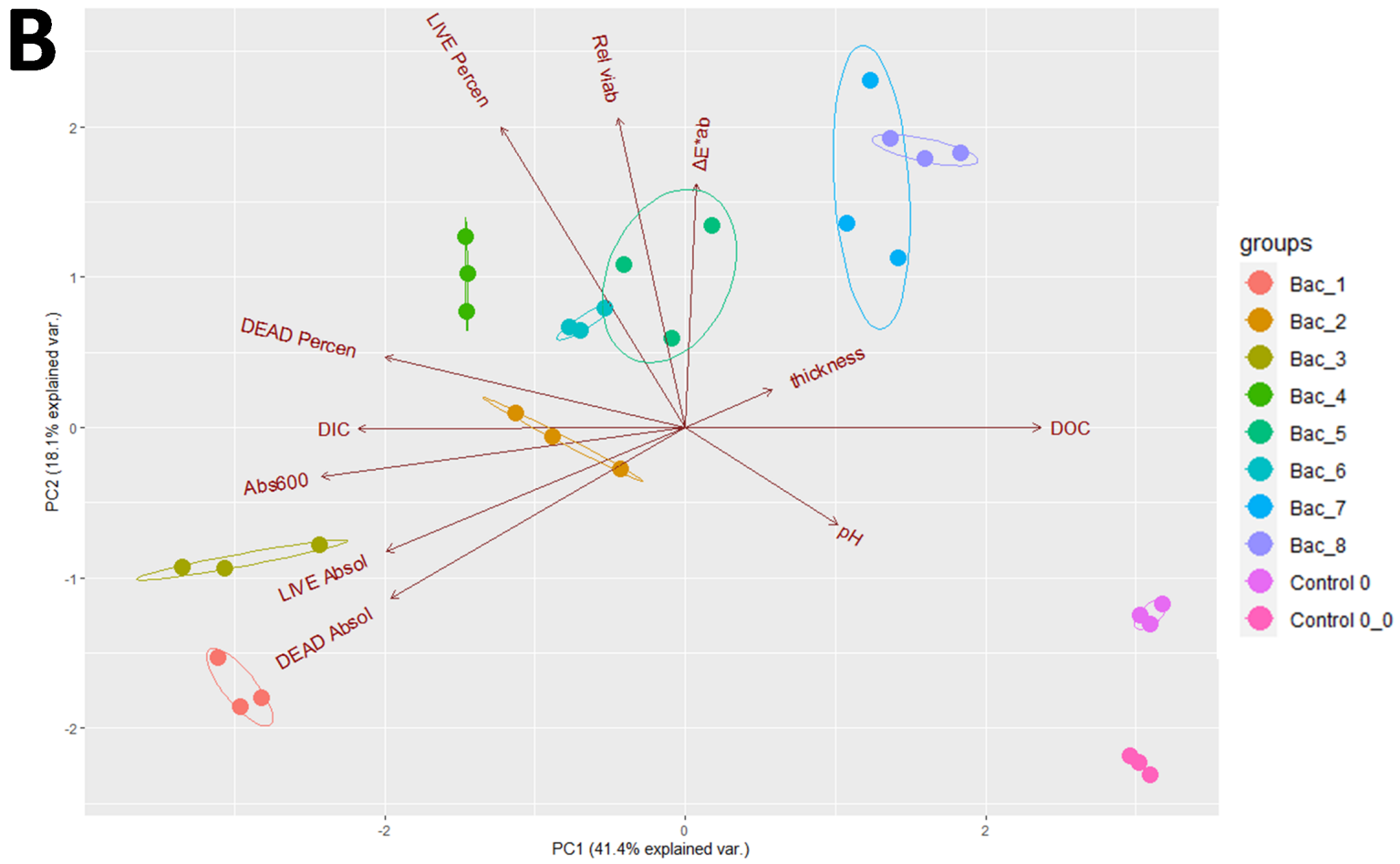

A
1720
1650
1580-1600
1256
1117
1068
707-740
BAC_5
C 0_0
C 0
Absorbance
1800
1600
1400
1200
1000
800
Wavenumbers (cm-1)
B
BAC_7
1650
1545
1080
BAC_3
1550 (s)
1720
1256
BAC_4
Absorbance
1800
1600
1400
1200
1000
800
Wavenumbers (cm-1)

Figure 4


A
Rel viab
LIVE Percen
thickness
DOC
ΔE*ab
FTIR ratio
LIVE Absol
Abs600
DIC
DEAD Percen
DEAD Absol
pH
PC2 (18.2% explained var.)
PC1 (47.4% explained var.)
groups
Bac_1
Bac_2
Bac_3
Bac_4
Bac_5
Bac_6
Bac_7
Bac_8
Control 0
Control 0_0


B
LIVE Percen
Rel viab
ΔE*ab
DEAD Percen
thickness
DIC
DOC
Abs600
pH
LIVE Absol
DEAD Absol
PC2 (18.1% explained var.)
PC1 (41.4% explained var.)
groups
Bac_1
Bac_2
Bac_3
Bac_4
Bac_5
Bac_6
Bac_7
Bac_8
Control 0
Control 0_0

A

BLACK	Control 0_0 vs 0			
Experiment	1	2	3	4
Abs_{600} (culture)				
Live/dead (culture)				
pH (culture)				
DOC(culture)				
DIC (culture)	+			+
Thickness (paint)				+
L*(paint)				+
a*(paint)				
b*(paint)				
FTIR (1721/1256 ratio) (paint)				

B

BLACK	Bac_1				Bac_2				Bac_3				Bac_4				Bac_5				Bac_6				Bac_7				Bac_8			
Experiment	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Abs_{600} (culture)	+	+	+	+	+				+	+	+	+	+	+	+	+	+						+	+					+	+		
Live/dead (culture)				+				+				+				+				+				+				+				+
pH (culture)	–				–				–							–	–						–						–		–	
DOC(culture)			–	–					–		–	–			–	–				–			–	–								
DIC (culture)		+	+	+		+				+	+	+		+	+	+			+					+								
Thickness (paint)																																
L*(paint)												+																				+
a*(paint)				–								–				–								–								–
b*(paint)																																
FTIR (1721/1256 ratio) (paint)				–								na*				–								–				na*				–

C

SILVER	Control 0_0 vs 0			
Experiment	1	2	3	4
Abs_{600} (culture)				
Live/dead (culture)				
pH (culture)				+
DOC(culture)				
DIC (culture)	+			
Thickness (paint)				
L*(paint)				–
a*(paint)				
b*(paint)				
FTIR (1721/1256 ratio) (paint)				

D

SILVER	Bac_1				Bac_2				Bac_3				Bac_4				Bac_5				Bac_6				Bac_7				Bac_8			
Experiment	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Abs_{600} (culture)	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	+			+	+					+	+		
Live/dead (culture)				+				+				+				+				+				+				+				+
pH (culture)	–				–			–	–			–	–	–		–	–	–					–	–				–	–	–	–	–
DOC(culture)	–	–	–	–			–	–	–	–	–	–	–	–	–	–			–	–			–	–								
DIC (culture)		+	+	+		+	+	+		+	+	+		+	+	+		+	+	+		+		+	–				–	+		
Thickness (paint)																																
L*(paint)				+				+				+				+								–								
a*(paint)																																
b*(paint)																																
FTIR (1721/1256 ratio) (paint)				na*				na*				na*				na*				na*				na*				na*				na*

Supplementary materials

Bioremediation of graffiti using novel commercial strains of bacteria

C. Cattò[1♯], P. Sanmartín[1,2*♯], D. Gulotta[3], F. Troiano[1], F. Cappitelli[1]

[♯]Both authors contributed equally to this work.

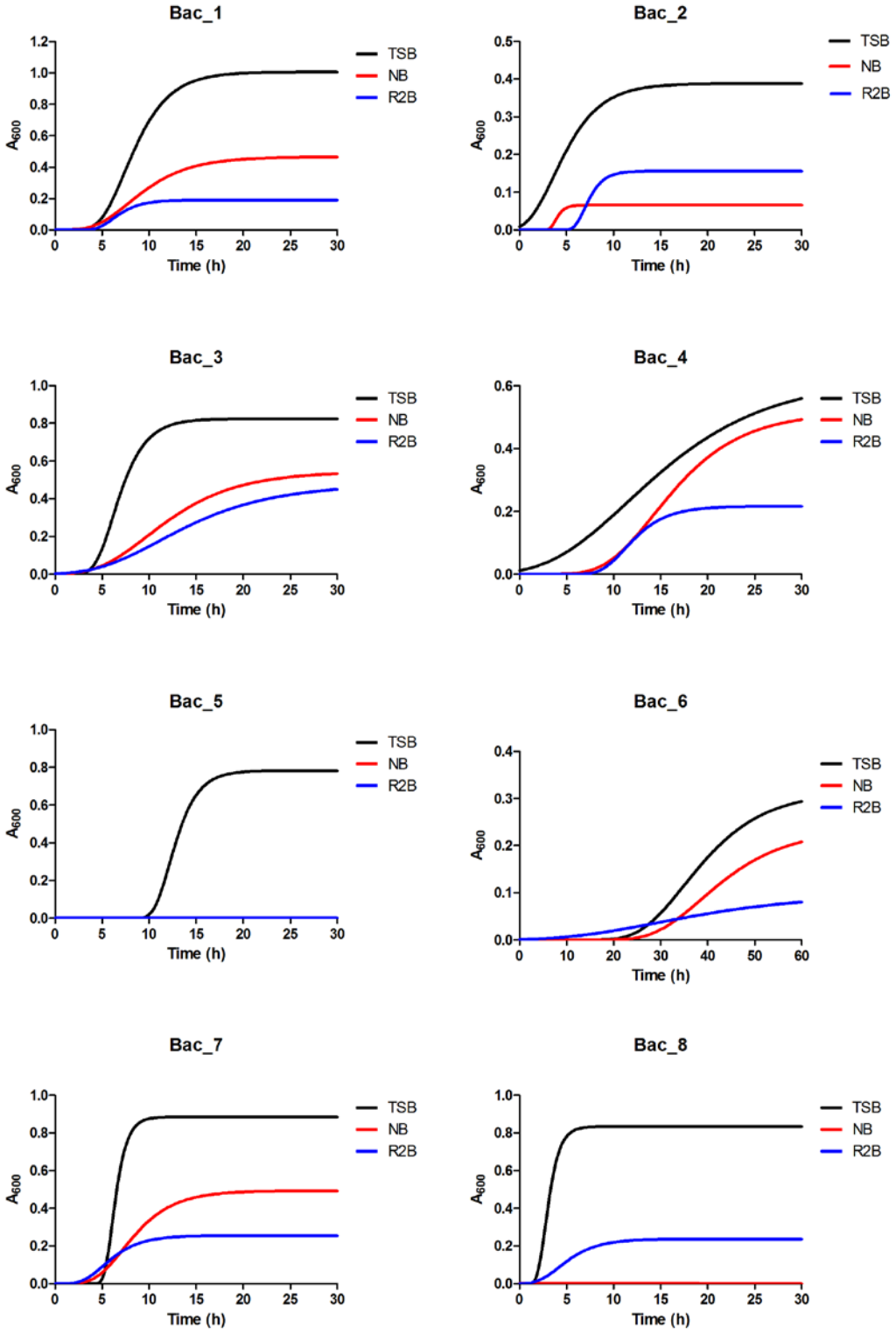


Figure S1. A_{600} planktonic growth curves of Bacs_1–8 in the presence of rich media TSB, NB and R2B.

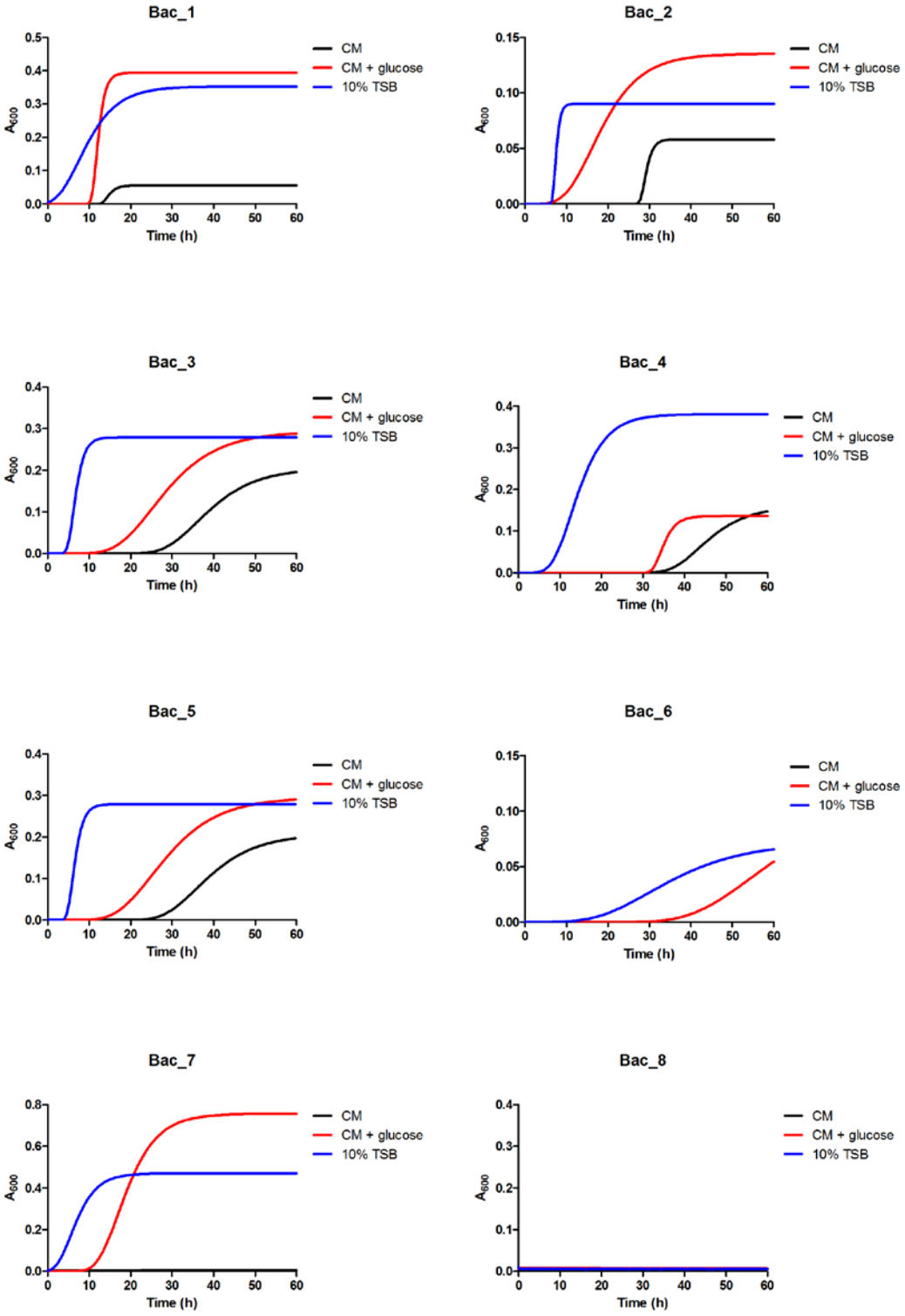


Figure S2. A_{600} planktonic growth curves of Bacs_1–8 in the presence of low nutrient media 10% TSB and CM-glu and the mineral medium CM.

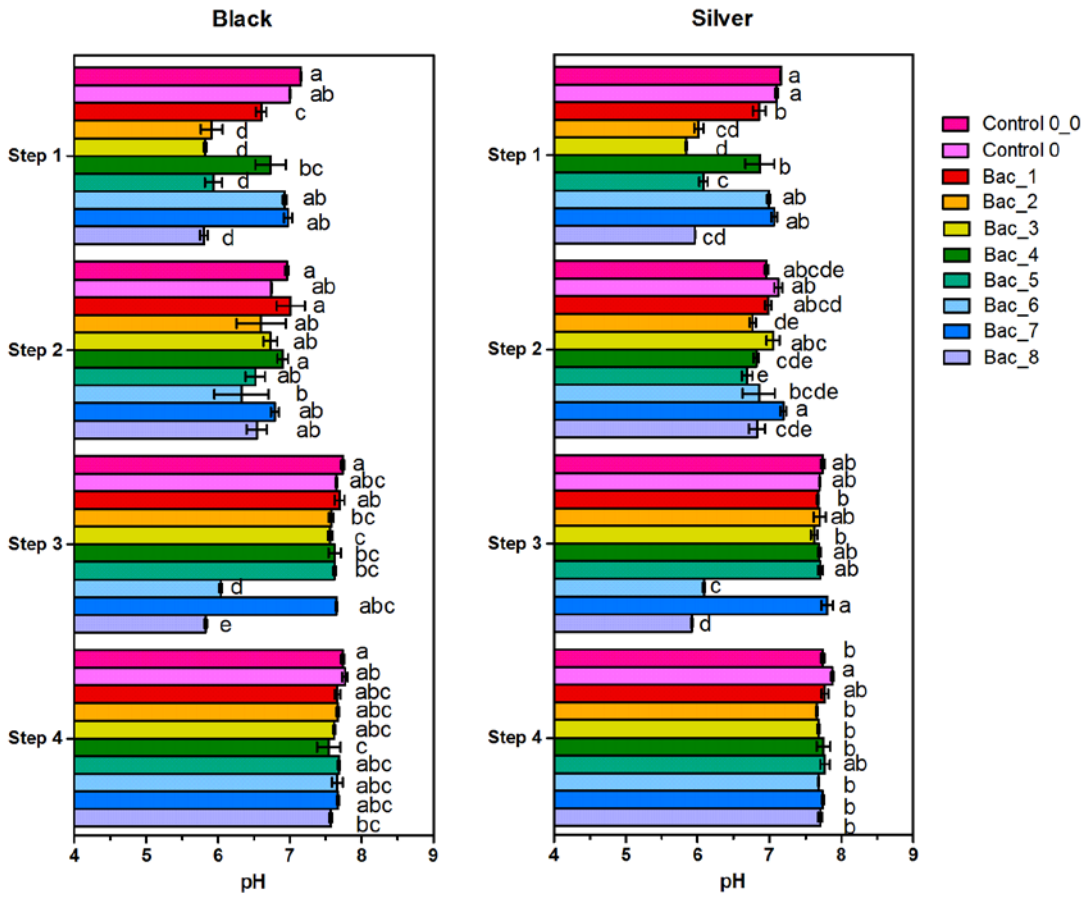


Figure S3. Analysis of pH in the liquid media without (Control 0_0) or with black and silver paints (Control 0) and the addition of bacteria (Bacs_1–8) from step 1 to step 4 of the experiment. The histograms represent mean values while black lines represent the standard deviation. Different letters indicate significant differences from Tukey's HSD ($p \leq 0.05$) between samples.

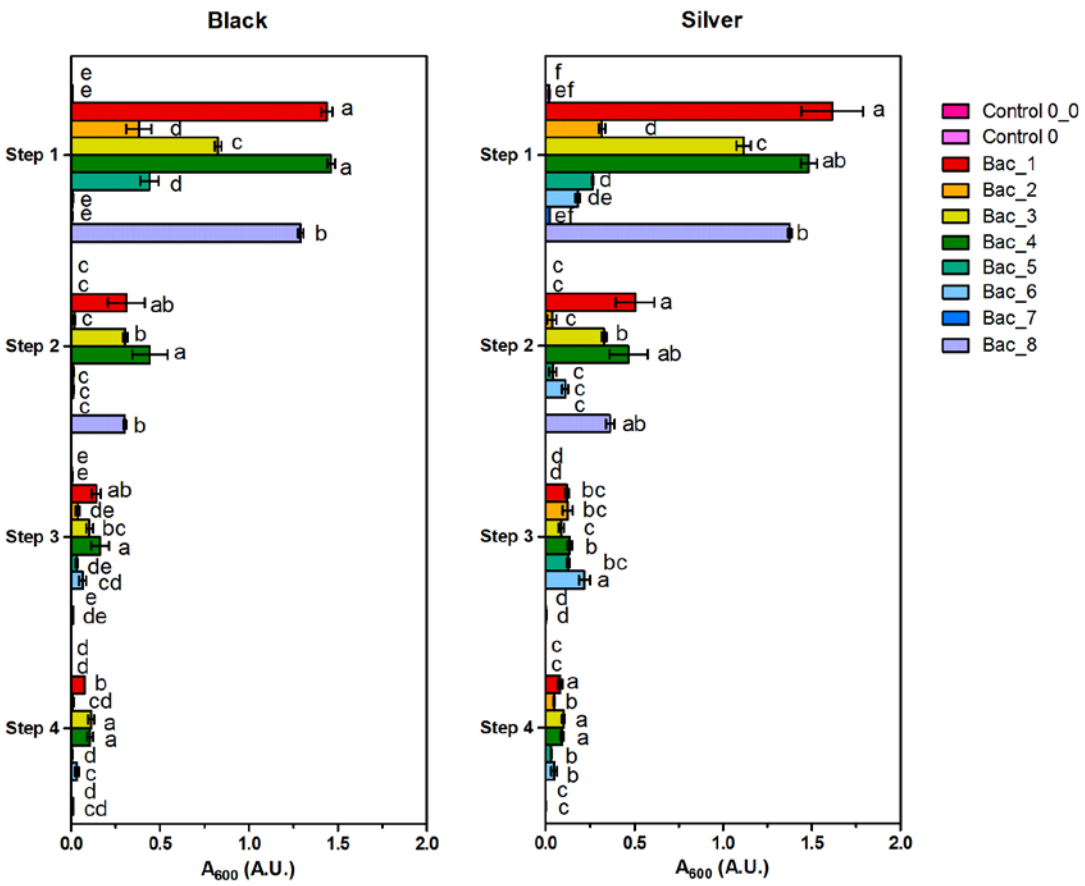


Figure S4. Analysis of A_{600} in the liquid media without (Control 0_0) or with black and silver paints (Control 0) and the addition of bacteria (Bacs_1–8) from step 1 to step 4 of the experiment. The histograms represent mean values while bar represent the standard deviation. Different letters indicate significant differences from Tukey's HSD ($p \leq 0.05$) between samples.

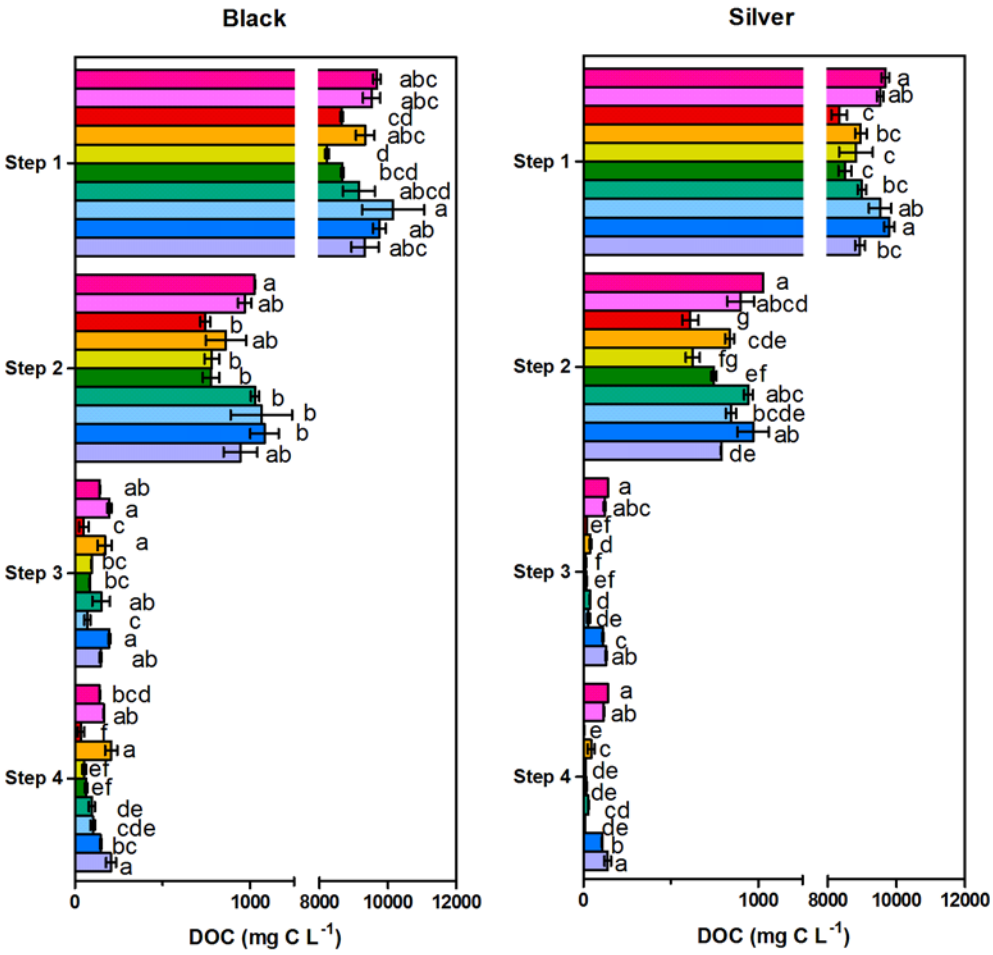


Figure S5. Analysis of DOC in the liquid media without (Control 0_0) or with black and silver paints (Control 0) and the addition of bacteria (Bacs_1–8) from step 1 to step 4 of the experiment. Histograms represent mean values while black lines represent the standard deviation. Different letters indicate significant differences from Tukey's HSD ($p \leq 0.05$) between samples.

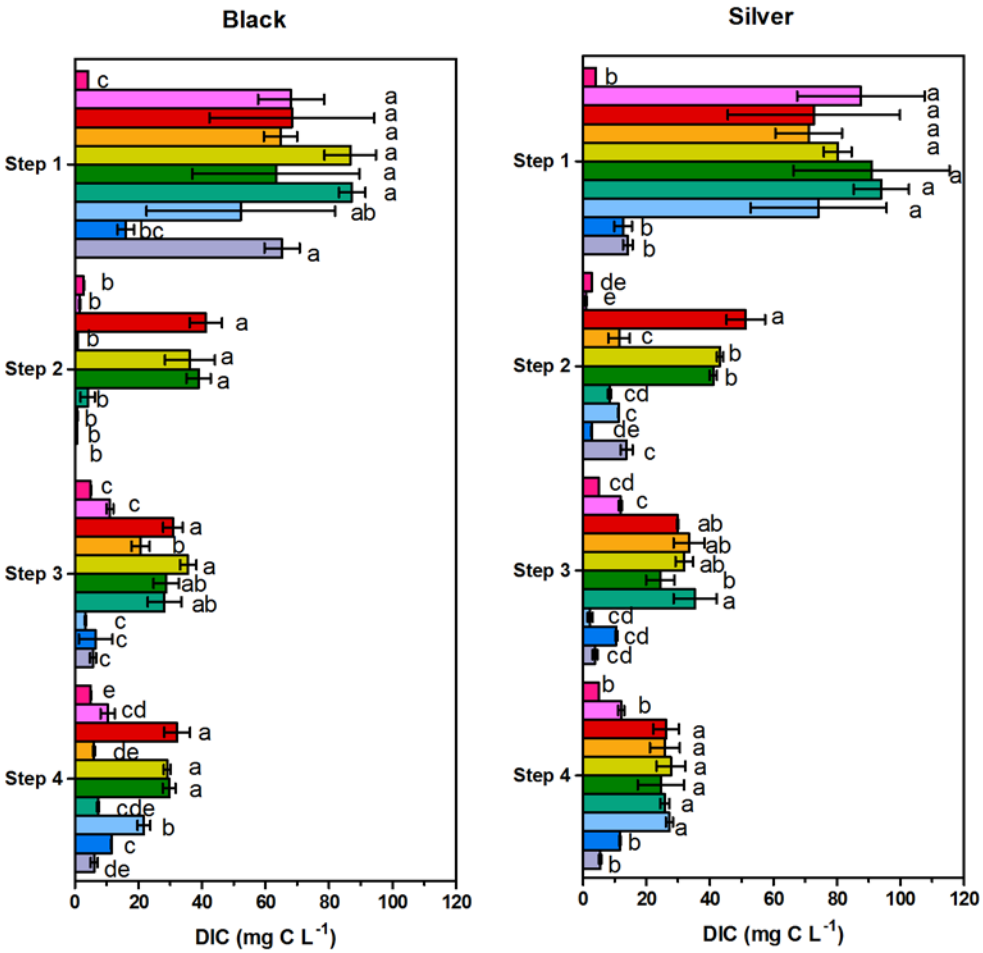


Figure S6. Analysis of DIC in the liquid media without (Control 0_0) or with black and silver paints (Control 0) and the addition of bacteria (Bacs_1–8) from step 1 to step 4 of the experiment. The histograms represent mean values and black lines represent the standard deviation. Different letters indicate significant differences from Tukey's HSD ($p \leq 0.05$) between samples.

		Lag Phase			Maximum specific growth rate		
Label	**Medium**	**Average (h)**	**St. Dev (h)**	**ANOVA**	**Average (A_{600}/h)**	**St. Dev (A_{600}/h)**	**ANOVA**
Bac_1	TSB	4.82	0.12	a	1.42E-01	9.29E-03	a
	NB	4.28	0.06	b	4.83E-02	1.84E-03	b
	R2B	4.50	0.17	b	4.35E-02	4.56E-03	b
	10% TSB	2.34	0.35	c	2.52E-02	2.15E-03	c
	CM + glucose	10.64	0.18	b	1.26E-01	4.37E-03	b
	CM	13.04	0.41	a	1.80E-02	7.66E-04	a
Bac_2	TSB	0.77	0.06	a	3.20E-02	6.31E-04	a
	NB	6.02	0.11	b	4.43E-02	8.13E-03	ab
	R2B	8.60	0.51	c	5.28E-02	1.69E-03	b
	10% TSB	6.64	0.70	c	5.11E-02	8.08E-03	c
	CM + glucose	9.58	1.24	b	7.71E-03	6.88E-04	b
	CM	27.55	1.00	a	2.24E-02	3.65E-04	a
Bac_3	TSB	4.17	0.37	a	1.53E-01	1.71E-02	a
	NB	4.75	0.30	a	4.03E-02	3.92E-03	b
	R2B	4.42	0.68	a	2.66E-02	3.83E-03	b
	10% TSB	4.72	0.43	c	8.07E-02	6.59E-03	b
	CM + glucose	16.62	0.74	b	1.26E-02	3.52E-04	a
	CM	27.89	2.49	a	9.63E-03	1.60E-03	a
Bac_4	TSB	3.21	0.41	a	2.77E-02	1.04E-03	a
	NB	9.16	0.62	b	3.71E-02	1.31E-03	b
	R2B	8.67	0.48	b	4.29E-02	3.44E-03	c
	10% TSB	8.07	0.34	c	3.03E-02	2.09E-03	c
	CM + glucose	31.87	0.87	b	2.25E-02	3.27E-03	14.55
	CM	38.75	1.98	a	9.12E-03	1.49E-03	16.37
Bac_5	TSB	10.49	0.48	a	2.65E-01	8.10E-02	30.53
	NB	Not converged			Not converged		
	R2B	Not converged			Not converged		
	10% TSB	4.59	0.43	c	8.20E-02	5.78E-03	7.05
	CM + glucose	16.55	0.69	b	1.26E-02	3.60E-04	2.85
	CM	27.74	2.34	a	9.61E-03	1.57E-03	16.30
Bac_6	TSB	26.50	2.08	a	1.51E-02	2.36E-04	1.56
	NB	28.60	1.36	a	8.57E-03	1.59E-04	1.85
	R2B	10.10	1.88	b	1.91E-03	1.34E-04	7.01
	10% TSB	17.04	2.87	a	2.09E-03	2.26E-04	10.84
	CM + glucose	Not converged			Not converged		
	CM	Not converged			Not converged		
Bac_7	TSB	5.23	0.05	a	3.76E-01	1.31E-02	3.48
	NB	4.26	0.15	b	6.16E-02	3.33E-03	5.40
	R2B	3.07	0.23	c	4.29E-02	4.45E-03	10.37
	10% TSB	2.96	0.57	b	4.94E-02	3.39E-03	6.87
	CM + glucose	11.94	0.54	a	5.44E-02	3.08E-03	5.66
	CM	Not converged			Not converged		
Bac_8	TSB	1.92	0.03	a	3.71E-01	8.81E-03	2.38
	NB	Not converged			Not converged		
	R2B	1.91	0.02	a	3.86E-02	1.31E-03	3.39
	10% TSB	Not converged			Not converged		
	CM + glucose	Not converged			Not converged		
	CM	Not converged			Not converged		

Table S1. Growth parameters, i.e., lag time (λ) and maximum specific growth rate (μ_m), obtained by the Gompertz model. Data represent the mean ± SD of four independent measurements. For each strain, different letters in each column indicate significant differences (Tukey's HSD, $p \leq 0.001$) in relation to the culture media.