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Reliability of color measurements for monitoring pigment content in a biofilm-forming cyanobacterium

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ABSTRACT

Pigment content provides valuable information about the growth and physiological status of organisms. This study investigated the relationship between pigment content (chlorophyll *a*, total carotenoids and phycobiliproteins) and reflectance color measurements, expressed by means of the CIELAB color coordinates. The experimental design was carried out with a biofilm-forming cyanobacterium of the genus *Nostoc* grown under different environmental conditions (nitrate and phosphate concentrations and light intensity). Analysis of the results revealed a close correlation between pigment content and CIELAB color coordinates and enabled formulation of predictive equations for estimating chlorophyll *a* and total carotenoid content. The interest in monitoring biofilm development on cultural heritage monuments has arisen within the field of preventive conservation and explains the importance of developing a simple non-destructive method of microorganism assessment based on color measurements, which can be performed rapidly on site and is cost-effective.

1. Introduction

Stone surfaces exposed to the open air are inevitably colonized by a variety of organisms (see Warscheid and Braams, 2000 for a review of stone biodeterioration). Photoautotrophic microorganisms, such as algae and cyanobacteria, can develop biofilms on stone surfaces in the presence or absence of organic matter. These microorganisms are therefore of great ecological importance as pioneer organisms in the colonization of stone materials. Biofilms cause the apparent staining of rocks and may also affect the physicochemical properties of mineral materials (e.g., Silva et al., 1997, 1999; Saiz-Jimenez, 1999; McNamara and Mitchell, 2005). Biofilm formation is of particular importance when the stone under consideration is the building material of monuments of historic and cultural interest. Thus, the implementation of techniques for assessing colonization is very important in the context of the preservation of stone buildings and monuments.

Biological colonization on building façades has been previously quantified by instrumental color measurements. Newby et al. (1991) were the first authors to relate measurements of reflectance (which can be obtained with a spectrophotometer or tristimulus colorimeter and the results expressed in CIE-L*a*b* color system units) to the color change caused by deposition of particles from natural sources, as well as pollutants, on a solid surface. On the basis of a previous study by Ball (1989), Newby et al. (1991) defined soiling of building materials as "an optical effect, a darkening of the surface that can be measured as a change in light reflectance, and is generally related to the deposition of airborne particulate matter onto the building surface". Several years later and taking this definition into account, Gorbushina and Krumbein (2000) stated that many of the spectral changes are related to the growth of massively pigmented biofilms. Krumbein (1995) considered that airborne particles, dust, pollen, particulate elemental carbon (PEC), fly ash, and microorganisms, often act concurrently in fouling (soiling) of the building, and noted that, as early as 1853, Ehrenberg had came to the conclusion that microorganisms can trap airborne particles in their slimy extracellular products more efficiently than the rock surface itself.

The use of portable tristimulus colorimeters and portable spectrophotometers to measure the color generated by phototrophic microorganisms growing epilithically on stony substrates came into common use in the late 1990s. As part of a larger research programme funded by Historic Scotland, researchers at Robert Gordon University (Aberdeen, Scotland. UK) conducted a series of experiments on sandstone buildings in Scotland. These researchers used a portable tristimulus colorimeter to monitor the color change brought about on building façades by biological growth (algae) after application of various biocide treatments (Urquhart et al., 1995; Young et al., 1995; Young, 1997). Around the same time,

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Urzì and Realini (1998) published a study in which the color of orange and grey patinas developed on the calcarenite stone from which the Baroque town of Noto is built (Sicily, Italy) was determined with a portable tristimulus colorimeter and correlated with the associated microflora of algae, bacteria and fungi. As part of the British National Materials Exposure Programme (NMEP), Viles and colleagues between 1987 and 1995 monitored microbial colonization, soiling and decay of limestone tablets located over 20 sites around the UK for periods of 1, 2, 4 and 8 years, depending on the climate and pollution conditions of the location. They used a portable spectrophotometer and characterization techniques such as scanning electron microscopy (SEM) (Viles et al., 2002). The study subsequently focused on limestone buildings located along roads with different levels of traffic in Oxford (England, UK), and the buildings were monitored for a period of three years (Viles and Gorbushina, 2003). After the Oxford Transport Strategy restricted access to vehicles (particularly private vehicles) in the centre of Oxford in 1999, Thornbush and Viles (2004, 2006) recorded the changing pattern of microbial growth and soiling on the building stones.

The reliability of colour measurements for estimating the biomass of phototrophic organisms in biofilms on stone surfaces was further demonstrated by Prieto et al. (2004) in a comparative study of the most common methods of quantifying phototrophic biomass, such as the in vitro measurement of chlorophyll *a* and fluorescein diacetate (FDA) hydrolysis. Prieto et al. (2002) carried out laboratory experiments that demonstrated a direct relationship between the amount of phototrophic organisms deposited on a surface and the colour generated. The same authors subsequently conducted a field study in which they used a portable spectrophotometer to monitor growth of a biofilm that was induced on quartz surfaces to mask the brilliant white color and thus mitigate the visual impact (Prieto et al., 2005). More recently, De Muynck et al. (2009) evaluated strategies for preventing algal fouling on two types of concrete (man-made stone), Tanaca et al. (2011) evaluated fungal colonization on three cement (man-made stone) formulations exposed to urban, rural and coastal environments and Sanmartín et al. (2012) established criteria for the early detection of phototrophic colonization (greening) and for monitoring its development on granite buildings using colour changes recorded with a portable spectrophotometer and represented in the CIELAB color space.

The application of reflectance colour measurements may thus provide an accurate method for monitoring the physiological state of biofilm-forming organisms (Prieto et al., 2002, 2005; Sanmartín et al., 2011a). The main advantages provided by this technique are the non-destructive nature of the procedure, allowing further analyses of the same sample, and the immediacy of the results. The technique focuses on cyanobacteria, since previous studies have indicated that cyanobacteria constitute a large part of the biomass on external surfaces of stone structures (Ortega-Morales et al., 2000; Tomaselli et al., 2000; Gaylarde and Gaylarde, 2005), and that they contribute significantly to accelerating the weathering of cultural monuments worldwide (Crispim and Gaylarde, 2005).

The characteristic blue-green colour of cyanobacteria is due to their photosynthetic pigment composition, which mainly consists of chlorophyll *a* (chl *a*) (greenish pigment), carotenoids (yellow-orange pigments) and phycobiliproteins, of which phycocyanin (PC) is responsible for the blue colour. Other phycobiliproteins in cyanobacteria include phycoerythrin (PE) (pink) and allophycocyanin (APC) (greenish-blue). As pigment content is closely associated with environmental conditions, such as the presence of nitrogen sources, light intensity, light quality, and nutrient availability, among other parameters (Tandeau de Marsac, 1977; Collier and Grossman, 1992; Kehoe and Gutu, 2006; Soltani et al., 2006), variations in environmental conditions give rise to visible changes in the colour of cyanobacterial cultures.

In a previous study, we evaluated the variation in colour of the cyanobacterium *Nostoc* sp. PCC 9104 in response to different environmental conditions, using the standardized CIELAB color system and determination of pigment (chl *a*, total carotenoids and PC) content (Sanmartín et al., 2010). We demonstrated that pigment content and the colour of this cyanobacterium are significantly affected by light intensity, availability of nitrates and macronutrient limitation, and we also established a close correlation between colour and pigment content. Considering relationships between pigment content and colour, and pigment content and environmental conditions, we now propose that the physiological status of the cultures or the influence of environmental changes could be assessed from their colour.

Taking into account previous results (Sanmartín et al., 2010), the first aim of the present study was to induce variations in colour and pigment content in the cyanobacterium *Nostoc* sp. PCC 9104, in response to other environmental conditions. Therefore, a source of phosphate was included as an environmental parameter (in addition to nitrate source and light intensity), with the aim of extending the colour and pigment content range obtained in the previous study (Sanmartín et al., 2010). Phosphate appears to play an important role in the colour of cyanobacteria (Collier and Grossman, 1992), and the presence of phosphate-containing compounds on stone surfaces may be increased by industrial sources or by air pollution (Ostroumov et al., 2003). For example, pigeon droppings contain phosphates and nitrates, and when the droppings are deposited on the surface of stone (e.g. a building facade), the salts can enter the pores, crystallize, increase in volume and cause deterioration of the stone. The salts may also remain on the surface, providing a source of nutrients that may encourage the growth of some microorganisms that form biofilms (Allsopp et al., 2004; Haag-Wackernagel, 2005).

The main objective of the present study was to improve our knowledge of the relationships between colour and pigment content in biofilms formed by cyanobacteria, in order to establish reflectance color measurements as a useful and reliable method of monitoring the growth and physiological status of the biofilms.

2. Materials and methods

In order to induce variations in color and pigment content, cyanobacterial cultures were exposed, in aerated tubes, to different environmental conditions (varying nitrate and phosphate levels and light intensity). This was achieved by a Box–Behnken design with 13 experimental points plus 2 additional experiments at the central point (three central replicates) (Montgomery, 1997). The experimental set-up was maintained for 14 days, during which the colour and pigment content of each sample was measured.

2.1. Culture conditions

A stock culture of *Nostoc* sp. PCC 9104, a filamentous N_2-fixing heterocyst-forming cyanobacterium, was grown in BG-11_0 medium (Rippka et al., 1979). Cells in the exponential phase of growth were collected and used as the inoculum for experiments.

The experimental procedure was carried out with a set of fifteen aerated batch cultures, containing 60 mL of various culture media and 20 mL of stock culture. The culture medium in each tube consisted of modified BG-11_0 medium containing 20 mM HEPES buffer, plus different amounts of nitrate (0, 1.5 and 3.0 g $NaNO_3$/L) and phosphate (0.04, 0.27 and 0.50 g K_2HPO_4/L). The batch cultures were maintained with aeration for 14 days under different light intensities (90, 135 and 180 μmol photon m^{-2} s^{-1}) and a 12 h:12 h