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Monitoring photosynthetic activity using *in vivo* chlorophyll *a* fluorescence in microalgae and cyanobacteria biofilms in the Nerja Cave (Malaga, Spain)

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Abstract: The characterization of the most common photosynthetic biofilms in the Nerja Cave by the continuous monitoring of the *in vivo* chlorophyll *a* (Chl *a*) fluorescence and the incorporation of the irradiance as a new environmental variable related to previous studies in the cave, have allowed us to improve our knowledge about the photosynthetic pattern of the biofilms of the cave. Effective quantum yield ($\Delta F/F_m$) and relative electron transport rate (rETR) were determined during periods of the light, whereas the maximal quantum yield (F_v/F_m) was determined during dark periods. Increases in the photosynthetic yields and productivity in summer period were found related to the highest values of the environmental variables, such as relative humidity, air carbon dioxide concentration and air temperature. According to the irradiance, the studied biofilms had an optimal growth with cave lighting, considered low in comparison with similar studies, perhaps because they can grow mixotrophically too. Moreover, when the irradiance increased, both the $\Delta F/F_m$ and the rETR decreased in springtime, suggesting photoinhibition of the photosynthetic yield in the biofilms within the cave, whereas in the summertime, the photosynthetic yield had a positive correlation with the irradiance, suggesting a decreased of the photoinhibition, possibly due to the increase of the environmental variables values which provokes an alleviate on the extent of photoinhibition.

Keywords: Biodeterioration, cyanobacteria, *in vivo* chlorophyll *a* fluorescence, microalgae, Nerja Cave

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INTRODUCTION

Cyanobacteria and microalgae biofilms can induce biodeterioration in natural and cultural heritage. Traditional techniques used to control photosynthetic biofilms damage in subterranean environments have been based on physical, mechanical, and chemical methods but most of them are considered risk factors for the conservation of the subterranean heritage (Mulec, 2009; Baquedano et al., 2019). Therefore, friendly environmental alternatives for combating the development of these biofilms are currently evolving towards the use of UV-C radiation (Borderie et al.,

2015; Pfendler et al., 2018), hydrogen peroxide solution (Faimon et al., 2003; Trinh et al., 2003; Baquedano et al., 2019) or lighting sources that reduce photosynthetic activity (Roldán et al., 2006; Hsieh et al., 2013; Álvarez-Gómez et al., 2015). However, photoautotrophic biofilms can survive in hostile environments such as the caves, frequently characterized by low lighting and desiccation periods (Potts, 1999; Luttge et al., 2009; Behrendt et al., 2020). In fact, among different biological members, cyanobacteria and microalgae have been highly adaptive throughout many eons and can grow autotrophically, heterotrophically or mixotrophically.

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Based on this, information about their biodiversity, ecology, physiology, structural organization and relationship with substrates is essential to control and prevent their growth and the biodeterioration (Albertano & Urzi 1999; Del Rosal et al., 2018b).

The Nerja Cave, in the southern of Iberian Peninsula (36°45'43"N 3°50'41"W, Spain), has been declared an Asset of Cultural Interest in the category of Archaeological Sites and an internationally recognized Heritage Site of Geological Relevance. The cave preserves a chrono-cultural and paleoenvironmental sequence from, at least, 25,000 to 3,600 years before the present and more than three hundred catalogued rock art paintings, belonging to the Upper Palaeolithic and Late Prehistory (Jordá-Pardo, 1986; Sanchidrián, 1994). The cave is also inhabited by several endemic species and has exceptional geological formations, constituting the natural heritage of the Nerja Cave (Del Rosal et al., 2014b). This natural and cultural monument is one of the most important tourist caves in Spain, with about 450,000 visitors annually. The monthly visitor numbers are largely consistent from year to year, with the minimum in winter and the maximum in summer (Carrasco et al., 2002). The cave has a surface area of 35,000 m² and a volume of about 300,000 m³ but only a third of the cave, the so-called Tourist Galleries, have been adapted for visitors.

The Nerja Cave is equipped with a complete network of environmental monitoring stations that automatically record air temperature, relative humidity, and CO₂ and ²²²Rn gas concentration in the air, amongst other parameters (Carrasco et al., 2002; Liñán & Del Rosal, 2015). In the Tourist Galleries the air temperature ranges between 13–20°C (average of 19°C), the relative humidity between 35–100% (average of 83%) and the CO₂ between 360–1552 ppm (average of 717 ppm) all of them with the highest values in the summer and the lowest in the winter (Liñán et al., 2018).

The tourist lighting system has favoured the development of photosynthetic microorganisms organized in biofilms in the cave, which colonise walls and speleothems and appears as green patinas around lighted substrate (Del Rosal et al., 2014). The most abundant biofilms grow on dry speleothems and are mostly formed by the cyanobacteria *Chroococcidiopsis* sp. and the red microalga *Cyanidium* sp., both with sheaths, especially large in the cyanobacteria (Del Rosal et al., 2018b). Jurado et al., (2018) established a 90% similarity at the molecular level between the *Chroococcidiopsis* sp. present in the Nerja Cave and a halotolerant strain of the genus *Halothece* (García-Pichel et al., 1998). Similarly, *Cyanidium* sp. in the Nerja Cave was found to be related to *C. chilense* Schwabe (Ciniglia et al., 2019) and was shown to have established 94.6, 95.2, and 92% molecular similarity with the *Cyanidium* of Monte Rotaro Cave (Italy) (Ciniglia et al., 2004), Atacama Desert (Chile) (Azúa-Bustos et al., 2009), and Sybil Cave (Italy) inhabitants, respectively, whilst the similarity with the extremophile *C. caldarium* (Tilden) Geitler was only 48.8% (Jurado et al., 2018).

Photosynthetically active radiation (PAR) is one of the most important limiting factors for photosynthetic

biofilms, although it is not the only one (Gómez-Bolea et al., 2014). Thus, light is directly related to the physiology of the microorganisms of caves (Roldán et al., 2006; Figueroa et al., 2017) presented a pioneer research on photosynthesis in the Nerja Cave by using *in vivo* Chl *a* fluorescence. They concluded that fluorimeters are useful tools to measure both photosynthetic yields and relative electron transport as non-intrusive procedure and established a preliminary relationship between environmental changes and the physiological status of the biofilms. However, their research was only carried out for several hours a day on different days.

The aim of this study is to increase our knowledge about the photosynthetic activity of biofilms in the Nerja Cave, by measuring of the effective quantum yields ($\Delta F/F_m$), maximal quantum yield (F_v/F_m) and relative electron transport rate (rETR) over completed days, and establish the relationship with the natural levels of CO₂, temperature, and relative humidity of the air and with the irradiance of the lamps, in this case, controlled by humans. The obtained information is useful in the design of strategies to control the biofilms growth and, therefore, in the adequate conservation of the natural and cultural heritage of the Nerja Cave.

MATERIAL AND METHODS

Study site and biofilms description

In this study, we selected two photosynthetic biofilms (Ne8 and Ne16) located in the inner section of the Tourist Galleries in the Nerja Cave, just a few metres apart and, therefore, affected by similar environmental factors (Liñán & Del Rosal, 2016; Liñán et al., 2018).

Both biofilms were bluish green, powdery and embedded into the dry substrate of the rock surface. Taxonomic studies identified biofilm Ne8 as mainly consisting of the cyanobacteria *Chroococcidiopsis* sp. (Del Rosal et al., 2018b) whilst in Ne16 the red microalga *Cyanidium* sp. was the most abundant (Del Rosal et al., 2015) (Fig. 1).

Bio-optical characteristics of the biofilms

To know the light absorption of the studied biofilms, *in vivo* transmittance (T_λ) spectra in the PAR of the spectrum ($\lambda = 400\text{--}700\text{ nm}$) tests were conducted in the laboratory on removed biofilms (area of 1.5 cm²). Spectral absorbance (A_λ) was determined as: $A_\lambda = 1 - T_\lambda - R_\lambda$, being $T_\lambda = T_{\lambda b} : T_{\lambda 0}$, the ratio of the transmittance of the biofilms ($T_{\lambda b}$) and the transmittance of the Petri dishes without biofilms ($T_{\lambda 0}$). Spectral reflectance (R_λ) was not considered.

The biofilms were transported in a cooled box and carefully placed in Petri dishes and illuminated by a solar simulator with a xenon lamp using dichroic filters to simulate the solar spectrum (Termo-Oriel, model 66.902). $T_{\lambda b}$ was determined by placing the sensor of the multidiode spectroradiometer Sphere Optics (model SMS 500) below the Petri dishes. To

test the dependence on humidity, samples were also moistened with a few drops of water.

The absorption spectrum generated with the solar

simulator does not generate spectral artefacts in the UV region and can be used to confirm the presence of accessory pigments absorbing in the UV region.

Fig. 1. Macroscopic image of biofilms Ne8 (A) and Ne16 (B). Confocal Laser Scanning Microscopy images with *Chroococcidiopsis* sp. (C) and *Cyanidium* sp. (D). Maximum intensity projection of the natural auto-fluorescence of pigments (red) and mucilaginous sheaths (green) and inorganic substrate reflection (white) (from Del Rosal et al., 2018b).

Nerja Cave lighting system

The studied biofilms were illuminated by white low-pressure lamps (model Roblan ES27, 18W with a colour temperature of 4,845 K and 1,030 lm), the same as used for the tourist lighting, and they stayed on for 8 hours approximately, depending on the development of activities inside the cave that need electric light (tourism, research or conservation). About these activities, they have different timings and, therefore, the period of darkness varies depending on the day. Each biofilm was lit by one lamp and no interference from other lamps was allowed. The spectral irradiance from 280 to 700 nm of the lamps was determined by using a multi-diode spectroradiometer (Sphere Optics model SMS 500). The incident irradiance (E_{PAR}) of photosynthetic active radiation (PAR, $\lambda=400-700$ nm) was measured using a spherical quantum PAR sensor (US-SQS/L, Walz GmbH) connected to a radiometer (Licor 250A). In order to evaluate the effect of the *in situ* irradiance produced by low pressure lamps and not by the actinic light provided by saturation pulses of the PAM fluorometer, different sampling sites were selected along a E_{PAR} gradient in both biofilms, named: “low”, “medium” and “high” (1, 6 and 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively) for Ne8 and “high” and “very high” (10 and 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively) for Ne16, where “high” and “very high” were the shortest distances between the biofilms and the lamps. These measurement areas were conditioned by the morphology of the surface where the biofilms grow, therefore, only one common area was measured in both biofilms, the so-called “high” (10 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Physico-chemical environmental variables

The temperature, relative humidity and CO_2 concentration in the air were determined using a monitoring station located near the study sites consisting of data-loggers CR1000 (Campbell Scientific) with special sensors selected for the range of measurements expected (Fig. 2). The CO_2 was measured using an infrared absorption sensor model GMM222-Vaisala (measuring range 0–5000 ppmv, accuracy $\pm 1.5\%$). Temperature and relative humidity were measured by the probe model HMP155A-Vaisala (measuring range 0–100% RH, accuracy $\pm 1.0\%$, measuring range -80 to $+60^\circ\text{C}$, accuracy $\pm 0.17^\circ\text{C}$). The environmental parameters were measured every 15 minutes, at the same time we measured the Chl *a* fluorescence.

In vivo Chl *a* fluorescence – physiological variables

Effective quantum yield ($\Delta F/F_m$) and maximal quantum yield (F_v/F_m) were determined *in situ* using a PAM fluorometer, Diving-PAM (Walz GmbH, Effeltrich, Germany) equipped with a red light as the measuring light and a halogen light as the actinic light. The actinic light and saturation pulses, with an irradiance of $>4,000 \mu\text{mol m}^{-2} \text{s}^{-1}$, were provided by a halogen lamp. The optical fibre used in this study has a length of 100 cm and a diameter of 5.5 mm. The optical fibre was placed around 1–2 mm away from the biofilm and care was taken to not shade the study site with the fibre. In each sampling site, measurements were also determined every 15 minutes, during both light ($\Delta F/F_m$) and dark periods (F_v/F_m), when the cave was open and closed to the public, respectively, during 24 hours a day for three days and in two seasons, spring and summer.

Fig. 2. Map of Nerja Cave where studied biofilms and monitoring station are located.

The effective quantum yield was calculated by $\Delta F/F_m'$, where $\Delta F = F_m' - F_t$ and F_m' was the maximum fluorescence yield of an illuminated sample whilst F_t was the instantaneous fluorescence of illuminated sample measured briefly before the application of a saturating pulse. The F_m' values were recorded after applying the saturating pulses (according to Figueroa et al., 2017). Maximal quantum yield was calculated by F_v/F_m , where $F_v = F_m - F_o$ and F_m was the maximal fluorescence after a saturating pulse during the dark period and F_o was the basal fluorescence of fully oxidized reaction centres. Finally, rETR through PSII, expressed as $\mu\text{mol electrons m}^{-2} \text{ s}^{-1}$, was determined as $\text{rETR} = \Delta F/F_m' * E_{\text{PAR}}$, where E_{PAR} was the incident irradiance of PAR, expressed in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Statistical analyses

PCO is a method to explore and to visualize similarities or dissimilarities of a data distribution in a distance matrix. It is a projection of points on the axis that minimizes the residual variation in the space of the chosen distance with the aim of producing a summarized geometric representation of the similarities between them. The eigen values are used to measure the amount of variance explained by the synthetic variables on each axis of the PCO diagram. A principal coordinate analysis (PCO) was carried out to detect patterns among photosynthetic responses throughout the seasonal gradient, which were compared in both spring and summer, based on the Euclidean distance using PERMANOVA for PRIMER6 package. The replicates, in both seasons, for each measured day were done with the monitoring of *in vivo* fluorescence parameters, i.e., F_v/F_m , $\Delta F/F_m'$ and rETR. Pearson correlation coefficients were calculated and tested amongst all the dependent variables. These analyses were performed using the Sigma Plot 12.0 (SYSTAT software Inc. USA). The overlay of the vectors onto the PCO was performed

using the Spearman correlation. This procedure is an equivalent ordination to a principal component analysis (PCA) and calculates the percentage variation explained by each or the axes in the multidimensional scale (Anderson et al., 2018).

In order to determine a general variation pattern between the photosynthetic variables and the abiotic variables, such as temperature, relative humidity, CO_2 and irradiance, a multivariate approach was followed, using a redundancy analysis (dbRDA). A preliminary distance based linear models with Akaike information criterion (AIC) selection criterion was carried out for each site. The redundancy analysis (dbRDA) and distance based linear model (DistLM) were made using PERMANOVA+ for PRIMER6 package (Anderson et al., 2018).

RESULTS

Environmental variables

During the spring, the average daily values of temperature ranged from 18.6 to 19.3°C, the relative humidity from 74.6 to 83.4% and the CO_2 levels from 570 to 1017 ppm. In the summer, the average daily values of temperature ranged from 19.4 to 19.9°C, the relative humidity from 92.4 to 97.1% and the CO_2 from 617 to 1472 ppm (Table 1).

The highest values of measured variables in summer were according with the lowest natural ventilation of the annual cycle in the Nerja Cave karstic system and the highest number of visitors (Liñán et al., 2018).

Spectral irradiance of the lighting system and spectral absorbance of biofilms

The spectral irradiance of the lamps showed maximal emission in the blue-green wavelength, with the highest peak around 430 nm and around 540 and 615 nm the second and third peak in decreasing order (Fig. 3).

Table 1. Statistical parameter of microclimatic data registered in the Nerja Cave. Legend: max = maximum value, min = minimum value, avg = average value, sd = standard deviation.

Variable	SPRING				SUMMER			
	max	min	avg	sd	max	min	avg	sd
Air temperature, T (°C)	19.3	18.6	19.0	0.1	19.9	19.4	19.6	0.1
Relative humidity, RH (%)	83.4	74.6	79.5	1.7	97.1	92.4	95.6	0.5
Air CO ₂ (ppm)	1017	570	717.1	88.1	1472	617	1023	179.2

Fig. 3. Emission spectrum of the white low-pressure lamps that illuminate the biofilms.

The *in vivo* absorption spectra ($\lambda = 280 - 700$ nm) was remarkably similar in both biofilms, with no significant differences between dry and humid measurements (Fig. 4). Both spectra showed overlapping absorption of the pigments in the range of Chl *a* (λ max. absorption around 670 nm), the phycobiliproteins phycocyanin (λ max. absorption =

610 – 625 nm) (Patel et al., 2005) and allophycocyanin (λ max. absorption = 650 – 660 nm) (Adir, 2005) and another overlapping peak-shoulder located in the UV region, at 340 – 360 nm, suggesting the presence of UV-absorbing pigments as mycosporine-like amino acids (MAAs) i.e., 310 – 360 nm and scytonemin, i.e., 370 nm (Dillon et al., 2002).

Fig. 4. Absorption spectrum of biofilms formed mainly by *Chroococcidiopsis* sp. (A) and *Cyanidium* sp. (B), respectively using a solar simulator in dry and humid conditions.

Physiological variables and photosynthetic activity

The photosynthetic activity of biofilms in the Nerja Cave was represented by $\Delta F/F_m'$, F_v/F_m' , and rETR. In Ne8 and Ne16 biofilms, these physiological parameters varied depending on the season and the irradiance but, in general, they showed the highest values in summer and presented oscillations in the quantum yield values with the temporal patterns such as natural diurnal variations.

$\Delta F/F_m'$ and F_v/F_m' averages increased with increased irradiance in Ne8 except in spring when the photosynthetic activity decreased at irradiance from 6 to 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. During the summer, $\Delta F/F_m'$

F_m' in Ne8 decreased rapidly when the lights were on but some hours later it increased again and continued throughout the dark period, when the maximum F_v/F_m' was reached. This pattern was not so clear in spring when, on the other hand, at a time before the lights came on, a decrease in F_v/F_m' was observed at 6 and 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 5A, B). rETR was also higher in summer than in spring when the average at 1 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was nearly 0 $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$. In the same way, during the spring and summer periods, rETR increased with the irradiance except from 6 to 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in spring, when rETR decreased (Fig. 5C, D).

Fig. 5. Values of effective ($\Delta F/F_m'$) and maximum (F_v/F_m') quantum yields (A, B), and relative electron transport rates (rETR) (C, D) at different irradiances in biofilm Ne8 during monitored days of spring (A,C) and summer (B,D). Legend: shaded: darkness, Avg $\pm$ SD: average values and standard deviation, E_{PAR} : PAR irradiance.

In biofilm Ne16, $\Delta F/F_m'$ and F_v/F_m' values showed the greatest differences between the summer and the spring, when they were lower. However, their averages were similar at different irradiances, both in the spring and summer. Similar average values of $\Delta F/F_m'$ and F_v/F_m' can be related to the low growth irradiance i.e., photosynthetic

yields in low irradiance are close to the maximum in darkness, under full oxidation of photosynthetic reaction centres. As in biofilm Ne8, a general decrease in $\Delta F/F_m'$ was observed after the light was on, but just in summer. After that, it slightly increased or was constant, also in darkness (F_v/F_m') to fall again during the next

light on period (Fig. 6A, B). As far as the rETR was concerned, it was also lower during the spring, when the average was close to 0 $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$ but

showed a strong increase in summer, with increases in irradiance, reaching maximum values close to 10 $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$ at 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 6C, D).

Fig. 6. Values of effective ($\Delta F/F_m'$) and maximum (F_v/F_m) quantum yields (A, B), and relative electron transport rates (rETR) (C, D) at different irradiances in biofilm Ne16 during monitored days of spring (A, C) and summer (B, D). Legend: shaded: darkness, Avg $\pm$ SD: average values and standard deviation, E_{PAR} : PAR irradiance.

Physiological variable similarity using principal coordinate analysis

This statistical analysis revealed that, in Ne8 biofilm, the first axis explained 83.6% of the variation of $\Delta F/F_m'$, F_v/F_m and rETR. The second axis only explained 11.5% of the variation. The combination of the first two axes explained 95.1% of the variation in these variables. Both dimensions (PCO1 and PCO2) had a relationship with the seasonal time, but the PCO1 had a negative correlation with low values for the $\Delta F/F_m'$, F_v/F_m , and rETR variables for the spring, in contrast, in summer all variables were higher, and they had a positive correlation with second dimension or PCO2 in both samples sites (see Fig. 7A, B). The points on the left side of the first axis

mainly corresponded to the summer samples and they showed higher values in the physiological variables than the points on the right, which corresponded to the spring samples (Fig. 7A). The same pattern was observed in PCO diagram for Ne16, where the first axis explained 92.6% of the $\Delta F/F_m'$, F_v/F_m and rETR variation whilst the second one only explained 5.5% of the variation. The first two axes explained 98.1% of the variation in these variables. This biofilm also showed higher physiological values in the summer compared to the spring (Fig. 7B). In both the Ne8 and Ne16 biofilms, the small angle between the vectors indicated a high correlation between all the physiological variables, in consequence, the seasonal factor had a very marked effect on these responses.

Fig. 7. Principal coordinates (PCO diagram) of biofilms Ne8 (A) and Ne16 (B) in the spring (values indicated with a circle) and summer (values indicated with a square); vectors overlay (Spearman rank correlation) indicates the relationship between the PCO axes and the monitoring photosynthetic variables; F_v/F_m (maximum quantum yield), $\Delta F/F_m'$ (effective quantum yield) and rETR (relative electron transport rate).

Physiological and environmental variables. Distance based redundancy analysis of biofilms

According to the results of the distance based linear models, which were performed as a preliminary analysis to redundancy, the best spatial arrangement of the samples was described by the (DistLM) with the AIC selection criterion.

In the preliminary DistLM analysis prior for Ne8 biofilm, the physiological variables and all the environmental variables studied showed the best spatial arrangement ($R^2 = 0.614$) whilst for Ne16, the DistLM analysis showed that just three categorical environmental variables: air CO_2 , relative humidity, and temperature, explained the best spatial arrangement of the physiological samples ($R^2 = 0.882$).

Fig. 8. Redundancy analysis (dbRDA diagram) of biofilms Ne8 (A) and Ne16 (B) for physiological variables in relationship to abiotic variables: CO_2 (carbon dioxide), T (temperature), RH (relative humidity) and irradiance, in spring (circle) and summer (square).

The dbRDA in Ne8 ordination explained 57.1% of the total variance of the physiological data and showed a season separation based on the abiotic factor response. Thus, the irradiance categorical factor showed a significant covariation for the samples in the summer, in consequence these variables increased with the irradiance in contrast with spring, when the physiological variables were better explained by the temperature and CO_2 in the air, and these results showed covariation amongst all the abiotic variables, explaining the distribution of the biotic samples (Fig. 8A). In Ne16, the dbRDA ordination explained 87.5% of the total variance of the physiological responses and showed a high separation between seasons. The physiological responses in the summer period showed a clear increase compared

to the CO_2 , temperature, and relative humidity, with a higher correlation in contrast to the spring, when the physiological response was lower compared to the abiotic variables (Fig. 8B).

DISCUSSION

The monitoring of the physiological parameters through the *in situ* measurements of the photosynthetic activity of the Nerja Cave biofilms was previously carried out by Figueroa et al. (2017). They used the fluorometer Diving PAM (measuring light: red-light) to monitor the *in vivo* fluorescence of the Chl *a* associated with photosystem II (PSII) to obtain relevant information about the physiological

behaviour of the biofilms. Their results allowed us to know about the dynamics of physiological variables F_v/F_m , $\Delta F/F_m$ and rETR. They also reported that increases in relative humidity, temperature and CO₂ in the air in summer, were highly related to increases in physiological variables of the biofilms. However, their research was only carried out for several hours a day on different days. In this study, longer-term monitoring of *in vivo* chlorophyll fluorescence than that by Figueroa et al. (2017) was conducted and, in addition, the effects of irradiance were analysed in order to increase the physiological pattern, i.e. photosynthesis, required to design useful strategies to control the development of the biofilms.

Despite the analysed biofilms being taxonomically different, the similarity of their absorption spectra was noteworthy. Both of them showed maximum absorption of Chl *a* and phycobiliproteins, phycocyanin and allophycocyanin. Phycobiliproteins are water-soluble proteins that are usually covalently bound with phycobilins which give them distinct absorption spectra ranging from 460 to 670 nm (MacColl, 1998). After capturing light energy, phycobiliproteins transfer this energy to chlorophyll P₆₈₀ during photosynthesis (Glazer, 1994). Normally, phycobiliproteins are found *in vivo* assembled in huge macromolecular complexes, which are known as phycobilisomes, the main light harvesting antenna systems found in cyanobacteria and red algae, located on the stromal face of the thylakoid membrane (Watanabe et al., 2013). Cyanobacteria can sense the wavelength and energy of the ambient light, process the information and modulate the phycobilisomes construction by changing the size, form and phycobiliprotein composition (Sanfilippo et al., 2019). However, the presence of phycoerythrin was not observed although the spectra of the lamps showed a maximum emission peak around 540 nm, the range maximum absorption of this phycobiliprotein.

As the absorption spectra generated with the solar simulator does not generate spectral artefacts in the UV region, the presence of accessory pigments absorbing in the UV region is reliable. In this way, UV radiation is considered a widespread environmental stressor with a positive influence on scytonemin biosynthesis, as adaptative strategy to survive in extreme environments like deserts (Orellana et al., 2020) and on MAAs which are considered to be photochemically stable molecules acting as sunscreens against harmful ultraviolet (UV) radiation. Moreover, MAAs can be quickly synthesized in response to light, nutrients or temperature stress (Moisan et al., 2009) whereas scytonemin is primarily a strategy of photoprotection against longer wavelengths solar irradiance than that MAAs (García-Pichel & Castenholz, 2004). Photoinhibition may favour the presence of MAAs and scytonemin as long-lasting protectants, but certain environmental stress, such as desiccation, can enhance synthesis (Fleming & Castenholz, 2007). Such absorbing molecules immobilised in EPS provided protection mainly against UV radiation both by UV-screen and antioxidant activity (De la Coba et al., 2009; Schenider et al., 2020; Vega et al., 2020). However, it has been demonstrated constitutively synthesis of scytonemin

under low visible light (no UVA), in *Chroococcidiopsis* CCME246, a cave strain never submitted to solar radiation (Fleming & Castenholz, 2007). Such strategy could be being used by the inhabitants Nerja Cave and protect them against photoinhibition, when other stress factors on the cave biofilms are preeminent.

Monochromatic narrow-band LEDs lights are showed as successfully light for touristic cave as they can be adjusted to wavelength emission. About the most adequate emission to prevent phototrophs biofilms growth, we must consider that their process of photosynthesis is enhanced at wavelength in the intervals 430-490 nm and 640-690 nm, where Chl *a* absorbs, and eliminating all blue light favouring yellow colours would deprive microorganisms of the most energetic photons absorbed by Chl *a*. Moreover, Roldán et al. (2006) concluded that green light could prevent the growth of phototrophic organisms except for those phycoerythrin-containing, which was not observed in Nerja Cave biofilms as Fig. 4 showed. Concerning Nerja Cave biofilms, a pioneer study about photobiological responses of biofilms illuminated by several light emitted diodes (LEDs) with different spectral compositions showed differences between the empirical and theoretical approach, concluding that blue light is more efficient to prevent the growth of cyanobacteria in the cave (Muñoz et al., 2021). Furthermore, according to the theoretical approach, the first empirical study in Nerja Cave and the absorption spectra in our study, in order to reduce the photosynthesis activity in the biofilms of the Nerja Cave we suggest to study the effect of narrow-band LED lights with maximum emission peaks around 590 nm (amber), 540 nm (green) and 480 nm (blue), respectively. The three different LED lights must be combined to obtain different white lights which are safe and esthetical satisfactory for visitors.

According to environmental research, continuous monitoring has shown that the photosynthetic activity in the Ne8 and Ne16 biofilms was very low during spring, but they increased in summer, as observed by Figueroa et al. (2017). Thus, although the cave environment can be considered inadequate for the survival of photosynthetic microorganisms during spring, they were able to survive. This result was also in accordance with Del Rosal et al. (2018a) who reported increases in the surface covered by biofilms in the summer period and related them with seasonal photosynthetic patterns. They also identified that water was key environmental factor in the growth of photosynthetic biofilms in the cave as they were mainly composed of microorganisms with mucilaginous sheaths (EPS), which protects them from unfavourable environmental conditions (Flemming et al., 2007; Rossi & De Philippis, 2015). Thus, the sheaths play a fundamental role during the adhesion of microorganisms to the substrate as well as during the driest periods, since they act as a water reservoir (Macedo et al., 2009; Keshari Adhikary 2013). Potts (2001), Alpert (2006) and Ramirez et al. (2011) showed that the microorganisms are able to dehydrate without dying withstanding moisture fluctuations in extreme habitats for long periods of time. In summer,

the high relative humidity in the Nerja Cave allows the presence of condensation water in the substrate holes and cracks (Liñán et al., 2021), which allows the microorganisms to recover quickly. However, the adhesion processes, together with changes in the volume of the sheaths due to the described storage and loss of water, can damage the most superficial layers of the substrate where the biofilms grow and favour their biodeterioration.

As far as the irradiance is concerned, it is one of the principal factors affecting the photosynthetic performance of the organisms (Halldal, 1980) and they can adapt and acclimatize to different irradiances, reaching an optimum growth (Nielsen et al., 1962). According to Lamprinou et al. (2012) and Popović et al. (2015) caves show a general pattern of diversity of algae and cyanobacteria forming biofilms in relation to PAR, but this has not been observed in the Nerja Cave (Del Rosal et al., 2014b). In our study, the PAR was the same in each sampling site throughout the whole monitoring time, as the lights were not changed throughout the year. The results showed an optimum activity of the biofilms at low irradiance ($<20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) compared with natural non-subterranean environment and according to Mulec (2008) whose results showed that the coccoid cyanobacteria are better adapted to low light intensity, compared to other organisms. Nevertheless, the cave irradiance is low, so this fact is an indicator of the high adaptability of the microorganisms at low irradiance. In Ne8 biofilm, increased irradiance showed a slight decay in quantum yield and rETR in the spring but in the summer quantum yield remained similar whilst the rETR increased with the irradiance. In contrast, in the Ne16 biofilm, photosynthetic yield remained similar with the increases of irradiances, during both the spring and summer, although in spring the values were extremely low. In this biofilm, because of the morphology of the surface, $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was the lowest monitoring irradiance and, at the same irradiance, the photosynthetic yield reached values closed to zero during the spring in the Ne8 biofilm. These results could be related to photoinhibition of both biofilms at irradiances from $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and higher as a decrease in F_v/F_m and maximum rETR are indicators of photoinhibition according to Figueroa et al. (2014). The non-photoinhibition and even the increase of rETR with the increasing irradiance in summer, in both biofilms, compared to the spring, can also be explained by the higher average CO_2 content in summer due to the increase of tourists. The breathing of tourist, together with other environmental variable increases and water availability, which has been linked to the alleviation of the stress caused by increased irradiance on photosynthesis in green microalgae, as García-Gómez et al. (2014) observed a decrease in reactive oxygen species (ROS) accumulation and DNA damage. In other microalgae lower photoinhibition at elevated CO_2 levels was also observed, greater than the current atmospheric CO_2 content (Yang & Gao, 2003; Iñiguez et al., 2017). On the other hand, the Nerja Cave biofilms showed oscillations in the quantum yield values with the temporal patterns

such as natural diurnal variations. Interestingly, despite the artificial irradiation in the light period being constant, in the summer period a daily pattern similar to that of microalgae submitted to solar daily variations was observed, i.e., a decrease of $\Delta F/F_m'$ at noon, similar to that which has been reported in other studies under natural daily solar radiation (Figueroa et al., 2014; Jerez et al., 2016). This pattern could be related to a circadian rhythm that is maintained in the biofilms although they have never been exposed to sun radiation. In addition, in Ne8 biofilm during spring, a decrease in F_v/F_m was also observed at a time before the lights came on, probably because the reaction centres closed before the light stimulation began, and this may act as a protection mechanism. The low values of F_v/F_m in cyanobacteria can be explained by plastoquinone pool being reduced in the dark and thus F_v/F_m values are not reflecting real maximum quantum yield in addition to the role of dark respiration, fluorescence emission spectra from phycobilisomes and Mehler reaction (Schuurmans et al., 2015). Lighted biofilms in the Nerja Cave showed a transient increase of $\Delta F/F_m'$ which have been also observed *in situ* measurements in the marine plant *Halophila stipulacea* (Forsskål) Ascherson (Runcie et al., 2009) and in terrestrial plants such as *Pinus silvestris* L. (Porcar-Castell et al., 2008). This transient has been related to osmotic and nutrient incorporation (Figueroa et al., 2014). The transient values of the $\Delta F/F_m'$ at the beginning of light period have been reported as an indication of the activation by light of enzyme activity i.e. Rubisco related to CO_2 (Maheshwari et al., 1992) and nitrogen metabolism through oxide-reduction processes mediated by ferredoxin/thioredoxin (Foyer et al., 2012). Diurnal variations of photosynthetic pigments and nitrogen metabolism have also been reported in other microorganisms as well as in green macroalgae (López-Figueroa & Rüdiger, 1991; Figueroa & Viñegla, 2001).

Similarly, the principal component analyses studies with the PCO diagram showed a high relationship between the physiological variables with the environmental variables. The redundancy analyses showed that the related environmental variables with the physiology of the biofilms were CO_2 , temperature, relative humidity, and irradiance, in decreasing significance for the Ne8 biofilm, whilst for Ne16 the irradiance had no effect on the photosynthetic pattern of this biofilm, probably due to the higher measurement irradiance of this biofilm and the photoinhibition effect noted above. Therefore, although irradiance is a key factor in photosynthesis, it has proved to be the least significant variable compared to the other natural variables due to the biofilms in the Nerja Cave being highly adapted to low irradiance, among other aspects, probably through the development of heterotrophy metabolism as Hauer et al. (2015) described in very deep caves. However, as the lighting system can be managed, light quality could be an important non-invasive factor for controlling biodeterioration. Based on this, we plan to study the effect of narrow-band LED lights with maximum emission peaks around 590 nm (amber), 540 nm (green) and 480 nm (blue), as we have

exposed before. The use of UV-B or UV-C radiation is another alternative to remove photosynthetic biofilms in caves, but it may be inefficient since cave microorganisms may have resistance mechanisms to extreme environments (Snider et al., 2009).

CONCLUSIONS

The continuous monitoring, over completed days, of *in vivo* Chl *a* fluorescence has allowed to increase the knowledge about *in situ* photosynthetic activity of the biofilms in the Nerja Cave. As Figueroa et al. (2017) proposed, our results confirmed an increase in photosynthetic activity in summer, when the natural values of temperature, relative humidity and CO₂ in the air were higher, whilst during spring, the photosynthetic activity was almost non-existent. Nevertheless, the biofilms could survive probably due to the presence of EPS which acts as a water reservoir under winter stress condition. About the irradiance, the studied biofilms had an optimal growth in the exceptionally low irradiance in the cave, possibly because they can grow mixotrophically too. Moreover, a photoinhibition of the photosynthetic performance has been suggested when the irradiance was increased, whereas in the summer period, the increased environmental variables probably alleviate the extent of photoinhibition as a supply in the system. Interestingly, despite the artificial irradiation in the light period being a constant, a circadian rhythm pattern could be observed in the photosynthetic activity, although the biofilms have never exposed to sun radiation. Thus, although irradiance is a key factor in photosynthesis, it is not an adequate key factor to control microorganism growth in the Nerja Cave where the use of narrow-band LED lights with lower photosynthetic effectiveness can be considered to control the development of the biofilms.

Finally, the possibility of an increase in the amount of data available due to the use of a PAM fluorometer together with the availability of analysis techniques based on artificial intelligence, could be used as an efficient tool to understand, explain, and resolve problems related to the research of lighting inside the cave, to prevent biodeterioration processes and contribute to the conservation of the subterranean heritage without compromising the visual quality and the safety of visitors.

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