The color of cyanobacteria: some methodological aspects

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Abstract

Although the color of cyanobacteria is a very informative characteristic, no standardized protocol has so far been established for defining the color in an objective way, and therefore direct comparison of experimental results obtained by different research groups is not possible. In the present study, colorimetric measurements and conventional statistical tools were used to determine the effects on the measurement of the color of cyanobacteria, of the concentration of the microorganisms and their moisture content, as well as of the size of the target area and the minimum number of measurements. It was concluded that the color measurement is affected by every factor studied, but that this can be controlled for by making at least 10 consecutive measurements/9.62 cm^2 at different randomly selected points on the surface of filters completely covered by films of cyanobacteria in which the moisture contents are higher than 50%.

Keywords: CIELAB system, colorimetry, cyanobacteria, minimum number of measurements, protocol.

Introduction

Although color is one of the defining characteristics of cyanobacteria, and provides the name of blue-green algae, it has so far not been defined in an objective way. Many studies have explored the color changes that cyanobacteria undergo in response to alterations in the energy distribution in the light spectrum, but none of these studies provide colorimetric data expressed in any of the standard CIE color spaces: CIE XYZ, CIELUV, CIELAB [1, 2]. Likewise, in the scientific literature on cyanobacteria, objective terms are not used to refer to the color of the microorganisms. Thus for instance, bleaching or chlorosis is defined as color change of the cells from “blue-green” to “yellow-green” by phycobilisome degradation, and nitrogen chlorosis is defined as the ‘yellowing’ of cyanobacterial cells following the onset of nitrogen starvation. In some cases the language used is more precise and more specific names of colors, such as red, green, blue and yellow are given. However, as communication of the color has an important cultural component sometimes is difficult to know the real meaning of the words.

The characteristic blue-green color of the cyanobacteria is due to their photosynthetic pigment composition: chlorophyll *a* which is a greenish pigment that makes photosynthesis possible by passing on charged electrons to other molecules to manufacture energy, and phycobiliproteins, which capture light energy and are exclusive of cyanobacteria. Between phycobiliproteins, phycocyanin is responsible for the blue color. Other colored photosynthetic pigments in cyanobacteria are phycoerythrin, pink, phycoerythrocyanin, purple, and allophycocyanin, greenish-blue, which are phycobiliproteins, and the yellow-orange pigments called carotenoids.

As pigment content is closely associated with environmental conditions such as nitrogen source, light intensity, light quality, and nutrient availability, among others parameters [3-6], variation in environmental conditions give raise to an appreciable change in the color of cyanobacteria cultures. Thus for example, in some cyanobacteria, the light quality, i.e. the relative number of photons of blue, green, red, far red and other portions of the light spectrum emitted from a light source, influences the composition of phycobilisomes. In green light, the cells accumulate more phycoerythrin, whereas in red light they produce more phycocyanin and hence the bacteria appear green in red light and red in green light. This process is known as complementary chromatic adaptation and is a way for the cells to maximize the use of available light for photosynthesis.

Taking into account relationships between pigment content and color, and pigment content and environmental conditions, the health of the cultures or the influence of environmental changes could be assess by their color. To date no objective color data has been report in relation to cultures because there is no a standardized protocol for the measurement of cyanobacterial color, even thought is a common practice in microbiological laboratories to make a first assessment of the health of the cultures by visual inspection.

Thus, the objective measurement of color is of great importance not only for a common understanding among researchers but also for carrying out scientific studies on ecology and physiology of cyanobacteria. In this sense, it is important to bear in mind that the objective measurement of color is affected by the color measuring devices used and the protocol applied, and comparable results should be obtained by different operators and instruments. As regards the devices, it must to be taken into account that the discrepancies in the color obtained by several contact-type color measuring devices are due to integration of the field of view, which is circular and of a size determined by the diameter of the measuring head (3-60 mm), and only highly homogeneous colors will be unaffected by this fact.

As regards the protocol, objective colorimetric characterization of cyanobacteria has been carried out in only two studies [7, 8]. In the first study [7], organisms were previously deposited on a 47 mm diameter acetate filter by means of vacuum filtration of 5 ml of culture, five times (i.e. one ml each time) to obtain a homogeneous distribution of the cells in a circular area (35 mm diameter); the color of the deposits was then measured with a contact-type color measuring device. To obtain a representative color of the cyanobacteria culture, 10 sequential circular measurements of 8 mm diameter were made and color was expressed as the average of the 10 measurements, in the CIELAB color space. The procedure yielded good results, and was used in a subsequent study [8]. However, the 10 measurements used in the former studies cannot be considered as standard since the diameter of the circular measurement head differs from one device to other, and as demonstrated with other types of surfaces [9], the minimum number of measurements required to characterize the color of a surface depends on the diameter of the measuring head, amongst other factors. Thus, to standardize the measurement of the color of cyanobacteria in order to obtain reproducible and comparable results, independently of the dimensions of the measuring head of the device, the number of measurements required in relation to the surface area must first be determined.

Furthermore, taking into account that in the first study [7], the moisture content of the cyanobacteria was found to affect the colorimetric characterization, relationships between moisture content and colorimetric parameters must be studied to obtain sufficient information to enable selection of the most appropriate conditions for standardization of the measurement of cyanobacterial color.

In view of above, and with the aims of encouraging communication between researchers and of obtaining an standardized protocol for the measurement of cyanobacterial color, the number of measurements, in relation to the surface area, required to characterize their color independently of the dimensions of the measuring head of the device and the amount of cyanobacteria, and the proper moisture content of the microorganisms to obtain reproducible and comparable results were established by a combination of colorimetric and statistical methodology.

Materials and methods

Experiments were carried out on a mixed culture (713 μg ml^{-1}) of three filamentous N_2-fixing heterocyst-forming cyanobacteria: *Nostoc* sp. strain PCC 9104, *Nostoc* sp. strain PCC 9025 and *Scytonema* sp. CCC 9801. All strains were grown in BG-11_0 medium [10], a diazotrophic culture medium commonly used for nitrogen-fixing strains, composed of: $K_2PO_4.3H_2O$ (0.04 g l^{-1}) + $MgSO_4.7H_2O$ (0.075 g l^{-1}) + $CaCl_2.2H_2O$ (0.036 g l^{-1}) + Citric acid (0.006 g l^{-1}) + Ferric ammonium citrate (0.006 g l^{-1}) + EDTA (0.001 g l^{-1}) + Na_2CO_3 (0.02 g l^{-1}) + Trace metal mix A5 (1 ml l^{-1}): H_3BO_3 (2.86 g l^{-1}) + $MnCl_2.4H_2O$ (1.81 g l^{-1}) + $ZnSO_4.7H_2O$ (0.222 g l^{-1}) + $Na_2MoO_4.2H_2O$ (0.39 g l-1) + $CuSO_4.5H_2O$ (0.079 g l^{-1}) + $Co(NO_3)_2.6H_2O$ (0.0494 g l^{-1}) + distilled water.

Depending on the experiment, five replicates of different aliquots of the culture were filtered under vacuum through nitrocellulose filter discs (0.45 μm and 47 mm diameter) to achieve 9.62 cm^2 of coated area (35 mm diameter). The color of the coated areas of the filters (Fig 1) was then measured with one or two contact-type color measuring devices.

To determine the appropriate range of humidity of the cyanobacteria for characterization of their color, a larger volume (20 ml) of the culture was filtered to achieve complete coating of the filter. In this case, the filters were first dried in an oven (105ºC) to constant weight. The filters with the cyanobacterial deposits were then immersed in water until saturated, then air-dried, and color and weight were measured at different times (10, 20, 30, 45, 60, 90, 120 and 150 minutes) until the filters appeared completely dry. Finally the filters were dried in an oven (at 105 º C) to constant weight to enable determination of the moisture content of the cyanobacteria.

The color was measured with a GretagMacbeth portable spectrophotometer (CE-XTH) with two diameter viewing apertures of 5 and 10 mm. The following measuring conditions were selected: 5 mm viewing aperture, illuminant D65, which represents a typically phase of daylight, with a CCT of approximately 6500K, and 2-degree observer (CIE 1931). The measurements were made by spectral reflectance, by use of diffuse illumination geometry, with an integration sphere, covered with a white material, so that the light is uniformly diffuse in all directions illuminating the sample, and is observed with the specular component included. A total of 20 measurements were made consecutively at different randomly selected points on the surface of the filters, in order to obtain sufficient data to characterize the color. The results were expressed as the average of only 10 measurements since this was established as the minimum number of measurements required for each 9.62 cm^2 of surface (see results section).

Color measurements were pointed in the CIELAB color space, the most perceptually uniform of the color spaces [11, 12, 13]. The CIELAB system is one widely used systems (since 1976) for calculating color differences for most practical applications. Use of the CIELAB system enables estimation of three color parameters: L*, a* and b*, where L* represents lightness (a value of 100 indicates white, and a value of 0, black), a* is associated with changes in redness-greenness (positive a* is red and negative a* is green) and b* is associated with changes in yellowness-blueness (positive b* is yellow and negative b* is blue). The three parameters are plotted on three orthogonal axes in a Cartesian coordinate system. In addition, the classic CIELAB formula (ΔE*ab) [2] and three CIELAB-based color-difference formulae (ΔE94 (1:1:1) [14], CMC (2:1) [15] and ΔE00 (1:1:1) [11]) were applied.

In order to determine the minimum number of measurements required to characterize the color of cyanobacteria, independently of the dimensions of the measuring head of the device and the concentration of the microorganisms, three dilutions (1/5; 1/2 and 1/1) of the culture were prepared (to provide relative concentrations of 20%, 50% and 100%), and 5 ml aliquots were filtered. As a control, a 5 ml aliquot of the culture medium was also filtered (0% concentration). In this case, the color was measured with a GretagMacbeth portable spectrophotometer (CE-XTH) with two diameter viewing apertures of 5 and 10 mm, and a Minolta colorimeter, with one measuring head (CR-300) with an 8 mm-diameter viewing area. Thus, the areas measured with the two devices were circular areas of 5, 8 and 10 mm diameter. The same measuring conditions as above were fixed in both devices so that the measurements were comparable.

A total of 20 measurements were made consecutively, with each of the three measuring heads, of 5, 8 and 10 mm diameter, at different randomly selected points on the surface of the filters within the appropriate range of moisture contents (see results section).

All of the data were subjected to multivariate analysis of variance (MANOVA) and Tukey's b multiple comparison test, by use of SPSS (version 15.0).

Results and discussion

Range of moisture contents

The variations in the L*, a* and b* values for the cyanobacteria deposits with moisture content (expressed as percentage) are shown in Fig 2. The values of the L* parameter were relatively low in 28.07-20.00 CIELAB units. The values of the a* chromatic parameter were negative, ranging from -1.21 to -2.51 CIELAB units, whereas the values of the b* chromatic parameter were positive, ranging from 10.46 to 4.25 CIELAB units, so that the color of the cyanobacteria was within the zone of the yellow-greenish colors, regardless of the moisture content (Fig 3).

On the other hand, L* initially decreased rapidly with increasing moisture content (Fig. 2). However, it remained practically constant for moisture contents higher than 50% since the variation in that part of the curve is only of 2 L* units, which is below the perceptibility threshold [16, 17]. This was not observed with the a* and b* parameters, for which differences between ranges of moisture content was not noticeable, since there was no variation in these parameters with moisture content. Moreover, the results of the MANOVA, with the CIELAB color parameters L*, a* and b* as dependent variables, and the moisture content as the independent variable confirmed statistically significant differences only among the L* values obtained at the different moisture contents (Wilks' lambda: 0.024; F: 26.294; df:69; Sig.: 0.000). Therefore, L* is the only CIELAB parameter related to moisture content, and decreased exponentially with increasing humidity, so that moisture content clearly affected the lightness values, as it caused a darkening of the color. A similar result was obtained in a previous study [7], in which variation in the L* parameter with moisture content was observed during the 15 days during which samples were left to dry, but not thereafter.

The above results clearly suggest that in order to obtain comparable results, color measurements must be made on test samples with a moisture content of more than 50% as in that range, the L*, a* and b* values remain fairly constant. For determining the color of liquid cultures deposited on filters, the measurements must be made immediately after deposition of cyanobacteria, while the microorganisms are still moist; when determining the color of cyanobacteria colonising surfaces, such as building facades, the measurement protocol should ensure that the moisture content of the surface is above 50%.

Minimum number of measurements

In order to determine the minimum number of measurements required to characterize the color of cyanobacteria, the cumulative averages of the CIELAB color parameters (L*, a* and b*) were plotted for each of the filters. The general shape of the graphs obtained was an inverted exponential decay model with a horizontal asymptote, with the stable section corresponding to the number of measurements after which the mean become constant; consequently, the first point of this section of the curve represents the minimum number of measurements required to characterize the L*, a* and b* coordinates for each case. By way of example, the plots obtained for one of the filters are shown in Fig 4. In each of the three graphs, the number of measurements after which the horizontal asymptote is reached and the mean becomes constant, is indicated by the marked segment. The minimum number of measurements required for characterization of each color coordinates, for the coated area of each filter and for each measuring head is shown in Table 1. The number of measurements required was different for each of the coordinates (L*, a* and b*).

The data were subjected to MANOVA, with the minimum number of measurements determined for L*, a* and b* as dependent variables, and concentration of the microorganisms and diameter of the measuring head as independent variables. The results of the analysis revealed that both the concentration of microorganisms and the diameter of the measuring head produced significantly different results in terms of the minimum number of measurements required to characterize L*, a* and b*. The results, including Wilks' lambda, the F-factor, the level of significance and the number of degrees of freedom are shown in Table 2 and Table 3, and the significant differences between the average value of number of measurements required to define L*, a* and b* for each organisms concentration and measurement head are indicated. There were significant differences in the minimum number of measurements required for color characterization of the filters with concentrations of 0 or 100% microorganisms and those with 20 or 50% microorganisms, for all three CIELAB color parameters (L*, a*, b*), with fewer measurements required for the former than the latter. This may be attributed to the greater heterogeneity of the color of the measured area corresponding to the filters with intermediate concentrations of microorganisms (20% and 50%): as the deposits did not cover the whole area (Fig. 1) this gave rise to an irregular layer of microorganisms in which two very different colors - the yellow-greenish, fairly dark color of the cyanobacteria, and the pure, bright white color of the filters - appeared together. Thus, depending on the concentration of filtered organisms, these chromatic patches on an achromatic support provided different scales of heterogeneity, which did not exist in the filters without cyanobacteria (0%) and was almost absent in the filters with 100% microorganisms, as a rather homogeneous layer of microorganisms was obtained. The minimum number of measurements required also increased with the heterogeneity of the color of the filters (Table 1).

Regarding the measuring head, the minimum number of measurement required to characterize b* was independent of this factor, while the significant differences for L* and a* were determined by the 5 mm measuring head, for which the number of measurements required was greater than for the 8 and 10 mm measuring heads (Table 3).

Since the color value of each of the five filters with the same concentration of microorganisms must be approximately equal, irrespective of the number of measurements, the greatest differences in partial and total color between filters with the same concentration of microorganisms were calculated. The number of measurements required to characterize the color of each filter (Table 4) was the highest number of measurements from among those for L*, a* and b* (Table 1) for each filter and each measuring head. Assuming a large tolerance of 5 CIELAB units [16,17,18], partial (ΔL*, Δa*, Δb*) and total (ΔE*ab, ΔE94(1:1:1), CMC(2:1), ΔE00(1:1:1)) color differences between filters with the same concentration of microorganisms were not perceptible (Table 5), and were far from the 6 CIELAB units considered as a perceptible but acceptable difference in color [19]. In addition, taking into account previous studies in which 3 CIELAB units are considered as the upper limit of perceptibility of the color [8,20,21], all values of the total color differences obtained, except those obtained with the 8 and the 5 mm measuring heads for filters with a covering of 20% microorganisms were below this threshold.

The total color differences were minimized by use of the newer and improved color formulae, i.e. ΔE94 (1:1:1), CMC (2:1) and ΔE00(1:1:1). It was also found that there was no equivalence of scale factor among the results obtained with the three formula considered. Moreover, the changes in total color were mainly produced by the partial differences of the lightness ΔL*, except for filters with 0% microorganisms, in which the changes were mainly due to Δb*, owing to the tendency to yellowing of nitrocellulose filters when they are exposed to ultraviolet light. The greatest effect of ΔL* on the filters with concentrations of microorganisms that gave rise to an irregular layer (20, 50 and 100%) was due to the textured nature of the samples, where L* is the color parameter that varied most between the white and green patches. A reduction in the total color differences would be unfeasible even if more measurements were made, as the values of L* a* b* would not change (Fig1).

Since the minimum number of measurements required varied with the color parameter, the concentration of microorganisms and the diameter of the measuring head, the minimum number required to characterize the color of cyanobacteria irrespective of the concentration of microorganisms would be the largest number obtained for all the concentrations tested for each measuring head. Thus, 10 measurements/9.62 cm^2 are required for measuring heads of 8 and 5 mm diameter and 8 measurements/9.62 cm^2 for a measuring head of 10 mm diameter.

Influence of the target area diameter

Once the minimum number of measurements was established in relation to the dimensions of the measuring head, the color values obtained with each measuring head were compared in order to establish whether the results were equivalent. The result of a MANOVA with values of L*, a* and b* as dependent variables and the diameter of the measurement head as the independent variable revealed no statistically significant differences ($p > 0.05$) among the values obtained with the different measuring heads. Therefore, the color characteristics obtained with the different measuring heads are comparable when the established minimum number of measurements is applied (see previous section).

From a practical point of view, and taking into account that in heterogeneous surfaces an increase in the field of view of the device reduces the effect of the different colors in the target area, with the consequent similarity in sequential measurements, the use of a 10mm measuring head is more convenient, as fewer measurements are required (8/9.62 cm^2) and the error derived from heterogeneity in the filter color is also reduced.

Conclusions

The results of the study demonstrate the influence of both the microorganisms and the measuring instrument on the characterization of the color of cyanobacterial biofilms. Instrument properties, such as the diameter of the measuring head, affect the minimum number of measurements required to characterize the color, and properties of the microorganisms, such as concentration and moisture content, affect both the minimum number of measurements required and the color obtained.

The minimum number of measurements required increased with increasing heterogeneity of the color of the area measured, which depended on the concentration of the microorganisms, and decreased, with the diameter of the measuring head. To control for the influence of the heterogeneity of the color of the area measured, the color of cyanobacteria should be measured on filters that are completely covered by the microorganisms, so that the white color of the filter is hided. The influence of the size of the measuring head is more difficult to control for, as researchers usually only have one color measuring device available. However, if the number of measurements corresponds to those established in this study in relation to the measuring head diameter, results thus obtained will be comparable. It was established that a total of 10 measurements/9.62 cm^2 are required for measuring heads of 8 and 5 mm diameter, and 8 measurements/9.62 cm^2 for a measuring head of 10 mm diameter. We therefore suggest that, in order to standardize measurement of the color of cyanobacteria, 10 measurements/9.62 cm^2 are sufficient for characterization of the color, regardless of the dimensions of the measuring head of the device.

However, we also recommend that when possible, a 10mm measuring head should be used, as fewer measurements are required (8/9.62 cm^2) and the error derived from heterogeneity in the color of the filter is reduced.

The L* values were greatly affected by the moisture content of the samples, whereas the chromatic parameters a* and b* were unaffected. Since the values of L* decreased exponentially with moisture contents up to 50%, which can be considered as the point from which a stable asypmtote is reached on the corresponding graph, characterization of the cyanobacterial color should be made with samples with moisture contents of more than 50%. Otherwise the results will not be comparable.

The methodology here proposed allows objective measure of color of cyanobacteria which would be very useful in those studies where the knowledge of pigment content variations lead to conclusions. Moreover it provides some advantages on traditional methodology as is non destructive and saves time and materials.

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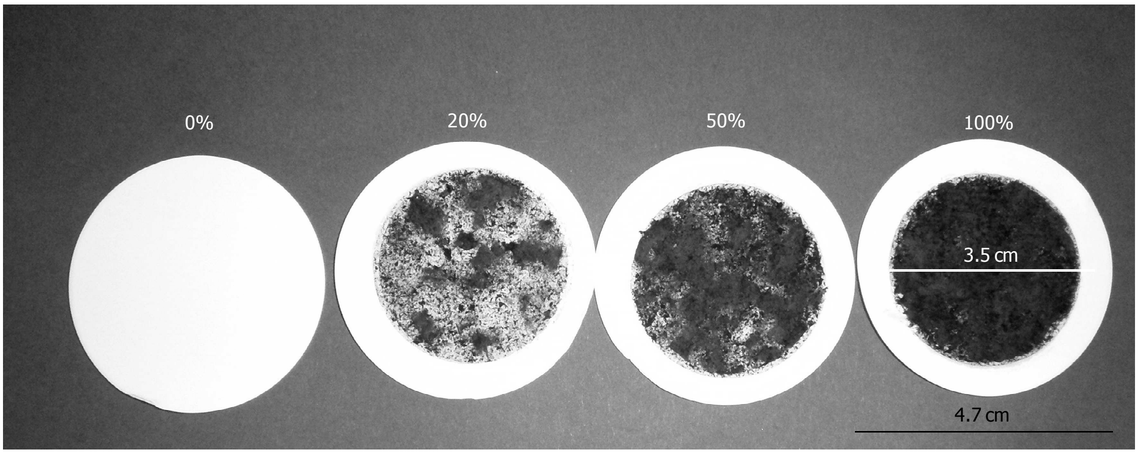

Figure 1. Appearance of the filters after depositing the aliquots of cultures containing 0, 20, 50 and 100% cyanobacteria.

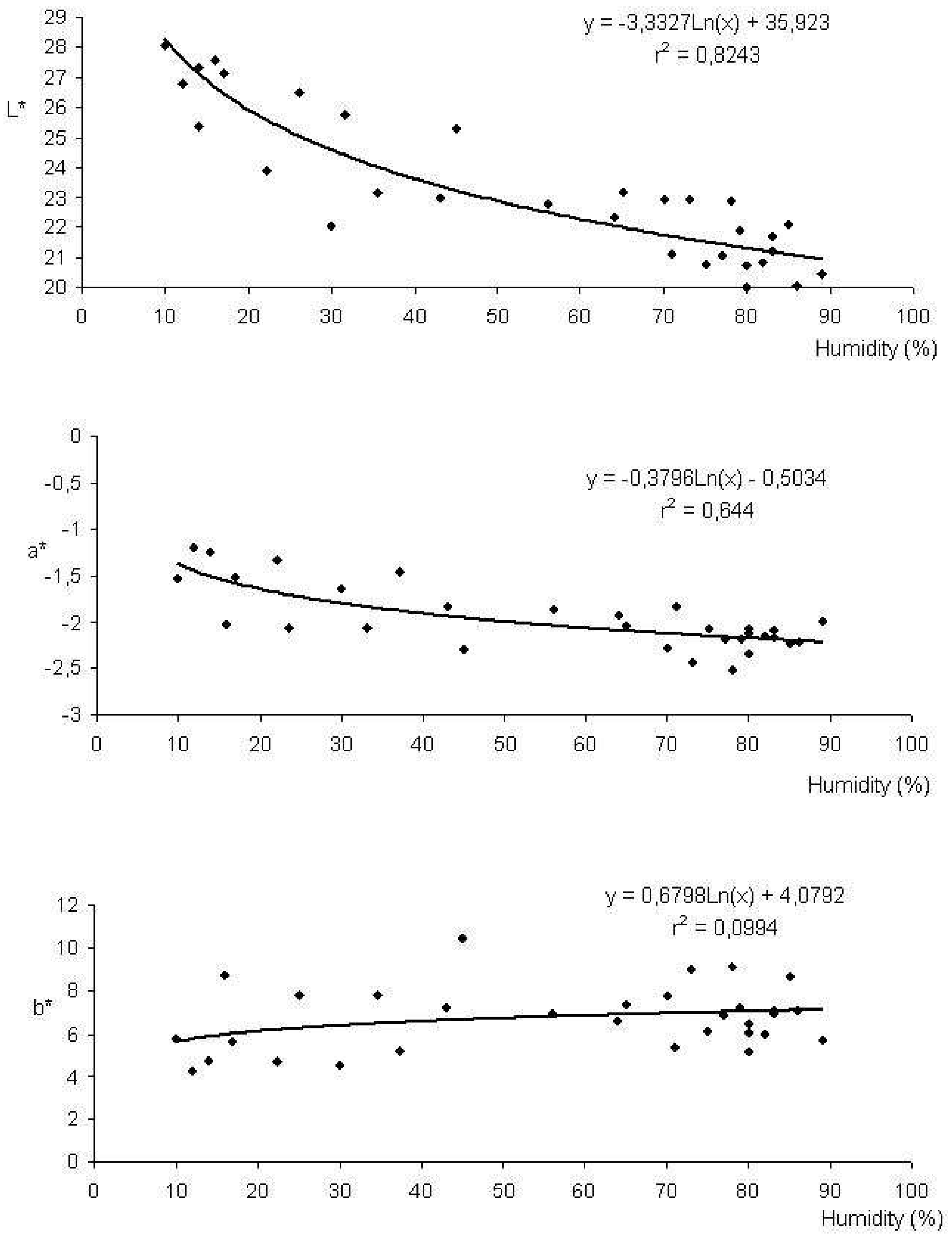

Figure 2. Variation in L*, a* and b* values of the filters containing cyanobacteria deposits with the moisture content (%).

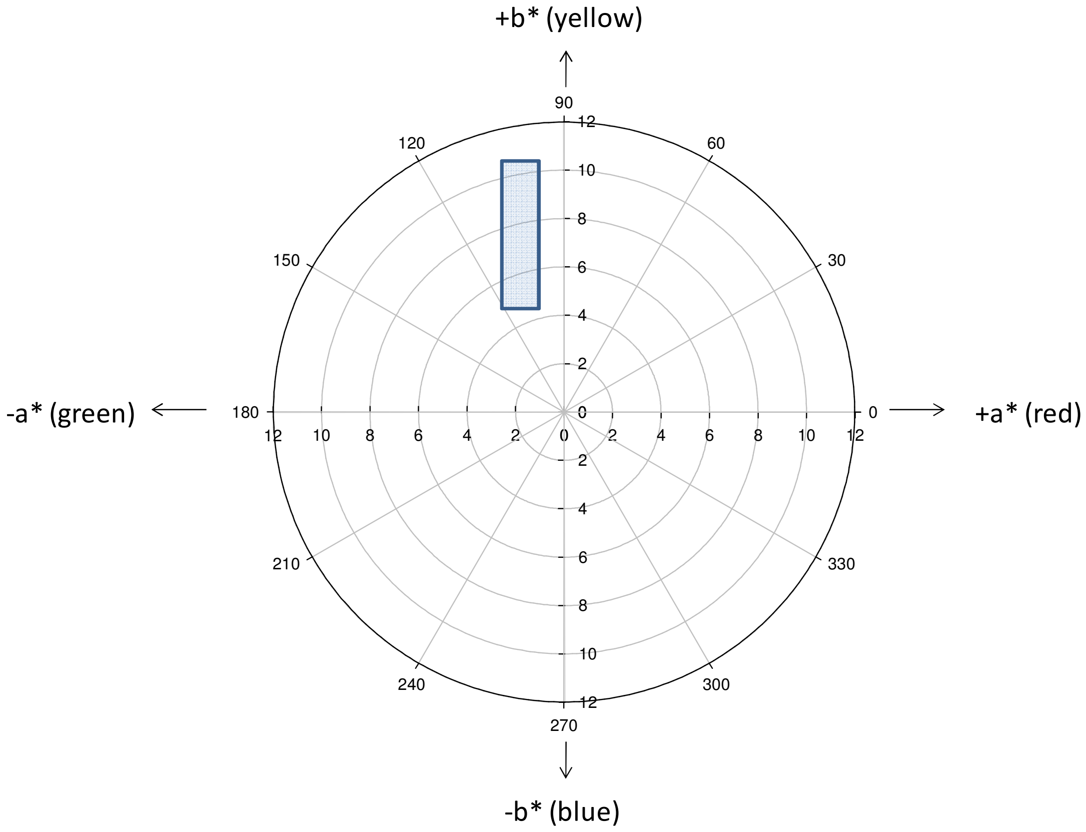

Figure 3. a*-b* diagram representing the yellow-greenish area corresponding to the filters containing cyanobacteria in relation to range of moisture contents.

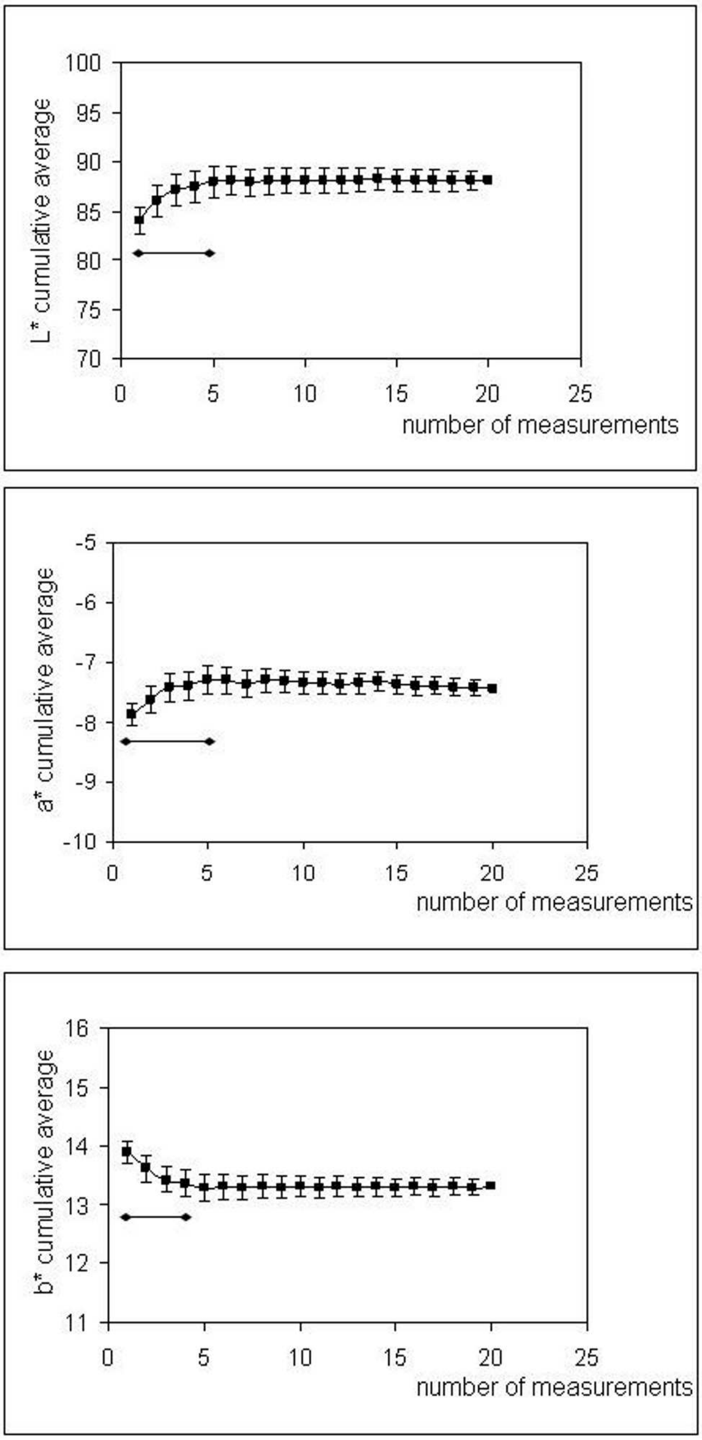

Figure 4. Example of the graphs used to determine the minimum number of measurements required. Cumulative averages of the CIELAB color parameters: L* a* b* in relation to the number of measurements, corresponding to filters containing 20% cyanobacteria, filter *b*, 10 mm measurement head.

Table 1: Minimum number of measurements determined for characterization of each CIELAB color parameter, for each filter with the different concentrations of cyanobacteria, and for different measuring heads.

Dilution (%)	**Filter**	**Diameter of the measuring head (mm)**								
		10			**8**			**5**		
		L*	**a***	**b***	**L***	**a***	**b***	**L***	**a***	**b***
0	a	2	3	4	2	2	2	2	3	3
	b	2	2	2	2	2	2	2	2	2
	c	2	2	2	2	2	2	2	2	2
	d	2	2	2	2	2	2	3	3	2
	e	2	2	2	2	2	2	2	2	2
20	a	3	5	3	3	3	3	6	5	3
	b	5	5	4	8	3	3	8	7	4
	c	6	6	5	4	4	4	3	3	3
	d	5	6	3	8	8	8	8	8	7
	e	4	6	5	2	7	6	3	5	5
50	a	4	4	4	3	2	3	7	7	5
	b	6	5	4	2	2	10	10	9	9
	c	8	8	5	5	9	5	8	8	3
	d	8	8	8	5	4	5	10	10	3
	e	4	4	3	3	3	3	9	10	10
100	a	2	2	2	4	4	3	3	5	2
	b	2	2	2	3	3	4	5	5	3
	c	4	2	2	4	3	3	4	4	3
	d	3	2	2	4	3	3	4	4	3
	e	4	3	3	3	3	3	5	5	3

Table 2. Three-way multivariate analysis of variance (MANOVA) of the number of measurements.

Source	Wilk's lambda	F-value	df.	Sig.
Organisms concentration	0.327	6.315	9	0.000
Diameter of the measuring head	0.676	2.882	6	0.014

Dependent: L*, a*, b*.

Table 3. Tukey-b test for the number of measurements in relation to different concentrations of cyanobacteria and diameter of the measuring head.

Dilution (%)	**L***	**a***	**b***
0	2.07^{a}	2.20^{a}	2.20^{a}
20	4.86^{b}	5.28^{b}	4.23^{b}
50	6^{b}	6.07^{b}	5.36^{b}
100	3.36^{a}	3.18^{a}	2.73^{a}
Diameter of the measuring head (mm)	**L***	**a***	**b***
10	3.67^{a}	3.83^{a}	3.33^{a}
8	3.53^{a}	3.53^{a}	3.84^{a}
5	5.12^{b}	5.35^{b}	3.94^{a}

Different superscript letters indicate significant differences (α: 0.05)

Table 4: Minimum number of measurements required to characterize each filter for each measuring head.

Dilution (%)	**Filter**	**Diameter of the measuring head (mm)**		
		10	**8**	**5**
0	a	4	2	3
	b	2	2	2
	c	2	2	2
	d	2	2	3
	e	2	2	2
20	a	5	3	6
	b	5	8	6
	c	6	4	5
	d	6	8	8
	e	6	7	5
50	a	4	3	7
	b	6	10	10
	c	8	9	8
	d	8	5	10
	e	4	3	10
100	a	2	4	5
	b	2	4	5
	c	4	4	4
	d	3	4	4
	e	4	3	5

Table 5. Maximum partial (ΔL*, Δa*, Δb*) and total (ΔE*ab, ΔE94 (1:1:1), CMC (2:1),) color differences for each concentration of cyanobacteria deposited on the filters.

Diameter of the measuring head (mm)	**Dilution (%)**	**ΔL***	**Δa***	**Δb***	**ΔE*ab**	**ΔE94(1:1:1)**	**CMC(2:1)**	**ΔE00(1:1:1)**
10	0	0.15	0.04	0.23	0.28	0.28	0.25	0.25
	20	2.31	0.47	0.66	2.45	2.37	1.28	1.56
	50	1.63	0.80	0.24	1.83	1.71	0.96	1.25
	100	2.84	0.57	0.77	2.99	2.88	1.50	2.47
8	0	0.06	0.12	0.29	0.32	0.29	0.29	0.29
	20	3.49	1.39	1.82	4.17	3.78	2.26	2.62
	50	2.37	0.86	-0.85	2.66	2.45	1.34	1.74
	100	1.92	0.64	0.47	2.08	1.97	1.05	1.55
5	0	0.16	0.06	0.34	0.38	0.37	0.35	0.35
	20	2.76	1.30	1.93	3.61	3.12	2.02	2.27
	50	2.62	0.94	0.91	2.93	2.70	1.46	1.91
	100	2.33	0.69	0.11	2.43	2.36	1.23	1.87