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Review

Microbial Diversity of Biodeteriorated Limestone Cultural Heritage Assets Identified Using Molecular Approaches—A Literature Review

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Abstract: A significant part of our stone heritage is made of limestone. Researchers are increasingly concerned about the risk of biodeterioration of these important objects. In this article, we present an up-to-date review of the microbial diversity of biodeteriorated limestone cultural heritage (CHL). This is based on an extensive bibliographic search of the literature investigating biodiversity using culture-dependent (CD) and culture-independent (CI) techniques. In the case of the former, only articles in which microorganisms were identified using molecular tools that generate DNA sequences were selected, with the aim of providing traceable identification based on the sequences submitted to public databases. The literature search resulted in the selection of 50 articles published between 2004 and 2023. The biodiversity data obtained from the CHL were organized into the following groups: fungi (626 records), bacteria and cyanobacteria (786 and 103 records, respectively), algae (51 records), and archaea (27 records). Within each group, the microbial diversity studied was compared according to results obtained using CD and CI techniques. Of all the articles selected, 12 used both approaches, demonstrating the growing effort to discover the total microbiome of biodeteriorated cultural heritage assets.

Keywords: limestone monuments; limestone buildings; algae; archaea; bacteria; cyanobacteria; fungi; lichens; Sanger sequencing; next-generation sequencing

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1. Introduction

The Mediterranean Basin countries are recognized to accommodate some of the world's most important cultural heritage sites, including stone monuments and lithic works of art (Figure 1). Due to their cultural, artistic, and religious meaning, the safeguarding of this precious cultural heritage is highly important. The wide availability of tertiary sedimentary rocks in Southern European countries, especially Spain, Portugal, and Italy, has made limestone the main source of creating stone monuments [1]. Limestone has undoubtedly been used since the Stone Age and even earlier, and the first records of its usage relate to the Egyptian Second Dynasty, which was about 5800 years ago, when it was used for the construction of the Giza pyramids [2].

Limestone is a sedimentary rock composed of two main calcium carbonate ($CaCO_3$) minerals, calcite and aragonite, assembled in trigonal and orthorhombic systems, respectively. It may also contain materials such as quartz, sand, chert, and clay. The bioreceptivity of limestone is determined by a number of factors, including its porosity, pH, texture, roughness, chemical composition, density, and moisture [3]. There is a growing concern that

microbial biofilms on cultural heritage buildings and monuments, especially on carboniferous stones such as limestone, impact their aesthetic and physical effects, since limestone is soluble in weak acid solutions and, therefore, particularly vulnerable to biocorrosion [1].

Figure 1. Biodeteriorated limestone outdoor monument of Aristotle, located at Dornava mansion (Slovenia) from the middle of 18th century. The statue shows discoloration phenomena and superficial disintegration of limestone. This photo was taken by Maja Gutman Levstek.

The locations from which the microbial samples discussed in this review originate range from the Giza pyramid complex [3], the archaeological site of the Herculaneum's House of the Bicentenary in Italy (1 CE) [4], the archaeological site of Copan, one of the most remarkable Classic Maya (250–900 CE) centers in Mesoamerica [5], the ancient Chinese Buddhist culture sites from West Lake Cultural Landscape dating back to the Northern Song (960–1219 CE) and Qing dynasties (1636–1912 CE) [6], the 15th century French Notre Dame Gothic Cathedral in Rheims [7], and some other selected examples, including prominent monuments, buildings, and landscapes of historical significance. Studied caves of CH significance from France [8–10] and Spain [11] are included as well. Carbonate rocks (limestone in particular) are easily biodeteriorated, yet it is the vulnerability of the substratum that made the limestone attractive for creating stone monuments and buildings, as it is soft and easy to carve [1]. Microorganisms, including bacteria, fungi, archaea, algae, and lichens, are major causes of damage to stone monuments and have, therefore, received significant attention from conservators, restorers, and conservation scientists. The microbial colonization of stones depends on environmental factors such as water availability, pH, exposure to wind and rain, nutrient sources, and petrologic parameters such as mineral composition, type of cement, and the porosity and permeability of the rock material [12]. Culture-dependent techniques have been adopted for many years for investigating microbial communities or for assessing specific patterns of degradation caused by certain microbial taxa. This strategy has the advantage of testing specific degradation problems, yet it fails us when a comprehensive view of the complexity of the microbial communities is needed, which is mainly due to the fact that approximately 99% of viable microorganisms cannot be cultured [13]. In this review, we refer to the deterioration of cultural heritage objects/items made of limestone (CHL) colonized by microbes that are identified and/or detected using molecular methods. Culture-dependent (CD) methods refer to all methods in which the identification of pure microbial cultures is based on selected target DNA regions, mostly obtained using Sanger sequencing, while culture-independent (CI) methods

include Illumina short-fragment and PacBio or Nanopore long-fragment sequencing on extracted DNA samples obtained directly from the CHL biodeteriorated sites.

2. Materials and Methods

Google Scholar, Scopus, and Web of Science engines were used for screening the taxonomic literature relating to the microbes involved in biodeterioration. Keywords such as "biodeterioration", "microorganism", "limestone", either "cultural heritage" or "historical buildings", and "molecular methods" were used. The bibliographic search was performed by the three authors independently.

The bibliography was selected if (i) the microbial samples were detected/isolated from CHL assets, either epi- or endolithically; (ii) identification of microorganisms was based on molecular marker genes; and/or (iii) the microbiomes were determined using culture-independent methods. Articles that included cultural methods with phenotypic identification only, or those describing microbial detection by microscopy only, or those that employed other physico-chemical analyses without molecular genetic approaches, were not included in order to make the taxonomic information precise. The only exception were lichens, since they are not culturable as such, and their identification mainly relies on morphology and some biochemical characteristics. In addition, the studies on samples taken from limestone mortar or wall paintings were not included because the chemical properties of these substrates differ from pure limestone.

After the bibliographic search, the names of all of the microorganisms mentioned in 50 eligible articles were entered into Supplemental Tables S1–S5. Since different articles presented results at different taxonomic levels, we used the NCBI (National Center for Biotechnology Information) Taxonomy database to standardize the taxonomic entries. After the literature review, the data on organisms obtained from CHL were organized into the following taxonomic/functional groups: fungi, bacteria, cyanobacteria, algae, and archaea, each being represented in its own table (Supplementary Material Tables S1–S5). The reason for this organization is that the vast majority of articles on cultural heritage asset biodeterioration separate organisms into these groups. However, according to NCBI taxonomy, archaea, and bacteria present independent monophyletic superkingdoms, and cyanobacteria are a monophyletic phylum within the superkingdom of bacteria. The reason for placing cyanobacteria in a separate table in our study is the general belief that these photoautotrophs, along with algae, are the primary colonizers of stone [14]. Fungi and algae belong to the monophyletic eukarya superkingdom. In contrast to the monophyletic kingdom of fungi, algae present a polyphyletic group. The data on unidentified microorganisms recognized by culture-independent techniques based on DNA sequences, which were not classified to the genus level, were not entered into the tables. In the 1st column of Tables S1–S5, the CHL objects are listed. The 2nd to 7th columns show the taxonomic classification according to the NCBI Taxonomy database. The 8th and 9th columns specify whether culture-dependent or culture-independent approaches were used. The 10th column indicates whether a taxon was derived from an indoor or outdoor environment or from both of these. The 11th column lists the countries where the specimens were collected, the 12th column contains the information about the aesthetic changes, e.g., color changes or other damages, and the 13th column lists the references.

3. Results

3.1. The Origin of Studies

Figure 2 provides information on the geographical distribution of the included studies. Most of the studies were performed in European countries. As evident, Portugal is the leading country in this field, with 13 studies having been carried out. It is followed by France with seven, China with six, Italy with five, and Israel with four studies. The microbiome of biodeteriorated limestone was also investigated in Egypt in three studies. For Mexico, Russia, and Spain, two studies were included, and, for Austria, Brazil, Germany, Iran, Serbia, Slovakia, and the United Kingdom (UK), a single research article was included.

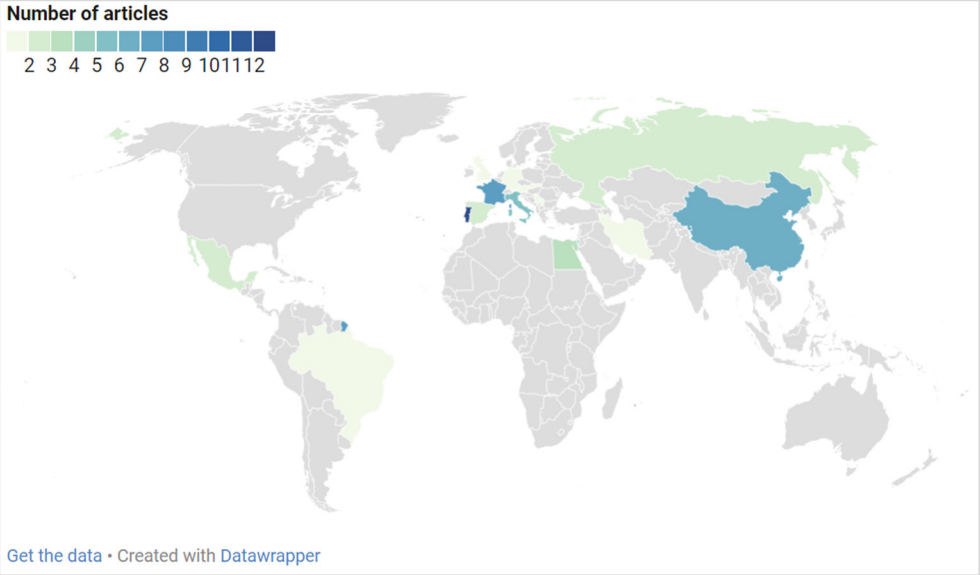

Figure 2. The number of studies on biodeteriorated limestone using molecular identification approaches by country marked up on the world map in different colors according to the number of articles.

3.2. Biodiveristy

3.2.1. Overall Biodiversity

As seen in Figure 3, a generous number of microbial isolates from limestone cultural heritage assets belong to bacteria (55.81%, of which 6.47% belong to cyanobacteria). The second most frequently isolated group identified by the molecular markers is fungi (39.30%). There are few research articles on the use of molecular methods identifying algae, so they represent only 3.20% of all microbes. Archaea are the least studied microbial group, representing only 1.69% of all microbial records.

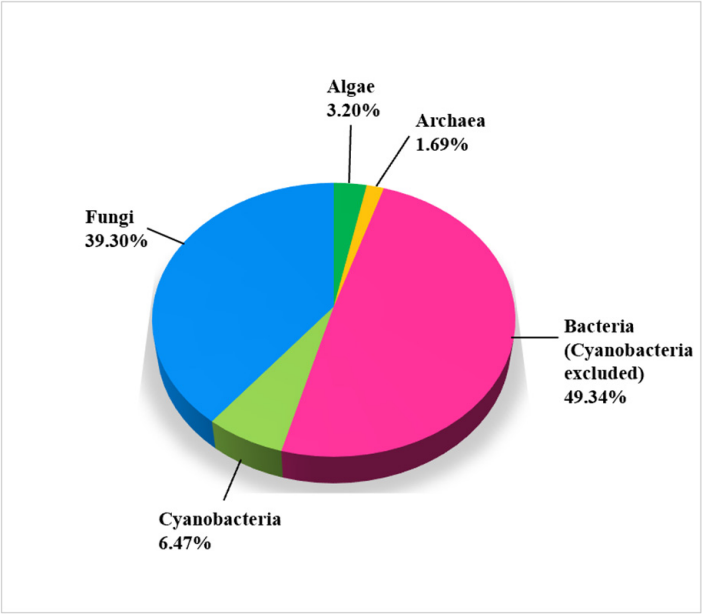

Figure 3. Biodiversity of microbial groups on damaged CHL with molecular identification (1403 records).

3.2.2. Fungi

Fungal taxa on biodeteriorated limestone (Figure 4) were extensively studied using CD methods (Figure 4A), as 397 out of 626 records were obtained this way, while the remaining 229 taxa were detected using CI approaches, as shown in Figure 4B, where we list all of the genera occurring in more than 1.5% of studies. All genera occurring in less than 1.5% were grouped in the category "Other". Among them, the genera *Clonostachys*, *Cyphelophora*, *Emericellopsis*, *Fusarium*, *Geosmithia*, *Mortierella*, *Ovicillium*, and *Stachybotrys* were recorded with the frequency of 1.0–1.5%, while the same frequency was noticed for the genera *Coniosporium*, *Exophiala*, and *Rhodotorula* using CI techniques. In the CI studies, 38% of fungal taxa remained unidentified at the genus level (Figure 4B), indicating that these taxa most likely have not yet been cultured, if sequences do not report "artifact taxa" due to the possibility of sequencing errors. Of the isolated cultures, only 1.26% of the taxa recorded remained unidentified and were assigned only to a higher taxonomic level.

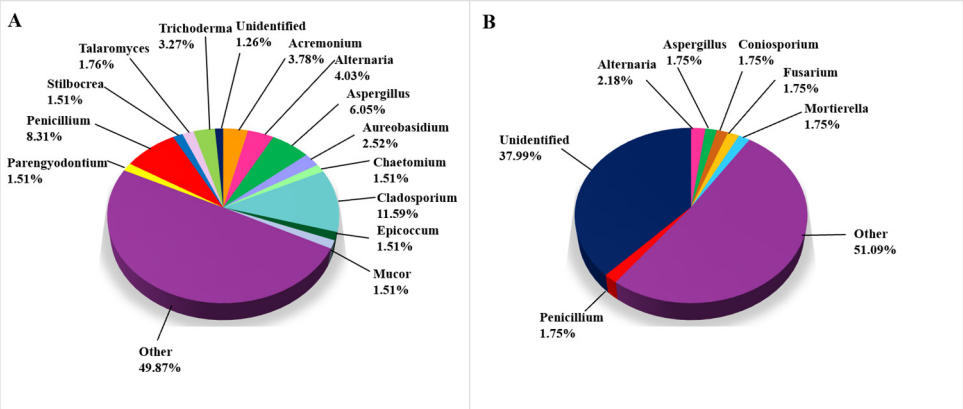

Figure 4. Fungi on damaged limestone cultural heritage. (**A**) Fungal genera identified using CD approaches (397 records obtained from 24 cultural heritage sites); (**B**) Fungal genera identified using CI approaches (229 records obtained from 13 cultural heritage sites).

Although *Penicillium* is the most often detected genus among fungi (33 records), *Cladosporium cladosporioides* is the most common fungal species on biodeteriorated CHL, having been detected in 10 studies. *Alternaria alternata* and *Penicillium chrysogenum* were detected in six articles, *Acremonium charticola* and *Epicoccum nigrum* in five, while *Penicillium brevicompactum* and *Stachybotrys chartarum* were detected in in four articles. All of the following fungal species occurred rather frequently (in three articles): *Alternaria infectoria*, *Aspergillus flavus*, *Aspergillus niger*, *Aureobasidium melanogenum*, *Aureobasidium pullulans*, *Cladosporium halotolerans*, *C. ramotenellum*, *C. sphaerospermum*, *Penicillium crustosum*, *P. glabrum*, *Stilbocrea macrostoma*, and *Talaromyces funiculosus* (Supplemental Table S1).

3.2.3. Bacteria

The bacterial taxa that were isolated and identified on biodeteriorated CHL are depicted in Figure 5. In contrast to the fungal taxa, the bacterial taxa were detected mainly using CI methods (530 of 786 records, Figure 5B), while the detection of 256 bacterial taxa relied on CD methods (Figure 5A). The percentage of genera determined is shown in Figure 5 for those with a frequency of more than 1.5%, while genera occurring in less than 1.5% are joined in the category "Other" for the taxa determined using CD approaches. For the taxa determined with CI approaches, the occurrence of genera specified under "Other" was less than 3%. Only 5.08% of cultivated bacterial taxa remained unidentified, while 30.0% of all taxa obtained using CI methods remained unclassified, possibly representing not-yet-cultivated organisms. The genus *Bacillus* was most often isolated from CHL (48 records), while *Pseudomonas* was most often detected in CI assets (45 records). *Arthrobacter agilis*, which was isolated and determined in six articles, is the most frequently occurring species

among bacteria in biodeteriorated limestone of cultural heritage significance. Frequent taxa include the members of *Bacillus cereus* and *B. subtilis* species complexes, mentioned in five different articles, and *B. licheniformis*, *B. safensis*, *Pseudomonas oryzihabitans*, and *Pseudomonas stutzeri*, which were mentioned in four articles. The following bacterial species were isolated in three articles: *B. aryabhattai*, *B. megaterium*, *B. muralis*, *B. simplex*, and *Rubrobacter radiotolerans* (Supplemental Table S2).

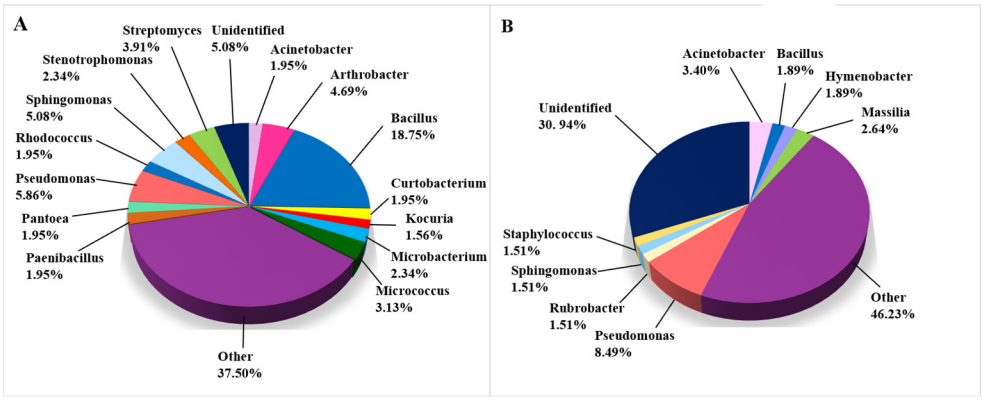

Figure 5. Bacteria on damaged limestone cultural heritage. (**A**) Bacterial genera identified using CD approaches (256 records obtained from 19 cultural heritage sites); (**B**) Bacterial genera identified using CI approaches (530 records obtained from 21 cultural heritage sites).

3.2.4. Cyanobacteria

Figure 6 represents the cyanobacterial taxa that were isolated and identified on biodeteriorated CHL. The genera that occurred in less than 3% of studies are categorized under "Other" (purple fraction). Research on the cyanobacterial microbiome was carried out very evenly using both CD methods (53 taxa, Figure 6A) and CI methods (50 taxa, Figure 6B). The trend of a higher percentage of unidentified taxa on the genus level obtained using CI methods persists, since there are 28.00% of unidentified cyanobacterial taxa, while the same in the CD-based approaches research represents only 1.89%. The percentage of determined genera is shown in Figure 6. The most often encountered cyanobacterial genera were *Leptolyngbia*, *Nostoc*, and *Pleurocapsa* (each genus mentioned in 13.2% of studies), while the species *Acaryochloris marina*, *Anabaena cylindrica*, *Loriellopsis cavernicola*, and *Nostoc punctiforme* occurred in two articles (Supplemental Table S3).

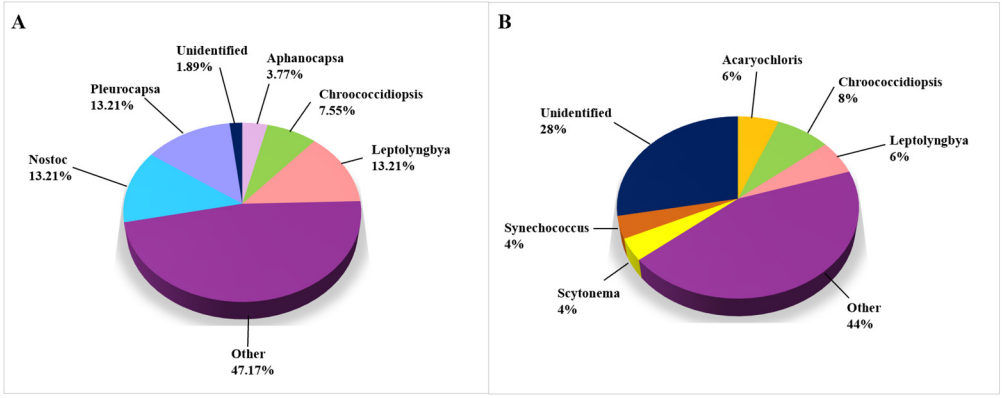

Figure 6. Cyanobacteria on damaged limestone cultural heritage. (**A**) Cyanobacterial genera identified using CD approaches (53 records obtained from 12 cultural heritage sites); (**B**) Cyanobacterial genera identified using CI approaches (50 records obtained from 14 cultural heritage sites).

3.2.5. Algae

Algal genera that were obtained from biodeteriorated CHL using CD approaches are depicted in Figure 7A, in which the genera that appeared in less than 5% are presented in the category named "Other". Since the number of genera detected using the CI approach was low, we have presented all of them in one graph (Figure 7B). Most taxonomic records on algae were obtained by the use of CD approaches, namely 39 of the 51 algal taxa, while 12 taxa were identified using the CI approaches. All algae were identified to the genus level in the CD studies. However, there were 41.67% of unidentified algal genera in studies conducted using CI approaches. The most frequently observed algal genus was *Bracteacoccus*, while the most frequently encountered species were *Acutodesmus bajacalifornicus* and *Pseudostichococcus monallantoides.* Both species were discovered in two articles (Supplemental Table S4).

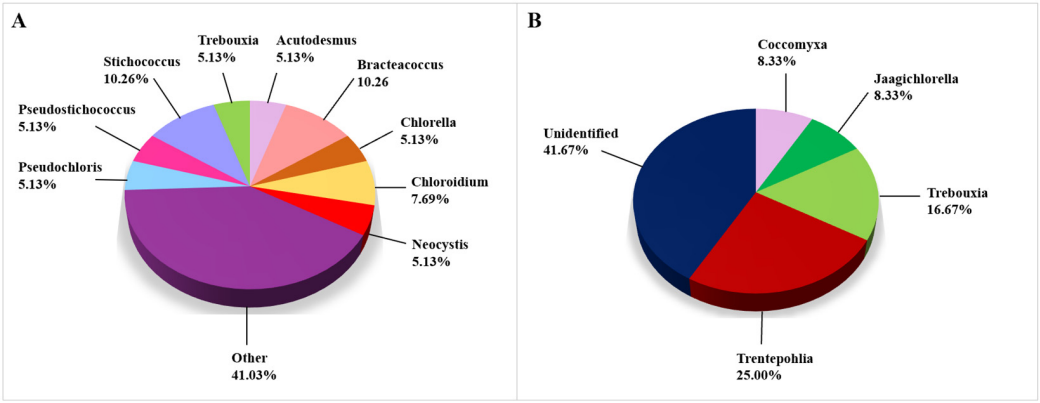

Figure 7. Algae identified on damaged limestone cultural heritage. (**A**) Algal genera identified using CD approaches (39 records obtained from 3 cultural heritage sites); (**B**) Genera of algae identified using CI approaches (12 records obtained from 5 cultural heritage sites).

3.2.6. Archaea

Figure 8 shows the genera of archaea isolated and identified on biodeteriorated CHL using CI approaches. Only a single species, *Haloferax mediterannei*, isolated from the Herculaneum's House of the Bicentenary, was identified on the basis of a culture (Supplemental Table S5).

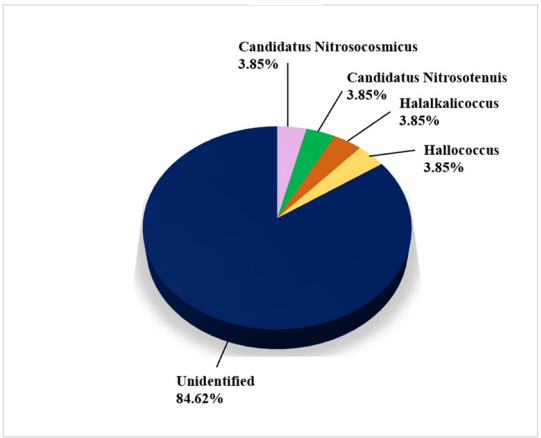

Figure 8. Archaea on damaged limestone cultural heritage identified using CI approaches (26 records obtained from 4 cultural heritage sites).

3.2.7. Lichens

Taxonomically, lichens, with a mutualistic association of fungi (mycobionts) and cyanobacteria or algae (photobionts), are classified according to the fungal partner. In this review, like in the biodeterioration studies, they are presented separately from fungi. Only 6 of the 50 selected articles present lichen identifications, while, in another 8 articles, there were mere observations of lichens using microscopy without any further identification being made [7,15–21]. Kirchhoff et al. [22] identified lichens at the species level using Sanger sequencing of PCR products obtained from DNA extracted from lichens, revealing *Xanthoria elegans*, *Caloplaca demissa*, *Texosporium sancti-jacobi*, *Caloplaca holocarpa*, and *Xanthomendoza hasseana*. Zhu et al. [6] recognized the genera *Leprocaulon*, *Botryolepraria*, *Pseudostichococcus*, *Lepraria*, and *Leprocaulon* as the dominating lichens on the studied statues and monuments. In the remaining four articles [23–26], the authors identified the lichens by their morphological characters, as follows: Gholipour-Shahraki and Mohammadi [23] identified the genera *Calogaya*, *Acarospora*, *Lecanora*, *Polysporina*, and *Verrucaria*; Nir et al. [24] identified *Caloplaca*; Ding et al. [26] recognized *Aspicilia*, *Caloplaca*, *Lecanora*, *Verrucaria*, *Dirina massiliensis*, and *Xanthoria*; and Pinheiro [25] mentioned *Aspicilia*, *Caloplaca*, *Caloplaca aurantia*, *Lecanora*, *Squamarina crassa*, *Thyrea*, *Verrucaria*, and *Xanthoria parietina*.

3.3. Culture-Dependent Methods

3.3.1. Isolation of Microorganisms

A variety of culture media was employed for the isolation of the different groups of microorganisms (Supplemental Table S6). These were either general enumeration media (e.g., DRBC or DG18 for fungi) or general cultivation media (e.g., MEA, OA, PDA, or CzA for fungi; NA, TSA, or BHI for bacteria; BG-11 for cyanobacteria and algae; and media with a high salt concentration for isolation of archaea). The majority of studies used at least two or even more culture media. For the isolation of fungi, the media were supplemented with antibiotics, such as chloramphenicol (100 mg/L) or streptomycin (0.5 g/L). In the media utilized for the isolation of bacteria, fungi were suppressed with cycloheximide (50 mg/L). Fluorescent illumination and light:dark cycles were applied when culturing phototrophic microbes, like algae and cyanobacteria. The incubation temperatures ranged from 15 to 30 °C.

Culture methods were also applied on communities. Kirchoff et al. [22], for example, took samples scratched from the stone surface and grew them in culture media (3N BBM+V medium and Z medium) prior to total DNA isolation, PCR amplification, and sequencing.

3.3.2. Identification of Microorganisms

The selection and diversity of isolated cultures was studied prior to sequencing in some studies in order to assess the diversity of the obtained cultures. For bacteria, repetitive extragenic palindrome PCR amplifications were used as fingerprint methods, while, for fungi, random amplified microsatellite polymorphism (RAMP) was utilized [27].

The identification of all microorganisms obtained in pure cultures, or even lichens as unculturable organisms growing on original substrata, was performed by employing Sanger sequencing (Table 1) as a standard technique for individual sequencing reactions using a specific DNA primer set for targeting the microorganisms on a specific template of DNA isolated from a pure culture [28]. The identification level depends on the specificity of the region defined by the PCR primer pair. For fungal identification, the ITS rDNA region was most often used as the specific barcode for identification [21]. Some older studies also employed 18S sequencing [27], which is less specific and may have resulted in a higher taxa identification level. Prokaryotes (bacteria, cyanobacteria, and archaea) were mostly identified by 16S rDNA sequences, while algae by 18S rDNA.

Table 1. Table with collected information on all included studies, including the microbial groups studied and the sequencing methods and other molecular tools used.

Object of Study	Period	Region	Target Microorganisms /Communities (com)	Sequencing Method	CD/CI Approaches	Reference, First Author, Year
EXTERNAL SURFACES OF CHL BUILDINGS						
The Chaalis Abbey	ME (1136 AD)	France	B	Sanger	CD	[17] Mihajlovski et al., 2017
Acropolis at Ek' Balam, Yucatan	100 BCE	Mexico	B (com)	Sanger (PCR products transformation into *E. coli*)	CI	[29] McNamara et al., 2006
Saaleck Castle (with outcropping rock)	ND	Germany	Al, F	Sanger	CD	[22] Kirchhoff et al., 2018
San Leonardo di Siponto Church	ME	Italy	Al, Cy (com)	Illumina MiSeq	CI	[30] Chimienti et al., 2016
Sacral and public buildings	Various (ND)	Portugal	Al, B, Cy, F	Sanger	CD	[25] Pinheiro et al., 2019 (review)
Santa Clara-a-Velha Monastery	1283 AD	Portugal	Al, Cy	Sanger	CD	[31] Miller et al., 2008
Monastery, cathedral, palace	Various (ND)	Italy, Portugal, Spain	B, Cy (com)	Sanger	CD	[32] Miller et al., 2009
Batalha monastery	1386 AD	Portugal	B, Cy (com)	Illumina MiSeq	CI	[1,26] Ding et al., 2021
Sacral and public buildings	Various (ND)	Worldwide (Brazil, Mexico)	Cy	Sanger	CD	[33] Crispim et al., 2006 (review)
Djoser and Lahun pyramids	2670–2650 BCE	Egypt	B, F (com)	Sanger Illumina MiSeq	CD CI	[34] Rizk et al., 2023
The West Lake stone cultural relics	ND	China	Al, Cy, B, F (com)	Illumina MiSeq	CI	[6] Zhu et al., 2023
Tel Megiddo	5000 BCE	Israel	B, Cy (com)	Illumina NovaSeq 6000	CI	[35] Zhang et al., 2023
Historic sacral buildings	ND	England	B, Cy B, Cy (com)	Sanger Illumina MiSeq	CD CI	[36] Skipper et al., 2022
CAVES						
Lascaux Cave wall	Paleolithic	France	F (quantitative analysis)	Real-time PCR	CI	[8] Martin-Sanchez et al., 2013

Table 1. *Cont.*

Object of Study	Period	Region	Target Microorganisms /Communities (com)	Sequencing Method	CD/CI Approaches	Reference, First Author, Year
Lascaux Cave wall	Paleolithic	France	B, F (com)	Illumina MiSeq	CI	[10] Alonso et al., 2018
Lascaux Cave wall	Paleolithic	France	B, F (com)	Illumina MiSeq	CI	[9] Bontemps et al., 2023
Sorcerer's Cave	13,000 BCE	France	B B (com)	Sanger Pyrosequencing	CD CI	[37] Lepinay et al., 2018
Pindal Cave walls	Paleolithic	Spain	B (com)	Illumina MiSeq	CI	[11] Martin-Pozas et al., 2023
OUTDOOR MONUMENTS						
Outdoor monuments (Lingyan Temple)	Period from the Tang Dynasties (618–907 AD) to the Republic of China (1912–1949 AD)	China	Al, Cy, B, F (com)	Sanger Illumina MiSeq	CD CI	[38] T. Li et al., 2023
Outdoor monuments (Mayan monuments in Uxmal)	700–1000 AD	Mexico	Cy, B (com)	Sanger (PCR products transformation into *E. coli*)	CI	[17] Ortega-Morales et al., 2004
Outdoor monuments (Qingxing Palace and Lingyin and Kaihua temples)	Northern Song (960–1219 AD) and Qing dynasties (1636–1912 AD)	China	Al, Cy, B, F (com)	Illumina MiSeq	CI	[39] Q. Li et al., 2016
Outdoor monuments (Ancient stone stela)	End of 3rd, beginning of 4th century AD	Serbia	F	Sanger	CD	[19] Savković et al., 2016
Outdoor monuments (Senusret I obelisk and Mosque of Elkadi Abd El Basset)	Ancient Egyptian era	Egypt	B	Sanger	CD	[3] ElBaghdady et al., 2019
Outdoor monuments (several)	12th–20th century	France	B	Sanger	CD	[7] Eyssautier-Chuine et al., 2021
Outdoor monuments and external site of a Cyrus the Great Tomb	558–529 BCE	Iran	F (com)	Sanger	CD	[23] Gholipour-Shahraki and Mohammadi 2017

Table 1. *Cont.*

Object of Study	Period	Region	Target Microorganisms /Communities (com)	Sequencing Method	CD/CI Approaches	Reference, First Author, Year
Outdoor tombstones in Cemetery of Milano	1885–1929 AD	Italy	Ar, B, Cy (com)	Sanger (PCR products transformation into *E. coli*)	CI	[40] Gambino et al., 2021
Outdoor statues (The Klippe statues in Hangzhou)	Yuan dynasty (1276–1368 AD)	China	Al, Cy, B, F (com)	Sanger Illumina MiSeq	CD CI	[20] Q. Li et al., 2017
Outdoor statues (Feilaifeng)	Five Dynasties period (907–960 AD)	China	F (com)	Sanger	CD	[21] T. Li et al., 2018
Outdoor statues and a cliffside inscription (West Lake Cultural Landscape of Hangzhou)	1038 and 1292 AD	China	B, Cy, F (com)	NGS (no further info)	CI	[16] Wu et al., 2021
Petroglyph sites from the Negev Desert	ND	Israel	Ar, B, Cy, F (com)	Metagenomic shotgun sequencing (Illumina) NextSeq500	CI	[24] Nir et al., 2022
Petroglyph sites from the Negev Desert	ND	Israel	B, Cy (com)	Illumina MiSeq Sanger	CI CD	[41] Nir et al., 2019
Austrian petroglyphs from Hallstatt-Dachstein/ Salzkammergut	ND	Austria	Ar, Al, B, Cy, F (com)	Nanopore (MinIon Mk1C)	CI	[15] Rabbachin et al., 2023
INDOOR SURFACES						
Internal walls of a building, archaeological site The House of the Bicentenary (Herculaneum)	Somewhere between 3rd century BCE and 1st century AD	Italy	Ar, B, Cy (com)	Sanger	CD	[4] Tescari et al., 2018
Indoor walls and columns of churches, cathedrals, and museums	12th–19th century AD	Russia	F (com)	Sanger	CD	[42] Ponizovskaya et al., 2019
Indoor statues	1400 AD	Slovakia	B, F (com)	Sanger	CD	[27] Pangallo et al., 2009
An indoor sarcophagus of D. Afonso I	16th century AD	Portugal	F (com)	Illumina MiSeq Sanger	CI CD	[43] Trovão et al., 2020

Table 1. *Cont.*

Object of Study	Period	Region	Target Microorganisms /Communities (com)	Sequencing Method	CD/CI Approaches	Reference, First Author, Year
Indoor tomb chamber of the Holy Aedicule	325/326 AD	Israel	Ar, B, Cy (com)	Nanopore (MinION Flow Cell R9.4.1 on a MinION Mk1C)	CI	[44] Delegou et al., 2022
Internal walls of a Santa Maria della Pietà church	13th century AD	Italy	Cy, F (com)	Sanger	CD	[45] Mascaro 2022
Indoor pillars of a church in Lemos Pantheon	16th century AD	Portugal	F (com)	Sanger Illumina MiSeq	CD CI	[46] Paiva et al., 2022
Internal walls of a church in Lemos Pantheon	16th century AD	Portugal	F (com)	Sanger Illumina MiSeq	CD CI	[47] Paiva et al., 2023
Internal walls of a church in a Lemos Pantheon	16th century AD	Portugal	F	Sanger	CD	[48] Paiva et al., 2023
Indoor statues	15th and 16th century AD	Portugal	F, B, Cy	Sanger Illumina MiSeq	CD CI	[49] Dias et al., 2020
SEMI-OPEN SITES						
Walls of a semi-open spaced Coimbra cathedral	12th–13th century AD	Portugal	Al, Cy (com)	Sanger Illumina MiSeq	CD CI	[50] Soares et al., 2019
Walls of a semi-open spaced Coimbra cathedral	12th–13th century AD	Portugal	F (com)	Sanger Illumina MiSeq	CI CD	[51] Trovão et al., 2019
Walls of a semi-open spaced Coimbra cathedral	12th–13th century AD	Portugal	Ar, B, Cy (com)	Sanger Illumina MiSeq	CD CI	[52] Coelho et al., 2021
Walls of a semi-open spaced Coimbra cathedral	12th–13th century AD	Portugal	Cy	Sanger	CD	[53] Soares et al., 2021
Internal and external sites of a Minaret of Prince Muhammad	Ottoman period	Egypt	B (com)	Sanger	CD	[54] Ahmed and Mohamed 2022
Coimbra's hypogean Roman cryptoporticus	Somewhere between 1st and 2nd century AD	Portugal	Al, Cy, F (com)	Sanger Illumina MiSeq	CD CI	[55] Soares et al., 2022
Coimbra's hypogean Roman cryptoporticus	Somewhere between 1st and 2nd century AD	Portugal	F	Sanger	CD	[56] Trovão et al., 2022
Outdoor walls of several buildings	ND	France	B, F (com)	Sanger	CD	[57] Balland-Bolou-Bi et al., 2023

AD = Anno Domini; Al = algae; Ar = archaea; B = bacteria; BCE = Before Common Era; CD = culture dependent; CI = culture independent; Com = community; Cy = cyanobacteria; F = fungi; ND = non-defined; ME = medieval.

All of the sequences were deposited to public databases (the National Center for Biotechnology Information or the China National Center for Bioinformation database) and are available for identification verification and sequence comparison.

3.3.3. Biodeterioration Studies on Pure Cultures

The usage of cultures extends beyond the isolation of specific microbes. Studies have also been conducted on microbial pure cultures to test their stone deterioration potential, such as the excretion of weak acids, the ability to solubilize $CaCO_3$, and the ability to precipitate $CaCO_3$. The former was tested on the Bruni medium, utilized to test $CaCO_3$ dissolution resulting in a clear cone around the colonies [54], and the ability to precipitate $CaCO_3$ on B4, where the positive strains produced crystals [27]. All of the cultures that were used in the 50 selected articles are listed in Supplemental Table S6.

3.4. Culture-Independent Methods

3.4.1. DNA Isolation

Prior to the application of these methods, total DNA was extracted from biodeteriorated CHL using different commercial kits. One of the most often cited was Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA) [6,20,21,24,34,39,41], followed by DNeasy PowerSoil (Qiagen, Hilden, Germany) [11,22,40,46,47], sometimes aiming at the epilithic microbiome, and sometimes also on the endolithic microbiomes [22].

3.4.2. Assessing Abundance and Diversity of Taxa by DNA Sequencing

In early studies, the total community of the biodeteriorated CHL was assessed after polymerase chain reaction (PCR) amplification from total DNA, and the sequence was obtained via cloning PCR products into *E. coli* (clone library preparation) and subsequential Sanger sequencing [18,22,29]. There was a wide variety of next-generation sequencing (NGS) methods used (second, third, and fourth generation), by which the outcome sequences were defined by the specificity of the used primer pairs in the PCR amplification reactions. The most often analyzed in the case of bacterial diversity assessment were amplicons of variable V3 and V4 regions of 16S rDNA, for fungi ITS 1 or ITS2 rDNA, and for algae the hypervariable regions V2-V4 of the 18S rRNA gene. In the second-generation sequencing, 454 pyrosequencing using GS FLX++ technology was used in a single article reviewed here [37], while the Illumina technology was far more utilized, especially the MiSeq platform (in 19 studies). In the third-generation sequencing, Illumina metagenomic shotgun sequencing (NextSeq500, Illumina Inc., San Diego, California, USA) was used in a single study [24], while the fourth-generation Nanopore (MinION Mk1C, Oxford Nanopore Technologies, Oxford, UK) was used in two studies, performed by Delegou et al. [44] and by Rabbachin et al. [15]. These studies revealed longer sequence reads and did not acquire prior PCR amplification; therefore, the outcome sequences were not biased by this intermediate step.

3.4.3. Following the Biodeterioration and Obtaining Insight into the Microbial Community Function

In the case of prior knowledge on the specific biodeteriogen and its genome, specific DNA probes were developed and real-time PCR was conducted, resulting in quantitative data on a selected species. By real-time PCR, *Ochroconis lascauxensis*, an invasive black fungus, was studied to score the extent of its colonization in Lascaux Cave [8].

Although the 16S rRNA gene does not provide direct evidence of a community's functional capabilities, there are tools that do allow such a prediction [6]. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt 1.1.4) can be used to predict the functional composition of a metagenome using the data of the marker gene and a database of reference genomes [58], while the Functional Annotation of Prokaryotic Taxa (FAPROTAX 1.2.10) can provide information on microbial functions [6].

As far as we know, sequencing of environmental RNA, which would reveal the actual functional genes being transcribed, has not been applied to biodeteriorated CHL yet.

3.4.4. The Use of Methodology Studying Biodeteriorated CHL Microbiomes

Our strategy of bibliographic search (see Section 2) detected 50 articles from 2004 to 2023 (Figure 9). Figure 9 corresponds to Table 1, with a slight modification, as follows: the entries are adjusted for cases where both CD and CI approaches were used and compared to the number of studies using one of these approaches only. A total of 17 articles were found using CI techniques. Starting in 2004, the CI methods appeared in all of the reported years, apart from the years 2008, 2009, 2013, 2017, 2019, and 2020, and increased over the last three years, as 11 out of 17 CI articles were published after the year 2021. However, the majority of studies, namely 21, were conducted with CD methods, which appeared rather constantly through the years, with the exception of the years 2004 and 2020, when there were no CD-based studies published. There were also 12 articles that employed both CI and CD approaches within a single study. The first one was published in 2017, and, in 2019, a peak of four articles used this combined approach. By 2020, the only article found in our bibliographic search belonged to this category. Over the past two years (2022 and 2023), six articles, three from each year, were identified. This trend may indicate that more researchers are recognizing the necessity of using both CI and CD approaches to gain comprehensive insights into the microbiota present in cultural heritage limestone.

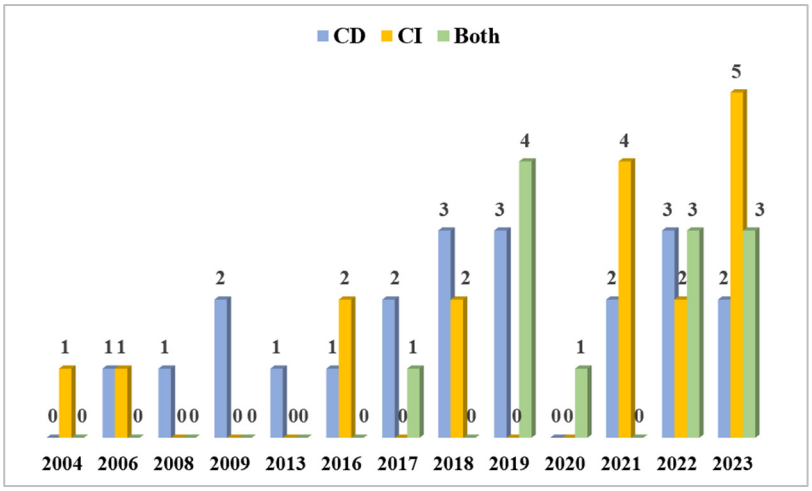

Figure 9. The number of molecular biology studies on CHL conducted using CD approaches, CI approaches, and both CD and CI approaches together through the years in ascending order.

4. Discussion

4.1. Microorganisms as the Agents of Biodeterioration

This work summarizes the literature results concerning microorganism biodiversity in a limestone substrate of cultural heritage significance and presents a crucial foundation for further explorations for further characterizing the diversity of the bacteria, cyanobacteria, fungi, algae, archaea, and lichens involved in the deterioration of limestone monuments or buildings. Several authors [26,59,60] consider lichens as one of the most severe stone biodeterioratiors and saxicolous crustose lichens as the most difficult organisms to remove from limestone. However, some authors [15,61–63] have also emphasized the bioprotective role that lichens possess when present on cultural heritage assets. Lichens can, for example, soothe the abrasive effect of airborne sand particles, reduce temperature fluctuations, especially where freeze–thaw cycles occur, and even limit the dissolution of stone material when in contact with water [15,61,62]. The different microbial communities coexisting in the biofilm have different metabolic functions (they can include phototrophs, chemolithotrophs, and chemoorganotrophs) and they cooperate to achieve substrate uti-

lization and the recycling of nutrients for their common survival [64]. On the surface of rocks, microorganisms always exist as a community and collaborate to perform various tasks. Therefore, to comprehend the impact of the microbial community on rock, studies should begin with a functional analysis of the entire population [6]. The alterations caused by microbial biodeterioration are, according to Mihajlovski et al. [18], classified into the following three different categories, namely: (i) biophysical, (ii) biochemical, and (iii) aesthetic changes, all of which can occur simultaneously or separately. Biophysical changes such as exfoliation and biopitting happen due to the penetration of fungal hyphae through the limestone surface [46], while biochemical alterations occur due to the production of organic acids, metabolites, exoenzymes, and metal-chelating compounds [65].

4.1.1. Biophysical and Biochemical Alterations

Stone substrates represent oligotrophic environments, since the nutrient availability is scarce, the ultraviolet exposure is high, and the moisture content is usually low [66], yet the formation of microbial communities occurs, starting with the occurrence of autotrophic microorganisms, such as cyanobacteria and algae, which can grow in the complete absence of organic matter [67]. The sulfur-oxidizing bacteria (SOB) and nitrifying bacteria involved in limestone weathering are autotrophic as well [57]. Fungi are also considered to be common limestone inhabitants, mainly due to the transportaition of their spores by air, atmospheric water, insects, and other biotic vectors [19] and because they possess the ability to excrete different types of acids, such as the following: citric, formic, fumaric, gluconic, lactic, malic, succinic, oxalic, and tartaric acids, which act as metal chelators or molecules involved in the dissolution of limestone [21,51]. Oxalic acid excreted from fungal hyphae has long been known for its ability to dissolve calcium carbonate and form secondary minerals such as calcium oxalates (whewellite and weddellite) on limestone cultural heritage assets, which causes the formation of a white patina on them [68,69]. Hyphae are not the only calcium oxalate that excrete fungal structure, as the crystalline can be excreted from fungal strands, cords and rhizomorphs, fruiting bodies, and lichen thalli as well [69]; however, they are the only fungal structures that are capable of enforcing mechanical pressure onto limestone by drilling into the substrate, due to the high mechanical resistance caused by the turgor pressure exerted by the protoplast on the inner side of the rigid fungal cell wall [70].

4.1.2. Aesthetic Changes Caused by Pigments

The most noticeable aesthetic changes are colored patinas, which can be of bacterial, cyanobacterial, fungal, algal, or even archaeal origin [4]. Black crusts are common for fungi-infested limestone, infested with namely *Aspergillus versicolor*, *Penicillium brevicompactum* [51], and the *Ochroconis* genera [8]. The black discoloration caused by fungi is often a consequence of strongly melanized cell walls. Melanin is a stress-protective pigment, which is beneficial for the survival of certain extremophilic fungi, among which belong the so-called "black fungi". Black fungi genera represent a heterogeneous taxonomic group, including orders within the classes Dothideomycetes and Eurotiomycetes [48]. Orange-colored pigments, carotenoids, serve the same purpose as melanin—the protection of the cell [48,71]—but they are mainly excreted by other inhabitants of CHL, such as the algal *Trentepohlia* genera [15] and some bacterial species, namely the following: *Staphylococcus aureus*, *Dietzia maris*, *Gordonia rubripertincta*, *Rhodococcus corynebacterioides* [4], *Rhodococcus cerastii*, and *Rhodococcus fascians* [7]. Pink discoloration, which was previously assigned to a non-biological origin only, was later found to be excreted by bacterial genera *Rubrobacter*, *Halobacillus* [4], *Micrococcus* [54], and *Arthrobacter* [4,7], and by taxons belonging to archaea, such as genera *Halobacterium*, *Halococcus*, and *Haloferax* [4]. Green patinas on CHL are a consequence of chlorophyll production by algae and cyanobacteria [1,6,39] or a consequence of different metabolite production by fungal genera such as *Aspergillus* and *Penicillium* [51]. White coloration can occur due to a calcium-oxalate-producing genera that include mainly *Streptomyces* [54], *Aspergillus*, *Penicillium*, and *Colletotrichum* [21], or due to the growth of white-looking lichen with a fungus from the *Leprocaulon* genera [6].

Interestingly Zhu et al. [6] found that some of the white patches in the West Lake Cultural Landscape were transitional.

4.2. Microbial Diversity Uncovered by the Application of Molecular Approaches

Metagenomics analyses typically provide diversity indexes such as Simpson's index, Shannon–Weiner Index, etc. They allow us to better circumscribe the diversity of species occurring in an environmental niche, but they fail to provide species level identifications [36,52,72]. Especially in bacteria, the above-species-level-identified taxa doubled in comparison to CD-based studies. Besides obtaining a greater depth into the microbial diversity, CD approaches allow species level identifications and results from culture-obtained metabolite profiles [26,36,73]. Pure cultures should best be obtained using the spread plate technique for obtaining the highest diversity. The isolation of the DNA from a pure culture can then be carried out, and later the isolated DNA can be amplified via PCR. A vast majority of articles using CI approaches used the Illumina MiSeq. One of the newer sequencing platforms, the so-called fourth-generation sequencing [28], is Nanopore sequencing, which was used only in two articles written by Delegou et al. and Rabbachin et al. [15,44]. It provides long reads and might, therefore, be the method of choice in future, as it also allows species-level identifications.

4.2.1. Discovered Microbial Biodiversity on CHL

As evidenced by this article, fungi and algae were mainly detected using CD methods, while archaea and bacteria were detected using CI methods. The identification of cyanobacteria was conducted with a relatively balanced employment of both kinds of techniques, while the identification of lichens was based on morphology and rarely employed molecular methods. Our results show that there were only two studies on lichen identification carried out using molecular approaches, one with CD [22] and one with CI methods [6]. Among the fungi in the cited articles (Table 1), which are also considered to play one of the major roles of limestone biodeterioration, *Cladosporium cladosporioides* is the most common fungal species found in biodeteriorated limestone, having been detected and identified using culture-dependent approaches. However, *C. cladosporioides* presents a complex of more than 200 species [62]. As this is obviously an important limestone colonizer, future research needs to focus on identifying the phylogenetic species involved. Algae were determined mainly by the use of CD methods. The most frequently found algae in the cited articles (Table 1) were *Acutodesmus bajacalifornicus* and *Pseudostichococcus monallantoides.* The bacterial taxa in the cited articles (Table 1) were identified mainly by using CI methods. *Arthrobacter agilis* was the most frequently identified species in limestone. Cyanobacterial biodiversity was identified using CD and CI methods (Figure 6A,B). As noticed by Ortega-Morales et al. [17] in the cyanobacterial community in cultural heritage stone biofilms in six different tropical and subtropical countries, statistically significant differences were calculated according to the type of stone (calciferous or siliceous), as well as according to the climatic regions and atmospheric pollution. This was the first meta-analytical study performed on stone biofilm data obtained using the same sampling, isolation, and identification techniques. Regarding the archaea that are usually considered difficult to grow on culture media, the only species able to grow on axenic culture was *Haloferax mediterannei*.

4.2.2. The Discovery of Novel Species on CHL

The exploration of microbial biodiversity on CHL resulted in the discovery of three new taxa, including one cyanobacteria and two fungi. During the identification of the phototrophic community of biodeteriorated cathedral walls, a hitherto unknown Nostoc/Komarekiella-like cyanobacteria was isolated, for which new genus and species, *Parakomarekiella sesnandensis* were proposed [53]. Similarly, the efforts of uncovering the fungal diversity on the walls of a Roman cryptoporticus gave rise to the discovery of a previously unknown strain related to *Bionectriaceae* (Hypocreales). Based on morphological and phy-

logenetic analyses, a novel genus and species, *Circumfusicillium cavernae* gen. et sp. nov. were proposed [56]. With a similar approach, a new genus, *Saxispiralis* gen. nov., and a new species, *Saxispiralis lemnorum* sp. nov., were discovered during the microbial study of a funerary art piece at the Lemos Pantheon [48].

5. Conclusions

There is a wide variety of methods that can be used to unravel microbial diversity with culture-dependent (CD) or/and culture-independent (CI) approaches. Out of the 50 articles that were included in our review, 12 articles contained both approaches, showing the ever-growing effort of discovering the overall microbiome of the biodeteriorated cultural heritage assets. The trend of utilizing only CD methods has persisted rather constantly through the years, while the usage of CI has gained its popularity in the last 4 years. The microbial biodiversity of CHL is mostly studied in bacteria and fungi, however, phototrophic cyanobacteria and algae are also studied well, and even previously poorly studied archaea are becoming a point of interest in some recent studies on CHL. The authors of this review article believe that it is crucial to keep exploring microbial biodiversity, preferably with a combined CD and CI approach, not only with the intention of uncovering the taxa and microbial communities responsible for the biodeterioration processes, but also with the curiosity that builds up the exiting knowledge of taxonomy, since the efforts of studying the microbial diversity on CHL have even resulted in the discovery and description of three new species in three novel genera, one cyanobacterial and two fungal, which would have otherwise remained unnoticed. The carrying out of studies on the topic of biodeterioration on CHL using molecular approaches is still rather limited to certain countries, even though limestone is widespread worldwide as a common building and artistic material. Therefore, we encourage conservators and biologists worldwide to keep exploring this field on their own cultural heritage assets; moreover, as for future directions, we encourage further studies on microbial biodeterioration to use CD and CI methods hand-in-hand and couple them with carefully monitored environmental and substrate characteristics. With the growing popularity of building predictive models that integrate biology datasets with data mining statistical algorithms to predict future outcomes of certain phenomena, we believe that predictive models could also be used in the field of microbial biodeterioration in the future to provide novel insights and answer questions such as how different combinations of microbial taxa commonly found on CHL alternate their substrate under different environmental factors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14167429/s1. Table S1: Fungi detected on limestone cultural heritage assets using molecular methods in the literature; Table S2: Bacteria detected on limestone cultural heritage assets using molecular methods in the literature; Table S3: Cyanobacteria detected on limestone cultural heritage assets using molecular methods; Table S4: Algae detected on limestone cultural heritage assets using molecular methods; Table S5: Archaea detected on limestone cultural heritage using molecular methods; Table S6: Culture media for microorganisms used in biodeteriorated culture heritage limestone studies.

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