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Article

Early Detection of Phototrophic Biofilms in the Polychrome Panel, El Castillo Cave, Spain

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Abstract: European caves contain some of the world's greatest Paleolithic paintings, and their conservation is at risk due to the use of artificial lighting. Both lighting and high CO_2 promotes the growth of phototrophic organisms on walls, speleothems and ground sediments. In addition, the combined effect of increases in CO_2, vapor concentration and temperature variations induced by visitors can directly affect the development of corrosion processes on the cave rock surfaces. An early detection of the occurrence of phototrophic biofilms on Paleolithic paintings is of the utmost importance, as well as knowing the microorganisms involved in the colonization of rocks and walls. Knowledge of the colonizing species and their ecology will allow the adoption of control measures. However, this is not always possible due to the limited amount of biomass available for molecular analyses. Here, we present an alternative approach to study faint green biofilms of *Chlorophyta* in the initial stage of colonization on the Polychrome Panel in El Castillo Cave, Cantabria, Spain. The study of the biofilms collected on the rock art panel and in the ground sediments revealed that the lighting of the cave promoted the development of the green algae *Jenufa* and *Coccomyxa*, as well as of complex prokaryotic and eukaryotic communities, including amoebae, their endoparasites and associated bacteria and fungi. The enrichment method used is proposed as a tool to overcome technical constraints in characterizing biofilms in the early stages, allowing a preliminary characterization before deciding for direct or indirect interventions in the cave.

Keywords: biofilms; caves; *Chlorophyta*; *Coccomyxa*; *Jenufa*; lampenflora; *Neochlamydia*; Paleolithic paintings; show cave conservation; *Vermamoeba vermiformis*

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1. Introduction

Caves are sites of remarkable geological, ecological and cultural interest, with diverse compartments that differ in physical, chemical and microbial composition. Caves are also reservoirs for biodiversity and several tens of novel species of organisms are discovered every year.

Microbial colonization in caves is a complex and dynamic process that is controlled by environmental and physicochemical factors (temperature, CO_2, nutrients availability, etc.). Caves are oligotrophic in nature, and the lack of natural light prevents the growth of phototrophic microorganisms [1]. However, one of the strongest impacts on show caves is the use of artificial lighting for visits. Lighting combined with high CO_2 enhances the growth of *Cyanobacteria*, *Chlorophyta* and other phototrophic organisms on walls, speleothems and

ground sediments [2–7]. In addition, a condensation–corrosion process occurs when humid airflow cools at the contact with rocks surfaces. Subsequently, the rate of CO_2 uptake by condensed water in the rock surfaces provokes pH decreases and, consequently, its capacity to dissolve calcite increases [8]. It can cause the dissolution of the rock substratum and detachment of rock art pigments.

In most caves, *Cyanobacteria* and *Chlorophyta* (green algae) coexist [3–5], although with distinct abundances, being *Cyanobacteria* dominant [6,7]. In fact, the conditions prevailing in most European caves lead to the colonization of characteristic communities of unicellular and filamentous *Cyanobacteria* [9]. *Cyanobacteria* are especially successful microorganisms in subsurface environments due to their tolerance to desiccation and low light intensity requirements.

European caves contain some of the world's greatest Paleolithic paintings. However, cave rock art is a cultural treasure at risk [10]. Biofouling of phototrophic organisms on paintings is a well-known problem that is difficult to manage in most caves. The adoption of measures for its control or elimination is not an easy task as many factors are involved: (i) a correct knowledge on the microbial taxa and their ecology; (ii) the difficulty of working in a site subjected to cultural protection and with no access to the paintings; and (iii) the sampling limitation.

In the past, Lascaux and Altamira caves were outstanding examples of incorrect management that provoked the deterioration of the paintings. A few authors have described the microorganisms and biodeterioration processes in these caves [11–14]. The first sign of rock art biodeterioration was observed in Lascaux Cave, France, in 1960, 12 years after the opening to the visits, with the occurrence of green biofilms on the walls [13]. At that time, the cave received 100,000 visitors per year with peaks of 1800/day and experienced a subsequent increase in CO_2 levels, water condensation and air temperature. The cave had to be closed to the public in 1963 and subjected to a biocide treatment that apparently eradicated the green biofilms. In 1969, treatments were resumed for fighting against new green biofilms [13]. Lefèvre [15] reported that the so-called "maladie verte" in Lascaux was due to the colonization and growth of the unicellular green alga *Bracteacoccus minor*.

Lascaux biodeterioration processes were reproduced in the Altamira Cave 40 years later, after decades of using artificial lighting [12]. The colonization of the paintings by phototrophic microorganisms should have been foreseen, since the cave ground was widely colonized by cyanobacteria, algae and mosses in the locations where lighting lamps were installed [14]. In 2002, the cave had to be closed to the visits due to the occurrence of green biofilms on the paintings [12]. Despite the closure and shutdown of light, metabolically active cyanobacteria were still detected on the paintings [16].

In this study, we present environmental and microbiological data on the early detection of green biofilms that threaten Paleolithic paintings in El Castillo Cave, Cantabria, Spain. The study was focused on both prokaryotes (bacteria) and eukaryotes (algae, fungi and other multicellular organisms), while the environmental study investigated the possible influence of the visiting regime.

2. Materials and Methods

2.1. The Site

Monte Castillo is a 385 m high hill that dominates the left bank of the wide fluvial plain of river Pas in Toranzo valley, Cantabria, Spain. The complex underground network that houses this mountain, made up of more than forty cavities excavated in Carboniferous limestone, includes five caves decorated during the Upper Paleolithic, of which two are among the richest in rock art known to date.

The current entrance to El Castillo Cave is through a big shelter. At the time of the discovery made by Hermilio Alcalde del Río in 1903, the morphology of the site was completely different and characterized by a steep slope in which the slope of the mountain was confused with the cone of debris and sediments that almost completely clogged the hollow [17]. The current appearance of the entrance hall is due to the enormous work of a

team led by Hugo Obermaier who, between 1910 and 1914, excavated most of the fillings accumulated over hundreds of thousands of years. These works, financed by the Institut de Paléontologie Humaine in Paris, also covered the study of the wall paintings by Henri Breuil [18]. Because of this fruitful period of research, El Castillo became one of the most important archaeological sites in Europe. A wide sequence that includes levels of the Lower Paleolithic, Middle Paleolithic, Upper Paleolithic, Azilian, Mesolithic and recent Prehistory (that is, from the first human presence in the region) more than 150,000 years ago until about 4000 years is represented in the cave [19].

The length of the cave is almost 800 m, with a descent of −16 m. The interior sector of the cavity begins with a great entrance hall, the "Great Hall", with a central area occupied by huge blocks, from which a long main gallery starts and is divided into several sections of changing directions and other secondary corridors of minor dimensions. The peculiar topographic organization of the cavity and the abundant and spectacular speleothems determine the existence of numerous compartmentalized places, side corridors, corners and hollows that play a fundamental role in the distribution of the numerous rock art paintings, distributed throughout the cave walls and ceilings. The parietal art extends throughout all cavity sectors but are rare in its deepest section, although they do not disappear.

The paintings and engravings in El Castillo Cave were made throughout the entire Paleolithic artistic cycle, in a period estimated in almost 30,000 years. Grouped in sets defined by different spatial areas or superimposed on the same panel, the paintings were performed in different periods. The oldest representations (red discs) have been associated with U/Th dates from 40,800 years ago; the most recent (figures of animals painted in black) date to 12,500 years ago, late in the Magdalenian period.

The panel studied is the so-called Polychrome, located on the north wall of the Great Hall of the cave (Figure 1). There are overlapping figures of different sizes, techniques and colors made at different times in the Upper Paleolithic. This panel has occupied a prominent place in the elaboration of "classic" chronological proposals for Paleolithic art [20].

Regarding the chronology of the panel, two major periods can be distinguished. First, a pre-Magdalenian phase that includes negative hands as older motifs, perhaps belonging to the Aurignacian period—ca. 37,000 years ago. These were followed in time by the red figures and signs that can be attributed to the wide temporal range that elapses between the Gravettian and the Solutrean (ca. 33,000–20,000 years ago). Second, a Magdalenian phase in which four bisons, painted in black, dated in a short time span, between 15,500 and 13,000 years ago [21].

2.2. Cave Monitoring

Environmental sensors were placed at the end of the descending passageway leading to the first panel decorated with negative hands, right at the connection to the narrow chamber hosting the tectiform signs on its walls (Figure 1). This location is within the tourist pathway, 20 m far from the cave entrance.

Air temperature data were obtained using a temperature logger with an external thermistor (SBE 56, Sea-Bird Electronics, Bellevue, WA, USA), with excellent performance in terms of measurement accuracy (±0.002 °C, from −5 to +35 °C) and resolution (0.0001 °C). Carbon dioxide concentration was recorded using NDIR sensors (Sensirion SCD30, Staefa, The Switzerland) coupled to an energy-efficient logger (Goodsell Systems, Weymouth, UK), specifically developed for long-term monitoring carbon dioxide in the range of 400 ppm–10,000 ppm with an accuracy of ±30 ppm. Radon concentrations were recorded using an AlphaE radon monitor (Bertin Technologies, Montigny-le-Bretonneux, France), based on a silicon diode diffusion chamber with a high-sensitivity detector (3 cph at 100 Bq/m^3), measuring from 20 Bq/m^3 to 10 MBq/m^3. Data were recorded at 5, 30 and 60 min intervals for temperature, CO_2 and radon, respectively. Cave monitoring was performed from 16 September 2020 to 27 September 2021, obtaining a full record of diurnal, synoptic and seasonal variations of these cave environmental parameters throughout a

complete annual cycle, except for a short data gap on CO_2 and radon series due to sensors or batteries failures (Supplementary Table S1).

Surface weather conditions of the study area were analyzed by using the hourly temperature and daily rainfall data series from a meteorological station at Torrelavega (21 m.a.s.l, 43°20′56.8″ N, 4°03′02.3″ W), belonging to the Spanish Meteorological Agency (Cantabria, Spain). This station is located at 8 km far from El Castillo Cave (150 m.a.s.l, 43°17′32.3″ N, 3°57′55.06″ W).

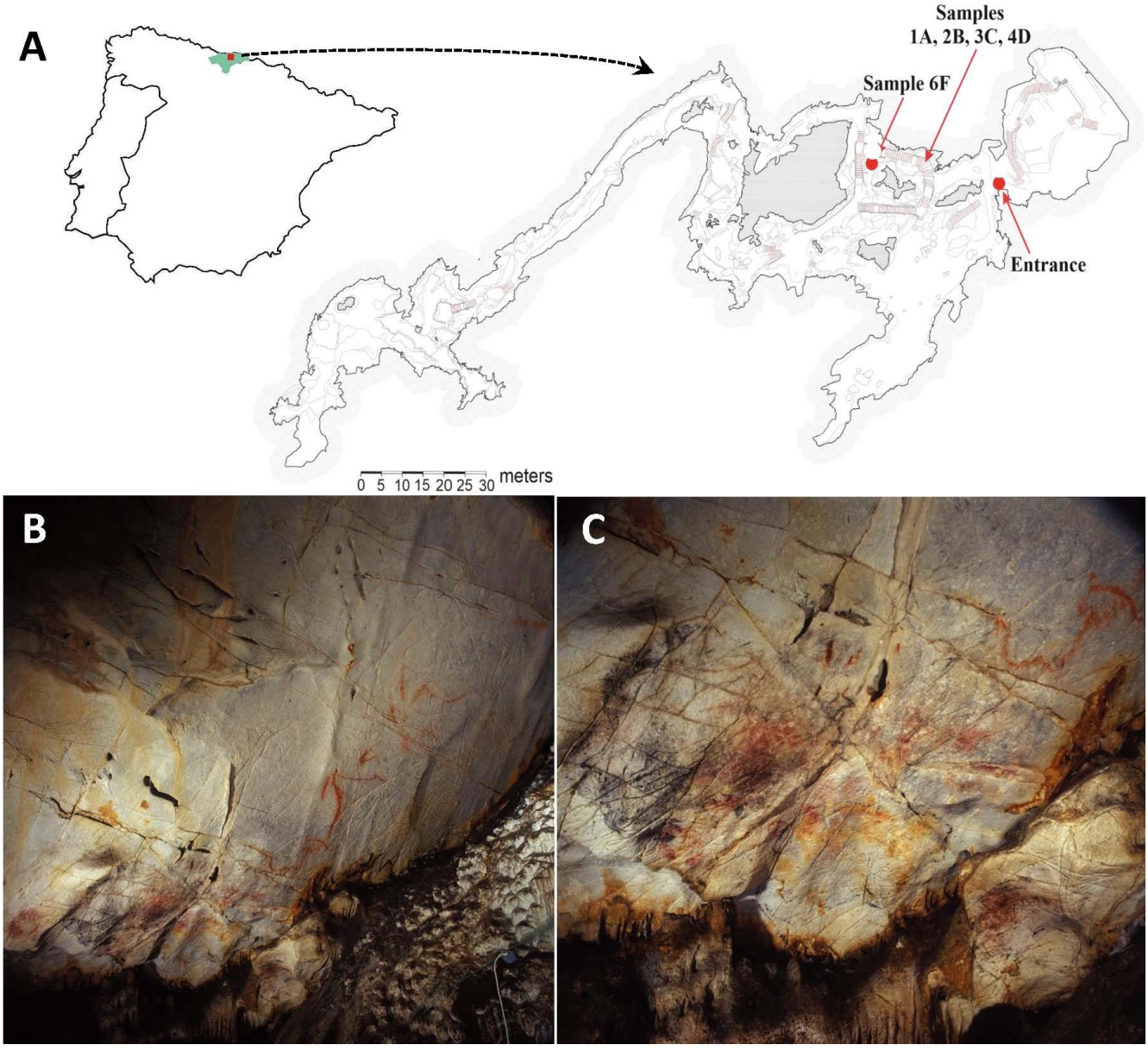

Figure 1. (**A**) Map with location of the samples in El Castillo Cave. (**B**) Polychrome Panel. (**C**) Magnification of the panel where the four green biofilms were collected. Figure 1B from Gobierno de Cantabria, Consejeria de Universidades, Igualdad and Cultura y Deporte/Pedro Saura Ramos.

2.3. Sampling Site

The early stage of biofouling was observed as faint green biofilms distributed along the Polychrome Panel in four different locations and biomass from the four biofilms were collected. For comparison, a fifth sample from a well-developed biofilm was taken from the ground in an area away from the panel and directly illuminated by a lighting lamp (Figures 1 and 2, Supplementary Table S2).

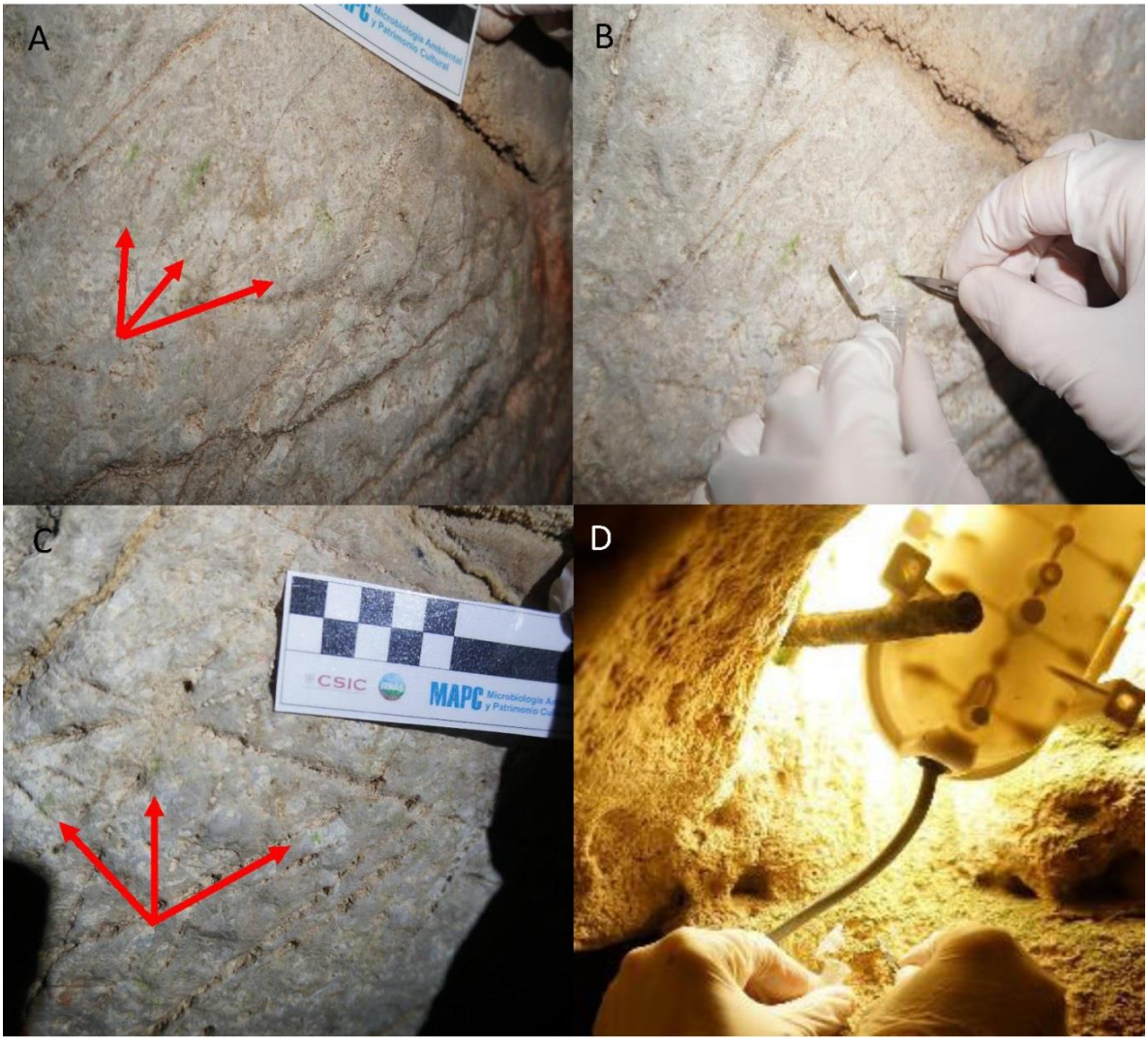

Figure 2. Polychrome Panel, El Castillo Cave. (**A**): Sample CAS1A. (**B**): Sampling in CAS1A. (**C**): Sample CAS2B. (**D**): Sampling in CAS6F. Red arrows mark the green biofilm's location.

The procedure used for sampling consisted in the careful removal of green biofilms with a scalpel that are then deposited in an Eppendorf tube. The amount obtained from each sample was very small. Subsequently, a swab was passed over the panel surface to remove the remaining green color and transferred to plates with culture medium (BG11, Sigma-Aldrich, St. Louis, MO, USA). This medium was commonly used for the cultivation of phototrophs. Eppendorf tubes were maintained at 4 °C and culture plates at room temperature (15–20 °C) until arrival at the laboratory.

2.4. Culture of Natural Samples

Due to the impossibility of applying molecular methods for the direct study of the biomass collected from the Panel (Supplementary Table S3), the study focused on the cultivation of the biomass collected in Petri plates. The plates were cultured in BG11 medium at 22 °C [22] and low light intensity in a shaded laboratory. Both cyanobacteria and chlorophytes can growth in this medium, as previously observed in laboratory tests. Microbial growth developed slowly with visible green colonies two to three months after sampling (Supplementary Figure S1). Biomass was collected from each plate in Eppendorf tubes for DNA extraction.

2.5. Extraction of Nucleic Acids

Nucleic acids were extracted using FastPrep matrix-E lysis tubes (Qbiogene Inc., Carlsbad, CA, USA) with glass beads. A physical breakdown was performed using a stirrer (Fast Prep-24, Solon, OH, USA) at a stirring speed of 5.5 m/s for 2 × 30 s.

DNA extraction was carried out from natural samples directly collected in Eppendorf tubes (E1A, E2B, E3C, E4D and E6F) and from samples grown for four months in BG11 culture medium (CAS1A, CAS2B, CAS3C, CAS4D and CAS6F).

The quality and concentration of the nucleic acids was measured by fluorometric quantification using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The concentrations are detailed in Supplementary Table S3. Samples of sufficient quality and quantity to perform optimal amplifications were only obtained from culture medium BG11.

2.6. Sequencing

Sequencing was performed using Next Generation Sequencing (NGS) of the V3 and V4 regions of the 16S rRNA gene, and the region V4 of the 18S rRNA gene. The sequencing of the 16S rDNA region allowed the identification of prokaryotes and the 18S rDNA region identified eukaryotic organisms. Takahashi et al. [23] showed that the prokaryotic universal primer (341F and 805R) used for the simultaneous analysis of prokaryotes had a coverage rate of 98% for *Bacteria* and 94.6% for *Archaea*.

The construction of libraries was carried out using the protocol of preparation of the 16S Metagenomic Sequencing Library (15044223 Rev.B). The primers used for the 16S rRNA library were 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 785R (5′-GAC TAC HVG GGT ATC TAA TCC-3′), and they were Reuk454FWD1 (5′-CCA GCA SCY GCGGTA ATT CC-3′) and V4r (5′-ACT TTC GTT CTT GAT-3′) for the 18S rRNA library. The DNA fragments generated (DNA libraries) were sequenced with the MiSeq V3 kit from the Illumina MiSeq platform using 300 paired-end base-pair reads.

2.7. Bioinformatics

The samples generated from 94,570 to 126,592 readings that were analyzed by eliminating sequences of low quality, small size and chimeras. The generated readings were analyzed using FastQC software [24] and processed with Quantitative Insights into Microbial Ecology, QIIME v2021.4 [25]. The elimination of primers, filtering of sequences according to their quality, elimination of chimeras and grouping of reads in variants Amplicon Sequencing (ASV) were performed with the DADA2 package [26]. Alpha diversity and evenness were calculated using Shannon diversity index, Faith's Phylogenetic Diversity and Pielou's evenness directly using QIIME2. The sequencing data were further imported into the R environment 3.6.0, and a Venn diagram was constructed to detect the exclusive and shared ASVs between samples. The 16S rRNA gene-based community profiles were represented in a barplot and heat maps using ggplot in R environment.

The taxonomic assignments were based on the database of sequences of SILVA reference version 138 [27], using the classification method implemented in QIIME2 [28]. Raw data from 16S–18S rRNA metabarcoding are available at the following website: https://www.ncbi.nlm.nih.gov/sra/PRJNA797223.

3. Results

3.1. Main Seasonal Pattern of Cave Environmental Conditions

Figure 3 shows the seasonal evolution of environmental parameters (air temperature and tracer gases concentrations; CO_2 and radon) in El Castillo Cave, in comparison with the weather outside and the daily influx of visitors to the cave. The average temperature of cave air was 12.49 °C, varying seasonally between 12.40 and 12.77 °C in absolute values. The average CO_2 concentration of the cave air was 1218 ppm, varying seasonally within a wide range between 694 ppm and 2164 ppm on absolute values.

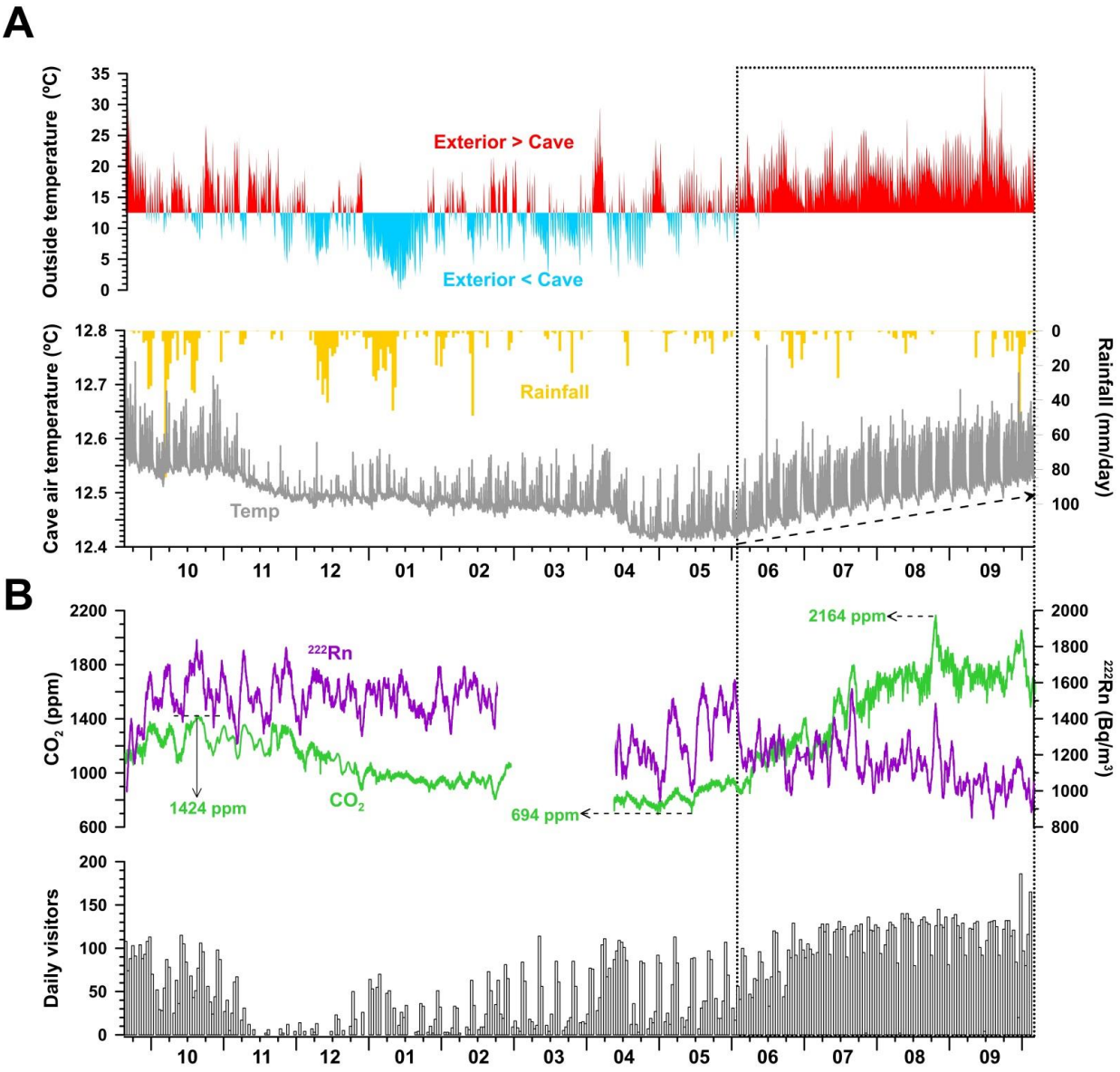

Figure 3. (**A**): Time evolution of air temperature in El Castillo Cave with the local meteorology (temperature and precipitation). (**B**) Short-term and seasonal variations of tracer gases (CO_2 and ^{222}Rn) and daily visitors in El Castillo Cave. X-axis comprises a hydrological year and is labeled by months (1: January–12: December). The short data gap on CO_2 and radon series was due to sensors or batteries failures. The dotted area defined the expected seasonal ventilation period characterized by a downward trend of radon concentration; however, a simultaneous cumulative effect on the temperature and CO_2 series was registered due to the increase in daily visitors.

Monte Castillo is an extensively karstified mountain above the water table of the local karst aquifer and topographically elevated and isolated from the surrounding carbonated outcrops. This geomorphologic configuration determines a tridimensional air exchange between El Castillo Cave and the exterior and likely with other nearby caves and smaller karst voids within this mountain, since air exchange is not intercepted by the deeper water drainage structures linked to the far-off saturated aquifer zone.

Under these geomorphological settings, the exchange of air with the outer atmosphere was mainly established during the summer months and short periods when the outside temperature sharply rises above the cave's air temperature (Figure 3). During summer and short warm periods, when the outside temperature is above the internal, a convective circulation of air because of a density gradient leads the air exchange. The colder and

denser subterranean air descends to deeper areas of the cave and is evacuated through the air-filled penetrative structures. The outer, warmer and less-denser air seeps through the same network of air-filled penetrative structures, renewing the cave atmosphere at once. The lower rainfall during summer also contributes to soils drying and an increment in the percentage of air-filled cracks and fractures of the host-rock over the catchment area of the cave. The air renewal process entails a noteworthy depletion of radon content in cave air levels of the subterranean atmosphere during summer, reaching minimum levels around 1000 Bq/m^3. This minimum radon activity is slightly lower than the annual mean values (1351 Bq/m^3) and far from the maximum levels of nearly 1800 Bq/m^3 registered in late fall.

A similar seasonal pattern should be expected for other tracer gases; however, this is not the case for CO_2. The noteworthy influence of visitors on this gas and cave air temperature masks this predictable natural dynamic, as described in the discussion section.

3.2. Microbial Community Composition

The samples analyzed were faint green biofilms corresponding to poorly developed stages, collected from the Polychrome Panel, and a mature biofilm on the ground sediments adjacent to a lighting lamp. Similar green biofilms were reported in the Polychrome Hall, Altamira Cave [12,14].

Due to the low DNA concentrations of the samples directly removed with a scalpel (Supplementary Table S3), analyses were only possible on the biomass cultured in Petri plates. Although recognizing the limitations imposed by this approach and the bias due to cultivation, there were no other options available for investigating biofilm compositions.

Supplementary Table S4 shows the number of amplicon sequence variants (ASVs), prokaryotic taxon richness and α-diversity indexes for the five samples. The α-diversity indexes ranged from 3.74 to 6.68 for Faith's Phylogenetic Diversity (Faith-pd) and 3.54–4.63 for Shannon with highest values in sample CAS3C and the lower in sample CAS1A. The evenness was also higher in CAS3C with the lowest values in the sample away from the Polychrome Panel (CAS6F). The α-diversity values were similar to those reported for other caves [29,30].

The Venn diagram of the ASVs distribution of prokaryotes in the five samples (Supplementary Figure S2) revealed the presence of 152 ASVs unique for each sample, representing 75.2% of the total assigned phylotypes (202). Most unique taxa were found in the ground sample (74), CAS3C (33), CAS4D (21) and CAS2B (19), whereas very limited numbers occurred in sample CAS1A (5). The common microbial core comprised only 1 distinct ASVs (0.35% of prokaryotic taxa), while 50 ASVs (24.8% of taxa) were shared between different samples. Among these, the largest phylotype number (7) was shared between CAS2B and CAS3C and between CAS4D and CAS6F (6).

3.3. Prokaryotes Community Structure

Supplementary Table S5 shows that the prokaryotic microbial communities of the samples are almost entirely composed of members of the *Bacteria* domain, with percentages ranging from 99.9% to 100%. The percentage of unassigned bacteria was 0.1% in two samples (CAS1A and CAS4D). *Archaea* were not detected. The absence of *Archaea* is common in caves, where their members do not seem to play a role in the community [30–35].

In the *Bacteria* domain, the microbial communities were composed of 10 phyla, with percentages greater than 1% (Figure 4). Taxa with relative abundances less than 1% were not included in the table.

The most abundant phylum by far is *Proteobacteria*, with a relative abundance of 26.0% in the sample taken from the ground (CAS6F) and between 51.4 and 83.0% in the Polychrome Panel. Other phyla with relative abundances higher than 10% in some samples were *Actinobacteriota*, *Bacteroidota*, *Cyanobacteria*, *Candidatus* Dependentiae, *Firmicutes* and *Chlamydiae*. Among them, the relative abundances of *Cyanobacteria*, *Proteobacteria* and *Actinobacteriota* in the ground sample were noticeable, while other three phyla showed relative abundances less than 5% (*Acidobacteriota*, *Thermomicrobia* and *Planctomycetes*).

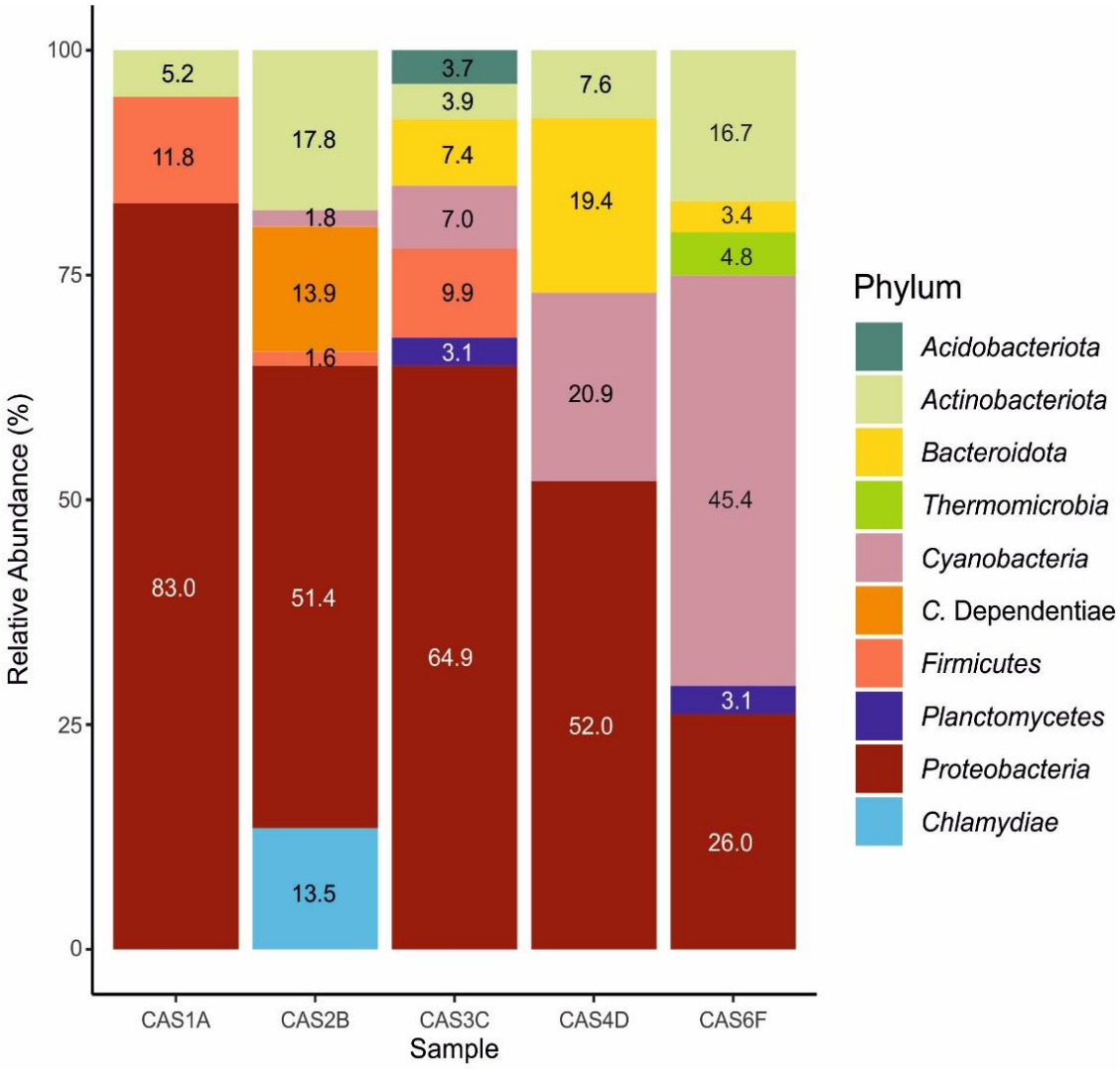

Figure 4. *Bacteria* distribution in El Castillo Cave samples at phylum level. The phyla are described in the right column and their respective relative abundances are included in the boxes. Unassigned ASVs at phylum level and sequences ASVs with relative abundances below 1% are not shown.

Figure 5 shows the heat map of the *Bacteria* classes, revealing that the most abundant class in the panel was *Alphaproteobacteria*, whose relative abundances ranged between 37.4 and 82.4%. The lowest value corresponded to the sample taken from the ground (16.0%). The relative abundances of *Gammaproteobacteria* ranged between 0 and 11.8% depending on the sample, while *Betaproteobacteria* disclosed relative abundances between 0.6 and 7.8%. In general, it was observed that *Proteobacteria* dominated the bacterial communities of El Castillo, as in other caves.

The *Proteobacteria* class was followed in relative abundance by chloroplasts (20.9% in CAS4D and 45.4% in CAS6F). In fact, in public 16S rRNA gene databases, the phylum *Cyanobacteria* includes not only free-living bacteria but also chloroplasts and mitochondria from eukaryotes. *Actinobacteria* reached percentages between 16 and 17.8% in CAS2B and CAS6F samples.

The class *Bacteroidia* was relatively abundant in three samples (CAS3C, CAS4D and CAS6F), while it was absent in another two. *Bacilli* were abundant in CAS1A and CAS3C, low in CAS2B and absent or practically null in CAS4D and CAS6F. These two classes are usually present in photosynthetic biofilms of anthropized caves or with a high number of visits (Jurado et al., 2020a).

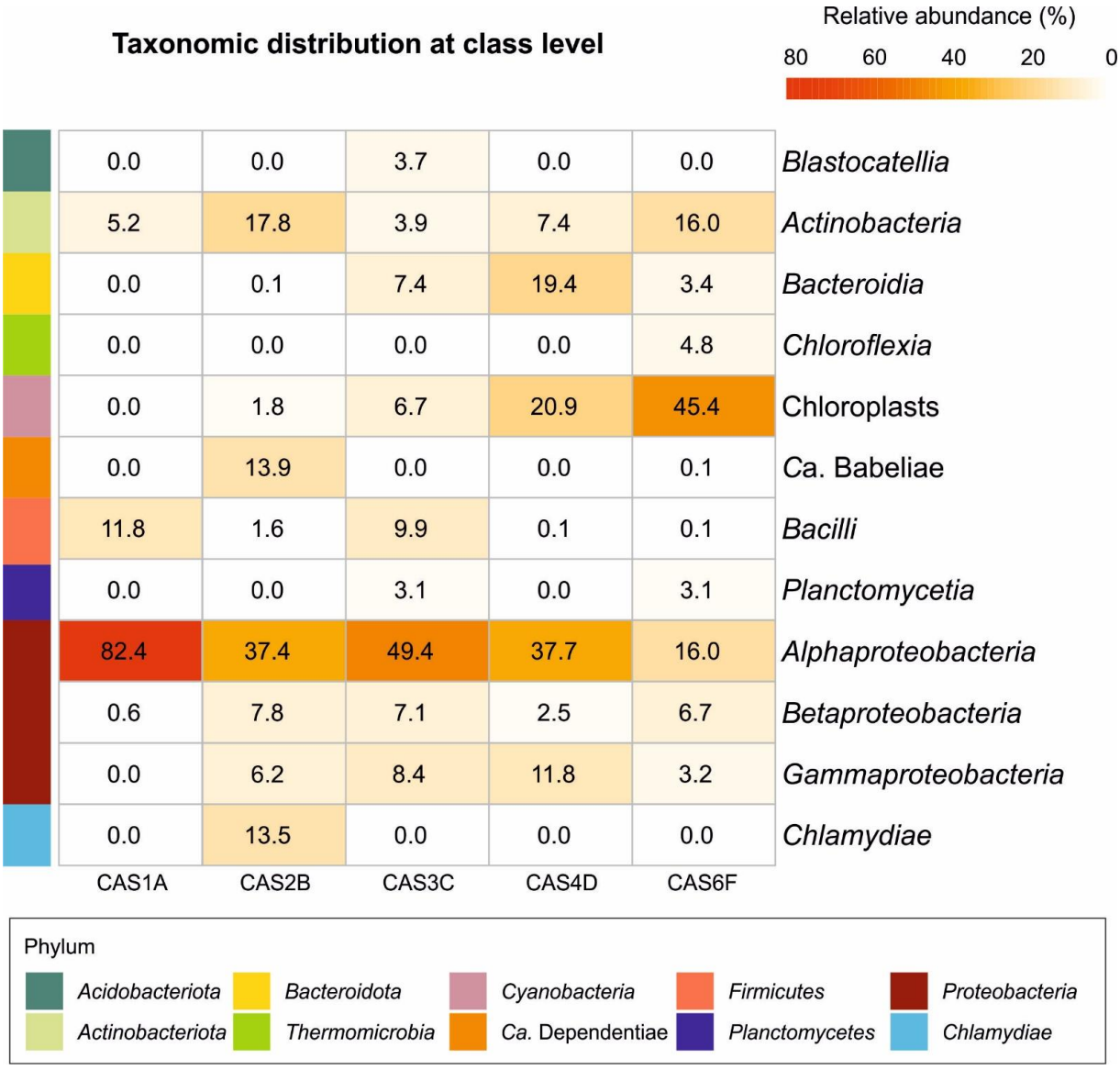

Figure 5. Heat map of El Castillo Cave samples with taxonomic identifications of *Bacteria* at class level. The classes are listed in the right column and their respective relative abundances included in the boxes. Scale shown in upper right corner of the figure represent color associated with the relative abundance of classes. Colored left bar groups the classification at phylum level.

Among the remaining classes, the occurrences of *Candidatus* Babeliae and *Chlamydiae* were noticeable in sample CAS2B, with relative abundances of 13.9% and 13.5%, respectively. Undoubtedly, in this sample, there is a relationship between these two classes and the phylum *Amoebozoa*, which includes amoebae. In *Candidatus* Babeliae, the only two isolates, *Candidatus* Babela massiliensis and *Vermiphilus pyriformis*, were obtained from amoebae [36]. The study of two representative sequences obtained in El Castillo Cave showed that one of them reached 90.2% similarity with *Candidatus* Babela massiliensis (HG793133) and 87.3% with *Vermiphilus pyriformis* (KT266866). This indicates that the sequence could correspond to a different species. However, another sequence showed 100% similarity with *Candidatus* Babela massiliensis and 86.7% with *Vermiphilus pyriformis*, which permitted assigning the sequence to *Candidatus* Babela massiliensis.

With percentages lower than 5%, the class *Chloroflexia* appeared and was only present in the CAS6F sample, and *Blastocatellia* appeared in CAS3C. *Planctomycetia* reached 3.1% in CAS3C and CAS6F samples but was absent in the other three samples.

Figure 6 shows the heat map of the genera found in El Castillo Cave. About half of the relative abundance in sample CAS6F from the ground was covered by chloroplasts, followed by *Streptomyces* (12.7%), *Phyllobacterium* (5.6%) and *Achromobacter* (4.8%). Sample CAS4D also showed a high relative abundance of chloroplasts (20.9%), *Dyadobacter* (16.6%), *Phyllobacterium* (13.1%) and *Reyranella* (12.8%). Less chloroplast relative abundances were found in CAS3C (7.0%) and CAS2B (1.8%). This last sample was characterized by their relative abundances in *Vermiphilaceae*, *Kribbella* and *Neochlamydia*, all three over 13%. Sample CAS3C showed high relative abundances for *Sphingopyxis* (13.8%) and unassigned *Xanthobacteraceae* (12.6%).

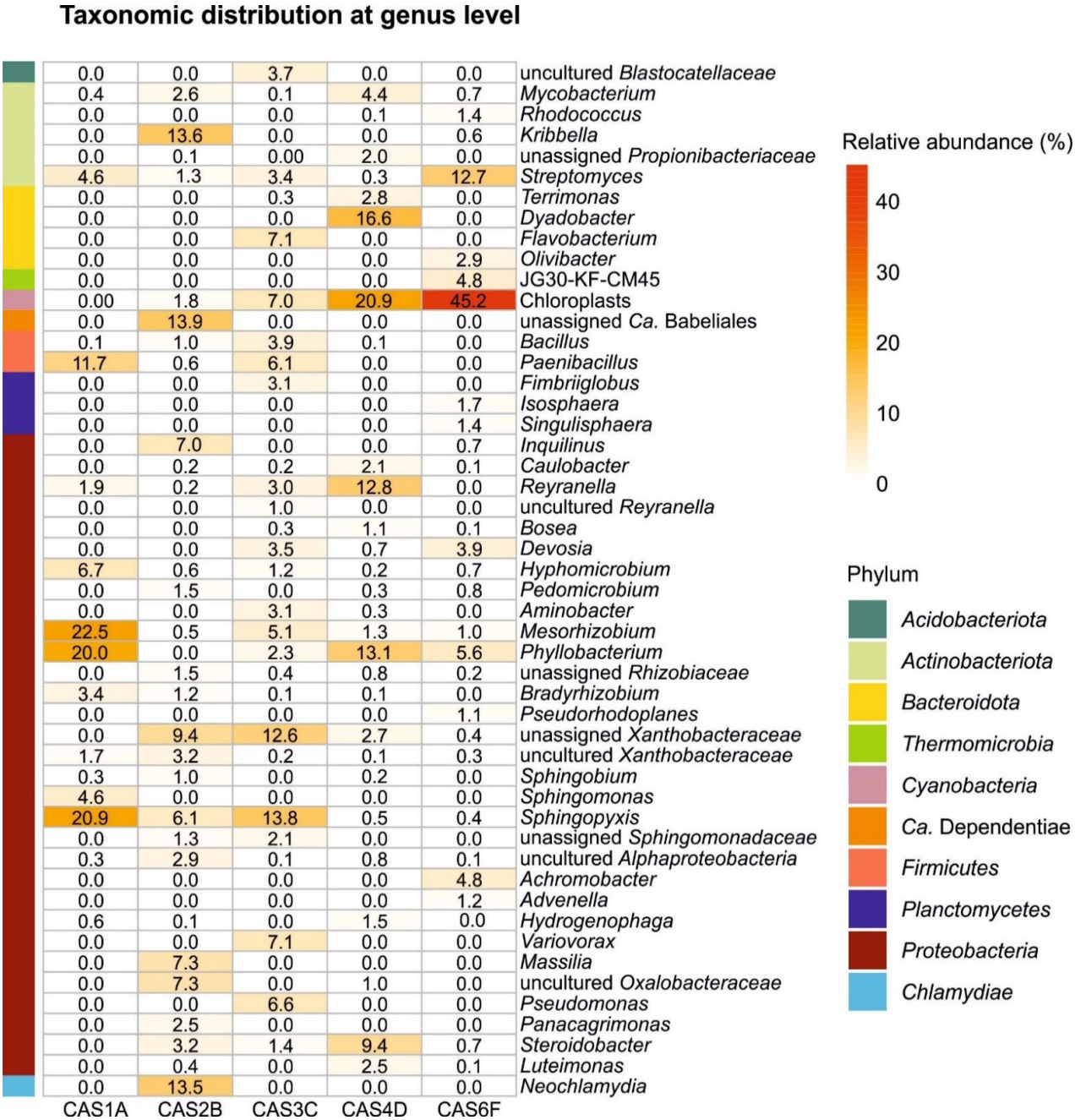

Figure 6. Heat map of El Castillo Cave samples with taxonomic identifications of *Bacteria* at the genus level. The genera are listed in the right column and their respective relative abundances included in the boxes. Scale shown in upper right corner of the figure represent color associated with the relative abundance of genera. Colored left bar groups the classification at phylum level.

3.4. Eukaryotes Community Structure

Supplementary Table S6 displays the relative abundances of *Eukaryota*. CAS1A and CAS3C samples showed unassigned percentages of 19.2 and 15.4%, respectively. In the other three samples, eukaryotes were distributed into five phyla: *Chlorophyta*, *Mucoromycota*, *Amoebozoa*, *Ascomycota* and *Protosporangiida* (Figure 7).

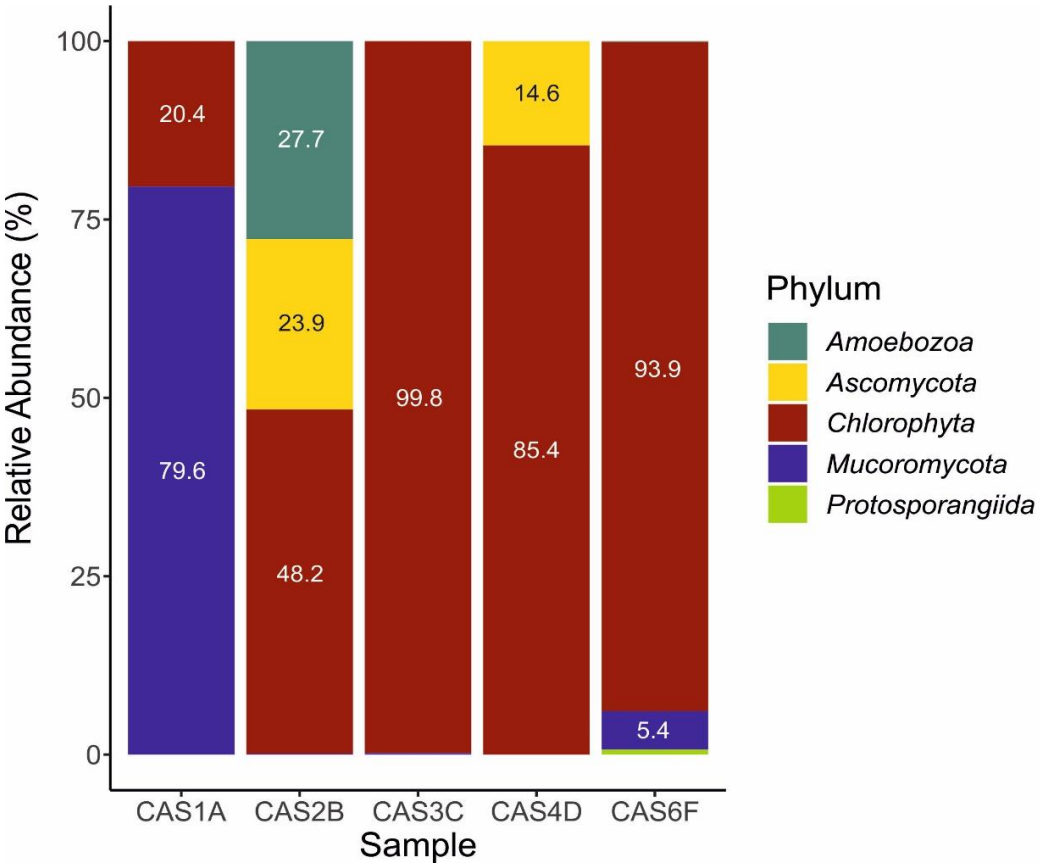

Figure 7. *Eukaryota* distribution in El Castillo Cave samples at phylum level. The phyla are described in the right column and their respective relative abundances included in the boxes. Unassigned ASVs at phylum level and sequences ASVs with relative abundances below 1% are not shown.

The highest relative abundance corresponds to *Chlorophyta*, with variable percentages in each sample, from 20.4% in CAS1A to 99.8% in CAS3C, but in all cases, except in CAS1A, *Chlorophyta* exceeds the other phyla. The relative abundance of *Mucoromycota* in CAS1A was noteworthy, with 79.6%, followed by 5.4% in the ground sample (CAS6F). In the remaining panel samples, this phylum was absent or not representative (0.1–0.2%).

Amoebozoa were only signified in sample CAS2B, which is, in turn, the one that shows the highest relative abundance of amoebae endosymbiont bacteria, an aspect that was highlighted above and with a negligible relative abundance (0.1%) in the ground sample. *Ascomycota* appeared in CAS2B and CAS4D samples, with 23.9% and 14.6% of relative abundances, respectively, while *Protosporangiida* was only found in the ground sample (CAS6F) with a relative abundance of 0.7%.

The *Chlorophyta* are divided into two classes: *Chlorophyceae* presented in all samples except in CAS6F, which is where *Trebouxiophyceae* appears, with 93.9% of relative abundance. *Chlorophyceae* reached the greater relative abundance in the CAS3C sample: 99.8%. The *Mucoromycota* and *Amoebozoa* classes presented the same relative abundances as the phyla, as well as the orders (Figure 8A), except *Ascomycota*, which were divided into two orders *Glomerellales* (in CAS2B) and *Hypocreales* (in CAS4D).

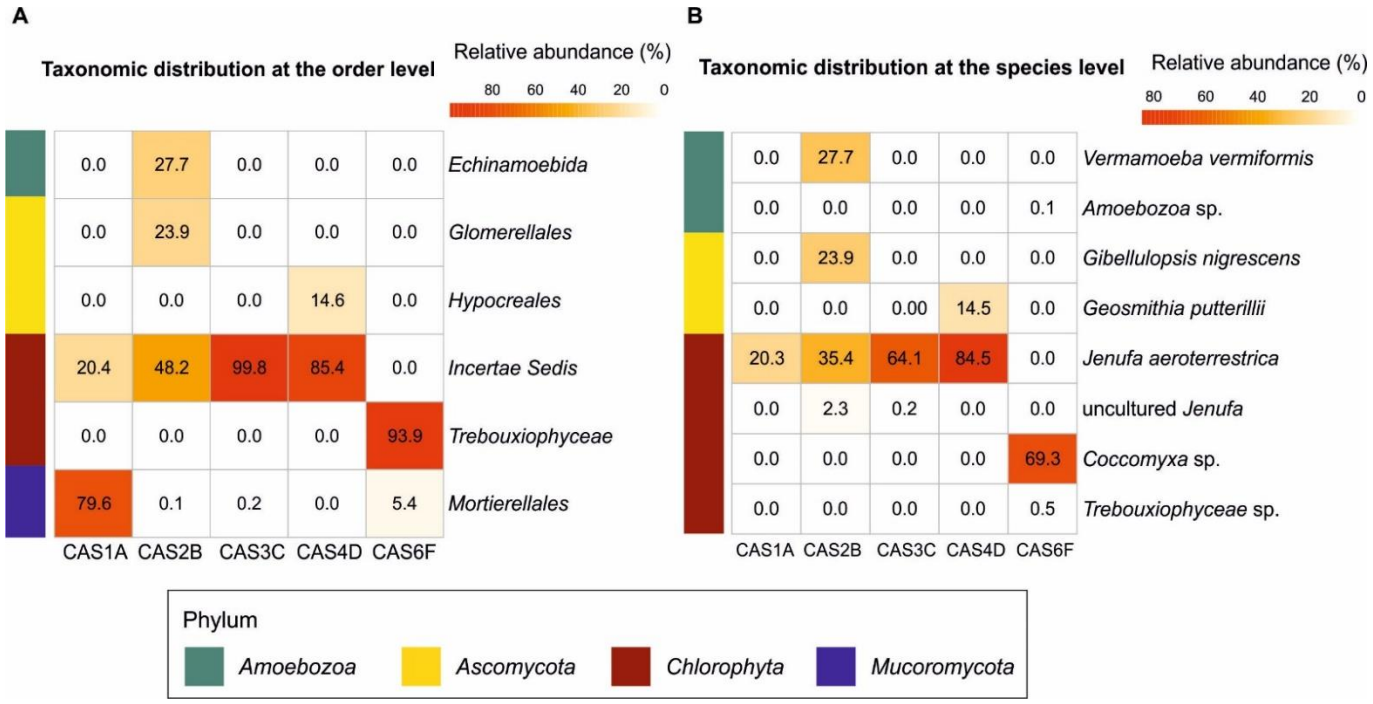

Figure 8. Heat map depicting *Eukaryota* distribution in El Castillo Cave samples at order (**A**) and species (**B**) level. Orders and species are described in the right column and their respective relative abundances included in the boxes. Scale shown in upper right corner of each figure represent color associated with the relative abundance. Colored left bar groups the classification at phylum level.

Figure 8B groups the species. In the Polychrome Panel were identified two species from the *Chlorophyceae* class: *Jenufa aeroterrestrica* and an uncultured *Jenufa*, but not in the ground sample, with members exclusively of *Trebouxiophyceae*: *Coccomyxa* sp. and an unidentified *Trebouxiophyceae* species.

4. Discussion

4.1. Microclimate Disruption by Daily Visitors and Cave Lighting

El Castillo Cave is a show cave with a large number of visitors. During the monitoring period, with limitations of visits imposed by the COVID pandemic, 18,528 people visited the cave. Visits are on a daily basis and the cave was closed every Monday. During a standard regime of tourist use, the average daily number of visitors to the cave ranges between 100 and 120 people/day, including cave guides who accompany groups of approximately 15 people. This visiting system implies that the painting panels receive hours of low intensity light daily. Visits were reduced between March and July 2020 to below 100 people/month, with groups of only five people due to limitations imposed by the pandemic, and increased progressively after July 2020 (Supplementary Table S1).

The cave's microclimate is affected by both daily visits and the seasonal increase in the number of visitors (Figure 3). The mean daily variation was 0.08 °C during visits and reduced to 0.02 °C/day without visits (Supplementary Table S1). The mean daily temperature variations caused by visitors exceed the daily thermal amplitude of the cave under natural conditions (without visits), presenting variations higher than 0.1 °C, on average, for more than 80 people/day (from June to September). It is just in the summer period, with a high number of visitors that a cumulative thermal effect was observed, with an increasing trend in temperature between weeks and a partial recovery during the days when the cave is closed to the public (Figure 3).

The average daily variation of CO_2 is 72 ppm when the cave was closed to the public, increasing to 85 ppm/day, on average, during days with visitors (Supplementary Table S1). The daily carrying capacity of the cave was estimated at approximately 60 people/day,

considering CO_2 as a key parameter, since the mean variations of CO_2 induced by influx below this value are usually of lesser magnitudes than the daily CO_2 oscillations recorded under natural conditions. The increase in the number of visitors above this average daily threshold generates variations in CO_2 concentrations that progressively cause a cumulative effect, very noticeable during summer. Thus, the levels of this gas experienced an increasing trend from early June linked to high daily visit rates, almost doubling the concentration during August and September (occasionally exceeding 2000 ppm).

These maximum CO_2 concentrations are quite distinct to those registered during winter (ranging 800–1000 ppm) or even compared to CO_2 during previous periods of cave air renewal but with lower visit rates (e.g., from October to November, ranging 1200–1400 ppm). The anomalous CO_2 concentrations and the upward trend during summer with prevailing air renewal are both obvious signs of the environmental disruption of the cave atmosphere, far from its expected natural dynamics.

4.2. Prokaryota

The high relative abundances of *Proteobacteria* and *Bacteroidota*, two well-known copiotrophic phyla, are consistent with the activity of El Castillo as a show cave. These abundances were also found in drip waters from Nerja, a cave that attracts about 450,000 visitors per year [32], and in a Chinese cave [37].

Proteobacteria was mainly composed by the class *Alphaproteobacteria*. *Betaproteobacteria* and *Gammaproteobacteria* reached lower percentages. In most caves, *Alphaproteobacteria* and *Gammaproteobacteria* were abundant [1,38], as well as *Gammaproteobacteria* and *Actinobacteria* in other caves [30,39,40]. *Actinobacteria* was very abundant in limestone and volcanic caves [40–43].

The class *Chlamydiae* was only found in sample CAS2B, as well as *Candidatus* Babeliae. Both classes form a rare community in this sample. The members of *Candidatus* Dependentiae and *Chlamydiae* are endosymbionts of amoebae [36].

What is more descriptive than a discussion on the distribution of *Bacteria* in different orders and families would be to focus the attention on the genera identified in El Castillo Cave with relative abundances higher than 10% (Figure 6).

The higher relative abundance corresponded to chloroplasts (20.9% in CAS4D and 45.2% in CAS6F). However, these sequences identified within the phylum *Cyanobacteria* (Figure 5) really corresponded to the chloroplasts of the genus *Jenufa* (*Chlorophyta*) that will be discussed latter within *Eukaryota*.

With relative abundances greater than 20%, *Mesorhizobium*, *Sphingopyxis* and *Phyllobacterium* were identified. *Mesorhizobium*, a nitrogen-fixing alphaproteobacterium present in root nodules, also has a free lifestyle [44] and reached a relative abundance of 22.5% in CAS1A and 5.1% in CAS3C samples, while in the other three samples, it ranged between 0.5 and 1.3%. This genus is well represented in all subterranean environments and caves, although often they appear as non-cultivable sequences. In the Nerja Cave, *Mesorhizobium* occurred both in phototrophic biofilms and in drip waters. In the last case, the highest abundance was related to the plants in the garden above the cave and the low rock thickness (5 m). Diaz-Herraiz et al. [45] reported the colonization of wall paintings in the Etruscan Tomba del Colle by *Hyphomicrobiales*, including *Mesorhizobium*. This was also attributed to the low thickness of the soil above the tomb and the occurrence of plant roots that invaded the tomb and hung from the ceiling.

The genus *Sphingopyxis* was abundantly represented in samples CAS1A and CAS3C (20.8 and 13.8%, respectively), decreasing to 6.1% in CAS2B, while in CAS4D and CAS6F, it only reached 0.5 and 0.4 %, respectively. *Sphingopyxis* have been found in different caves [46–49].

Phyllobacterium is a genus of *Hyphomicrobiales* frequently isolated from root nodules [50], although it is also found in subterranean environments, such as *Phyllobacterium catacumbae*, isolated from a Roman catacomb [51]. Species of *Phyllobacterium* have been collected in

the air of caves, such as Lascaux [52] and Ardales [53]. Likewise, different species of *Phyllobacterium* were found in an Etruscan tomb [54].

Among the bacteria with relative abundances between 10 and 20%, several bacteria were retrieved: *Dyadobacter*, a member of the order *Candidatus* Babeliales, *Kribbella*, *Neochlamydia*, *Reyranella*, *Streptomyces*, *Paenibacillus* and an unidentified *Xanthobacteraceae*.

The genus *Dyadobacter* was only found in CAS4D sample. Species of this genus have been reported in soils [55] and in biofilms on historical monuments [56].

An unidentified genus included in the order *Candidatus* Babeliales appeared in the CAS2B sample. This order comprises endosymbiotic bacteria that naturally infect free-living amoebae such as *Vermamoeba vermiformis* [57], which is also present in El Castillo Cave (Figure 8).

Candidatus Babela massiliensis is an intracellular bacterium not only found in *Acanthamoeba polyphaga* [58] but also in biofilms at 1470 m depth in an American gold mine [59]. Possibly, in this mine, it was associated with amoebae, although this aspect was not studied by Thompson et al. In support of this assumption, the occurrence in the mine biofilms of other bacteria related to amoebae can be argued, such as *Afipia*, *Legionella*, *Nordella*, *Bosea*, etc. [60].

A similar relationship with amoebae was found in the genus *Neochlamydia*, which is equally abundant in CAS2B sample (13.5%). Members of this genus are obligate endosymbionts of amoebae and particularly of *Vermamoeba vermiformis* [61,62]. Interestingly, one of the genomes studied by Köstlbacher et al. [62] was isolated from a biofilm in a cave wall in Hawaii. The data shown in this work indicate the close relationship between phototrophic biofilms, endosymbiotic bacteria of the order *Candidatus* Babeliales, the genus *Neochlamydia* and amoebae (*Vermamoeba vermiformis*).

Kribbella is common in subterranean environments. Maciejewska et al. [63] found *Kribbella* in the moonmilk of a Belgian cave, Alonso et al. [48] in Lascaux Cave and Diaz-Herraiz et al. [54] observed it in an Etruscan tomb. In El Castillo Cave, it was abundantly represented in CAS2B sample (13.6%) but in low quantities in the ground sample CAS6F (0.6%).

The genus *Reyranella* was found in all samples, except for CAS6F. The relative abundances were highly variable, from 12.8% in CAS4D to 0.2% in CAS2B. The first species of the genus *Reyranella massiliensis* was obtained from amoeba co-cultures from different places, river water and water from cooling towers [64].

There are a few bacteria with relative abundances below 10% but are of interest due to their occurrence and association with amoebae: *Massilia*, *Inquilinus*, *Pseudomonas*, *Bosea*, *Nordella*, *Bordetella*, *Candidatus* Procabacter, *Legionella*, *Bacillus*, *Mycobacterium*, *Rhodococcus* and *Achromobacter* [62,65–70]. This denotes the importance of amoebae in phototrophic biofilm communities.

The high relative abundance of *Streptomyces* in the ground sample CAS6F (12.7%) is noticeable, while in other samples, it ranges between 0.3 and 4.6%. *Streptomyces* was the genus most abundantly isolated in Altamira, Tito Bustillo, La Garma and Grotta dei Cervi caves [41,71,72]. Species of the genus *Streptomyces* are frequently found in the air in a large number of cavities due to their ability to form spores. In a study on the aerobiology of three Andalusian caves (Ardales, Tesoro and Gruta de las Maravillas), 19 species of *Streptomyces* were identified. This diversity was directly related by Dominguez-Moñino et al. [53] to the abundance of phototrophic biofilms, as is the case in the ground sample.

An unidentified genus of the *Xanthobacteraceae* family was represented in CAS2B, CAS3C and CAS4D samples (with relative abundances of 9.4, 12.6 and 2.7%, respectively). *Xanthobacteraceae* were also found in the drip waters of the Nerja Cave with relative abundances from 0.4% to 9.4% (unpublished work).

Van der Kooij et al. [73] found *Alphaproteobacteria*, mainly *Xanthobacteraceae*, such as *Bradyrhizobium*, *Pseudorhodoplanes* and other bacteria resistant to *Acanthamoeba* spp. and *Vermamoeba vermiformis* in drinking water supplies in The Netherlands. Among other resistant bacteria, these authors identified *Reyranella*, *Bosea* and *Blastocatellaceae*, all of them identified in El Castillo Cave.

Paenibacillus, together with *Bacillus*, is frequently observed in caves. In El Castillo Cave, *Paenibacillus* had important relative abundances in CAS1A (11.7%) and CAS3C (6.1%) and was absent in CAS4D and CAS6F; in the CAS2B sample, it only reached 0.6% of relative abundance. *Paenibacillus* has been found in many Spanish caves, such as Altamira [74,75], Ardales, Tesoro and Gruta de las Maravillas [53] and is generally observed to be together with *Bacillus*. *Bacillus* was only present in samples CAS3C (3.9%) and CAS2B (1.0%).

If we compare the phototrophic community of El Castillo Cave with that of the Nerja Cave [32], notable differences can be observed, as they correspond to initial and matures stages, respectively. While in Nerja, the established community is highly developed due to years of growth and *Cyanobacteria* taxa were abundant in the mature stage of lampenflora, in El Castillo, the faint green color on the panel and the low diversity of phototrophs point to an initial stage, but it is capable of progressing and evolving into more complex communities over time, as observed in Nerja and other caves [9,32].

The absence of *Cyanobacteria* taxa in El Castillo is noticeable, as opposed to that reported for other caves [3–7,9,32]. In El Castillo Cave, *Cyanobacteria* did not appear in CAS1A sample but showed high relative abundances in two other samples. However, these sequences were assigned to chloroplasts of *Chlorophyta* (CAS4D and CAS6F). In fact, in public 16S rRNA gene databases, the phylum *Cyanobacteria* includes not only free-living bacteria but also eukaryote chloroplasts and mitochondria.

From our data, it is evident that the cultivation of the green biofilms in the laboratory provided a representative picture on the diversity of microbial communities thriving on the Polychrome Panel once direct NGS analyses were not possible.

4.3. Eukaryota

Regarding *Eukaryota*, the phylum *Protosporangiida* includes protosteloid amoebae, whose habitats are the remains of dead plants and soils, and due to their predatory activity, they feed on bacteria and fungi decomposing organic matter. Aguilar et al. [76] found 21 species of protostelids in the Somiedo Reserve (Asturias) and comprised 65% of all the species described in the world in those years, which made the studied area the richest in Europe. Given the geographic proximity of Somiedo to El Castillo Cave and the similar weather conditions, the occurrence of *Protosporangiida* in the ground sediments with an abundance of chlorophytes was not suprising. Aguilar et al. [77] proved the influence of the microhabitat on the development of these amoebae, which are abundant in places with high rainfall, low temperature in summer months and low minimum temperatures in winter months. The high relative humidity and the low and constant cave temperature seem to be an ideal niche for the development of protosteloid amoebae.

Jenufa aeroterrestrica was described as a new species of *Chlorophyta* for Europe in the biofilms on tree bark and on the walls of buildings [78]. It has recently been found in the biofilms on limestones from Coimbra Cathedral [79]. Previously, Hallmann et al. [80] published the sequence of an unidentified *Jenufa* that developed as epilithic biofilm on the rocks of a medieval castle in Germany, and in 2006, we deposited two sequences of chloroplasts in NCBI that were collected in Altamira Cave, which have later been revealed to belong to *Jenufa* [81].

Del Rosal Padial et al. [82] observed *Jenufa* on the speleothems and walls in Nerja, a cave with abundant phototrophic biofilms. Chlorophytes (*Jenufa* and *Desmococcus*) were mostly identified in areas with liquid water (occasional or habitual), while cyanobacteria were abundant in dry areas.

The *Jenufa* species from Nerja Cave showed molecular differences with the two described species, *J. perforata* (96%) and *J. minuta* (98%), pointing out that it could be a new species. Subsequently, Procházková et al. [78] described *Jenufa aeroterrestrica*, which is predictably the species from the Nerja, Altamira and El Castillo caves. The occurrence of another unidentified species of *Jenufa*, in CAS2B and CAS3C samples, likely indicates the existence of a novel species. A few authors mentioned the occurrence of non-cultivable

Jenufa on leaves [83] and limestones [79], which suggests that the genus would have more species than those currently described.

Song et al. [83] described a fourth species of *Jenufa*, *J. lobulosa*, isolated from a sandstone surface, which differed morphologically and molecularly from the three previously described *Jenufa* species.

It is interesting that the species of the genus *Jenufa*, as well as other epilithic chlorophytes, are capable of surviving long periods of desiccation and have a high capacity to adapt to long periods of darkness [81] or to very low light intensities. Kol [84] reported that some species of cyanobacteria and chlorophytes can tolerate the complete absence of light for more than one year and that they can even develop under these conditions. However, the ability to adapt to the absence of light is not a general characteristic of algae but rather of some strains within certain species.

Zammit [85] studied the composition of epilitic phototrophic biofilms in catacombs and other subterranean constructions excavated in limestone in Malta. There, filamentous cyanobacteria predominated, along with heterotrophic bacteria and chlorophytes. However, in Hal-Saflieni Hypogeum, *Jenufa* predominated and remained viable and capable of growing in cultivation even when the availability of light was drastically reduced or completely eliminated.

Coccomyxa sp. and an unidentified species, the first with a great relative abundance (69.6%) and the second with only 0.5%, that are members of *Trebouxiophyceae* were found in the ground adjacent to the lighting lamp (CAS6F).

Coccomyxa is a genus with a wide geographic distribution, which forms biofilms and dominates in some ecosystems due to its versatility of habitats and life forms [86]. It can be found free in terrestrial biofilms and is associated with mosses or in symbiotic association with lichens [87]. A recent study has showed that *Coccomyxa* is capable of growing in a wide range of habitats, including acid lakes or metal-polluted waters, and can grow mixotrophically, which explains its dominant position in extreme conditions, even in extremely low light conditions [88]. The presence of *Coccomyxa* is not rare in caves. It was found in the Mammoth Cave in Kentucky [89], and more recently, it was reported in European caves [90,91].

Concerning chlorophytes, Mulec et al. [92] and Piano et al. [93] reported that the first colonizers in caves with artificial light are green algae; in mature stages, the biofilms are mainly composed by cyanobacteria that overgrow green algae. In addition, Muñoz-Fernández et al. [94] found that *Jenufa* dominated the wet areas in the Nerja Cave. These statements are in agreement with our findings and the occurrence of an initial stage of colonization by *Jenufa* in the panel. *Jenufa* is well adapted to growth in subterranean environments, as evidenced by its wide distribution in Spanish, French and Swiss caves and in Italian catacombs. This behavior is due to its high capacity to adapt to long periods of darkness or very low light intensity. The presence of *Jenufa* on the panel is worrying. In addition to their characteristic adaptation to adverse environmental conditions, their autospores can be easily transported through the air and colonize new substrata and other panels. *Coccomyxa* is capable of growing in a wide range of habitats, even mixotrophically, which explains its occurrence close to the lamp (Figure 2D), a place more warm and dry than the panel. In this respect, Abe et al. [95] reported that *Coccomyxa* adapts to stress conditions, including desiccation, high temperatures and nutrient deficiency.

The *Mucoromycota* with the genus *Mortierella* was very abundant in the CAS1A sample (79.7%) and less in CAS6F (5.4%). In the remaining samples, it was absent (CAS4D) or did not exceed 0.2% of relative abundance. This fungus was associated with bat guano [96]. Jurado et al. [97] found *Mortierella* in the sediments of Castañar de Ibor Cave after a fungal outbreak. The high abundance of *Mortierella* in the panel is surprising, although its occurrence in the ground sample is not.

The *Amoebozoa* are represented in El Castillo Cave by two classes: *Tubulinea* and *Discosea*. Within *Tubulinea*, the species *Vermamoeba vermiformis* is included and reached 27.7% relative abundance and was only present in sample CAS2B (Figure 8B). *Vermamoeba*

vermiformis, previously classified as *Hartmannella vermiformis*, is widely distributed in various environments: water, soil and air [98].

Amoebae and their possible endosymbionts are relatively common in caves. Garcia-Sanchez et al. [69] identified *Acanthamoeba astronyxis*, *Acanthamoeba castellanii*, *Acanthamoeba* sp. and *Hartmannella vermiformis* in the sediments of the Lascaux Cave, and Bastian et al. [67] reported the occurrence of their endosymbiontic bacteria. Previously, the occurrence of cave amoebae was reported by a few authors [99,100]. More recently, Jurado et al. [32] (2020a) found a high abundance of amoebae in the phototrophic biofilms of Nerja Cave, including *Acanthamoeba*, *Balamuthia*, *Dactylopodida*, *Echinamoebida* and other unidentified members.

Vermamoeba vermiformis has been associated with bacteria, and it hosts (*Legionella pneumophila*, *Vermiphilus pyriformis*, *Neochlamydia hartmanellae* and others) fungi and viruses [98]. It is also a predator of bacteria in natural biofilm communities [101].

In the ground sample (CAS6F), another amoeba appeared, with a very low relative abundance (0.1%), and is identified as *Amoebozoa* sp. of the order *Dactylopodida*, without being able to accurately discriminate the genus and species. This order includes the species *Paramoeba*, *Korotnevella*, *Vexillifera*, *Neoparamoeba* and *Pseudoparamoeba* [102]. More recently, new species have been described, such as *Cunea* [103]. However, unidentified *Dactylopodida* sequences appeared in phototrophic biofilms from other caves. These lobose amoebae predate bacteria, cyanobacteria, algae, yeasts and particulate matter, as did the amoebae of class *Tubulinea* [32].

Within *Ascomycota*, two fungi appeared in the cave: *Gibellulopsis nigrescens* and *Geosmithia putterillii*. The first was observed in the CSA2B sample, and the second was observed in CAS4D, both with significant relative abundances (23.9 and 14.5%, respectively). *Gibellulopsis nigrescens* accommodates previous isolates of *Verticillium nigrescens* [104]. The latter species is a plant pathogen colonizing the roots and lower parts of the stem. Its role in this cave is unknown, unless it is related to the chlorophyte *Jenufa aeroterrestrica*. It is interesting that it appears almost exclusively in the CAS2B sample, with an abundance of *Jenufa*, *Vermamoeba vermiformis* and their endosymbionts.

The genus *Geosmithia* was erected by Pitt [105] to accommodate *Penicillium lavendulum* and related species. *Geosmithia putterillii* is a recognized fungus associated with tree bark beetles [106] and the sole reference to its occurrence in a cave was in Lascaux [107], where this fungus and *Geosmithia* (=*Penicillium*) *namyslowskii* represented 17.6% of the clones. The finding of *Geosmithia putterillii* in El Castillo Cave could be hypothetically related to the occurrence of insects on the panel.

The wide compositional diversity of the different biofilms in the panel must be remarked. This was probably due to distinct environmental, nutritional and light irradiance conditions in the niches sampled.

A wide array of treatments has been proposed to control and/or eradicate biofouling in caves, namely, sodium hypochlorite, hydrogen peroxide, benzalkonium chloride, monochromatic light and UV irradiation [2,6,94,108,109]. Mulec and Kosi [110] reviewed the pros and cons of physical, chemical and biological methods to control phototrophs in caves, but there is no ideal solution currently.

In light of the diversity of microorganisms thriving on the Polychrome Panel, its great extension and irregular surface (Figures 1 and 2), the overlapping of figures and their spatial distribution prevent the application of any of these treatments, because there is not enough information about the possible effects on microorganisms, the paintings and the substratum.

5. Conclusions

El Castillo Cave's microclimate is affected by the current visiting regime. The high number of visitors during the summer season, in which the cave enters the ventilation phase, prevents the decrease in CO_2 to its natural levels. Overall, the cave maintains abnormally high temperatures and CO_2 concentration values throughout the year, which are favorable for the development of phototrophic microbial communities. The artificial

lighting of the cave has promoted the development of green algae, both on the panel and on the ground.

Although the data were obtained from cultures (that is, by an indirect method), the biomass grown in the laboratory revealed a relatively similar composition to that found in other cave biofilms analyzed directly by NGS.

Judging by the occurrence of a few chlorophytes in the rock art panel, the establishment of the phototrophic community was in their initial stages without considerable progress, as can be deduced from the low diversity of phototrophic taxa. We do not believe that this is a result of bias in methodology because most cave *Cyanobacteria* and *Chlorophyta* grow very well in laboratory conditions.

It is worth noting the different species were found in each niche: *Jenufa* in the panel and *Coccomyxa* in the ground. This was undoubtedly caused by different ecological conditions (substrata, light intensity, temperature, relative humidity and nutrient availability) in both locations.

The presence of amoebae and associated pathogenic bacteria in the cave could be a risk for visitors, and the occurrence of green algae, bacteria, fungi and amoebae in the Polychrome Panel requires full attention and the adoption of measures that prevent their growth. Unfortunately, the treatments and elimination of these microbial communities in a panel with rock art paintings are a delicate issue and previous experiences do not advise approaching a treatment with biocides, which led to a serious deterioration in the entire Lascaux Cave. Other treatments shed doubts on its effectiveness and safety and have not been reliably verified on complex microbial communities such as those found in El Castillo Cave. Therefore, two types of measures seem advisable in El Castillo Cave at short term. First of all, it is crucial to eliminate the artificial and permanent lighting system, particularly in the panel areas and to implement visits with flashlights. If possible, this action should be extended to the entire cave. Subsequently, the panel areas colonized by biofilms should be removed and cleaned manually by an expert restorer, either with swabs or delicately with the tip of a scalpel. It is convenient to periodically control the panel and to survey the behavior of the remaining *Chlorophyta* if it is not effectively removed over time.

On the other hand, the ground requires a treatment including the removal of the first centimeters of sediments and adjacent areas, as well as of the microorganisms adhering to the lighting lamps. Subsequently, a treatment of the ground with hydrogen peroxide is advisable. This treatment oxidizes the remaining microbial organic matter and leaves no chemical residues that could be susceptible of use as nutrients by other microorganisms. It would be desirable to carry out this treatment in all the lit areas located on the ground that developed phototrophic biofilms, since bacteria, algae and fungi can disperse to the rest of galleries and halls from these areas.

At mid- and long-term periods and depending on the evolution of *Chlorophyta* colonization, the pros and cons of other treatments should be thoroughly investigated in the laboratory and the response of the biofilm components should be tested before any other interventions in the cave.

Finally, this research shows that, in cases of poorly developed green biofilms corresponding to initial stages of colonization by phototrophic microorganisms, a careful sampling of the rock art panel and the cultivation of the biomass in the laboratory results in the growth of a characteristic microbial community where most of the original organisms were likely represented, as denoted by the wide and complex microbial communities. This may be a method that can overcome limitations imposed both by the cultural heritage authorities for sampling Paleolithic paintings and by the low amount of DNA available, which is difficult for direct molecular analytical approaches.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applbiosci1010003/s1, Figure S1: Samples grown in BG11 medium. A: Sample CAS1A. B: Sample CAS2B. C: Sample CAS3C. D: Sample CAS4D. E: Sample CAS6F. Figure S2: Prokaryotic community distribution in El Castillo Cave. Venn diagram showing the overlap between the ASVs generated by the five samples. Table S1: Monthly data on visits to the cave (total, average and maximum daily visits) compared with the mean daily variations of temperature and CO_2 concentration for cave air. Table S2: Description of samples from the Polychrome Panel, El Castillo Cave. Table S3: DNA concentration of samples from the Polychrome Panel, El Castillo Cave. Table S4: Number of ASVs, taxon richness and α-diversity indexes for El Castillo Cave. Table S5: Prokaryotic ASVs distribution in El Castillo Cave. Table S6. Eukaryotic ASVs distribution in El Castillo Cave.

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