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In situ photosynthetic yields of cave photoautotrophic biofilms using two different Pulse Amplitude Modulated fluorometers

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ABSTRACT

In order to evaluate the photosynthetic efficiency of cave photoautotrophic biofilms, *in vivo* chlorophyll *a* fluorescence was measured using two different fluorometers, in two biofilms in the touristic karstic Nerja Cave (Spain). The study was done for entire days in summer and in winter during the first year and repeated for a second year, in order to cover the widest range of environmental conditions, *i.e.*, atmospheric CO_2, temperature, seeping water and relative humidity levels. Effective quantum yield and relative electron transport rate (rETR) were determined during periods of light whereas maximal quantum yield (F_v/F_m) was determined *in situ* during dark periods. On summer days, *in situ* photosynthetic yields in cyanobacterium biofilms (*Chroococcidiopsis* sp.) increased 7–16 times compared to that of winter days, whereas in biofilms comprised of green and red microalgae and various cyanobacterium species, no seasonal or yearly variations were observed. In contrast, maximal rETR in the two biofilms increased in the periods with the highest values of both CO_2 and relative humidity. Positive correlations between all environmental variables and rETR were found. According to Redundancy Analysis, all environmental variables, mainly CO_2 and relative humidity were related to photosynthetic variables. The effective quantum yields showed different values depending on the measuring light of the PAM. The values were higher with red light (Diving PAM) compared to blue light (Junior PAM) mainly in the site dominated by cyanobacteria. Nerja Cave is shown as an excellent place to study the effects of light and CO_2, among other environmental variables of biofilm photosynthetic activity. The monitoring of photosynthetic activity by *in vivo* chlorophyll *a* fluorescence could be used to follow the effects of the treatments applied by the touristic cave managers to reduce the proliferation of biofilms composed of various species, and, consequently, the biodeterioration of speleothems could be reduced.

1. Introduction

Autotrophic biofilms can grow in different environments, such as the soil of deserts, coastal intertidal systems, estuaries, in sediments of deep waters, and in the entrance caves, among others [1–4]. Biofilms can also proliferate on speleothems inside of touristic or "show caves" since they are illuminated by artificial light and they present favorable conditions for photosynthetic activity due to the high humidity, relative constant temperature, and high levels of CO_2 [3–6]. Biofilms alter the natural color of the stones, producing the biodeterioration of speleothems [7–9], *i.e.* "maladie verte" [10] or "lampenflora" [11]. There is high interest in avoiding or decreasing the biofilm cover in tourist caves by using physical, mechanical, and chemical methods [12]. One strategy is to apply light/dark treatments to reduce the photosynthetic activity and consequently the biomass proliferation. For example, photosynthetic activity can be decreased by increasing the dark period during the cave's visiting hours, the use of weaker-intensity lamps, the application of LEDs with light qualities enriched in the wavelength with less photosynthetic quantum efficiency for microalgae and cyanobacteria, and, finally, the application of UVC radiation ($\lambda = 200$–280 nm) in order to damage the biofilms [12,13]. This research field is a great opportunity for photobiologists to conduct *in situ* studies on the photophysiology of the biofilms under different environmental conditions and evaluate the bio-optical procedures done to eradicate, reduce, or control the biofilm growth. Generally, in touristic caves, physical and chemical environmental conditions are monitored and thus large-scale tests of the relation between biofilms physiology and environmental variables can be conducted.

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However, only a small number of photosynthetic studies have been done on biofilms of natural or artificial illuminated caves [14,15]. The photosynthetic activity of biofilms has been studied using oxygen evolution or by *in vivo* chlorophyll *a* fluorescence mainly in microphytobenthos [16–18]. Primarily developed to evaluate physiological status and photosynthetic activity in terrestrial higher plants [19,20]. Pulse Amplitude Modulation (PAM) chlorophyll fluorescence of photosystem II (PSII) has been used in phytoplankton [21,22], microphytobenthos [17,18,23], or soil biofilms in deserts [24]. In spite of the great advantages of *in vivo* chlorophyll fluorescence determination, the use of PAM florescence in cave biofilms is only very recent [6]. A PAM fluorometer emitting light pulses at three different wavelengths (470, 525 and 610 nm) within the range of a specific spectrum of chlorophyll *a* fluorescence was used [6]. Several authors showed the relation between fluorescence parameters and O_2 evolution or CO_2 fixation in terrestrial plants and algae [16,21,22,23,24]. In addition, diversity, growth, and primary production of cave biofilms have been tested by using different techniques, such as image analysis and confocal microscopy [5,8,13].

In order to reduce bio-degradation, it is necessary to identify the species of the biofilms, their structural organization, and their relation to the substrate and the bio-optical and physiological function [26–28]. The taxonomic composition of the biofilms and the bio-optical characteristics are known in the case of Nerja Cave [29], and this can help in the interpretation of the effect of light quality on photosynthetic activity. In the last few years, taxonomical and ultrastructural studies of the photosynthetic biofilms associated with natural and artificial light have been conducted in Nerja Cave [29]. However, no information on the yields and photosynthetic capacity is available yet. The caves could be good natural laboratories to evaluate the effect of environmental variables on primary productions of algae and cyanobacteria, *i.e.*, increasing CO_2 and temperature levels. This requires us to have CO_2 variations within the expected rise according to the climate change models [30]. This is the case of Nerja Cave since the variations of CO_2 are maintained throughout the year between 480 and 1000 ppm [31].

The aim of this study is to get information on the physiological activity of autotrophic biofilms in caves, where biofilms are exposed to optimal (temperature, relative humidity, or CO_2 levels) or stress conditions, such as desiccation periods, low irradiances, and low nutrient availability, among others. In this study, the photosynthetic activity of *in vivo* chlorophyll *a* fluorescence was evaluated *in situ* in algal and cyanobacterium biofilms exposed to different natural levels of CO_2, temperature, and the relative humidity of the air. The use of PAM fluorescence in cave biofilms is an innovative aspect in this study as the online measurements under the environmental conditions of the cave. Two Pulse Amplitude Modulated (PAM) fluorometers equipped with different light sensors as well as actinic light were used: Junior PAM (blue light, as both the measuring light and actinic light) and Diving PAM (red light as the measuring light and halogen light as the actinic light). Thus, it was possible to compare the photosynthetic yield of cyanobacteria and algae, with different bio-optical characteristics, and consequently different fluorescence responses depending on the expected light quality [25,32]. By using *in vivo* chlorophyll *a* fluorescence, the physiological knowledge of the biofilms can be increased and the possible cultivation of biofilms for the extraction of interesting bio-active substances could be successfully conducted.

2. Material and methods

2.1. Study site description, measuring sites, and the species in the biofilms

Nerja Cave was opened in 1960, and it is visited annually by 485,541 persons per year, the average number of visitors per year for the period 1988–2013 [33]. The control of CO_2 levels is crucial for the cave's conservation as well as for public health, so an adequate air quality is monitored for the visitors. The cave has a fixed microclimatic station and several mobile stations, including several sensors to measure temperature, relative humidity, and concentration of CO_2 in the air and ^{222}Rn at hourly intervals [34–36]. In addition, a weather station measures the environmental parameters outside the cave being the level of CO_2 the highest in August, which is the month with the greatest number of visitors in the cave (100,000) [31]. August, which is the month with the highest number of visitors in the cave (100,000) [31]. The average annual CO_2 concentration in the cave is 505 ppmv during autumn and winter, whereas in spring and summer the average increases to 750 ppmv with peak values 850 ppmv [31]. Only during a short period of the year (one week in July due to the increase of visitors related to recreational activities), CO_2 levels can reach values up to 1200–1500 ppmv, but after 7 days the CO_2 levels recover again to the normal, summer level of 800 ppmv [37]. In a study from 2008 to 2013, the reported maximal and minimal level of temperature was 15.6 and 19.91 °C, respectively, whereas the relative humidity range was from 53 to 100% and, finally, CO_2 ranged from 357 to 1423 ppmv [33]. In the Nerja Cave, the external atmosphere enters the cave due to good ventilation, mainly in winter, and human activities are considered the main sources of CO_2 [38]. In Nerja Cave during autumn, winter and spring, a positive correlation between the CO_2 content of the air inside in the cave and the difference between the external and internal air were observed, such that when this difference increased, there was a higher level of CO_2 within the cave [31]. In contrast, during summer, there was a negative correlation between CO_2 and temperature difference between the air inside and the air outside of the cave. At this time in the year, the renovation of the air is much slower due to lower ventilation. On the other hand, a positive correlation between the CO_2 concentration in the air of the cave and the number of visitors was observed.

Two sites in the touristic galleries were selected, each with different stable biofilms (Fig. 1). Site Ne8 is located at the Hall of Phantasms whereas Ne12 is located deeper in the cave, at the beginning of the Hall of Cataclysms (Fig. 1). The biofilms have not previously been submitted to any cleaning treatments. At the Ne8 site, there is no flowing water on the rocks (seeping water) whereas in Ne12, in summer, drops of water run over the speleothems (Fig. 1). The biofilms present different morphologies. Ne8 has a powdered consistency while Ne12 is gelatinous and very easy to cut from the rock in contrast to the biofilms of the Ne8 site. Del Rosal [29] identified only cyanobacteria at the genus level in the Ne8 biofilms, *i.e.*, *Chroococcidiopsis* sp. whereas, in Ne12, several cyanobacteria taxa were identified: *Pseudophormidium* sp., *Leptolyngbya* sp., *Aphanothece saxicola* Nägeli, *Gleocapsa atrata* Kützing and *Chalicogloea cavernicola cavernicola* M. Roldán, M. Ramírez, J. del Campo, M. Hernández-Mariné & J. Komárek, red alga *Cyanidium* sp., and green algae as *Desmococcus endolihicus* P.A., Broady & M. Ingerfeld and *Jenufa* sp.

2.2. Bio-optical characteristics of the biofilms

In order to know the bio-optical characteristics of the biofilms, *i.e.*, *in vivo* transmittance (T_λ) spectra in the photosynthetic active radiation region of the spectra (PAR, $\lambda = 400–700$ nm) tests were conducted in the laboratory on removed biofilms (area of 1.5 cm^2). The biofilms were transported in a cooled box. For the transmittance measurement, biofilms were carefully placed in Petri dishes and then illuminated by a solar simulator with a xenon lamp using dichroic filters to simulate the solar spectrum (Termo-Oriel, model 66,902). The spectral transmission of the biofilms ($T_\lambda b$, transmittance of biofilms) was determined by placing the sensor of the multidiode spectroradiometer Sphere Optics model SMS 500 below the Petri dishes. Spectral absorptance (A_λ) was determined as:

$$A_\lambda = 1 - T_\lambda - R_\lambda \quad (1)$$