Rapid preliminary evaluation of three biocide treatments against the cyanobacterium *Nostoc* sp. PCC 9104 by color changes

Patricia SANMARTÍN[(1,2)]*; Federica VILLA[(1)]; Andrea POLO[(1)]; Benita SILVA[(2)]; Beatriz PRIETO[(2)]; Francesca CAPPITELLI[(1)]

[(1)] *Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS). Università degli Studi di Milano, Via Celoria 2, 20133 - Milano, Italy*
[(2)] *Departamento de Edafología y Química Agrícola. Facultad de Farmacia. Universidad de Santiago de Compostela, 15782 - Santiago de Compostela, Spain*

*Corresponding author: E-mail address: patricia.sanmartin@usc.es
Telephone: 617-495-4180. Fax: 617-496-1471

Abstract Repeated short–term exposures to: (i) a commercial isothiazoline biocide (Biotin T®), (ii) constant temperature (37ºC) and (iii) UV-C germicidal irradiation (254 nm) on planktonic culture of an aeroterrestrial cyanobacterium of the genus *Nostoc* was carried out to assess (a) the effectiveness of the three trial treatments, referred to here as biocide strategies, and (b) the usefulness of CIELAB color coordinates (L*, a*, b*, C^*_{ab} and h_{ab}) in monitoring their effectiviness. After each exposure, spectrophotometric measurements of chlorophyll-a, phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin) and total carotenoids were conducted together with CIELAB color measurements and the adenosine triphosphate (ATP) assay. In terms of effectiveness of biocide treatments, Biotin T® was the most effective, followed by UV-C irradiation. Constant 37ºC temperature showed no biocidal effect, as ATP, chlorophyll-a, phycocyanin and allophycocyanin values increased. Results showed that L*, a* and h_{ab} CIELAB parameters could be usefully used to monitor the effectiveness of these three biocide strategies being all the CIELAB color coordinates significantly correlated with chlorophyll-a, phycocyanin, allophycocyanin and ATP contents. In particular, for the first time linear regression equations were calculated to predict chlorophyll-a and ATP from parameters L* and a*, and phycocyanin and allophycocyanin from parameters L* and h_{ab}, obtaining values of adjusted R^2 close to 0.9. Future considerations will include the application of this technique to cyanobacterial biofilms present on stone surfaces, since the evaluation presented in this study is limited to planktonic cultures.

Key words: Biocide; planktonic culture; CIELAB; color measurements; non-destructive methods; pigments.

Findings

Cyanobacteria contribute significantly to the acceleration of weathering processes of cultural properties worldwide (Crispim and Gaylarde 2005; Cappitelli et al. 2012). *Nostoc* are particularly involved in a range of effects on stone buildings and have long been recognized as cyanobacterial biodeteriogens. Consequently, a massive presence of *Nostoc* cells on stone works and structures must be eradicated using the most adequate

treatment in each specific case. In this sense, microbial abatement is commonly achieved with chemical biocides, however these often have detrimental effects on stone (Warscheid and Braams 2000, and references therein). Besides, questions concerning the ecotoxicity of commercial biocides may make them poor candidates for use in outdoor environments, and many countries have forbidden the use of some of the previously most common (and effective) biocides; thereby biocide use has been discouraged (Scheerer et al. 2009; Villa and Cappitelli 2013) and alternative methods being thus invoked to control biofouling (Villa and Cappitelli 2013). These alternative methods include treatment with UV germicide light and temperature change.

The selection of a trial treatment used as a biocide strategy, and its application, against a particular microorganism or microbial consortium requires testing of it, first rapidily, in the laboratory (e.g. Cappitelli et al. 2009: Prieto et al. 2014), and then under more realistic conditions for extended periods (e.g. de los Ríos et al. 2012). Previous laboratory tests are absolutely necessary, although the effectiveness of biocidal strategy on microorganisms associated with the lithic substrate can be reduced significantly, compared to the sensitivity of microorganisms observed in lab efficacy tests (Warscheid and Braams 2000; Nugari and Salvadori 2008). To assess the effectiveness of a biocide strategy, methods based on detecting changes in the biomass and identifying the reduced activity of the microorganisms are the most frequently used (Warscheid and Braams 2000; Scheerer et al. 2009). Although they have certain advantages and their use is widespread in research, these methods also have some disadvantages. The main disadvantages are the associated costs and the length of time they take. Moreover, most of the techniques are destructive and require prior sample preparation and acquisition, which is always difficult or even impossible task in the case of cultural heritage monuments. For these reasons, identifying innovative tools for testing the effects of biocides on biodeteriogens surfaces is an important goal in the area of cultural heritage (Tretiach et al. 2010). Techniques based on optical methods as the instrumental color measurement with a spectrophotometer or a tristimulus colorimeter (for details about this technique, see e.g., Wyszecki and Stiles 1982; Prieto et al. 2010; Vázquez-Nion et al. 2013) could be an interesting option.

In recent research, we demonstrated the suitability of the instrumental color measurements and the CIELAB color system as a reliable method for monitoring the effectiveness of the chemical biocide Biotin T® against a mesophilic and filamentous N_2-fixing heterocyst-forming cyanobacterium, *Nostoc* sp. PCC 9104, in both planktonic and biofilm mode of growth (Sanmartín et al. 2011). In the present study, the effectiveness of three trial treatments referred to here as biocide strategies: the aforementioned Biotin T®, constant 37ºC temperature and UV-C light, against the same cyanobacterial strain (*Nostoc* sp. PCC 9104) in planktonic mode of growth were tested, with the aim of determinate their activity against the studied strain. Considering the relationships previously obtained between biocide response and color; and biocide response and physiological state of a microorganism, we now propose that the effect of the treatments could be assessed from color measurement of microorganisms. Importantly, in natural conditions, monospecies biofilms are relatively rare; thus most biofilms are composed of mixtures of microorganisms. For this preliminary study we select a single cyanobacterium, however successive studies should be applied to microbial consortia isolated from monuments in natural conditions.

Cells in the exponential phase of growth from axenic cultures of *Nostoc* sp. PCC 9104 (1.55 mg L^{-1}, diazotrophic conditions) were collected and used as the inoculum for experimental testing of biocides. Biotin T® stock solution was added to planktonic samples and its effect was determined as in Sanmartín et al. (2011); resulting cell suspension was analysed for color, pigments and ATP contents as reported in detail below, immediately after biocide-treatment. To our best knowledge, in cyanobacteria low survival rate after a short heat treatment at 10-15°C above the optimum growth temperature have been several times reported (Eriksson et al. 1996; Tuominen et al. 2008; Sheng et al. 2011). It seems reasonable to assume therefore, that an increase in temperature to 15°C in *Nostoc* sp. PCC 9104 in planktonic lifestyle could inhibit its growth. So, eighteen 4-ml logarithmic-phase samples were put in plastic tubes capped with aluminium sheet for at least 30 min at room-temperature (18-20°C) before the heat treatment. After that time, the samples were put in an oven at 37ºC. Triplicate samples were withdrawn after 0, 5, 10, 15, 20 and 25 min. Cells were harvested by centrifugation

at 4000 g for 15 min and the resulting cell suspension was analysed for color, pigments and ATP contents as reported in detail below. All treatments and measurements were performed in the dark. For the UV-light treatment, eighteen 4-ml logarithmic-phase samples were pipetted into Petri dishes and placed open in the Petri dishes 5 cm below a 30-W germicidal UV-C lamp (model G30T8; 2.7 ± 0.3 mW/cm^2 irradiance at 254 nm wavelength; Sankyo Denki, Tokyo, Japan) for 0 to 15 min. UV exposures of 0, 1, 2, 5, 10 and 15 min were equivalent to 0, 1870, 3190, 7150, 13750, 20350 J/m^2 respectively. Note that UV light has been proposed several times for the treatment of biodeteriored stones in terrestrial environments, even fairly recently (e.g. Borderie et al. 2012; Eklund and Young 2013). After exposure, the Petri dishes were covered immediately with an aluminium sheet in order to create dark condition and incubated at room temperature (18-20°C) for 24 hours for reset the cyanobacterial circadian clock and subsequently block the biocide activity of UV-C. Subsequently, cells were harvested by centrifugation at 4000 g for 15 min and the resulting cell suspension was analysed for color, pigments and ATP contents as reported in detail below. Experiments were performed in triplicate.

The color, amount of chlorophyll-a and ATP content, from each one of the treated samples of *Nostoc* sp. PCC 9104 was determined as in Sanmartín et al. (2011). Total carotenoids content was calculated using the equation of Wellburn (1994) from the same chlorophyll-a samples. Phycobiliproteins were determined by a modified osmotic shock method: 1.5 ml aliquots were centrifuged 10 min and the pellets homogenized in 150µl of glycerol (10% total volume) and incubated in the dark at 5.2 °C for 24 h. Distilled water was then added to osmotically lyse the cells. Moreover, Na-acetate (200mM) was added, in order to separate the phycobiliproteins, obtaining more resolutive and clear absorbance spectrum. Centrifuged 10 min and the supernatant was collected with phycobiliproteins. It was measured targeting as control a solution of glycerol to 10% and 200 mm Na-acetate in distilled water. Extracts were measured using the 6705 UV/VIS Spectrophotometer (JENWAY, Italy). Phycobiliproteins content was calculated according to the equations of Bennet and Bogoard (1973).

Data were subjected to multivariate analysis of variance (MANOVA) followed by the Tukey's-b post hoc analysis. Differences were considered significant at $p < 0.05$. The

relationships between the data were assessed by two tailed Bivariate Pearson's correlations and stepwise linear multiple regression models. Statistical analyses were performed with SPSS (SPSS v21.0 for Windows).

The temporal variation in the different parameters in relation to exposure to the trial treatments is summarized in **Figure 1**. Exposure to constant 37ºC temperature was the least effective treatment. Indeed, the contents of most of the compounds (chlorophyll-a, ATP, PC and APC) increased significantly on exposure to constant 37ºC temperature. Although the other two treatments were effective against *Nostoc* sp. PCC 9104, by the end of the experiment, treatment with the biocide Biotin T® proved most effective as it caused the greatest decrease in the contents of all of the compounds. The overall effect of Biotin T® (i.e., throughout the entire exposure period) was also significantly higher than that of UV-C light and constant 37ºC temperature, for all of the compounds except the carotenoids and ATP. The latter exceptions were probably caused by the effectiveness of UV-C light in reducing the content of both compounds, rather than an ineffective response of Biotin T® for the same purpose. Biotin T® significantly decreased the carotenoids and ATP contents, although the decrease was highly variable. This can be interpreted as a typical response of a microorganism such as *Nostoc* sp. PCC 9104 to a chemical biocide such as Biotin T® because phototrophs can adjust the intracellular concentration of pigments in response to external stress, despite the high energy cost (Sanmartín et al. 2011). Increased concentrations of carotenoids in the cells of algae/cyanobacteria have been reported as adaptive responses to biocides (Krinski 1989).

Separate consideration of each parameter revealed that chlorophyll-a content was reduced by all three treatments (significantly in the case of Biotin T® and UV-C light) after the second exposure (**Figure 1**). Likewise, all treatments significantly reduced the content of carotenoids after the second exposure time. Exposure to UV-C light caused a strong and significant decrease in the carotenoids contents from the beginning of the experiment, and the values remained low until the end of the experiment. This contrasts with findings with other cyanobacteria, such as *Synechocystis* sp. PCC 6803, in which the short-term effect of UV-C radiation decreased the chlorophyll-a content, but had no effect on carotenoids content (Jantaro et al. 2011). ATP content increased significantly

after the second exposure to constant 37ºC temperature and Biotin T®, whereas it decreased, although not significantly, under UV-C light. PC and APC contents varied in similar ways in response to the different treatments. The decrease in the contents of both phycobiliproteins after exposure to Biotin T® was strong and significant from the second exposure time onwards. Constant 37ºC temperature and UV-C light had very similar effects on the PC and APC contents, causing a significant increase in both until the fourth exposure time, after which the contents stabilized and did not vary significantly with constant 37ºC temperature, although they decreased significantly under UV-C light. PE content, which was lower, only decreased significantly at the beginning of the experiment in the *Nostoc* sp. PCC 9104 samples treated with UV-C light, and the level then remained similar until the end of the experiment. The change in PE content induced by Biotin T® was only statistically significant at the end of the experiment (sixth exposure time), whereas constant 37ºC temperature did not significantly affect the PE content throughout the experiment.

The temporal variation in the color in response to exposure to the different treatments is shown in **Figure 2**. Biotin T® had the strongest effect on all of the CIELAB color parameters, causing a large color difference that was significantly different from the changes caused by the other two treatments. The L* parameter (color lightness) varied between 43.1 ± 0.9 and 85.2 ± 2.8 CIELAB units, indicating lighter colors. The widest range of variation in L* was observed in the *Nostoc* sp. PCC 9104 samples treated with Biotin T®. The large variation in the L* coordinate contrasts with the smaller variations in L* caused by the other two treatments. Previous studies have demonstrated the suitability of the L* parameter for estimating cell population growth (Sanmartín et al. 2010, 2011) as the decrease in L* is closely related to population growth, and an increase in the parameter indicates end of growth. The value of a* (associated with greenness (-) to redness (+) changes) varied from -24.0 ± 0.9 to -0.3 ± 0.3 CIELAB units, conferring the strain a greenish color. Biotin T® also caused the largest color change with respect to this parameter; smaller although also significant were the decrease in response to constant 37ºC temperature and the increase in response to UV-C light. The changes in b* (associated with blueness (-) to yellowness (+) changes) and C^*_{ab} (color chroma) were

very similar; in both cases the effect of Biotin T® on *Nostoc* sp. PCC 9104 was very different from the effects generated by the other two treatments. Exposure to Biotin T® caused a significant decrease in the values of b* and C^*_{ab}, whereas exposure to constant 37ºC temperature caused a significant increase in the values of both color parameters; UV-C light generated a very variable response. The hue angle, h_{ab}, fell within the interval 91.9º ± 1.9º to 142.9º ± 6.1º, so that all the *Nostoc* sp. PCC 9104 samples were located from the yellow hue to the very slightly bluish green hue area. This range corresponded to the change caused by Biotin T® between the second exposure time and the end of the experiment. After application of the chemical biocide, the hue angle increased significantly (by approximately 16°) to a color indicating a better physiological state (Sanmartín et al. 2010, 2011); the h_{ab} value then decreased significantly from the third exposure time onwards. For the other two treatments, the h_{ab} values were quite similar, and although they varied significantly throughout the exposure time (like the other colorimetric parameters), the variations were small. The overall changes in color or total color differences, ΔE^*_{ab}, resulting from the application of the three treatments exceeded (in most cases, greatly) the value considered as the general limit of perceptibility, i.e., 3 CIELAB units (Wyszecki and Stiles 1982; Prieto et al. 2010), which is the upper limit of rigorous color tolerance. Even when a higher threshold of perception of 6 CIELAB units, considered as an evident color change (Prieto et al. 2010; Giacomucci et al. 2012), was taken into account, the value was still exceeded. Therefore, the total color change was visually evident and noticeable at first glance.

The changes in many of the studied parameters were consistent with the color differences. Hence, the hue angle (h_{ab}) values, which are good indicators of the changes in the microorganism under study (Sanmartín et al. 2011), are consistent with the ATP measurements. The graphs of the coordinates a*, b* and C^*_{ab} (**Figure 2**) were very similar to those of the bluish pigments (**Figure 1**), such as PC, which causes the blue color in cyanobacteria, and APC, which confers a greenish-blue color. The L* parameter reflected the response of all of the physiological parameters to the test treatments. Statistical tests were applied to analyze these relationships in further detail.

The correlation matrix showing the Pearson coefficients for the physiological parameters and the CIELAB color coordinates is summarized in **Table 1**. Except for phycoerythrin and carotenoids, all CIELAB coordinates were closely correlated ((**), $p < 0.01$) with some of the physiological parameters studied. Regarding the phycoerythrin, the poor correlation is probably due its too low amount ($< 7 \times 10^{-4}$ μg mL^{-1}). For the carotenoids, this is probably because the total carotenoids were measured, comprising the oxidized forms (the xanthophylls) and the reduced forms (the carotenes), so there was possibly a different response from each carotenoid forms to the trial treatments. The strongest correlations were between the phycocyanin and allophycocyanin contents and the L* value (Pearson's coefficients: -0.92** and 0.95**, respectively), followed by those between the chlorophyll-a and ATP contents and the L* value (Pearson's coefficients: -0.89** and 0.88** respectively). The L* parameter was previously found to be the most informative CIELAB color parameter for chlorophyll degradation in the specific case of the filamentous cyanobacterium *Nostoc* sp. PCC 9104 (Sanmartín et al. 2010).

Stepwise multiple linear regression was applied to obtain simple expressions for estimating the values of physiological parameters from the CIELAB color coordinates (**Table 2**). Adjusted R^2 values close to 0.9 were obtained, which validates the CIELAB color coordinates as a useful tool for assessing the effectiveness of biocide treatments in *Nostoc* sp. PCC 9104. Thus, the physiological parameters studied here may be best quantified by parameters L*, a* and h_{ab}. The closest relation corresponded to the ATP content with an adjusted R^2 value of 0.90, and $ATP = 27.72 - 0.33L^* + 0.34a^*$ as a predictive equation.

In summary, the effectiveness of the three strategies studied against *Nostoc* sp. PCC 9104 in planktonic lifestyle was assessed: Biotin T® was the most effective biocide treatment, followed by UV-C irradiation. Constant 37ºC temperature showed no biocidal effect, as four of the six physiological parameters studied significantly increased their content after applying this treatment. The CIELAB color coordinates were significantly correlated with physiological parameters in *Nostoc* sp. PCC 9104. For the first time, linear regression equations were used to predict ATP, chlorophyll-a, phycocyanin and

allophycocyanin from parameter L* (lightness of the color), a* (redness-greenness of the color) and h_{ab} (hue angle of the color), and values of adjusted R^2 were close to 0.9. This preliminary research that was focused on planktonic mode of growth needs of further extension towards biofilm mode of growth, monitoring of epilithic phototrophic biofilms on stone surfaces and testing of the presented method on case studies.

Acknowledgements

Dr. Patricia Sanmartín is supported by a postdoctoral contract within the framework of the 2011-2015 Galician Plan for Research, Innovation and Growth (Plan I2C) for the year 2012. Dr. Federica Villa is currently a Marie Curie fellow (FP7-PEOPLE-2012-IOF) under the grant agreement no. 328215.

References

Bennet A, Bogoard L (1973) Complementary chromatic adaptation in a filamentous blue-green alga. The Journal of Cell Biology 58:419–435.

Borderie F, Alaoui-Sehmer L, Bousta F, Orial G, Rieffel D, Richard H, Alaoui-Sosse B (2012) UV irradiation as an alternative to chemical treatments: a new approach against algal biofilms proliferation contamining building facades, historical monuments and touristic subterranean environments, in Algae: Ecology, Economic uses and Environmental Impact, Nova Science Publishers, Inc., Editors: Dagmar Krueger, Helga Meyer, pp. 1-28.

Cappitelli F, Abbruscato P, Foladori P, Zanardini E, Ranalli G, Principi P, Villa F, Polo A, Sorlini C (2009) Detection and elimination of cyanobacteria from frescoes: the case of the St. Brizio Chapel (Orvieto Cathedral, Italy). Microbial Ecology 57(4):633–639.

Cappitelli F, Salvadori O, Albanese D, Villa F, Sorlini C (2012) Cyanobacteria cause black staining of the National Museum of the American Indian Building (Washington, D.C., USA). Biofouling 28(3):257-266.

Crispim CA, Gaylarde CC (2005) Cyanobacteria and biodeterioration of cultural heritage: A review. Microbial Ecology 49(1):1-9.

de los Ríos A, Pérez-Ortega S, Wierzchos J, Ascaso C (2012) Differential effects of biocide treatments on saxicolous communities: case study of the Segovia cathedral cloister (Spain). International Biodeterioration and Biodegradation 67:64 – 72.

Eklund JA, Young ME (2013) Biological Growth on Masonry: Identification & Understanding. Historic Scotland, January 2013. Available in: http://conservation.historic-scotland.gov.uk/bio-growth-masonry-inform.pdf.

Eriksson MJ, Clarke AK (1996) The heat shock protein ClpB mediates the development of thermotolerance in the cyanobacterium *Synechococcus* sp. strain PCC 7942. Journal of Bacteriology 178:4839–4846.

Giacomucci L, Toja F, Sanmartín P, Toniolo L, Prieto B, Villa F, Cappitelli F (2012) Degradation of nitrocellulose-based paint by *Desulfovibrio desulfuricans* ATCC 13541. Biodegradation 23(5):705-716.

Jantaro S, Pothipongsa A, Khanthasuwa S, Incharoensakdi A (2011) Short-term UV-B and UV-C radiations preferentially decrease spermidine contents and arginine decarboxylase transcript levels of *Synechocystis* sp. PCC 6803. Current Microbiology 62(2):420-426.

Krinsky NI (1989) Antioxidant functions of carotenoids. Free Radical Biology and Medicine 7(6):617-635.

Nugari MP, Salvadori O (2008) Stone materials. In: Caneva, G., Nugari, M.P., Salvadori, O. (Eds.), Plant biology for cultural heritage. Biodeterioration and conservation. The Getty Conservation Institute, Los Angeles, pp. 326-335.

Prieto B, Sanmartín P, Aira N, Silva B (2010) Color of cyanobacteria: some methodological aspects. Applied Optics 49:2022–2029.

Prieto B, Sanmartín P, Silva C, Vázquez-Nion D, Silva B (2014) Deleterious effect plastic-based biocides on back-ventilated granite facades. International Biodeterioration and Biodegradation 86: 19-24.

Sanmartín P, Aira N, Devesa-Rey R, Silva B, Prieto B (2010) Relationship between color and pigment production in two stone biofilm-forming cyanobacteria (*Nostoc* sp PCC 9104 and *Nostoc* sp PCC 9025). Biofouling 26:499–509.

Sanmartín P, Villa F, Silva B, Cappitelli F, Prieto B (2011) Color measurements as a reliable method for estimating chlorophyll degradation to phaeopigments. Biodegradation 22:763–771.

Scheerer S, Ortega-Morales O, Gaylarde C (2009) Microbial deterioration of stone monuments – an updated overview. Advances in Applied Microbiology 66:97–139.

Sheng J, Kim HW, Badalamenti JP, Zhou C, Sridharakrishnan S, Krajmalnik-Brown R, Rittmann BE, Vannela R (2011) Effects of temperature shifts on growth rate and lipid characteristics of *Synechocystis* sp. PCC 6803 in a bench-top photobioreactor. Bioresource Technology 102(24): 11218–11225.

Tretiach M, Bertuzzi S, Salvadori O (2010) Chlorophyll *a* fluorescence as a practical tool for checking the effects of biocide treatments on endolithic lichens. International Biodeterioration and Biodegradation 64:452-460.

Tuominen I, Pollari M, von Wobeser EA, Tyystjarvi E, Ibelings BW, Matthijs HC, Tyystjarvi T (2008) Sigma factor SigC is required for heat acclimation of the cyanobacterium *Synechocystis* sp. strain PCC 6803. FEBS Letters 582(2):346-350.

Vázquez-Nion D, Sanmartín P, Silva B, Prieto B (2013) Reliability of color measurements for monitoring pigment content in a biofilm-forming cyanobacterium. International Biodeterioration and Biodegradation 84:220-226.

Villa F, Cappitelli F (2013) Plant-derived bioactive compounds at sub-lethal concentrations: towards smart biocide-free antibiofilm strategies. Phytochemistry Reviews 12(1):245-254.

Warscheid T, Braams J (2000) Biodeterioration of stone: a review. International Biodeterioration and Biodegradation 46:343–368.

Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolutions. Journal of Plant Physiology 144:307–313.

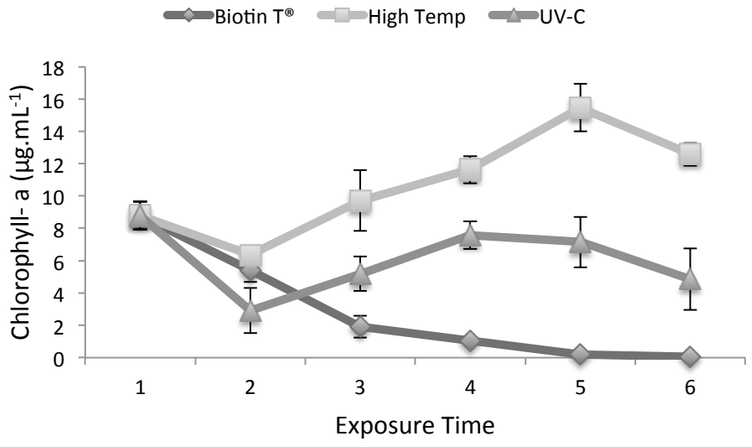
Wyszecki G, Stiles WS (1982) Color Science. Concepts and Methods, Quantitative Data and Formulae, John Wiley and Sons, New York.

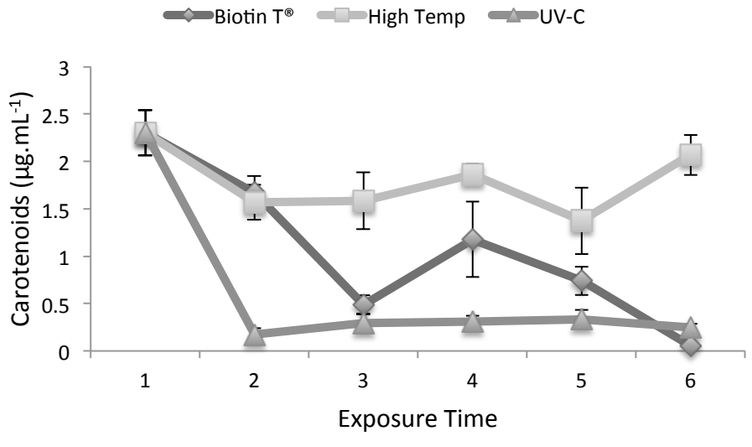
Capture figures

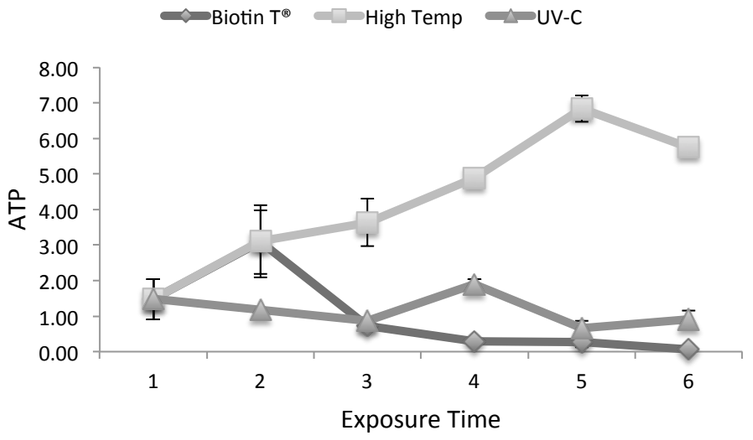
Figure 1. Variation in the time of the physiological parameters studied: chlorophyll-a, total carotenoids, adenosine triphosphate (ATP) and phycobiliproteins (phycocyanin, allophycocyanin and phycoerythrin) with the exposure to different biocides.

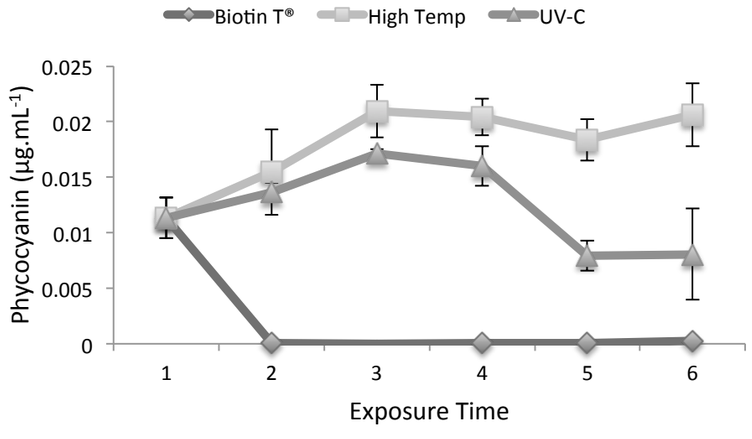
Figure 2. Variation in the time of the color, based on the CIELAB coordinates: L*, a*, b*, C^*_{ab} and $h_{ab,}$ and the total color difference (ΔE^*_{ab}) with the exposure to different biocides.

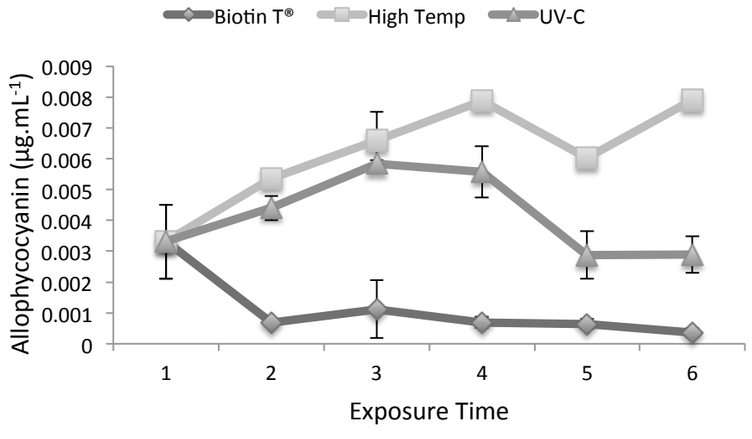
Figure 1

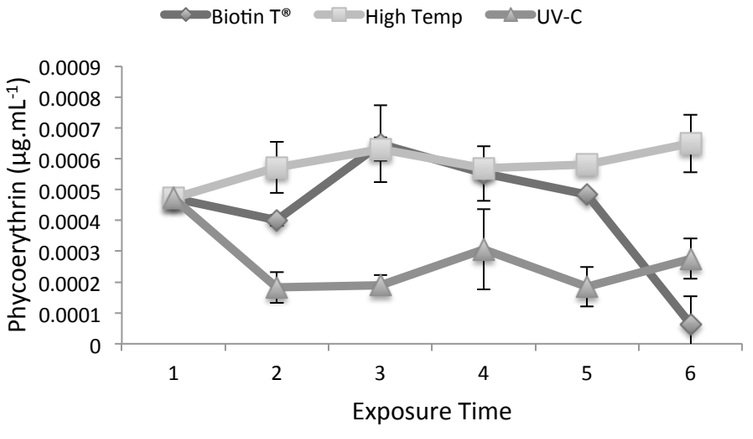






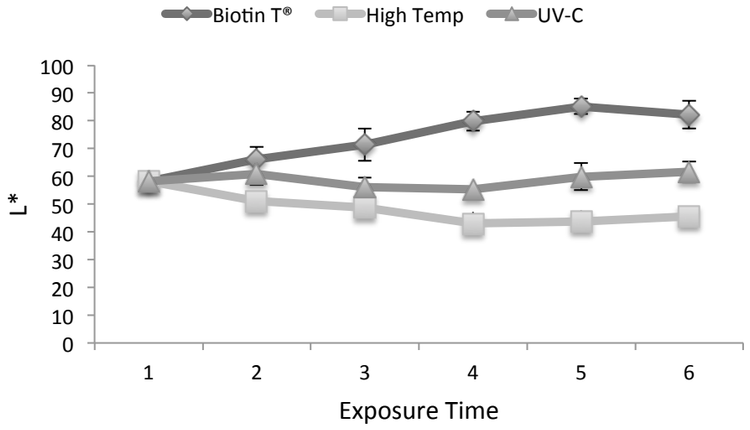


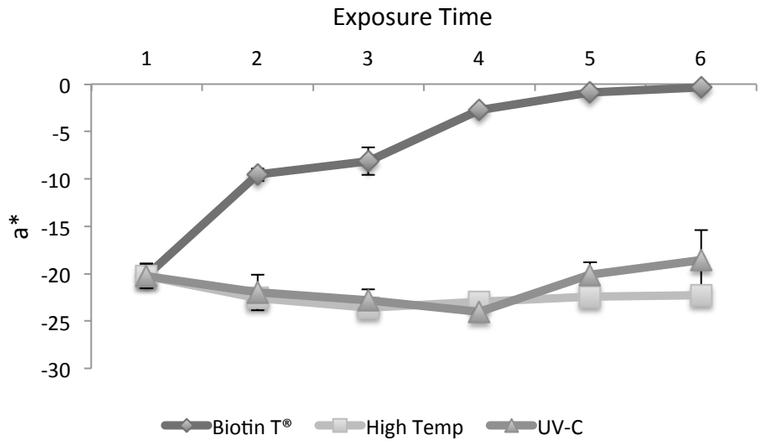


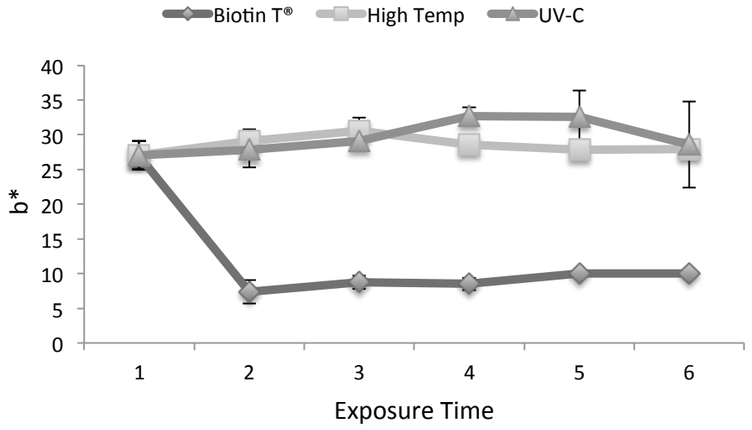


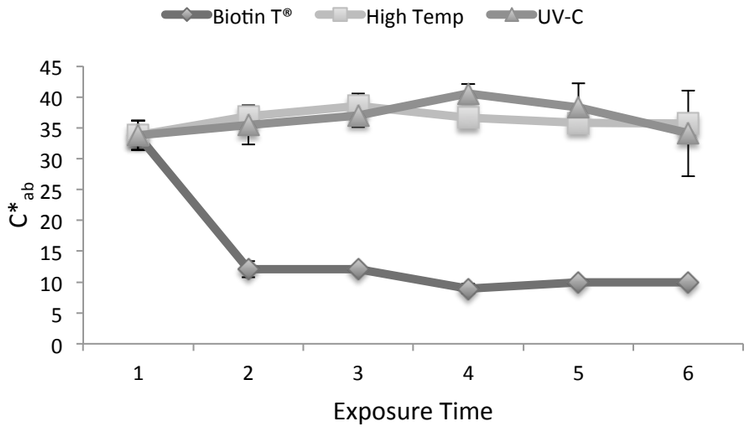
Exposure Time	Biotin T® (hours)	High Temp (minutes)	UV-C (minutes)
1	0	0	0
2	1	5	1
3	3	10	2
4	9	15	5
5	24	20	10
6	48	25	15

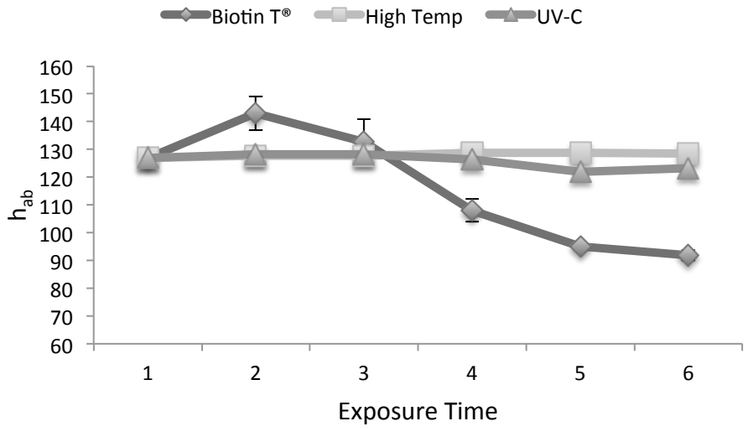
Figure 2

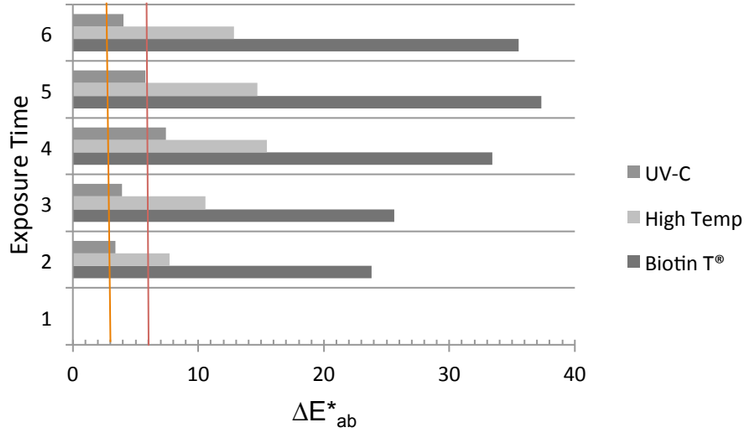












Exposure Time	Biotin T® (hours)	High Temp (minutes)	UV-C (minutes)
1	0	0	0
2	1	5	1
3	3	10	2
4	9	15	5
5	24	20	10
6	48	25	15

Table 1. Correlation matrix showing the Pearson's coefficients for the physiological parameters and the CIELAB color coordinates.

	CIELAB color coordinates				
Physiological parameters	L*	a*	b*	C^*_{ab}	h_{ab}
Chlorophyll-a content	-0.89**	-0.72**	0.38	0.59*	0.58*
Carotenoids content	-0.45	-0.24	-0.12	0.04	0.39
Adenosine triphosphate content	-0.88**	-0.72**	0.32	0.53*	0.67**
Phycocyanin content	-0.92**	-0.84**	0.63**	0.72**	0.40
Allophycocyanin content	-0.95**	-0.86**	0.60*	0.71**	0.50
Phycoerythrin content	-0.38	-0.18	-0.18	-0.03	0.48

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

Table 2. Stepwise multiple linear regression equations for the prediction of the contents in chlorophyll-a (Chl-a), adenosine triphosphate (ATP), phycocyanin (PC) and allophycocyanin (APC) by use of some CIELAB color parameters.

Parameter Estimated	*Predictive Equation*	R^2	*adjusted* R^2
Chlorophyll-a content (Chl-a)	Chl-a = 38.82 – 0.46 L* + 0.30 a*	0.85	0.83
Adenosine triphosphate content (ATP)	ATP = 27.72 – 0.33 L* + 0.34 a*	0.92	0.90
Phycocyanin content (PC)	PC = 15.94 – 0.14 L* – 0.04 h_{ab}	0.89	0.87
Allophycocyanin content (APC)	APC = 5.05 – 0.05 L* – 0.01 h_{ab}	0.91	0.90