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Effects of changes in UV-B radiation levels on biofilm-forming organisms commonly found in cultural heritage surfaces

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ABSTRACT

Accurate measuring and monitoring methods available since the 1980s have shown that the amount of Ultraviolet B (UV–B) radiation reaching the Earth's surface has increased as a result of degradation of the ozone layer. Since the adoption of the Montreal Protocol in 1987, ozone levels have been recovering successfully. However, in the context of the current climate change, other factors such as changes in cloud patterns and an increased incidence of natural disasters (e.g. fires) may be disrupting this recovery. The present study aimed to investigate the effects of different UV-B radiation levels on biofilms colonising heritage monuments. For this purpose, the effects of current UV-B levels on a biofilm composed of *Synechocystis* sp. (a cyanobacterium), *Bracteacoccus minor* (a green alga) and *Fusarium* sp. (a fungus) were compared at three points along a South-North transect: Portugal, Galicia (NW Spain) and Ireland (from highest to lowest UV-B radiation, respectively). Increased levels of UV-B radiation caused changes in the growth, physiological state and composition of subaerial biofilms, with cyanobacteria being more resistant than green algae to high levels of UV-B. A reduction of fungal growth and extracellular polymer substances (EPS) production was also observed, related to the reduction of biofilm aggregation at high UV-B levels.

1. Introduction

In the stratosphere, sunlight and oxygen react to form ozone, which absorbs 99% of the ultraviolet radiation that enters the atmosphere, thus playing a crucial role in protecting life on Earth. The ozone layer was increasingly threatened during the second half of the 20th century as a result of the emission of chlorofluorocarbons (CFCs) (Prinn et al., 2000). Since the implementation of the Montreal Protocol (1987), the stratospheric ozone layer has been recovering (Burkholder et al., 2015). However, possible disruption of this recovery by natural disasters such as large wildfires, which produce smoke and burned biomass that destroy ozone in the stratosphere, is of concern (Bernath et al., 2022). A return to the ozone levels of 1980 could lead to a greater increase in the amount of Ultraviolet (UV) radiation reaching the Earth's surface (Fang et al., 2019; Williamson et al., 2014). UV radiation is divided into three ranges: long-wave radiation between 320 and 400 nm (UV-A), medium wavelength radiation between 280 and 320 nm (UV–B) and short-wave radiation between 100 and 280 nm (UV–C). Of the total amount of UV radiation that reaches the Earth's surface, 5% is UV-B. It is expected to be the type of UV radiation most affected by changes in stratospheric ozone. Regardless of changes in stratospheric ozone, climate change *sensu stricto* may lead to variations in the amount of radiation reaching the Earth's surface. On the one hand, cloud cover is undergoing changes, increasing in some regions (usually wetter areas), while decreasing in others (usually drier regions). On the other hand, changes in vegetation cover as a result of climate change may also alter the exposure of organisms to UV-B radiation (Bais et al., 2018; Bornman et al., 2019).

The existence of a latitudinal gradient of UV radiation, and modification of this gradient in response to climate change, could cause a shift in the geographical distribution of many organisms towards different latitudes (Bornman et al., 2019). The diversity, structure and function of different ecosystems, as well as the metabolism of the constituent organisms, may be modified by variations in exposure to UV-B radiation. However, not all organisms are affected in the same way by variations in radiation. Phototrophic aquatic microorganisms are protected from these variations by the overlying water column, unlike terrestrial organisms, which must cope with much more extreme conditions, such as large variations in radiation on both diurnal and seasonal scales. Thus, small variations in light can trigger substantial changes in the behaviour of these species. The biofilms colonizing cultural heritage artefacts

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constitute an example of terrestrial species that are exposed to high levels of solar radiation.

Of the organisms that make up biofilms colonizing cultural heritage, phototrophic organisms, especially algae and cyanobacteria, are the most strongly affected by radiation, which directly affects photosynthesis. The organization of microbial organisms in biofilms protects the cells from environmental damage. The structure of biofilms, in which the cells are grouped in layers embedded in a matrix of exopolymers and water, protects the cells of the deeper layers from the action of environmental factors and specifically from the action of light, as ultraviolet (UV) radiation does not generally penetrate far into the biofilm matrix, and only the upper layers of cells are exposed to its harmful effects (de Carvalho, 2017). UV-B radiation is biologically active and, at low doses, induces changes in gene expression, physiology, metabolism, and cell morphology and leads to the production of reactive oxygen species (ROS). This type of radiation has been reported to cause stress in photosynthetic organisms by damaging DNA and modifying molecules such as lipids and proteins, which are particularly vulnerable because they preferentially absorb UV-B (Beardall et al., 2002; Ma et al., 2012; Hartmann et al., 2020). The exposed cells can produce photoprotective compounds, whose cellular content increases in proportion to the intensity of ultraviolet light (Castenholz and Garcia-Pichel, 2012; Geraldes and Pinto, 2021). If exposure to UV-B increases, the species will face greater stress than they are used to, which could lead to harmful physiological changes, as UV-B is known to cause photodamage by modifying vital biochemical reactions such as transcription. Increased exposure to UV-B radiation can alter many of the characteristics of the organisms that colonize heritage monuments and affect their capacity to deteriorate the substrate. As a survival strategy, it is expected that increased levels of UV radiation will cause organisms to shift towards higher latitudes. Moreover, the expected increase or change in secretion of protective substances will lead to changes in the colour of organisms, leading to both aesthetic and physicochemical changes in the substrate.

The effects of UV-B radiation on phototrophic aquatic organisms have been widely reported in the scientific literature. However, little is known about how a complex consortium of subaerial organisms would respond to an increase in UV-B radiation or how the possible effects on any of component organisms might affect the overall integrity of the biofilm, modifying its biodeterioration capacity. In this context, the present study aimed to analyse the effects of changes in the UV radiation levels on the physiological state, inter-species relationship and general composition of a subaerial biofilm composed by *Bracteacoccus minor* (a green alga), a *Synechocystis* sp. (a cyanobacterium) (both phototrophic species) and *Fusarium* sp. (a fungus), all of which are typical colonizers of stone cultural heritage.

2. Materials and methods

In order to study the effect of changes in UV-B radiation on organisms colonizing cultural heritage monuments, we used a model biofilm composed of *Synechocystis* sp. (a cyanobacterium), *Bracteacoccus minor* (a green alga) and Fusarium sp. (a fungus). The selected species were chosen because they are typically present in biofilms colonising granitic heritage (Vázquez-Nion et al., 2016; Noguerol-Seoane and Rifón-Lastra, 1996). Samples of the biofilm were exposed to prevailing UV-B conditions in three locations along a South-North transect, including Ireland, Galicia (NW-Spain) and Portugal: the samples were exposed to UV-B radiation for 0 (control), 1, 2 or 3 h per day (respectively), for 42 days (sufficient time for the biofilm to mature). The effect of UV-B on growth, organism dominance, photosynthetic efficiency, pigment and EPS production was monitored every 15 days.

2.1. Biofilm preparation

The study was carried out using replicate samples of a subaerial biofilm grown on polycarbonate membranes from a mixed culture of *Synechocystis* sp. and of *Bracteacoccus minor* (1:1 cell ratio) plus *Fusarium* sp. The subaerial biofilm samples were grown on polycarbonate membranes (0.22 μm pore, 25 mm diameter, Millipore) previously sterilized for 1 h on both faces under UV-C lighting (Philips TUV F17T8). A total of 156 membranes were prepared and placed in Petri dishes containing 25 mL of previously sterilized (110 °C, 25 min) agarized BG11 medium, in groups of 3 (n = 108), and the rest in groups of 4 (n = 36). All membranes were inoculated in a laminar flow hood (IndeLab, IDL-48RV) with a suspension of exponentially growing cells of the culture (250 μL). The membranes were gently transferred with sterile forceps to fresh agar plates every 2 days, to ensure optimal growth of the biofilms, and were exposed for 42 days under the test conditions.

2.2. Experimental design and lighting set-up

The biofilm samples were exposed to the four different lighting treatments for 42 days with a customized illumination system, in a light cabinet with four compartments (Fig. 1). In all cases the same 8h light/16h dark cycle was applied. The duration of each type of UV-B treatment was adjusted according to the annual average daily UV-B radiation for each of the locations considered (Ireland, Galicia and Portugal) (EUROSUN database). In addition, a correction was applied in order to simulate UV exposure on a North-facing wall. It results in a duration of 1, 2 and 3 h per day of UV-B treatment to reach the daily cumulative dose, considering the power of the lamps used (0.000075W) (Table 1). Thus, the lighting period varied for each treatment: 8 h of daylight only (OSRAM L18/865), considered control conditions; 8 h of daylight plus 1 h of UV-B (Philips Ultraviolet-B TL-20W/12RS), simulating conditions in Ireland; 8 h of daylight plus 2 h of UV-B, simulating conditions in Galicia; 8 h of daylight plus 3 h of UV-B, simulating conditions in Portugal. The quality (spectrum) and quantity (intensity of radiation) of light that reached the inoculated membranes was measured with a StellarNet Blue-Wave spectrometer, with an operating range of between 350 nm and 1050 nm.

Nine Petri dishes each containing 3 inoculated membranes and three Petri dishes each containing four inoculated membranes were placed in each compartment of the lighting cabinet. On day 0 and after 14, 28 and 42 days, groups of 3 replicate plates were removed for monitoring (chlorophyll fluorescence, pigment extraction, colour measurements and EPS production). On day 42, plates with 4 membranes were used to analyse biofilm structure by confocal laser scanning microscopy (CLSM), in addition to the previously mentioned techniques (Fig. 1).

2.3. Monitoring biofilm formation

2.3.1. Quantification of Synechocystis sp. PCC 6803 and Bracteacoccus minor cells and Fusarium sp. growth rate

The numbers of green algae and cyanobacteria cells at the beginning and at the end (day 42) of the experiment were estimated using a 0.1 mm deep counting chamber or haemocytometer with a graduated Neubauer scale. The growth rate of *Fusarium* sp. was calculated by measuring the diameter of the fungal colony every two days (after each change of culture plate).

2.3.2. Chlorophyll fluorescence

To characterize the effect of UV dose on biofilm development, the chlorophyll fluorescence was monitored, as a proxy for growth (Eggert et al., 2006) and physiological state (Genty et al., 1989), immediately after inoculation of the samples (day 0) and on days 14, 28 and 42. Fluorescence signals in the samples were determined at wavelengths of 470, 645 and 665 nm by pulse-amplitude modulated (PAM) fluorometry, using a Phyto-PAM system (Heinz Walz GmbH) equipped with a Phyto-EDF fibre optics emitter-detector unit. The samples were held in darkness for 30 min to ensure the reduced state of the Photosystem II (PSII) centres (closed) before measurement. Five readings were taken to quantify the variables: minimal fluorescence in the dark- and

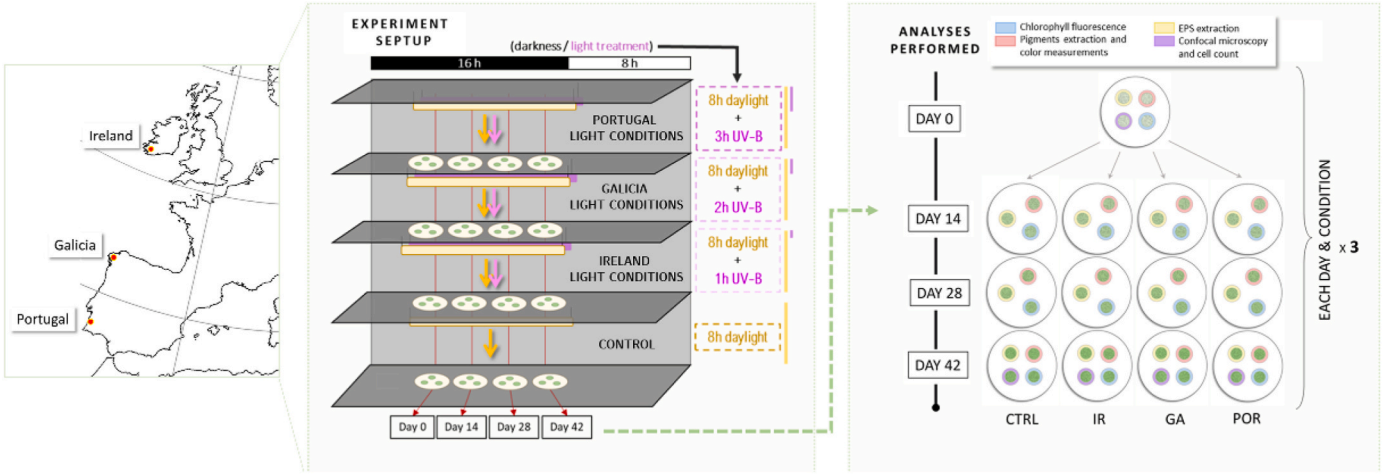

Fig. 1. Diagram of the experimental set-up and analytical systems used.

Table 1
Annual mean of cumulative UV and UV-B radiation per day on a roof and on a northern façade at three locations along a North-South transect (South Ireland, North-West Spain and South Portugal), and the corresponding exposure hours applied in the experiment considering the lamp power.

Site	Cumulative energy/day (J/cm^2)			Set up conditions***
	Total UV*	UV-B*	UV-B rad. on a North-facing wall **	UV-B lamp exposure (h)
South Portugal	110.08	2.55	0.71	3
North-West Spain	85.77	2.03	0.57	2
South Ireland	57.25	1.38	0.39	1

* EUROSUN database.
** Percentage of mean annual insolation of a northern façade in relation to a horizontal surface (28%) at a at the latitude of Galicia (NW Spain) (Díez-Mediavilla et al., 2019).
*** Using a UV-B lamp with 0.000075 J/seg.

light-adapted states (F_0 and $F_{0'}$, respectively), with all the PSII centres open; maximal fluorescence in the dark- and light-adapted state (F_M and $F_{M'}$, respectively), with all the PSII centres closed. These variables were used to calculate both Y_{MAX} (maximum quantum efficiency of PSII photochemistry, as $(F_M–F_0)/F_M$) and Y_{EFF} (effective quantum efficiency of PSII photochemistry, as $(F_{M'}–F_{0'})/F_M$) (Roháček, 2002). In addition, in order to study the variation in the relative microbial abundance, the $F_0$470nm/$F_0$645nm ratio was calculated as the signal at 470 nm, related to chlorophyll *b* (algae), and the signal at 645 nm, related to allophycocyanin (cyanobacteria). High values of the ratio indicate a predominance of green algae, while low values indicate a predominance of cyanobacteria (Fuentes and Prieto, 2021).

The Chl fluorescence decrease ratio or vitality index, RFd, defined as the F_d/F_s ratio, measured at saturation irradiance, is correlated with the potential CO_2 fixation rate (Brown, 1967; Lichtenthaler et al., 2005). The RFd was therefore measured at the end of the experiment as an indicator of CO_2 fixation by using a Handy FluorCam FC 1000-H (PSI, Brno, Czech Republic), with the following measurement parameters: shutter, 1; sensitivity, 14%; super, 30%; far, 30%; act, 100%).

2.3.3. Quantification of photosynthetic pigment contents

The chlorophyll *a*, chlorophyll *b*, scytonemin and total carotenoid contents and the phaeophytinization quotient (PQ) of all the samples exposed to different doses of UV-B were determined. Phytopigments were extracted on days 0, 14, 28 and 42, with dimethyl sulfoxide (DMSO). For this purpose, membranes with the biofilm were suspended in 1.5 mL of DMSO for 1 h at 65 °C with agitation. The samples were centrifuged for 10 min at 7000 g (Centric 150 Tehtnica), and the supernatant was measured at different wavelengths in a UV–visible spectrophotometer (Varian Cary 100). The chlorophyll *a (Chl a)*, chlorophyll *b (Chl b)*, scytonemin and carotenoid contents and the PQ were calculated using the following equations (Mushir and Fatma, 2012; Wellburn, 1994):

Chlorophyll-a = 12.47 - (Abs664-Abs750) - 3.62 - (Abs647-Abs750).
Chlorophyll-b = 25.06 - (Abs647-Abs750) - 6.5 - (Abs664-Abs750)
Scytonemin = 1.04 - Abs384–0.79 - Abs665.1–0.27 - Abs490
Total carotenoids = (1000 - Abs480–1.29 - Chla - 53.78 - Chlb)/220
PQ = Abs435/Abs415

2.3.4. Quantification of extracellular polymeric substances (EPS)

Three replicate biofilm samples per treatment and day were used to quantify the EPS and to determine the carbohydrate (polysaccharides) and protein contents. The EPS was extracted following the method described by (Yang et al., 2019), with a slight modification. Biofilm was scraped from the membrane and resuspended in 2 mL of NaCl. Samples were vortexed and sonicated for 2 min. The samples were then shaken at 150 rpm for 10 min and sonicated for another 2 min. The samples were then centrifuged for 15 min at 5000g. To separate the cell debris from the supernatant containing the EPS, samples were filtered through 0.45 μm nitrocellulose membranes (Millipore). The supernatant containing the LB-EPS (loosely bound EPS) was retained. The pellet was resuspended in 2 mL of NaCl and sonicated for 10 min. The samples were then centrifuged twice, and supernatants containing the TB-EPS (tightly bound EPS) were retained. Carbohydrate content of the EPS was measured by the phenol-sulfuric method (Dubois et al., 1951), with glucose as standard, and the protein content was measured by the Bradford assay (Bradford, 1976), with albumin as standard.

2.3.5. Biofilm structure

In order to analyse the effect of UV-B on the biofilm structure, biofilm samples were examined by CLSM at the end of the experiment. Biofilms were stained with lectin ConA-TMR (concanavalin A, tetramethylrhodamine conjugate) and fluorescein isothiocyanate isomer I (FITC) (Invitrogen) following the method described by Wang et al. (2018). Observations were made on random areas of each biofilm: ConA-TMR (excitation, 552 nm; emission, 578 nm) targeting polysaccharides were recorded in the red channel; FITC (excitation, 495 nm; emission, 519 nm) targeting proteins were recorded in the green channel; and chlorophyll autofluorescence from biofilm cells was recorded in the blue channel for green algae (excitation, 664 nm; emission, 725 nm) and in the pink channel for cyanobacteria (excitation, 570–580 nm; emission, 695 nm). The captured images were analysed using Adobe Photoshop™ CS v.13.0.1 software (Adobe Systems, San Jose, CA, USA).

2.4. Statistical analysis

The data obtained were subjected to ANOVA, with UV-B exposure (0, 1, 2 or 3) as a factor, and a post-hoc Duncan's test ($p \leq 0.05$) was applied. All statistical analyses were conducted using the SPSS statistical program (version 23.0).

3. Results

Fig. 2 shows the results obtained for the growth of biofilms exposed to different UV-B levels and of the control sample. After 14 days of treatment there was no great difference in the appearance of the biofilms, although the control sample was slightly lighter in colour. After 28 days of treatment, the biofilms were darker: the samples exposed to UV-B radiation appeared bright and the control samples were dull in appearance. The biofilm samples exposed to UV-B radiation for 1 and 2 h covered a much larger area than the other samples. However, despite covering a smaller area, the control sample was denser (more biomass per area). This trend was maintained until the end of the experiment. Moreover, except for the sample exposed to the highest dose of UV-B, the other samples showed darkening, appearing almost black.

Fig. 3a shows the proportions of the algal and cyanobacteria cells conforming the biofilm at the end of the experiment. Variation in biofilm composition was related to exposure to different UV-B levels. The composition of the control sample, which was not exposed to UV-B radiation, did not change. However, in the other samples, the proportion of cyanobacteria (*Synechocystis* sp.) and green algae (*Bracteacoccus minor*) varied relative to the initial proportion (50-50%). The decrease in the proportion of green algae was significant in all samples exposed to UV-B radiation, with final percentages of 7.6%, 4.5% and 3.2% for samples exposed for 1 h, 2 h and 3 h respectively. Fig. 3b shows the rate of growth of *Fusarium* sp., in which it can be seen that UV-B radiation had a negative effect on its development. Fungal growth was greater in the control sample (not exposed to UV-B radiation) than in samples exposed to UV-B. Samples subjected to low (Ireland) and medium (Galicia) intensity UV-B radiation showed similar growth, which was respectively, 44 and 47% lower than in the control sample. Fungal growth was reduced by 60% in samples subjected to high levels of UV-B radiation (Portugal).

In all biofilms, except those exposed to UV-B radiation for 3 h, the minimum fluorescence (F_0) increased with biomass (Fig. 4). The control biofilms (not exposed to UV-B radiation) underwent a linear increase in growth from day 0 to the end of the experiment, while samples exposed to UV-B radiation underwent initial higher rate of growth followed by a decrease in the rate. At the end of the experiment, the highest biomass corresponded to the control samples, and the lowest to biofilm exposed to the highest UV-B dose (3 h). Biofilms exposed to UV-B for 1 and 2 h showed intermediate values (not statistically significantly different). The F_0645/F_0470 ratio showed a general decrease in the proportion of signal related to cyanobacteria, with no statistically significant differences between biofilms exposed to different UV-B levels, although differing from control from day 14 onwards.

The Y_{MAX} underwent an initial decrease for the 14 first days, with a slight recovery thereafter to the end of the experiment. The highest recovery was observed in the control sample, in which values reached levels similar to the initial values. The other samples show photosynthetic efficiency of around 40%. The Y_{EF} parameter varied in the same way as Y_{MAX}, with fewer differences between UV-B exposed and control

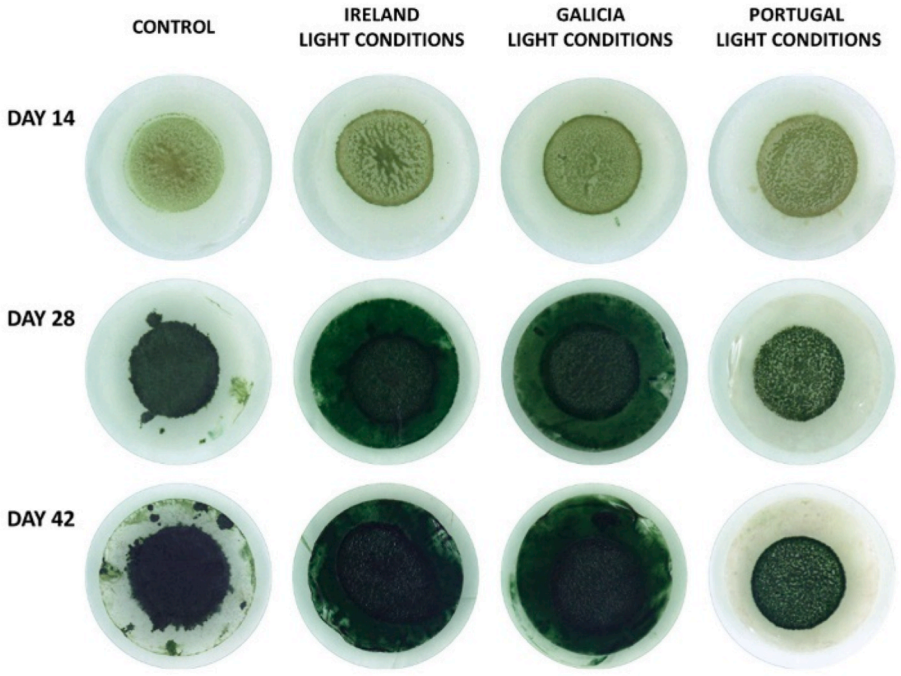

Fig. 2. Photographs of the changes in biofilms exposed to different UV-B levels: 0 h (Control), 1 h (simulating light conditions in Ireland), 2 h (simulating light conditions in Galicia) and 3 h (simulating light conditions in Portugal).

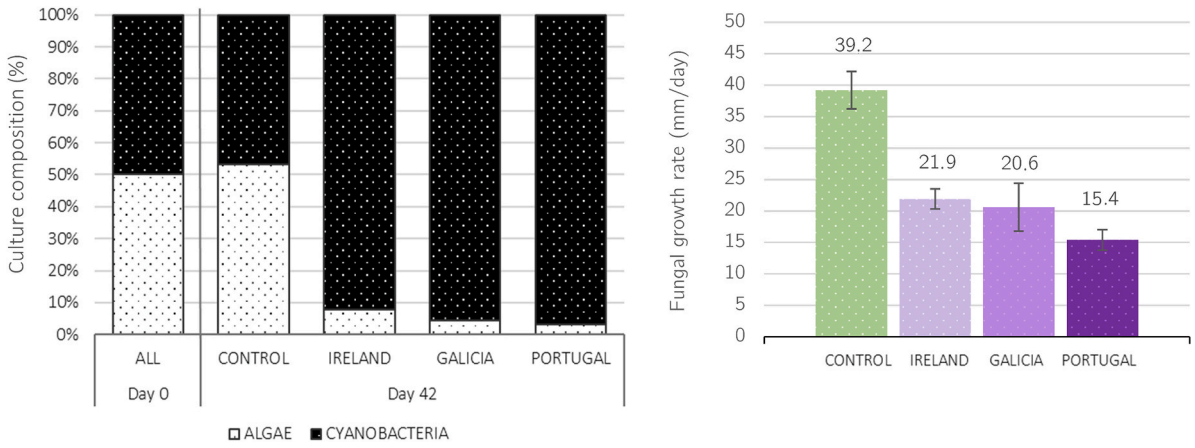

Fig. 3. **a)** Percentage composition of *Bracteacoccus minor* and *Synechocystis* sp. at the beginning and end of the experiment, and **b)** growth rate of the fungus *Fusarium* sp. under the different UV-B conditions.

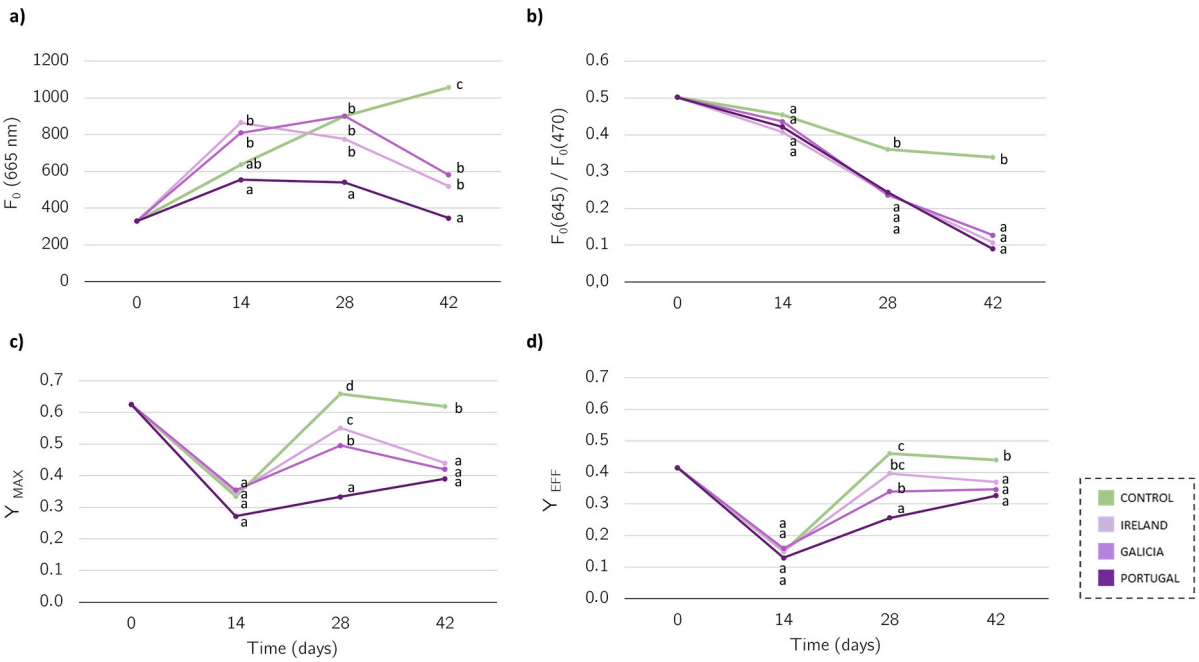

Fig. 4. Changes in fluorescence parameters over time: **a)** F_0, **b)** F_0470nm/F_0645nm ratio, **c)** Y_{MAX} and **d)** Y_{EFF} for the different levels of UV-B exposure throughout the experiment. Different letters indicate significant differences ($p < 0.05$) between samples in relation to the different conditions.

samples at the end of the experiment. Both parameters show that the effective photosynthetic efficiency was inversely related to the UV-B dose.

The values obtained for the vitality index (Rfd) are shown in Fig. 5. All Rfd values were below 1, which is consistent with the findings of Orekhova et al. (2018) and Johnson et al., (2022), who reported that the values are usually lower for cyanobacteria and green algae than for plants. The present results clearly indicated a decrease in the vitality in samples exposed to UV-B dose. Compared to control values, a reduction at the end of the experiment of 53%, 59% and 74% was observed for simulated for conditions of Ireland, Galicia and Portugal, respectively.

Fig. 6a-d show the pigment production in the different biofilm samples. Chl-a production increased over time. At the end of the experiment, the biofilm samples exposed to UV-B contained the highest quantities of Chl a. Although Chl b production also increased over time, at the end of the experiment it was highest in the control samples and much lower in the biofilms exposed to highest levels of irradiation. Chl b production was different in samples exposed to UV-B for 2 and 3 h. Total chlorophyll (Chl *a* + Chl *b*) followed a similar pattern to that observed for chlorophyll *b*. Although total carotenoid production was much lower than chlorophyll production, it increased over time. At the end of the experiment, carotenoid production was null in control biofilms and highest in biofilms exposed to UV-B for 3 h. Scytonemin was not produced in any case, and the PQ did not differ in relation to time or treatment (data not shown).

Fig. 6e-f show the results obtained for production of TB- and LB-carbohydrate components. TB-carbohydrates decreased over time after day 0. At the end of the experiment, there were no differences between any of the samples in relation to TB-carbohydrates. However, the LB component of carbohydrates increased from the beginning of the experiment, and by the end of production was higher in the control than in the UV-B-exposed samples. The samples exposed to UV-B for 1 or 2 h produced more carbohydrates than those exposed for 3 h.

Fig. 7 shows the confocal microscopy images obtained at the end of the experiment, with carbohydrates shown in red, proteins in green, green algae in blue and cyanobacteria in pink. The control samples

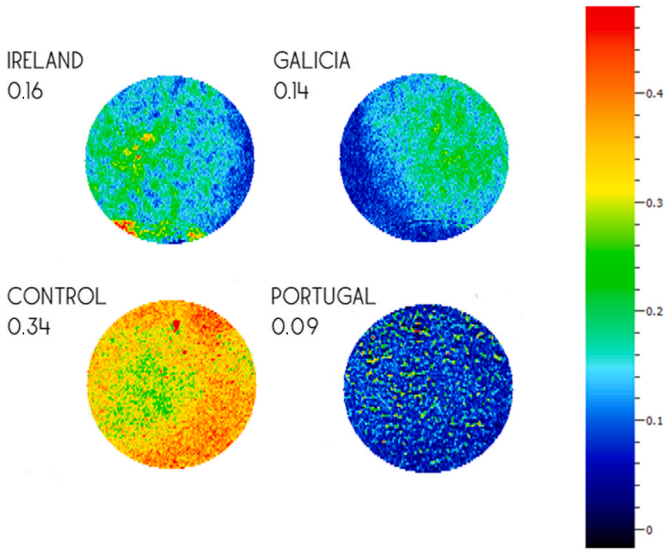

Fig. 5. The Rfd index calculated for the entire surface of biofilms exposed to different UV-B levels, at the end of the experiment.

produced greater amounts of carbohydrates and proteins, while in the samples exposed to UV-B, especially those exposed for 3h per day, production of both compounds decreased. In general, the density of both EPS components decreased as the length of UV-B exposure increased, with a higher proportion of clusters in the control samples. Proteins, in particular, were mainly found surrounding the cells. Both proteins and carbohydrates were associated with green algae to a much greater extent than with cyanobacteria. Furthermore, in addition to a decrease in the presence of green algae (indicated by the bluish colour) the cell size tended to increase in response to the increased radiation (red circles).

4. Discussion

UV-B radiation is known to affect growth, survival, pigmentation, general metabolism, photosynthesis, nitrogen fixation and assimilation of nitrogen in several cyanobacteria and microalgae (Sinha et al., 1995; Xue et al., 2005). In this study, UV-B radiation induced physiological, metabolic and structural changes in the organisms conforming the biofilms. All UV-B doses induced differences in the parameters measured, relative to those in the control sample, which were not exposed to UV-B radiation. The control biofilm grew faster than biofilms developed under any of the other conditions studied. The different UV-B levels studied, representative of three different latitudes in Europe, i.e. Ireland, Galicia and Portugal, had a gradual effect on the biofilms that directly proportional to the cumulative radiation dose. Although there were no significant differences in the studied parameters between samples exposed to the cumulative doses representing conditions in Ireland or Galicia (1- or 2-h UV-B/day, respectively), the parameters differed significantly from those obtained in samples subjected to the cumulative dose representing conditions in Portugal (3 h UV-B/day), indicating a more harmful effect.

In general, species typically found on exposed heritage surfaces are expected to tolerate UV-B radiation, as terrestrial species generally have a higher tolerance to UV-B radiation (Rastogi et al., 2014) than, for example, aquatic green algae, which are protected by the upper water column and are thus able to recover their efficiency up to 80% after 12 days under favorable conditions (Karsten et al., 2007). In this study, UV-B exposure had different effects on algae and cyanobacteria and clearly affected the fungal growth rate. Compared to cyanobacteria, green algae were more affected at higher UV doses. The proportion of green algae in the biofilm was reduced in all samples exposed to UV-B,

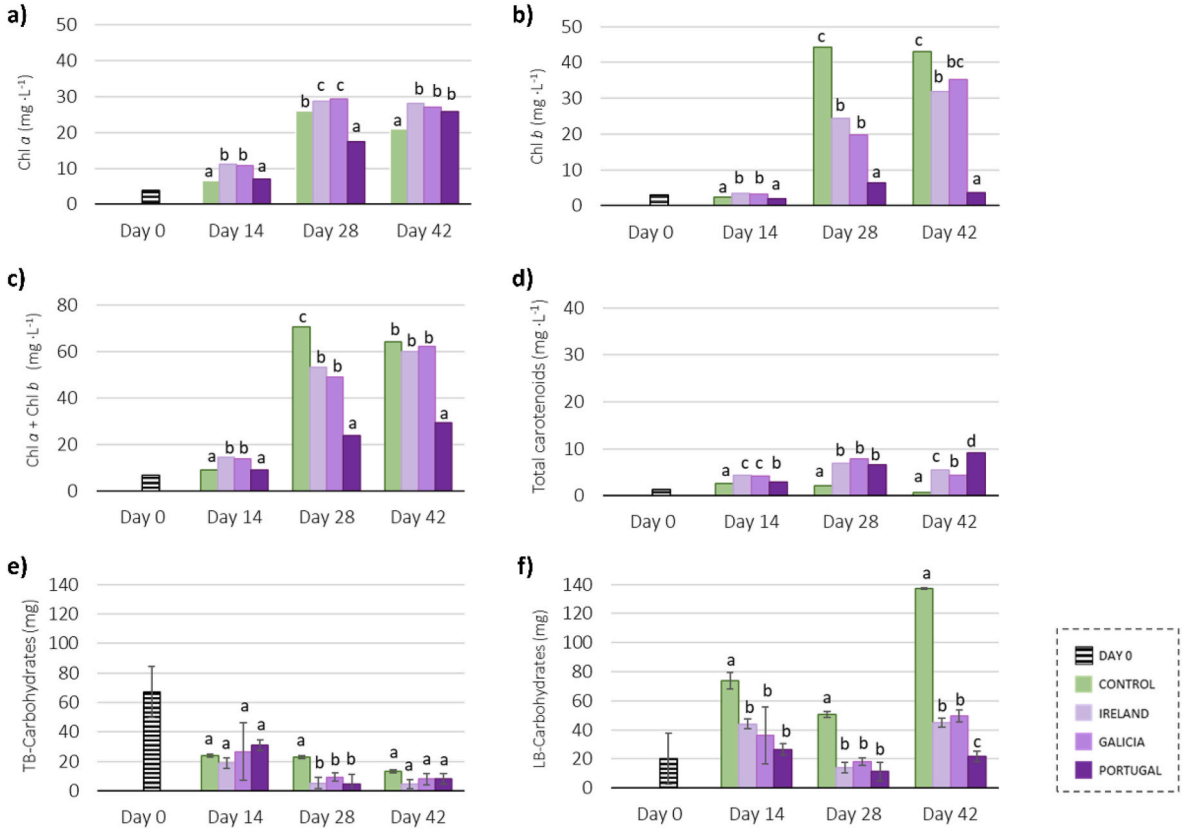

Fig. 6. **a)** Chl *a*, **b)** Chl *b*, **c)** Chl *a* + Chl *b*, **d)** total carotenoids, **e)** TB-Carbohydrates and **f)** LB-carbohydrates produced under the different UV-B levels throughout the experiment. Different letters correspond to significant differences ($p < 0.05$) between the samples in relation to the different conditions.

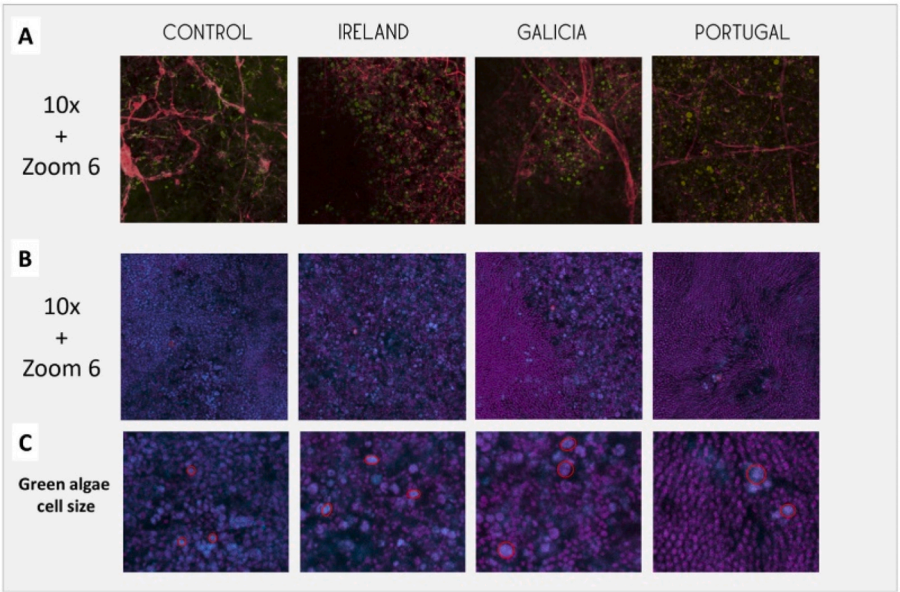

Fig. 7. **A**) Carbohydrate (red) and protein (green) matrix production and **B)** green algae (blue) and cyanobacterial (pink) cells under the different UV-B levels at the end of the experiment. **C)** Magnified image showing the size of green algae cells in red circles.

but not in the control sample, where the initial proportion of 50% was barely affected. This indicates a greater effect of the radiation on the green algae than on the cyanobacteria, i.e. lower resistance of the former, which is further evidenced by the greater presence of cyanobacteria on monuments in warm zones, whereas green algae are more common in temperate and cold zones, where radiation is generally lower (Rindi, 2007). In addition, the growth rate of *Fusarium* also varied with exposure to UV-B radiation, which led to a reduction in the growth ratio, but not death of the fungus. This is consistent with the previously reported sensitivity of the fungus to UV radiation, including effects on conidia viability, conidia germination and fungal growth (Fargues et al., 1996; Fernandes et al., 2007; Fernández-Bravo et al., 2016). Numerous studies have investigated the effect of UV-B radiation on cyanobacteria, but fewer have addressed the effects on green algae or terrestrial fungi (Yadav et al., 2017), and studies involving combinations of the organisms are almost non-existent. This is the first study to compare the effect of small variations in UV-B levels on a terrestrial biofilm conformed by a green alga, a cyanobacterium and a fungus, which is more realistic than model biofilms composed by only phototrophic organisms.

Several studies have also shown that UV-B radiation can damage photosystem II of phototrophic organisms, reducing the photosynthetic efficiency and the vitality index (Rfd) (He and Häder, 2002; Holzinger and Lütz, 2006; Karsten et al., 2007; Teramura, 1983). Chloroplasts are very sensitive to UV-B radiation, and extreme levels of UV radiation may lead to oversaturation of the light reactions of photosynthesis, which may eventually cause photo-inhibitory damage to the photosynthetic apparatus (Kataria et al., 2014). In this study, organisms underwent an initial decrease in photosynthetic efficiency in all cases, possibly in response to the stress generated by the transition from the planktonic way of life to the biofilm state, followed by recovery of the photosynthetic efficiency to the initial levels only in the control samples. In biofilms exposed to UV-B radiation, the photosynthetic efficiency decreased gradually at higher levels of radiation, although the differences were not statistically significant. In all biofilms, the photosynthetic efficiency was around 40%, i.e., 20% less than optimal, indicating symptoms of stress as a result of UV-B exposure. Moreover, the vitality index (Rfd) of the UV-B irradiated organisms decreased significantly with the UV-B dose relative to the control. Thus, under the current light conditions in Portugal the vitality index was almost half that in current light conditions in Ireland. Other works show a reduction in the Rfd parameter as a consequence of UV-B radiation, indicating a possible effect on the activity of rubisco, an enzyme controlling the CO_2 assimilation process and the cooperation between the light and dark photosynthesis reactions (Skórska and Murkowski, 2018).

Another effect of the UV-B radiation detected was the increase in the size of green algae cells. This response, which can be attributed to DNA damage and the consequent cessation of the processes involved in the cell cycle, has previously been observed (Jin et al., 2017; Li and Gao, 2013; Xiong et al., 1996). It was also suggested as a strategy to decrease penetration of UV-B into the nucleus and chloroplasts in an attempt to reduce damage (Segovia et al., 2015). The increase in cell size was only observed in green algae and not in cyanobacteria. This is consistent with the aforementioned higher tolerance of cyanobacteria, as in situations of high tolerance there is no need for the cell size to increase as a protective strategy. Hessen et al. (1997), also related the increase in cell size in the green algae *Selenastrum capricornutum* to UV-B exposure. In addition, reduction of fungal growth was previously found by Moody et al. (1999), who reported a reduction in mycelial extension ratio and spore germination of terrestrial fungi exposed to UV-B radiation.

The stress undergone by biofilms exposed to UV-B radiation was also related to the production of different pigments and components of the extracellular matrix. These products are particularly important in the field of cultural heritage conservation, as they produce both physicochemical and aesthetic changes in the substrate. Pigments and EPS also play a role in protecting against some type of stress. In general, enhanced UV-B generally decreases the pigment content (Singh et al., 2022; Xue et al., 2005). This occurred in this study for all pigments, except chlorophyll *a*, production of which was higher in biofilms subjected to low UV-B levels than in the control biofilms. This may be due to a strategy used by the organisms to protect themselves and to increase the photosynthetic system in order to produce more light energy: cells produce greater amounts of this pigment to increase the chances of survival and enable the cells to survive longer under UV-B exposure. This was also observed in *C. vulgaris* and *C. humicola,* which showed enhanced production of chlorophyll *a* in both species exposed to up to 2 h of UV-B radiation, relative to the control (Singh et al., 2019), and was also observed in the cyanobacterium *N. flagelliforme* (Yu and Liu, 2013). Nonetheless, the total chlorophyll production, considering Chl *a* plus Chl *b*, is consistent with the changes in biomass, which was highest in the control biofilms, followed by the samples exposed to 1 or 2 h of UV-B

radiation. This is due to the increase in chlorophyll *b* production from day 28 (except in the samples subjected to highest radiation), with production of higher levels of chlorophyll *b* than of chlorophyll *a* by the end of the experiment. Samples exposed to the highest levels of UV-B radiation did not show this chlorophyll *b* production, mainly because chlorophyll *b* is only found in green algae, but not in cyanobacteria, so that the reduction in the number of green algae cells in response to higher UV-B exposure involves a reduction in the amount of this pigment. The great increase in chlorophyll *b* production in control samples and samples exposed to UV for 1 and 2 h (even higher than chlorophyll *a* production) is not a common finding. For the green algae used in this work, however, there are no previous studies regarding the normal pigment composition, although a change was observed after inoculation (where the organisms come from a planktonic culture state) to the formation of the biofilm. As the density and depth of subaerial biofilms increase, the upper layers protect the lower ones, reducing solar damage. The reduction in the amount of energy reaching the lower part of the biofilms could lead to higher chlorophyll *b* production, as one of the functions of the pigment is to capture light energy, which is then transferred to chlorophyll *a*. An increase in the amount of chlorophyll *b* promotes production of light-harvesting complex proteins, which is a possible adaptation to low light conditions. Although light was found to have a harmful effect on the organisms studied, the pigment scytonemin, produced by cyanobacteria to provide some protection against light stress, was not detected in this study. This may be due to the previously commented lower level of damage to the cyanobacteria, or to the low absorbance of this pigment in the UV-B-region (280–315 nm). The highest peaks of scytonemin absorbance are in the UV-A-(315–400 nm) and UV-C-regions (100–280 nm), (Ekebergh, 2014), which were not applied in the present study.

The carbohydrate component of the EPS differed for TB-carbohydrates and LB-carbohydrates. These classes of carbohydrates have different functions in aggregation behaviour: an increase in the LB-carbohydrate composition is related to cell aggregation, while the association between TB-carbohydrates and aggregation is not clear (Eboigbodin and Biggs, 2008). In the present study, the amount of TB-carbohydrates decreased from the planktonic to the biofilm state, while the LB-carbohydrates increased towards the end of biofilm development in all cases, except in the samples exposed to the highest level of UV-B radiation (Portugal). Thus, the reduction in TB-carbohydrates may be an important indication of reduced aggregation. Regarding the differences in the UV-B radiation levels, carbohydrate production was relatively high in the control biofilm, while under UV-B exposure (at realistic levels) the carbohydrate production decreased as the dose increased. Furthermore, the closer association between carbohydrate and green algae, revealed by confocal microscopy, also explains the higher carbohydrate production in the non-irradiated control sample, with greater survival of green algae; by contrast, biofilms exposed to UV-B radiation, mainly composed of cyanobacteria, showed lower carbohydrate production. This implies that green algae, regardless of exposure, naturally produce more carbohydrates than cyanobacteria. However, proteins were barely detectable but were observed to decay through the confocal images as UV-B radiation increased. Proteins detected by confocal microscopy were mainly associated with algal cells and were probably proteins from inside the cell and not from the extracellular matrix (and therefore not detected by EPS quantification after sonication). The amount of protein, regardless of its origin (matrix or cellular), was always lower than the amount of carbohydrate in both the UV-exposed and control samples, particularly in the UV-exposed samples. A relationship between the presence of fungus and biofilm aggregation was observed. It is possible that the fungus itself increased the production of EPS, or that the hyphae acted as a mesh that makes the biofilm more cohesive (Fig. 8). The intrinsic capacity of fungi to produce EPS was previously described as being linked to an increase in desiccation resistance (Blankenship and Mitchell, 2006; Breitenbach et al., 2016; Gorbushina, 2003). It is therefore possible that the fungus provided greater protection to the phototrophic organisms, contributing to the composition of a stronger matrix that would prevent more disaggregated growth, but without impairing the growth or photosynthetic efficiency of the phototrophs. This different growth expression (Fig. 8) may have implications for heritage conservation.

Radiation may also interact with other factors involved in climate change, such as humidity, temperature and CO_2. In a review paper, Caldwell et al. (2007) pointed out that an increase in UV-B radiation reaching higher plants subjected to drought has a more detrimental effect than an increase in UV-B radiation reaching well-watered plants. However, an increase in temperature within the optimal range may counteract the damaging effect of UV-B radiation by promoting activity of DNA repair enzymes. Furthermore, an increase in UV-B radiation may counteract the effects of other environmental factors, such as the fertilizing effect of CO_2. Although similar findings have been reported for higher plants, the effects on biofilm-forming organisms of any interactions between UV-B and other environmental parameters have not previously been investigated. Such studies are complex as biofilms can be composed of diverse species of microorganisms, whose responses to interactions between different environmental factors may vary widely.

In the context of Cultural Heritage conservation, changes in the diversity and proportion of different species in a biofilm will modify the overall capacity of the biofilm to degrade the underlying substrate. In this study, it was found that an increase in UV-B radiation reduced

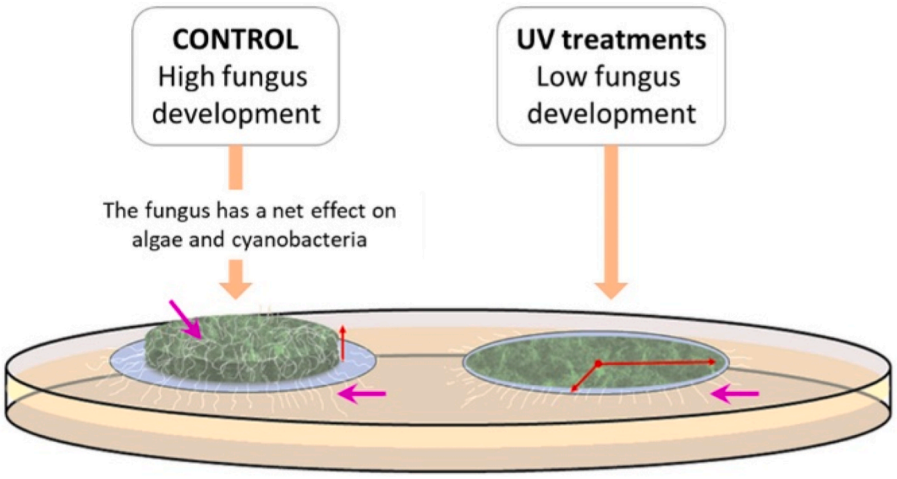

Fig. 8. Model hypothesis for biofilm development with or without fungi.

phototrophic biomass and fungal hyphal length, so that the ability of the organisms to penetrate the substrate would be expected to be diminished. This, together with the lower EPS production, may affect the consistency of the biofilm and modify its resistance to other climatic factors and also to cleaning treatments. EPS are known to have a particularly high capacity to deteriorate substrates as they undergo volumetric changes with variations in humidity and temperature, giving rise to cracks. This research provides a new approach to evaluating the response of a biofilm composed of a microalga, a cyanobacterium and a fungus to increased UV-B radiation. Based on these findings, future research should include other factors involved in climate change in order to address the combined effects on biofilm-forming organisms, the trophic relationships and the impact on stone-built cultural heritage.

5. Conclusions

From the results obtained in this study, it can be concluded that increased levels of UV-B radiation will lead to changes in the growth, physiological state and composition of subaerial biofilms.

In the biofilm under study, the cyanobacteria were more resistant to UV-B radiation than the green algae as they were more abundant and did not vary in size, even after exposure to the highest dose of UV-B radiation, whereas the algal cells increased in size in response to UV-B radiation.

This is the first study of the effect of radiation on a SAB comprising phototrophic organisms (cyanobacteria and green algae) and also a fungus. The inclusion of fungus in the biofilm produced interesting results as it seemed to affect the aggregation state: in the samples exposed to UV-B radiation the fungus grew less, and the consortium cells were also less aggregated, probably due to the reduction of EPS. The reduced aggregation under higher UV-B exposures may be due to the detrimental effect of radiation on the organisms (implying a reduction in EPS production), or to the reduction in fungal growth by radiation (limiting the network effect produced by the fungal hyphae on the phototrophs), or to a combination of both. Further studies are needed to clarify this point.

This is the first time that the effect of UV light on SABs has been studied in the context of latitudinal shifts promoted by climate change. From this point of view, biofilms exposed to current UV-B light conditions in Ireland and Galicia responded in the same way, while those exposed to UV-B light conditions in Portugal were more strongly affected. Therefore, if global change causes an increase in radiation, changes (integrity, ecology and physiological state) in biofilms colonising cultural heritage in areas latitudinally similar to Portugal will also be expected to occur in subaerial biofilms at higher latitudes. This situation provides an opportunity to take advantage of the development of conservation strategies both from the point of view of the organisms and of the cultural heritage elements that they colonize.

Consent to participate

All authors have been personally and actively involved in the work.

Consent for publication

All authors approve its submission and publication.

Ethics approval

All authors assure that this is an original work, which has not been previously published elsewhere.

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Credit author statement

Conceptualization: E.F.; D.P.V; B.P; Methodology: E.F.; D.P.V; B.P; Software: E.F.; D.P.V; Formal analysis: E.F.; D.P.V; Investigation: E.F.; D.P.V; Resources: B.P; Writing - Original Draft; Preparation: E.F.; D.P.V; Writing - Review & Editing: B.P; Supervision: B.P; Project administration: B.P; Funding acquisition: B.P

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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