

1 **Shaping colour changes in a biofilm-forming cyanobacterium by modifying the culture**
2 **conditions**

3 B. Prieto, D. Vázquez-Nion, B. Silva, P. Sanmartín*

4 Departamento de Edafoloxía e Química Agrícola, Facultade de Farmacia, Universidade de
5 Santiago de Compostela, 15782 Santiago de Compostela, Spain.

6

7 *Corresponding author: Patricia Sanmartín.

8 E-mail address: patricia.sanmartin@usc.es

9 Telephone: +34 881 814984

10 Address: Departamento de Edafoloxía e Química Agrícola. Facultade de Farmacia. Campus
11 Vida. Universidade de Santiago de Compostela. 15782, Santiago de Compostela (A Coruña)

12

13

14

15 **Abstract**

16 Cyanobacteria, pioneering microorganisms that create a conditioning layer on substrates and
17 act as a source of nutrients for successive colonization by heterotrophic microbes, are
18 commonly studied in relation to the microbial ecology of stones in indoor and outdoor habitats.
19 They are well known for producing greenish-yellow, bluish-green or occasionally pink or pinkish-
20 orange discolouration of surfaces. Such discoloration may be desirable for integrating new
21 elements in landscapes and could be achieved by inoculating surfaces with cyanobacteria of a
22 specific colour. In the present study, with the aim of producing cultures of perceptibly different
23 hues, we modelled the colorimetric response of the biofilm-forming cyanobacterium *Nostoc* sp.
24 PCC 9104 to variations in light intensity (L) and the concentrations of phosphorus (P) and
25 nitrogen (N). The model obtained, which was validated from both mathematical and perceptual
26 perspectives, enables production of cultures of a particular hue, within a range of 18° and with
27 an efficacy of 92%. Coloured cultures of hue between 129° and 147°, corresponding to
28 yellowish-green to bluish-green tones, were obtained by modifying nutrients inputs and the
29 amount of light, without the need to resort to genetic manipulation.

30

31 **Keywords:** modelling; response surface methods; cyanobacteria; light and nutrients.

32 **1. Introduction**

33 Cyanobacteria are ecologically and economically important in environmental research and also
34 in bioindustrial processes and the field of material conservation [1]. Cyanobacteria have simple
35 metabolic requirements, tolerate marginal environments and undergo rapid genetic changes.
36 They are easy to handle in the laboratory and large amounts of biomass can be produced at low
37 cost. Because of their considerable metabolic versatility and flexibility they have been
38 engineered to produce valuable bioindustrial compounds, such as polysaccharides [2] and
39 hydrocarbons [3], as well to generate hydrogen [4] and treat polluted water [5]. Environmental
40 applications in which cyanobacteria are used to remediate water and soils have also been
41 widely investigated. For example, cyanobacteria are used to remove excess fluoride from
42 industrially polluted water [6], to trigger land rehabilitation in arid and semiarid environments [7],
43 and to remove nitrogen from aquatic ecosystems [8].

44 Cyanobacteria, along with other phototrophic and heterotrophic microorganisms, play an
45 important role in the formation of subaerial biofilms (SAB) on solid surfaces [9]. Biofilm formation
46 can be induced on the surface of new elements in landscapes to mitigate the corresponding
47 visual impacts. This is done in open cast quartz mining, in which the bright white colour of the
48 mine walls has a strong visual impact against a background of wooded areas. Mining
49 companies are required by law to restore the appearance of any landscape affected by
50 opencast mining, and assessment of the visual impact considers two aspects: (i) the area
51 occupied by the quarry as observed from a specific location, and (ii) the chromatic contrast
52 between the landscape and the mining exploitation [10]. Traditional revegetation techniques are
53 unsuccessful on quartz surfaces because of the resistance of quartz to weathering, and the
54 surrounding areas thus become strongly eroded. Our research group has carried out studies
55 aimed at evaluating the induction of epilithic photoautotrophic biofilms on quartz surfaces as an
56 alternative remediation technique [11-13]. Successful results, in terms of biofilm development
57 and reduction on the visual impact, were obtained in laboratory tests with quartz samples.
58 Biofilm formation was induced on quartz despite the low porosity, smooth surface and purity of
59 the material, which lacks elements considered nutrients. Moreover, the biofilm caused a
60 substantial change in the colour of the surface of the quartz samples, turning them greenish-
61 yellow or reddish-yellow.

62 The colour of organisms is determined by the type and abundance of biological pigments that
63 they possess, i.e. chlorophyll *a*, carotenoids and phycobiliproteins, which in turn vary with
64 environmental factors such as nutrient availability, light, temperature, ultraviolet radiation and
65 pH. Numerous studies have demonstrated the relationship between the colour of cyanobacteria
66 and the surrounding environment [14-17]. Our research group was the first to report the
67 objective quantification of the colour of cyanobacteria [18] and to later develop a standardized
68 protocol for defining the colour [19]. Use of the protocol enabled our group to establish
69 relationships between i) the colour of cyanobacteria and the pigment content and ii) the
70 changes in both colour and pigment contents and environmental variable including adverse
71 conditions such as those resulting from the application of biocidal products [16, 20-21].
72 Moreover, the close relationship between the pigment content and the colour of cultures was
73 considered in developing linear equations to estimate the pigment contents (chlorophylls and
74 carotenoids) in the cyanobacterium *Nostoc* sp. PCC 9104 via measurement of colour
75 parameters [17].

76 The objective of the present study was to produce cultures of cyanobacterial cells of specific
77 hues, without the need to resort to genetic manipulation, with the overall aim of using the
78 cultures to mitigate the chromatic contrast of new elements in landscapes. With this aim, we
79 analysed the effects of light intensity and nutrient concentration on the colour of the biofilm-
80 forming cyanobacterium *Nostoc* sp. PCC 9104 and used response surface methods to produce
81 a model that could be used to control the colour in the colorimetric range of the organism. We
82 designed screening experiments to determine which the parameters initially considered (light
83 intensity and concentrations of N, P, S, C and Fe) had the greatest influence on the colour of
84 the cultures. We then designed an optimization experiment to generate a model relating the
85 colour and the culture conditions. The model was validated using experimental data, and a
86 psychophysical study was also carried out to validate the model from a visual perspective.

87 **2. Material and methods**

88 **2.1. Batch experiments with cyanobacterial cultures**

89 The experimental work was carried out with *Nostoc* sp. PCC 9104, an aerobic, terrestrial,
90 mesophilic, filamentous N₂-fixing heterocyst-forming cyanobacterium. The growth, pigment

91 content and colour of this strain have already been characterized by our research group [16-18].
92 Thus, regarding the pigment contents, chlorophyll *a* contents of between 10.76 and 72.63 mg l⁻¹,
93 total carotenoids contents of between 1.20 and 4.36 mg l⁻¹ and phycocyanin contents of
94 between 0.3·10⁻² and 6.7·10⁻² mg l⁻¹ were determined in 14 day-old cultures subjected to
95 different growth conditions in terms of nutrients and light intensity [16]. The colour data obtained
96 in the same study also indicated the area of CIELAB space in which this strain defines its
97 colour: from 41.47 to 86.34 for L*, from -23.92 to -2.50 for a*, from 1.30 to 23.57 for b*, from
98 2.94 to 41.00 for C*_{ab} and from 113.60 to 158.80 for h_{ab} [16]. The close relationship between the
99 pigment contents and colour of this cyanobacterium has also been reported [16,17].

100 Axenic cultures of *Nostoc* sp. PCC 9104 were grown in N-free BG11₀ liquid medium [22] in a
101 climatic chamber (SCLAB PGA-1228/2 HR) under stationary conditions at 20°C and with a 12 h
102 light/dark photoperiod (100 μmol photon m² s⁻¹). Cells in the exponential growth phase were
103 collected and used as the inoculum in the experiments.

104 **2.2. Experimental procedure for modelling the colorimetric response of cyanobacterial** 105 **cells under different culture conditions**

106 The response surface methodology [23] was used to model the colorimetric response of the
107 cyanobacterial cells to different culture conditions.

108 Initial screening was carried out with the aim of identifying the most important factors influencing
109 the colour of the cyanobacterial cells. An optimization experiment was then carried out to
110 generate a model relating the colour of the cyanobacteria and the culture conditions. The
111 experimental procedure was carried out with aerated batch cultures (Figure 1), each containing
112 60 mL of various different culture media and 20 mL of the *Nostoc* sp. PCC 9104 stock culture.
113 The culture medium consisted of modified BG-11₀ medium (containing 20 mM HEPES buffer),
114 to which different amounts of nutrients were added in each tube (see Table 1). The batch
115 cultures were maintained with aeration for 14 days under different light intensities (see Table 1)
116 and a 12h:12h light:dark photoperiod. Lighting was provided by fluorescent lamps (Mazda Fluor
117 Lumiere du Jour C9 TF 65, 85 W), and the light intensity was measured with a radiometer (DHD
118 2302.0, HERTER). Water loss by evaporation was corrected daily by the addition of sterilized
119 distilled water.

120 a) Initial screening

121 An incomplete factorial design was used to analyse the influence of environmental conditions on
122 the colour of the cyanobacterial cultures [24]. The design enabled the number of experiments
123 required to estimate the effect of each considered factor on the response (in this case the
124 cyanobacterial colour) to be minimized. The design included 6 factors, each with 2 levels, and a
125 total of 8 assays were carried out (with 2 independent replicates of each assay).

126 On the basis of previous findings on the relationship between environmental conditions and
127 colour changes in cyanobacteria [14,15], including our own findings [16, 17], the following
128 factors were selected for study: light intensity (L) and the concentrations of the various nutrients
129 in the culture medium: NaNO_3 (N), K_2HPO_4 (P), MgSO_4 (S), Na_2CO_3 (C) and
130 $(\text{NH}_4)_5[\text{Fe}(\text{C}_6\text{H}_4\text{O}_7)_2]$ (Fe). The different intensities/concentrations of each factor are shown in
131 Table 1. The ranges are also based on the findings of the aforementioned studies.

132 The cyanobacterial biomass and colour were determined after 14 days. The optical density of
133 each culture was measured at 750 nm (OD_{750}) in a spectrophotometer (UV-Vis Varian Cary
134 100) and used to determine the biomass by applying the linear relationship previously
135 determined for this cyanobacterium ($\text{cell density (g dry biomass L}^{-1}) = 0.002 + 0.441\text{OD}_{750}$, $R^2 =$
136 0.990). Each sample was diluted with BG11₀ medium to yield $0.478 \text{ g dry biomass L}^{-1}$ (value of
137 the sample with minimal cell density) so that the cell density was equal for all samples. Aliquots
138 (1.5 mL) of each sample were filtered through the nitrocellulose discs (pore size, $0.45 \mu\text{m}$ and
139 diameter, 25 mm) on which the cells were deposited and which were used for the colour
140 measurements (Figure 2).

141 Colour measurements were carried out with a Konica Minolta portable spectrophotometer (CM-
142 700d) on the cyanobacterial cell layer deposited on the still humid filters [19]. A total of 8
143 readings were taken from different zones of each filter, under the following conditions: illuminant
144 D65, observer 2° and a 3 mm diameter target area. Equalling the biomass deposited by filtration
145 of the different samples prior to taking the colour readings minimizes the variations in colour due
146 to the white colour of the nitrocellulose discs, as the same baseline can be assumed for all the
147 samples.

148 Colour measurements were analysed using the CIELAB colour system, which represents each
149 colour by means of three scalar parameters or Cartesian coordinates: L*, lightness or luminosity
150 of colour; a*, associated with changes in redness-greenness; and b*, associated with changes
151 in yellowness-blueness. Each colour can also be represented by means of three angular
152 parameters or cylindrical coordinates that are closely related to the psychophysical perception
153 of the colour: L*, lightness or luminosity of colour, also defined in both scalar and angular colour
154 sets; C*_{ab}, chroma or saturation, related to the intensity of colour; and h_{ab}, hue angle or tone of
155 colour, which refers to the dominant wavelength and represents redness, yellowness,
156 greenness, or blueness on a circular scale.

157 Linear regression analysis was used to determine the significance of the effect of each factor
158 and the p values were calculated for each. Statistica 10 software (StatSoft Inc., Tulsa,
159 Oklahoma, USA) was used to design the experiment and to analyse the data obtained.

160 b) Optimization experiment

161 In order to optimize the levels of the variables and to produce a model relating cyanobacterial
162 colour and culture conditions, the results of the initial screening experiment were taken into
163 account (see Results and Discussion). The experiment thus aimed to optimize three factors:
164 light intensity (L), and the concentrations of NaNO₃ (N) and K₂HPO₄ (P). A Box-Behnken design
165 with 3 independent variables (each with 3 levels), measured at 13 experimental points plus 2
166 additional central points (3 central replicates), was applied. This design requires a total of 15
167 experiments, and it is thus more economical and efficient than a 3³ design (27 experiments).
168 The independent variables studied and their levels are listed in Table 3. The colour parameters,
169 measured as described above, were considered the dependent variables.

170 The experimental data were analysed by the response surface methodology (Box & Wilson,
171 1954), implemented using Statistica 10 (StatSoft, Inc., Tulsa OK, USA) software. Results were
172 fitted to a second order equation:

$$173 \quad y = \beta_0 + \sum_{j=1}^k \beta_j x_j + \sum_{j=1}^k \beta_{jj} x_j^2 + \sum_{i < j} \sum \beta_{ij} x_i x_j$$

174 where k is the number of variables (3 in this study: L, N and P), and β_0 , β_j , β_{jj} and β_{ij} are the
175 interception, linear, quadratic and interaction coefficients, respectively.

176 **2.3. Validation of the model**

177 The fitted model produced in the optimization experiment and which relates the culture
178 conditions to the colour of the cyanobacterial cells was validated using experimental data. Eight
179 additional assays were carried out for this purpose (with 3 independent replicates of each). The
180 light intensity (L) and concentrations of NaNO_3 (N) and K_2HPO_4 (P) in the medium were
181 selected in order to yield cyanobacterial cultures of specific colours, according to the model
182 predictions and with the aim of achieving as wide a range of colours as possible. The
183 intensity/concentrations of L, N and P tested are shown in Table 6. The colours of the
184 cyanobacterial cultures (measured as explained above) obtained in these 8 assays were
185 compared with the colour estimates produced by the model, in order to test the effectiveness of
186 the model.

187 **2.4. Psychophysical study**

188 In order to validate the model from a perceptual point of view, a visual sorting task (psycho-
189 physical experiment) was conducted to relate the quantitative values of the different colours of
190 the cultures. The colours were subjectively evaluated by several assessors.

191 Twenty assessors (12 women and 8 men aged between 23 and 65) with normal colour vision,
192 i.e. trichromatic observers (see 25, for details of colour vision examination), were asked to rank
193 a set of photobioreactors containing *Nostoc sp.* PCC 9104 according to the colour hue, from the
194 yellowest to the bluest. The photobioreactors used were obtained in the experiment designed to
195 validate the model and consisted of 4 photobioreactors with h_{ab} values of 128.62° , 137.37° ,
196 144.69° and 147.49° , covering the range of hues produced. If assessors were not able to
197 differentiate between two or more photobioreactors, they were asked to indicate those with the
198 same hue (Figure 3).

199 In order to prevent errors due to a lack of awareness about colour parameters, the observers
200 were previously trained to distinguish colour hues. Training consisted of showing observers
201 Munsell colour chips with the same value and chroma (equivalent to L^* and C^*_{ab} CIELAB

202 parameters) values and with different hue values (equivalent to the h_{ab} CIELAB parameter), all
203 included in the range of hues achieved (Figure 3).

204 The data obtained were analysed using descriptive statistics.

205 **3. Results and discussion**

206 Initial screening was carried out to determine which variables have the strongest influence on
207 the colour of the cyanobacterial cells. Thus, an incomplete factorial design was used to
208 determine the relative importance of the variables light intensity (L) and the concentrations of
209 the main nutrients (N, P, S, C and Fe) in the culture medium (BG11₀). Table 1 shows the
210 colorimetric response of the *Nostoc* sp. PCC9104 culture to the environmental conditions tested
211 in the screening experiment. The wide range of colour obtained indicates the suitability of the
212 parameters selected. The values of the different CIELAB colour parameters ranged from 25.41
213 to 48.55 for L^* , between -24.29 and -18.93 for a^* , between 12.77 and 26.76 for b^* , between
214 23.21 and 34.14 for C^*_{ab} and between 128.40 and 146.63 for h_{ab} (Figure 4). These colour
215 differences, of up to 23 CIELAB units in the case of lightness (ΔL^*) and 9 CIELAB units in the
216 case of the hue (ΔH^*_{ab}), were visible to the naked eye (Figure 5).

217 The regression models fitted to the data obtained in the screening experiment for each of the
218 five CIELAB colour parameters (L^* , a^* , b^* , C^*_{ab} and h_{ab}) indicate that all factors studied (L, N, P,
219 S, C and Fe) significantly affected ($p < 0.05$) at least one of the colour parameters in the cultures
220 (Table 2). The factors C and Fe only affected the lightness (L^*) of the culture and the factor S
221 only affected parameter a^* . By contrast, the variables L, N and P had a stronger effect on the
222 final colour of the cultures, as they had significant effects on several of the CIELAB parameters.
223 In addition, L exerted the opposite effect on the parameters L^* , b^* , C^*_{ab} and h_{ab} than produced
224 by N and P, and the combined effects may therefore be expected to generate a wider range of
225 colours. Considering the goodness-of-fit of the regression models, adjusted R^2 values of 0.90,
226 0.88 and 0.93 were obtained for respectively L^* , b^* and h_{ab} , with L, N and P having the greatest
227 effects on the fits for these three parameters. The factors L, N and P were thus considered the
228 most suitable for inclusion in the optimization experiment as they had the strongest influence on
229 the colour parameters of most value in relation to the overall study objective.

230 In this respect, previous studies have demonstrated that L, N and P are closely related to
231 pigment production in cyanobacteria [see, 14,16,26]. Thus, Collier and Grossman [14] showed
232 that production of chlorophyll-a and phycobiliproteins in *Synechococcus* sp. PCC 7942 is halted
233 almost immediately after N deprivation, but continues for several hours after P deprivation.
234 Limited conditions of light and nitrogen lead to important variations in the concentrations of
235 chlorophyll-a and phycoerythrin in *Cryptomonas* sp., with a significantly greater variation in
236 phycoerythrin under nitrogen stress [26]. Studies carried out with the cyanobacteria *Nostoc* sp.
237 PCC 9025 and *Nostoc* sp. PCC 9104 (the strain used in the present study) have shown that, for
238 both strains, the concentrations of chlorophyll-a and phycocyanins were higher in the cultures
239 grown in the presence of nitrate, although under low light intensity for *Nostoc* sp. PCC 9104 and
240 high light intensity for *Nostoc* sp. PCC 9025. However, the carotenoid concentration was
241 affected by different factors depending on the strain: for *Nostoc* sp. PCC 9104, nutrient
242 availability was the determining factor, whereas for *Nostoc* sp. PCC 9025, the presence of
243 nitrates and high light intensity were more important [16].

244 A Box-Behnken design was used to generate a model relating the colour of the cyanobacteria
245 under study and the most influential environmental parameters (light intensity (L) and NaNO_3
246 (N) and K_2HPO_4 (P) concentrations). The independent variables studied and their levels are
247 listed in Table 3. Colour parameters were considered the dependent variables.

248 The results obtained for the CIELAB colour parameters in the cyanobacterial cultures (Table 3)
249 revealed colour ranges similar to those obtained in the initial screening (Table 1), with values
250 varying between 24.02 and 40.37 for L^* , between -22.89 and -15.02 for a^* , between 10.66 and
251 22.92 for b^* , between 18.42 and 30.19 for C^*_{ab} and between 130.62 and 147.01 for h_{ab} (Table 3,
252 Figure 4). The variations in colour produced in the different assays are visually perceptible
253 (Figures 1 and 6).

254 The resolution of the Box-Behnken design is greater than that of the incomplete factorial design
255 used in the initial screening experiment, enabling estimation of the direct effect of the factors on
256 the response variable (colour) and also of the combined effects and these factors and their
257 interactions. The regression models fitted to the data obtained (Table 4) reveal hue (h_{ab}) to be
258 the only colour parameter on which the factors considered have a significant effect (at $p < 0.05$).

259 Significant fits were obtained for all of the parameters studied; however, in the case of L^* , a^* , b^*
260 and C^*_{ab} , the mean square error, calculated from the three replicates from the central point of
261 the design, was very high. This indicates that for these parameters, the variability inherent in the
262 system (i.e. the variability due to causes other than those controlled in the experiment) was too
263 high to allow any significant effects to be attributed to the factors that were controlled. This, in
264 turn, caused a strong decrease in the adjusted R^2 relative to R^2 , which occurs when variables
265 that do not provide information relevant to the fit are included. For these reasons, the results
266 obtained with the Box-Behnken design cannot be used to generate a model that could reliably
267 control the colour parameters L^* , a^* , b^* and C^*_{ab} in the *Nostoc* sp. PCC 9104 cultures from the
268 factors studied, although they could be used to determine the range of values within which
269 these parameters vary.

270 The regression model for h_{ab} , however, yielded $R^2 = 0.96$, and all of the factors had a significant
271 effect on this parameter, except for the interaction between N and P. As the effect of this
272 interaction was not significant, it was included in the error term of the regression with the aim of
273 generating a better fitting model. Inclusion of the interaction produced a slight increase in the
274 adjusted R^2 , from 0.89 to 0.90, in the new model (Table 5).

275 The new model was used to obtain the following equation for predicting the hue (h_{ab}) acquired
276 by the cyanobacterium *Nostoc* sp. PCC 9104 from the culture conditions (L, N and P):

277

$$278 \quad h_{ab} = 124.754 + 10.559N - 1.742N^2 + 11.550P - 36.181P^2 + 0.181L - 0.001L^2$$
$$279 \quad \quad \quad - 0.024LN + 0.146LP$$

280 where L is the light intensity (in $\mu\text{mol photon m}^2 \text{ s}^{-1}$), N is the concentration of nitrates (in g
281 $\text{NaNO}_3 \text{ L}^{-1}$) and P is the concentration of phosphates (in g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O L}^{-1}$).

282 This four-dimensional model can be visualised using three-dimensional plots in which one of the
283 three factors is held constant (Figure 6).

284 The equation enables cyanobacterial cells of a particular colour to be obtained by varying L, N
285 and P in cultures. The colour is selected on the basis of its hue (the parameter included in the

286 equation). Selection of the colour by using hue as the differentiating parameter has practical
287 advantages, as psychophysical and psychological studies have demonstrated a greater
288 sensitivity to differences in hue than to differences in other parameters such as lightness or
289 colour saturation in test subjects [27, 28]. Moreover, this parameter most closely matches the
290 idea that colour-blind people have of “colour” as it corresponds to the so-called “dominant
291 wavelength” of luminous radiation, which serves the observer to establish similarities with
292 known colours in the spectrum (green, yellow, red, blue, etc.).

293 The validity of the model was tested by carrying out a further 8 assays (with 3 independent
294 replicates of each). The conditions (L, N and P) in these tests were selected (within the ranges
295 studied) from the predictions generated by the model, with the aim of generating specific hues
296 of cyanobacteria within as wide a range as possible (Table 6, Figure 7).

297 Of the 24 experimental values obtained, only two of these (replicate 1 from assay 2 and
298 replicate 2 of assay 7) did not fall within the 95% confidence interval for the model estimates.
299 These results confirm the 92% effectivity of the model for predicting the colour that the
300 cyanobacteria will acquire due to modifications in the culture conditions. The hue ranged
301 between 129° and 147° (minimum and maximum values respectively, generated by the model).
302 The colour range of $h_{ab} = 129^\circ\text{-}147^\circ$ implies a difference in h_{ab} (Δh_{ab}) of 18° and in ΔH^*_{ab} of 12.5
303 CIELAB units. From a colorimetric perspective, colours are considered different when they differ
304 by 3 CIELAB units [see, 28]. The ΔH^*_{ab} difference of 12.5 CIELAB units therefore indicates a
305 significant change in colour (from yellowish-green to bluish-green hues). In this respect,
306 psychophysical research has shown that greens are perceived more readily than any other
307 colour because of the combined colour perception of rods and cones [29]. This is probably an
308 adaptation brought about by human interaction with the natural world, in which greens are
309 predominant.

310 A psychophysical study was carried out with the aim of determining whether the different hues
311 of the cultures can be distinguished by the human eye as well as by a colour-quantifying device.
312 The study was carried out by a group of 20 different observers with normal colour vision. The
313 assessors were asked to rank 4 cultures on the basis of the hue (from yellow to blue) (Figure 3).
314 The results of this study are shown in Table 7.

315 The percentage of correct responses in assigning the hues of the cultures to a scale ranging
316 from yellow to blue was 100% for cultures A and B, and 95% for cultures C and D, in which the
317 correct order was that defined by the experimentally determined value of h_{ab} . Moreover, all
318 assessors were capable of distinguishing between the four cultures according to the hue. These
319 findings demonstrate that the range of values of h_{ab} that the *Nostoc* sp. PCC 9104 culture can
320 acquire in response to modifications in the culture conditions is sufficiently large to include hues
321 that can be differentiated by the human eye and that can be visually assigned to a scale.

322 Thus, in the present study we demonstrated that the colour of cyanobacterial cells can be
323 determined by controlling certain culture conditions. This study reports of a model that can be
324 used, with an efficacy of 92%, to manipulate and predict the colour of the cyanobacterium
325 *Nostoc* sp. PCC 9104 by varying the light intensity and concentrations of N and P in cultures of
326 the organism. Use of the model has enabled modification of the colour of a strain of
327 cyanobacteria, within a range of 18° of the hue, without the need for genetic manipulation of the
328 microorganism. The colour modifications induced were clearly visualized and differentiated by a
329 group of observers who validated the model from a perceptual perspective.

330 The findings presented here represent progress in the field of environmental technology, as the
331 range of colour of cyanobacterial cells can be altered by modifying only nutrient inputs and the
332 amount of light received. This new biotechnological resource can be used in natural
333 environments to protect and restore environments. In this respect, control of the colour of
334 cyanobacterial cultures has applications in correcting visual impacts as well as in designing
335 landscapes and harmonious urban environments, as it enables surface discolouration (or other
336 unwanted effects) to be masked by using live organisms and taking into account the colour of
337 the natural surroundings.

338 **4. Conclusions**

339 Here, we report the use of a mathematical model to shape the colour of cyanobacterial cells by
340 modifying only nutrient inputs and the amount of light, without the need to resort to genetic
341 manipulation. Use of the model enabled us to produce cultures of perceptibly different hues
342 ranging from yellowish-green to bluish-green. Control of the colour of cyanobacterial cultures
343 has applications in correcting visual impacts as well as in the design of landscapes and

344 harmonious urban environments, as it enables surface discolouration (or other unwanted
345 effects) to be masked by using live organisms and taking into account the colour of the natural
346 surroundings. The model was validated using experimental data, and a psychophysical study
347 was also carried out to validate the model from a visual perspective.

348 **Acknowledgements**

349 This study was partly financed through grant GRC2014/028 (Xunta de Galicia) and project
350 CGL2016-79778-R (Ministerio de Economía, Industria y Competitividad). D. Vázquez-Nion is
351 financially supported by a postdoctoral contract ED481B/2017/016 (Xunta de Galicia). P.
352 Sanmartín is financially supported by a postdoctoral contract POS-B/2016/030 (Xunta de
353 Galicia). The authors thank all the observers who generously agreed to collaborate in the
354 psychophysical study.

355 **Declaration of authors' agreement to authorship and submission of the manuscript for** 356 **peer review**

357 The authors wish to declare our agreement to authorship and submission of the manuscript for
358 peer review.

359 **Declaration of authors' contributions**

360 B. Prieto and P. Sanmartín conceived of the idea and designed the experiments. D. Vázquez-
361 Nion performed the research and analysed the data. B. Prieto, D. Vázquez-Nion and P.
362 Sanmartín wrote the manuscript. B. Silva supervised the research.

363 **Conflict of Interest Statement**

364 The authors declare no conflicts of interest.

365 **Informed consent, human/animal rights**


366 No conflicts, informed consent, human or animal rights applicable.

367

368 **References**

- 369 1. Díez B, Ininbergs K. 2014. *Ecological importance of cyanobacteria*, in *Cyanobacteria: An*
370 *Economic Perspective*. In *Cyanobacteria*; Sharma, N. K.; Rai, A. K.; Stal, L. J., Eds. John Wiley
371 & Sons: Chichester, U.K.
- 372 2. De Philippis R, Sili C, Paperi R, Vincenzini M. 2001. Exopolysaccharide-producing
373 cyanobacteria and their possible exploitation: A review. *J Appl Phycol* 13: 293–299.
- 374 3. Ladygina N, Dedyukhina EG, Vainshtein MB. 2006. A review on microbial synthesis of
375 hydrocarbons. *Proc Biochem* 41: 1001-1014.
- 376 4. Rupprecht J, Hankamer B, Mussgnug JH, Ananyev G, Dismukes C, Kruse O. 2006.
377 Perspectives and advances of biological H₂ production in microorganisms. *Appl Microbiol*
378 *Biotechnol* 72: 442–449.
- 379 5. Abed RMM, Dobretsov S, Sudesh K. 2009. Applications of cyanobacteria in biotechnology. *J*
380 *Appl Microbiol* 106: 1-12.
- 381 6. Biswas G, Thakurta SG, Chakrabarty J, Adhikari K, Dutta S. 2018. Evaluation of fluoride
382 bioremediation and production of biomolecules by living cyanobacteria under fluoride stress
383 condition. *Ecotoxicol Environ Saf* 148:26–36.
- 384 7. Rossi F, Hua L, Liu Y, De Philippis R. 2017. Cyanobacterial inoculation (cyanobacterisation):
385 perspectives for the development of a standardized multifunctional technology for soil
386 fertilization and desertification reversal. *Earth-Sci Rev* 171:28–43.
- 387 8. Huang Y, Li P, Chen G, Peng L, Chen X. 2018. The production of cyanobacterial carbon
388 under nitrogen-limited cultivation and its potential for nitrate removal. *Chemosphere* 190:1-8.
- 389 9. Gorbushina AA, Broughton WJ. 2009. Microbiology of the atmosphere–rock interface: how
390 biological interactions and physical stresses modulate a sophisticated microbial ecosystem.
391 *Annu Rev Microbiol* 63: 431–450.

- 392 10. Pinto V, Font X, Salgot M, Tapias J, Mana T. 2002. Image analysis applied to quantitative
393 evaluation of chromatic impact generated by open-pit quarries and mines. *Environ Geol* 41:
394 495–503.
- 395 11. Prieto B, Silva B, Aira N, Laiz L. 2005. Induction of biofilms on quartz surfaces as a means
396 of reducing the visual impact of quartz quarries. *Biofouling* 21(5-6): 237-46.
- 397 12. Prieto B, Silva B, Aira N. 2006. Methodological aspects of the induction of biofilms for
398 remediation of the visual impact generated by quartz mining. *Sci Total Environ* 370(1): 254-261.
- 399 13. Prieto B, Silva B, Aira N. 2008. Recycling of waste products in the induction of biofilms to
400 remediate the visual impact generated by quartz mining. *Bioresour Technol* 99(8): 2887-2892.
- 401 14. Collier JL, Grossman AR. 1992. Chlorosis induced by nutrient deprivation in *Synechococcus*
402 sp. strain PCC 7942: not all bleaching is the same. *J Bacteriol* 174: 4718-4726.
- 403 15. Kehoe DM, Gutu A. 2006. Responding to color: The regulation of complementary chromatic
404 adaptation. *Annu Rev Plant Biol* 57: 127-150.
- 405 16. Sanmartín P, Aira N, Devesa-Rey R, Silva B, Prieto B. 2010. Relationship between color
406 and pigment production in two stone biofilm-forming cyanobacteria (*Nostoc* sp. PCC 9104 and
407 *Nostoc* sp. PCC 9025). *Biofouling* 26(5): 499-509.
- 408 17. Vázquez-Nion D, Sanmartín P, Silva B, Prieto B. 2013. Reliability of color measurements for
409 monitoring pigment content in a biofilm-forming cyanobacterium. *Int Biodeterior Biodegradation*
410 84: 220-226.
- 411 18. Prieto B, Rivas T, Silva B. 2002. Rapid quantification of phototrophic microorganisms and
412 their physiological state through their colour. *Biofouling* 18: 229–236.
- 413 19. Prieto B, Sanmartín P, Aira N, Silva B. 2010. Color of cyanobacteria: some methodological
414 aspects. *Appl Optics* 49: 2022–2029.
- 415 20. Sanmartín P, Villa F, Silva B, Cappitelli F, Prieto B. 2011. Color measurements as a reliable
416 method for estimating chlorophyll degradation to phaeopigments. *Biodegradation* 22(4): 763–
417 771.

- 418 21. Sanmartín P, Villa F, Polo A, Silva B, Prieto B, Cappitelli F. 2015. Rapid evaluation of three
419 biocide treatments against the cyanobacterium *Nostoc* sp. PCC 9104 by color changes. *Ann*
420 *Microbiol* 65(2): 1153-1158.
- 421 22. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Genetic assignments,
422 strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* 111: 1–61.
- 423 23. Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. 2008. Response surface
424 methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* 76: 965-977.
- 425 24. Montgomery DC. 1997. *Design and Analysis of Experiments*, 4th Edition, New York, NY,
426 Wiley.
- 427 25. Fletcher R, Voke J. 1985. *Defective colour vision: fundamentals, diagnosis and*
428 *management*. Boston (MA): Adam Hilger Ltd. 624 pp.
- 429 26. Sciandra A, Lazzara L, Claustre H, Babin M. 2000. Responses of growth rate, pigment
430 composition and optical properties of *Cryptomonas* sp to light and nitrogen stresses. *Mar Ecol*
431 *Prog Ser* 201: 107-20. 
- 432 27. Martínez-Verdú F. 2002. Revisión de los aspectos cromáticos sobre la captura de imágenes
433 (II). *Óptica Pura Aplic* 35: 61–75.
- 434 28. Berns RS. 2000. *Billmeyer and Saltzman's principles of color technology*. 3rd ed. New York
435 (USA): John Wiley & Sons. 272 pp.
- 436 29. Gordon J, Abramov I. 1977. Color vision in the peripheral retina: II. Hue and saturation. *J*
437 *Opt Soc Am* 67: 202–207.

438 **Figure captions**

439 Figure 1. Appearance of the photobioreactors cultured for 14 days under the conditions
440 considered in the optimization experiment (Box-Behnken design). The numbers correspond to
441 the different assays.

442 Figure 2. Appearance of cultures after being filtered at the same cell density for colour
443 measurements. Differences in colour can be appreciated for photobioreactors 8 (top right), 9
444 (bottom right), 10 (top left) and 11 (bottom left) from the Box-Behnken design.

445 Figure 3. Set-up of the psychophysical study. Left: photobioreactors to be ranked by observers.
446 Right: Munsell chips used to train observers to distinguish colour hues.

447 Figure 4. Polar diagram representing the colours of the cultures with the C^*_{ab} and h_{ab}
448 coordinates.

449 Figure 5. Appearance of the photobioreactors cultured for 14 days under the conditions tested
450 in the screening experiment. The numbers correspond to the different assays.

451 Figure 6. Representations of the response surface for values of (A) $P = 0.26 \text{ g K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$
452 L^{-1} , (B) $N = 1.5 \text{ g NaNO}_3 \text{ L}^{-1}$ and (C) $L = 135 \mu\text{E m}^2 \text{ s}^{-1}$.

453 Figure 7. Representation of the results obtained for the model validation. The bars represent the
454 95% confidence intervals for the expected values.

455