

Color measurements as a reliable method for estimating chlorophyll degradation to phaeopigments

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Abstract The application of biocides is a traditional method of controlling biodecay of outdoor cultural heritage. Chlorophyll degradation to phaeopigments is used to test the biocidal efficacy of the antimicrobial agents. In the present study, the usefulness of color measurements in estimating chlorophyll degradation was investigated. An aeroterrestrial stone biofilm-forming cyanobacterium of the genus *Nostoc* was chosen as test organism, comparing its different behaviour in both planktonic and biofilm mode of growth against the isothiazoline biocide Biotin T[®]. Changes in $A_{435\text{ nm}}/A_{415\text{ nm}}$ and $A_{665\text{ nm}}/A_{665\text{ a nm}}$ and in the chlorophyll *a* and adenosine triphosphate (ATP) cell content were compared with the variations in the CIELAB color parameters (L^* , a^* , b^* , C^*_{ab} and h_{ab}). Our findings showed that both the phaeophytination indexes are useful in describing degradation of chlorophyll *a* to phaeopigments. Moreover, the CIELAB color parameters represented an effective tool in describing chlorophyll degradation. L^* CIELAB parameter appeared to be the most

informative parameter in describing the biocidal activity of Biotin T[®] against *Nostoc* sp. in both planktonic and biofilm mode of growth.

Keywords Chlorophyll · Phaeophytination · Non-destructive methods · CIELAB color measurements · Biocide

Introduction

It is widely recognised that all the cultural heritage, defined as the totality of tradition-based creations of a cultural community (UNESCO 2000), exposed to open air is susceptible to biodeterioration, the undesirable change in the properties of a material caused by the activities of organisms (Hueck 1965). Phototrophs, like algae and cyanobacteria, are pioneer microorganisms able to readily colonize outdoor surfaces and develop biofilms, which, in turn, causes aesthetic, chemical and physical decay. Microbial abatement is commonly achieved using biocides (Cappitelli et al. 2009; De Saravia et al. 2008; Fonseca 2009; Gladis et al. 2010; Young et al. 2008).

The effectiveness of biocides towards phototrophic organisms can be tested as the degree of chlorophyll degradation (Silkina et al. 2009; Underwood and Paterson 1993). When chlorophyll degrades, it forms a series of degradation products, the nature of which depends on the part of the molecular structure of chlorophyll affected. Chlorophyll molecule consists

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of a flat hydrophilic ring-like structure called “porphyrin”, which has in the center a magnesium molecule, and a lipid-soluble hydrocarbon phytol, “tail”. If the degradation causes the loss of the magnesium from the center of the molecule, the resulting molecule is termed *phaeophytin*, while if the degradation causes the loss of the phytol tail, the former molecule is *chlorophyllide*. Further degradation of either *phaeophytin* or *chlorophyllide* produces *phaeophorbide*: *phaeophytin* is degraded by the loss of the phytol tail and a *chlorophyllide* loses its magnesium ion (Fig. 1). The three degradation products of chlorophyll: *phaeophytin*, *chlorophyllide* and *phaeophorbide* are called phaeopigments.

Although the measurement of chlorophyll degradation to phaeopigments has been well investigated in lichens (Barnes et al. 1992; Manrique et al. 1989; Pisani et al. 2007; Ronen and Galun 1984) and aquatic bryophytes (Lopez et al. 1997; Martínez-Abaigar and Núñez-Olivera 1998; Martínez-Abaigar et al. 2008), knowledge about this process is currently very limited in algae and cyanobacteria (Agrawal 1992; Louda et al. 1998). In all these studies, the ratio of absorbances at 435 and 415 nm (A_{435}/A_{415}) was interpreted as the phaeophytinization quotient, which reflects the ratio of chlorophyll to phaeopigments. Similarly, the ratio between A_{665} and A_{665a} , obtained after acidification with hydrochloric acid, was used to indicate phaeophytinization of chlorophyll. In both cases a decrease in that ratio indicates the degradation of chlorophyll to phaeopigments.

However, the spectrophotometric methods are often time- and labor-intensive and require invasive manipulation of the attached microbial community and destructive sample preparation. Furthermore, due to the need of sampling, they can only be applied

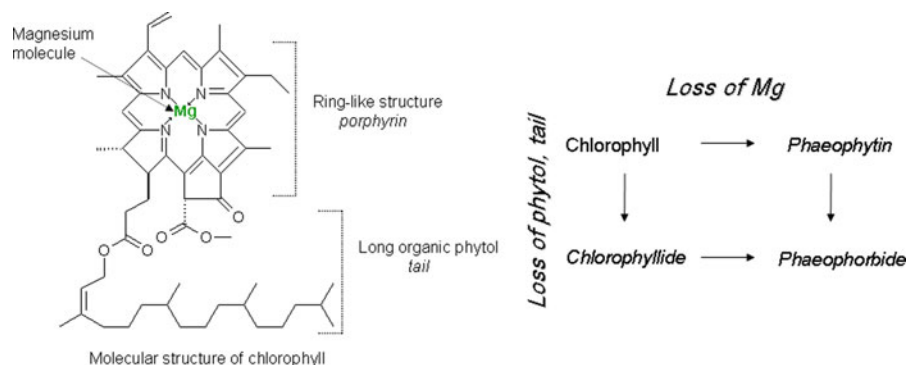
when the amount of sample is enough to take sample which never occurs before the biofilm is visible to the naked eye. By contrast, on site color measurements do not present these disadvantageous features as they can be applied even before the naked eye detects the presence of biofilm. In fact, they have successfully been applied for quantifying the growth and the physiological state of phototrophic organisms on solid substrata (De Muynck et al. 2009; Dubosc 2000; Escadeillas et al. 2009; Prieto et al. 2002, 2005) and to study the effect of environmental parameters, such as light and nutrients on the pigment content of cyanobacteria (Sanmartín et al. 2010). Since there are relationships between some color parameters and the amount of chlorophyll (Prieto et al. 2002; Sanmartín et al. 2010), relationships between color and chlorophyll degradation should exist. Thus, the main aim of the present work was to investigate whether color measurements could be used as an indicator of chlorophyll degradation in order to develop non-invasive and non-destructive study methodologies which can be applied to the cultural heritage. Furthermore, the validity of both phaeophytinization ratios ($A_{435 \text{ nm}}/A_{415 \text{ nm}}$ and $A_{665 \text{ nm}}/A_{665a \text{ nm}}$) for evaluating the chlorophyll degradation in *Nostoc* sp. PCC 9104 both in planktonic and biofilm lifestyles will be assessed.

Materials and methods

Cyanobacterial strain, medium and antimicrobial agent

Nostoc sp. PCC 9104, a filamentous N_2 -fixing heterocyst-forming cyanobacterium was chosen as

Fig. 1 Molecular structure and degradations pathways of chlorophyll (from Carlson and Simpson 1996, with permission of North American Lake Management Society)



test organism. This strain is an aeroterrestrial species isolated in Galicia (NW Spain). It has previously been characterised with respect to its growth, pigment content and color (Acea et al. 2001, 2003; Prieto et al. 2002; Sanmartín et al. 2010). PCC 9104 was routinely grown in liquid and solid N-free BG11₀ medium (Rippka et al. 1979), at room temperature under sunlight illumination.

A biocide based on *N*-octyl Isothiazolinone (OIT) and quaternary ammonium salt (cationic surfactant), Biotin T[®] supplied by CTS (Italy), was employed as active antimicrobial agent. A volume of Biotin T[®] stock solution was added to culture broth for the planktonic experiments or was added to molten culture medium supplemented with Bacto Agar to create a biocide-amended agar for biofilm experiments. The final Biotin T[®] concentration in both planktonic and biofilm assays was 2% v/v following the recommendations of the commercial supplier. The isothiazoline-based biocide Biotin T[®] exhibits a broad-spectrum activity against phototrophs and it is often used in buildings (Fonseca 2009; Gladis et al. 2010) and water systems (Batista et al. 2000). It interacts with thiol-groups of amino acids, degrading proteins that play an important role in protecting the organism cells (Collier et al. 1990).

Planktonic culture and biocide susceptibility assay

Logarithmic-phase planktonic culture (1.55 mg L^{-1}) was divided into two equal volumes and Biotin T[®] was added to one aliquot and an equal volume of water was added to the second aliquot. Both cultures were placed in an orbital shaker at room temperature and sampled at 0, 3, 9, and 24 h. Cells were harvested by centrifugation at 4000g for 15 min, rinsed with phosphate-buffered saline (PBS, Sigma-Aldrich, Italy) and resuspended in the buffers. The resulting cell suspension was analysed for color measurements, pigments and ATP contents as reported in detail below. Experiments were performed in triplicate.

Biofilm culture and biocide susceptibility assay

Sub-aerial biofilms of this strain were prepared following the procedure described by Anderl et al. (2000). Fifty microliters of cell suspension (0.32 mg L^{-1}) were used to inoculate sterile black

polycarbonate filter membranes (0.22 μm pore size and 25 mm diameter, Millipore, Italy) resting on agar BG-11₀ culture medium (Rippka et al. 1979). Using a sterile forcep, membranes were transferred to fresh agar plates every 3 days. Biomass of 40 days-old mature biofilms was assessed gravimetrically. Mature biofilms were transferred to Biotin T[®]-containing agar and incubated at room temperature for 24 h. Biofilms were sampled at 0, 3, 9 and 24 h and each membrane-supported biofilm was placed in 2 mL of PBS. Biofilm was dispersed by vortexing at 2,000 rpm for 2 min. The resulting cell suspension was analysed for color measurements, pigments and ATP contents as reported in detail below. Additional control samples were performed using biofilm-free polycarbonate filter membranes. Experiments were performed in triplicate.

Staining, cryosectioning and microscopy

Forty days-old membrane-supported biofilm was stained with the Alexa fluor[®] 488-labelled Concanavalin A (ConA, Invitrogen, Italy) as per the manufacturer's instructions to study the extracellular polymer substances (EPS). Stained biofilms on polycarbonate membrane filters were covered carefully with a layer of Killik (Bio Optica, Italy) and placed on dry ice until completely frozen. Frozen samples were sectioned at -19°C using a Leitz 1720 digital cryostat (Leica, Italy). The 10- μm thick cryosections were mounted on a poly-L-lysine coated slides (VWR International srl, Italy), examined by fluorescence microscopy using Leica DM 4000 B microscope at a magnification of 400 \times . Digital images were acquired using the CoolSNAP CF digital camera (Photometrics Roper Scientific, Germany) and elaborated using the Imagej ver. 1.34s software (Rasband 1997–2007, downloaded from <http://rsbweb.nih.gov/ij/>).

Color measurements

Two millilitres and 0.6 mL of homogeneous cell suspensions from planktonic and biofilm assays, respectively, were filtered under vacuum, through nitrocellulose filter discs (0.22 μm and 25 mm diameter, Millipore, Italy), and reflectance color measurements were taken directly on the still humid filters (Prieto et al. 2010). A Konica Minolta colorimeter with a measuring head CR-300 (8-mm-diameter viewing area) was used. The measuring conditions

were: (a) 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 in 0° ($d/0^\circ$) in relation to normal, (b) illuminant D65, (c) observer 2°.

A total of five readings were taken at different randomly selected zones on each filter (Prieto et al. 2010). Color measurements were analyzed by considering the CIELAB color system (CIE Publication 15-2 1986), which is organized with three axes in a spherical form: L^* , a^* and b^* . The L^* axis is associated with the lightness of the color and moves from top (value: 100, white) to bottom (value: 0, black), whereas the a^* and b^* axes are associated with changes in redness-greenness (positive a^* is red and negative a^* is green) and in yellowness-blueness (positive b^* is yellow and negative b^* is blue); both move in the two axes that form a plane orthogonal to L^* , and do not have specific numerical limits. Furthermore, the color parameters most closely related to the psychophysical characteristics of color, i.e., more related to color perception, and which correspond to the angular coordinates of chroma [$C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$] and hue angle [$h_{ab} = \arctan(b^*/a^*)$] were also calculated. C^*_{ab} is the relative strength of a color, chroma or saturation of color, and h_{ab} refers to the dominant wavelength, starts with 0° and increases counterclockwise (Wyszecki and Stiles 1982).

ATP cell content

ATP contents were measured by a luminescence assay employing the luciferine–luciferase-based biomass test kit (Promicol, The Netherlands) with a luminometer Berthold Detection System (R-Biopharm, Italy). ATP was extracted and measured on 200 μ L of cell suspension following the instructions of the manufacturer and relative luminescence units were converted to ATP concentrations with the use of a standard ATP curve as previously described by Principi et al. (2006). The values obtained were normalised for dry biomass.

Chlorophyll *a* quantification and phaeophytinization indexes

After color measurements, the cellular lawns deposited on membrane filters were resuspended into 2 mL of

dimethylsulfoxide (DMSO, Sigma-Aldrich, Italy) and heated to 65°C for 1 h according to Bell and Sommerfeld 1986. The samples were centrifuged 10 min at 7,000g and the supernatant was measured using the 6705 UV/VIS Spectrophotometer (JENWAY, Italy) at 665, 435 and 415 nm. Chlorophyll *a* content was calculated using the equation of Wollenweider (1969). The ratio of absorbances at 435 and 415 nm ($A_{435\text{ nm}}/A_{415\text{ nm}}$), and the ratio of absorbances at 665 and 665a nm obtained after acidification with 100 μ L HCl 1 M ($A_{665\text{ nm}}/A_{665a\text{ nm}}$) were used to indicate phaeophytinization of chlorophyll *a* (Martínez-Abaigar and Núñez-Olivera 1998). The values obtained were normalised for dry biomass.

Statistical analyses

Statistical analyses were performed with SPSS (SPSS v17.0 for Windows). Data were subjected to MANOVA at the 5% significant level (p value <0.05) and two-tailed-Bivariate Spearman's correlations.

Results and discussion

The effectiveness of Biotin T[®] was tested against *Nostoc* sp. PCC 9104 in both planktonic and biofilm mode of growth. The biocide activity of 2% Biotin T[®] in planktonic biocide susceptibility assays led to the majority of the population being killed within 24 h as demonstrated by the ATP cell content (82% reduction). Chlorophyll *a* content significantly decreased over biocide time exposure (99.8% reduction) following the same trend of ATP (Table 1).

After 40 days of growth as a colony biofilm, *Nostoc* sp. 9104 organisms accumulated to level of 3.70 ± 0.57 mg of dry weight per membrane. Alexa Fluor-labelled Concanavalin A staining was used to investigate the extracellular polymer substances (EPS) (Fig. 2). The fluorescently-labelled ConA mainly accumulated inside the cell-free void of mature microcolonies, demonstrating the presence of the EPS fraction and the growth of a biofilm defined as a complex community embedded in a self-produced polymeric matrix attached to a surface (Costerton 2007). Membrane-supported biofilms method might be an attractive and simple model system for recreating cyanobacterial sub-aerial biofilms. As expected, biofilm retained its relative

Table 1 Values of chlorophyll *a* content, phaeophytination indexes: ($A_{435\text{ nm}}/A_{415\text{ nm}}$ and $A_{665\text{ nm}}/A_{665a\text{ nm}}$), and ATP cell content of *Nostoc* sp. PCC 9104 strain in planktonic state and forming biofilm

Exposure time (h)	Planktonic assay				Biofilm assay			
	0	3	9	24	0	3	9	24
μg chlorophyll <i>a</i> / μg biomass	2.84 ± 0.28^a	0.62 ± 0.22^b	0.12 ± 0.22^c	0.01 ± 0.01^d	5.78 ± 0.29^a	9.07 ± 0.07^b	9.64 ± 1.81^b	3.91 ± 1.25^a
pmol ATP/ μg biomass	4.76 ± 1.84^a	2.34 ± 0.45^b	0.91 ± 0.31^c	0.86 ± 0.47^c	62.85 ± 16.81^a	0.05 ± 0.01^b	0.06 ± 0.02^b	0.04 ± 0.00^b
($A_{435\text{ nm}}/A_{415\text{ nm}}$)/ μg biomass	0.39 ± 0.02^a	0.31 ± 0.01^b	0.33 ± 0.02^b	0.31 ± 0.05^b	2.73 ± 0.03^a	3.02 ± 0.01^b	2.87 ± 0.01^c	1.93 ± 0.00^d
($A_{665\text{ nm}}/A_{665a\text{ nm}}$)/ μg biomass	0.65 ± 0.02^a	0.32 ± 0.10^b	0.20 ± 0.04^b	$0.43 \pm 0.18^{a,b}$	1.60 ± 0.10^a	1.62 ± 0.04^a	1.55 ± 0.05^a	1.27 ± 0.02^b

Different superscript letters indicate significant differences ($p < 0.05$) between the means of three independent replicates for the values of chlorophyll *a*, both phaeophytination indexes and ATP in planktonic and biofilm assays, in relation to the exposure time to biocide

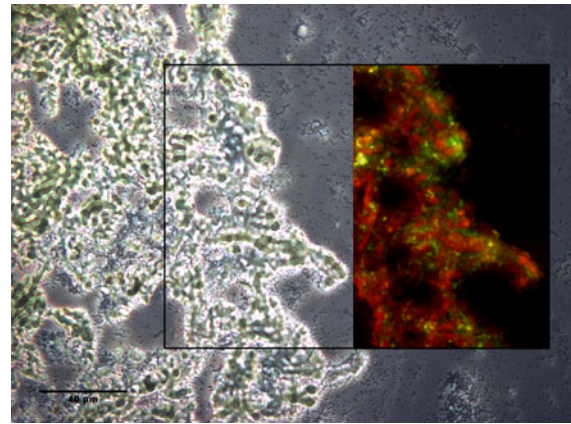


Fig. 2 Bright-field and fluorescence cryosectioning image from *Nostoc* sp. PCC 9104 sub-aerial biofilm. Biofilm matrix was stained in green (bright) with Alexa fluor[®] 488-labelled Concanavalin A. The red (dull) autofluorescence of individual cells showed the total biomass. Scale bar represents 40 μm (Color figure online)

resistance to Biotin T[®] compared to that of its planktonic counterpart after 24 h of treatment. Degradation of chlorophyll *a* occurred by 32% (Table 1). It is well known that biofilms are less susceptible to biocidal agents due to: (a) the barrier properties of the EPS matrix; (b) the physiological state of biofilm organisms and (c) the presence of subpopulations of resistance phenotypes called persisters (Xu et al. 2000; Hall-Stoodley et al. 2004; Costerton 2007). Interestingly, in the biofilm assays, the chlorophyll *a* content increased within 9 h of biocide treatment. Phototrophs may adjust their intracellular concentration of pigments in response to external stress in spite of the energy cost (Ricart et al. 2009). It is worth noting that this behaviour occurred only in the biofilm lifestyle, suggesting a better response to external stress. The ATP cell contents were not correlated with the chlorophyll *a* degradation and probably underestimated because the ATP extraction and detection were strongly hindered by the presence of the EPS matrix. In contrast, DMSO treatment used in the pigment extraction protocol disrupting polymer chain aggregation in EPS polysaccharides (Herasimenka et al. 2008) might improve cell release from biofilm and lysis leading to an efficient recovery of chlorophyll *a*. The colorimetric measurements showed that the biocidal activity of Biotin T[®] against both *Nostoc* sp. planktonic and biofilm lifestyles

Table 2 Values of CIELAB color parameters (L^* , a^* , b^* , C^*_{ab} , h_{ab}) of *Nostoc* sp. PCC 9104 strain in planktonic state and forming biofilm

Exposure time (h)	Planktonic assay					Biofilm assay				
	0	3	9	24	24	0	3	9	24	24
L^*	58.21 ± 2.92 ^a	71.37 ± 5.77 ^b	79.86 ± 3.38 ^c	85.17 ± 2.77 ^d	85.17 ± 2.77 ^d	60.51 ± 6.32 ^a	60.68 ± 7.33 ^a	62.04 ± 0.42 ^a	74.06 ± 4.78 ^b	74.06 ± 4.78 ^b
a^*	-20.23 ± 1.29 ^a	-8.14 ± 1.44 ^b	-2.73 ± 0.41 ^c	-0.88 ± 0.17 ^d	-0.88 ± 0.17 ^d	-15.09 ± 1.82 ^a	-9.45 ± 0.65 ^b	-5.80 ± 0.60 ^c	-2.39 ± 0.39 ^d	-2.39 ± 0.39 ^d
b^*	27.03 ± 2.03 ^a	8.72 ± 0.96 ^b	8.49 ± 0.87 ^b	9.96 ± 0.29 ^c	9.96 ± 0.29 ^c	11.78 ± 1.44 ^a	6.70 ± 0.91 ^b	11.76 ± 0.15 ^a	4.35 ± 1.41 ^c	4.35 ± 1.41 ^c
C^*_{ab}	33.77 ± 2.39 ^a	12.04 ± 0.29 ^b	8.94 ± 0.74 ^c	10.00 ± 0.29 ^c	10.00 ± 0.29 ^c	19.15 ± 2.23 ^a	11.63 ± 0.37 ^b	13.12 ± 0.35 ^c	4.98 ± 1.40 ^d	4.98 ± 1.40 ^d
h_{ab}	126.82 ± 0.38 ^a	132.85 ± 8.15 ^b	108.05 ± 4.08 ^c	95.04 ± 0.93 ^d	95.04 ± 0.93 ^d	142.01 ± 1.95 ^a	144.66 ± 5.14 ^a	116.19 ± 2.20 ^b	120.16 ± 5.57 ^c	120.16 ± 5.57 ^c

Different superscript letters indicate significant differences ($p < 0.05$) between the means of three independent replicates (the reported color parameters for each replicate are the mean values of five measurements) for the values of CIELAB color parameters in planktonic and biofilm assays, in relation to the exposure time to biocide

resulted in a significant color change (Table 2). The L^* parameter, associated to lightness, increased significantly with the exposure time of biocide. The increase of L^* was higher in planktonic assay, with values ranging from 58.21 to 85.17 CIELAB units, whereas biofilm assay had a slighter range of variation between 60.51 and 74.06 CIELAB units. Previous studies evidenced the suitability of L^* parameter to estimate the cell population growth (Prieto et al. 2002; Sanmartín et al. 2010), being the decrease in L^* close related with the population growth, and its increase with the end of growth. Thus, in both planktonic and biofilm biocide susceptibility assays, cell activity and growth were affected, which could have been followed by a degradation of pigments. This process was more severe in planktonic cells where the reduction of L^* parameter was two times higher with respect to biofilm assay (Table 2).

Regarding a^* , associated to greenness (–)–redness (+) changes, ranged between –20.23 and –0.88 for planktonic assay, and between –15.09 and –2.39 for biofilm assay. b^* values, associated to blueness (–)–yellowness (+) changes, ranged between 27.03 and 8.49 for planktonic assay, and between 11.78 and 4.35 for biofilm assay (Table 2). Thus, application of the biocide gave rise to a decrease in the green and yellow components of the color, being higher in the planktonic culture. The latter indicates a better physiological state (Prieto et al. 2002; Sanmartín et al. 2010) of *Nostoc* sp PCC 9104 forming biofilm at the end of the experiment.

Results for chroma C^*_{ab} , showed a similar pattern to that observed for b^* , with values ranging between 33.77 and 8.94 CIELAB units for planktonic assay and between 19.15 and 4.98 CIELAB units for biofilm assay. In both cases *Nostoc* sp. PCC 9104 cells lost chroma in their color.

Hue angle (h_{ab}) of *Nostoc* sp. PCC 9104 cells decreased significantly with the exposure time of biocide, varied in planktonic assay between a yellow–greenish hue (126.82°) and a yellow hue (95.04°); and in biofilm assay between a very slightly bluish green hue (142.01°) and a yellow–greenish hue (120.16°). h_{ab} offered a clear and comprehensive vision of color changes occurred in *Nostoc* sp. PCC 9104 in both planktonic and biofilm biocide susceptibility assays. It described the lower chlorophyll *a* degradation occurred in the biofilm tests.

Table 3 Correlation matrices showing the Pearson coefficients (*r*) found among the phaeophytination indexes, the chlorophyll *a* content, ATP values and CIELAB color parameters

	Planktonic assay					Biofilm assay								
	Chl <i>a</i>	ATP	L*	a*	b*	C* _{ab}	h _{ab}	Chl <i>a</i>	ATP	L*	a*	b*	C* _{ab}	h _{ab}
A _{435 nm} /A _{415 nm}	0.615**	0.690**	-0.603**	-0.735**	0.088	0.467	0.541*	0.916**	0.081	-0.676*	-0.408	0.292	0.204	0.317
A _{665 nm} /A _{665a nm}	0.727**	0.564*	-0.547*	-0.536*	0.269	0.620**	0.387	0.578*	0.534	-0.613*	-0.697*	0.574	0.613*	0.368

Significance level **p* < 0.05; ***p* < 0.01

Spearman correlation coefficients were calculated to evaluate relationships among phaeophytination indexes and CIELAB color parameters, chlorophyll *a* and ATP cell contents (Table 3). The statistical analysis revealed that the phaeophytination indexes A_{435 nm}/A_{415 nm} and A_{665 nm}/A_{665a nm} were related to chlorophyll *a* content in both planktonic (*r*_{435/415}: 0.615**; *r*_{665/665a}: 0.727** Pearson correlation test) and biofilms tests (*r*_{435/415}: 0.916**; *r*_{665/665a}: 0.578* Pearson correlation test). These findings demonstrated the validity of the phaeophytinization ratios in evaluating the chlorophyll *a* degradation for both *Nostoc* sp. planktonic and biofilm lifestyle. Pearson correlation tests also showed that the L* CIELAB color parameter represents an effective tool in describing chlorophyll degradation as is correlated to both phaeophytinization ratios in both lifestyles. Moreover, a* and C*_{ab} are correlated to the A_{665 nm}/A_{665a nm} index in both lifestyles.

Conclusions

The suitability of color measurements as a reliable method for estimating chlorophyll degradation to phaeopigments has been stated in this work. The determination of the phaeophytination indexes A_{435 nm}/A_{415 nm} and A_{665 nm}/A_{665a nm}, which have been proved as useful in describing degradation of chlorophyll *a* to phaeopigments in both *Nostoc* sp. PCC 9104 planktonic and biofilm biocide susceptibility assays, could be substituted by color measurements as there is a closed relation between them and the color parameters. Among the CIELAB parameters L* appeared to be the most informative parameter in describing the biocidal activity of Biotin T® against *Nostoc* sp. in both planktonic and biofilm mode of growth.

The methodology tested in this study will be very useful in those fields where sampling is a critical aspect, as in the field of conservation of works of art, since it will allow to monitorize the effectiveness of biocides as the degree of chlorophyll degradation to phaeopigment avoiding sampling.

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