Biological Risk for Hypogea: Shared Data from Etruscan Tombs in Italy and Ancient Tombs of the Baekje Dynasty in Republic of Korea

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Abstract: Biological growth represents one of the main threats for the conservation of subterranean cultural heritage. Knowledge of the conditions which favour the various taxonomic groups is important in delineating their control methods. Combining our experience regarding hypogea in Italy and the Republic of Korea, we aim to perform a critical review and comparison of the Biodeterioration Patterns (BPs) found, the materials used, and the conservative treatments applied. For this purpose, we focused on Etruscan tombs (Italy, 7th to 3th century BC) and the ancient tombs of the Baekje Dynasty (Republic of Korea, 6th to 7th centuries AD), most of which have been designated UNESCO World Heritage Sites, collecting original and bibliographic data as well as official documents. Results highlight the rich biodiversity of the bacterial and fungal species. Phototrophs were observed only in niches with sufficient light and the development of roots was also detected. Changes in humidity and temperature, the nature of the soil, nutrient accumulation, and vegetation above the hypogea along with human activities explain the different BPs. The effects of biocide treatments are also discussed, such as the emergence of dangerous fungal species. The shared data also enhance the role of overlaying tumuli and vegetation as well as protective barriers to reduce biological risk.

Keywords: cultural heritage conservation; eco-friendly biocides; ecology of biodeterioration; mural paintings; stone biodeterioration

1. Introduction

Subterranean sites such as caves and catacombs are generally characterised by limited air circulation, a limited variation in temperatures throughout the year, a high level of humidity, and at times, pronounced gradients of light [1]. Hypogea show similarities but also differences with caves due to their more limited size which leads to greater fragility also on the basis of the variability of materials used by human activities [2,3]. In fact, they were often embellished by wall paintings, stuccos, ceramics, bricks, and mosaics for decorative and augural purposes, as well as by statues, sarcophagi, and funerary objects which need to be preserved as testimony of their cultural heritage [4,5]. Moreover, the tombs which often maintained an environment cut off from the exterior world for hundreds of years, undergo substantial environment changes after excavations and consequent weathering [6,7].

Literature on biological colonisation in caves is becoming highly relevant, with hundreds of papers describing the microbial diversity and the relationship with environmental conditions, whereas much

less is available for tombs. The most common inhabitants of subterranean sites are oligotrophs (bacteria, represented mainly by *Actinobacteria*, and fungi), accustomed to long periods of starvation and, at times, phototrophs (cyanobacteria, green algae, diatoms, mosses, ferns) that have adapted to scarce light [4–6,8–13]. The current knowledge of the ecological succession of microbial communities in hypogea in relation to environmental factors, such as the efficiency or risks of different control methods seems insufficiently addressed. The peculiarities of hypogea, which vary according to different cultures also deserve special attention, as they give rise to distinct biodeterioration phenomena. There are relatively few studies on the biodeterioration of tombs. The first tombs were in Egypt [14,15] and Japan [16–19], then in Italy and other European countries, and more recently in Korea and China [6,7,13,20–22].

Environmental conditions of subterranean tombs give rise to physic-chemical weathering especially when water evaporation occurs which leads to efflorescence (carbonation, nitrification, sulfatation) or when it reaches condensation, causing a loss of pigment and lithic effects of materials [2,23,24]. They also greatly increase the biological risk since the levels of humidity, temperature, input of nutrients, light, and the nature of the substratum, have long been considered facilitating factors for microbial colonisation [16,18,25–28]. Knowledge of microbial biodiversity and their metabolic and ecological profile, such as the environmental conditions which favor the various taxonomic groups, are important in defining the risk for artefacts and in creating both indirect (preventive) and direct (biocide treatments) control methods [4,29]. Knowledge of the tolerance range of each limiting factor for the various species has a great practical relevance in stopping or reducing undesirable growth [4,30]. Such information is also relevant when biocide treatment is taken into consideration, due to the need to choose the most efficient one against specific microorganisms [31].

Within an international cooperation project between Italy and Korea, we selected Etruscan tombs (mainly in Tarquinia but also at other sites of ancient Etruria), along with the ancient tombs in Songsan-ri (Gongju) and in Neungsan-ri (Buyeo), respectively. They are of interest also because most of them constitute relevant UNESCO World Heritage Sites. The aim of this paper is to analyse and provide a critical review and comparison of recurrent biodeterioration phenomena observed in the Etruscan tombs in Italy and the ancient tombs in Republic of Korea, as well as the respective conservative treatments carried out at these sites, with a special focus on preventive intervention and biocides. Combining experience from countries with significant differences in bioclimatic conditions and historical traditions, we also discuss the ecological relationships and successions among the communities thus making the results more relevant on an international scale.

2. Materials and Methods

In order to assess recurrent biodeterioration phenomena observed in Etruscan tombs in Italy and the ancient tombs in the Republic of Korea, we carried out an extensive study among peer-reviewed literature and other bibliographic source in particular from ICCROM (International Centre for the Study of the Preservation and Restoration of Cultural Property) as well as from the Italian ICR named later ISCR (Istituto Superiore per la Conservazione e il Restauro) and from the Korean KNUCH (Korea National University of Cultural Heritage), CHA (Cultural Heritage Administration), NRICH (National Research Institute of Cultural Heritage), and KNU (Kongju National University, Research Institute for Basic Science).

Further information was gathered from our recent field studies carried out on selected tombs in both countries to assess the microflora associated to some deterioration phenomena [32–34]. In this context, culture methods were used to isolate microorganisms and sequencing of some DNA barcode markers for their identification. Biochemical screening test were also performed to assess the detrimental potential of isolates [32–34]. We also created a taxonomical database on the species/genera/families that were identified, which took into consideration both qualitative and quantitative data, if present, along with documentation on the surrounding ecological conditions. As this work covers over 40 years

of research, the identification of microorganisms has different level of taxonomical details being performed partly by morphological and partly by molecular approaches.

Such data were also examined to understand the ecological relationships and successions among the communities. Any data on conservative treatment (mainly biocides) were also considered and discussed.

2.1. Etruscan Tombs in Italy

The practice of building hypogea and decorating them with mural paintings was developed in several Etruscan cities especially in northern Latium, Tuscany, Umbria and Romagna covering a period from the 7th to the 3rd century BC. Since 2004, both the Necropolises of Cerveteri and Tarquinia (Latium) have become UNESCO World Heritage sites in consideration of their painted tombs which have an extraordinary importance as they reflect the daily life and burial practices of the Etruscans and bear witness to the achievements of their culture [35,36].

Our data set collected information on biodeterioration phenomena in 23 Etruscan tombs (Figure 1) present in three necropolises in Latium (in Tarquinia: Lotus, Jugglers, Mercareccia, Pulcella, Hunting and Fishing, Bulls, Shields, Ogre, Moretti, Blue Demons, Lions, Animas, Hunters, Elderly Men, Painted Vases, Giustiniani, Bartoccini, Lionesses, Caronti, in Cerveteri: Triclinium, Well, Reliefs) [25,26,28,32,37–43], and in Veio (Tomb of the Ducks) [44,45]. A further two tombs that were studied belong to a necropolis in Chiusi, Tuscany (Tombs of Hill and of the Monkey) [46–48].

Figure 1. Location of the Italian study cases and details of two tombs belonging to the UNESCO World Heritage sites of the Necropolis of Tarquinia (Monterozzi) (**a**) external view, (**b**) mural paintings, and of Cerveteri (Banditaccia): (**c**) external view, (**d**) mural paintings. (**b**,**d**: Courtesy of Amici delle Tombe di Tarquinia).

These tombs were dug in different geological substrata to obtain the sepulchral chambers, which lie at depths that vary from 2 m to 8 m. In Tarquinia, the geological features were calcarenites banks (Macco), which is an organogenic limestone rich in small shells. This bank constituted the top of the "Monterozzi" hill which was in contact with the underlying Pliocene clays that were more pliable and erodible. Both in Cerveteri and Veio, the characteristic tuffs, containing fragments of pumice arising from ignimbrite deposits, were preferred by sculptors and builders thanks to their softness and low permeability [49]. In the Chiusi necropolises, the tombs are situated in a lithological complex made from Pliocene sands (quartz, feldspars and a smaller amount of calcite) with clay cement [44].

From a bioclimatic point of view, the Cerveteri, Tarquinia and Veio sites fall within the Mediterranean macrobioclimate, with lower mesomediterranean thermotype and a lower subhumid ombrotype, whereas the Chiusi area has temperate characteristics [50]. In most of the Etruscan tombs of this study, the microclimate was found to be quite stable, with temperatures ranging from 10–15 °C

to 17–20 °C and relative humidity (RH) between 90% and 100%. Such values sometimes led to dew point temperature, with water condensation on surfaces, especially in the cooler tombs [23].

The necropolises were often characterised by the presence of a large number of tumuli (circular tombs covered by earth mound, varying in diameter and height) (Figure 1) and sometimes of different shapes such as "dadi" (cube tombs). In the various necropolises, the original tumuli were often dismantled and only sometimes rebuilt for the purpose of protection, without considering the original shape [36,51].

The materials of Etruscan mural paintings were made utilising several mineral pigments, such as hematite, Egyptian blue and charcoal black which were then deposited on a previously prepared thin clay layer on the surface [46,51].

Several weathering phenomena of these tombs have accentuated both the physic-chemical weathering as well as biological issues. Each tomb has a specific history and problem, and we will subsequently describe the recurrent phenomena as well as some peculiar ones.

2.2. The Ancient Tombs of Baekje Dynasty in Republic of Korea

Many of the tombs with wall paintings were built by the people of Goguryeo in the Korean Peninsula. Goguryeo tombs are found throughout North Korea and in the Jian region, China. Sixty-three of these tombs have been designated as UNESCO World Heritage Sites since 2004. There are approximately ten tombs of this type in Republic of Korea, constructed from the 5th to 15th centuries. They have been managed as historical sites for their cultural importance and currently, access to these tombs is restricted due to damage caused by exposure to the external environment and influx [52].

During the Three Kingdoms period (Goguryeo: 37 BC–668 AD; Baekje: 18 BC–660 AD; and Silla: 57 BC–668 AD), three tomb styles developed in the Baekje Kingdom: "(1) a stone mound tomb (tomb made by piling up the stone) in the Hansung period, (2) a brick chamber tomb (a tomb style made by laying bricks for the tomb's corridor and the main chamber and constructing a tumulus on top of them) in the Ungin period, (3) a corridor-style stone chamber tomb (a tomb style where the corridor and main chamber were made of stone then covered with soil on top) in the Ungin/Sabi period" [53,54]. In lieu of previous reports and investigations [55–62], we selected Ancient Tomb No. 6 in Songsan-ri, Gongju, a brick-chamber tomb, and Ancient Tomb No. 1 in Neungsan-ri (Buyeo), a corridor-style stone chamber tomb, in Baekje Dynasty, listed as UNESCO World Heritage Sites for this study (Figure 2).
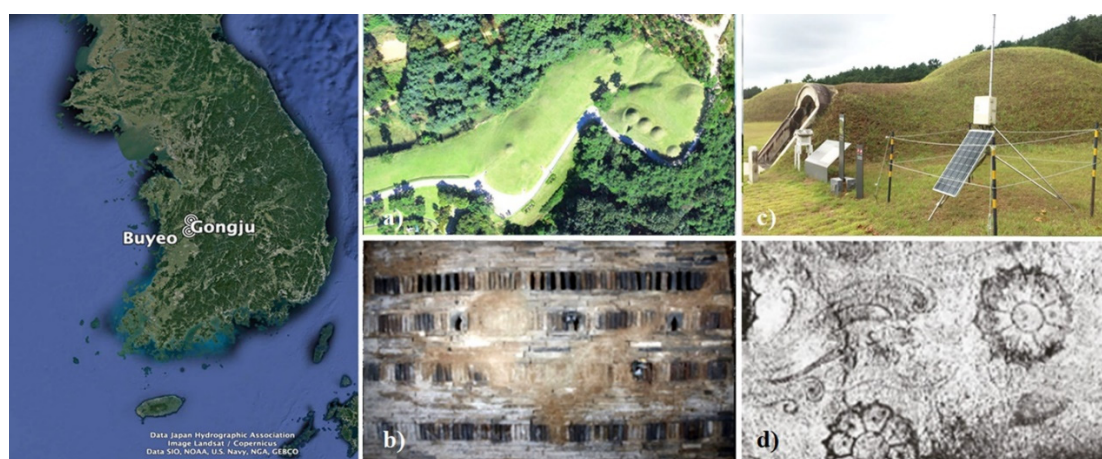

Figure 2. Location of South Korean study cases and details of the UNESCO World Heritage sites of the Ancient Tombs in Songsan-ri, Gongju (**a**) external view, (**b**) mural paintings [63], and the Ancient Tombs in Neungsan-ri, Buyeo: (**c**) external view [64], (**d**) mural paintings [64].

Tomb No. 6 was built in the mid-6th century as a royal tomb pertaining to the Baekje Dynasty. It has double corridors under an arch-shaped ceiling and a long rectangular brick chamber. It was discovered

in 1933 and opened to the public after its excavation. Four guardian deities (east: blue dragon; west: white tiger; south: red phoenix; and north: black tortoise) are painted on the four walls. In addition, the "sun and moon" were also painted on the southern wall. Plastering the white lime upon the black ground on surface of the bricks, the only white pigments were painted on top of the ground layer made with soil [63]. The mural paintings were executed utilising only white pigment which was obtained either from oyster shells ($CaCO_3$) or chalk ($CaCO_3$) [65]. Today, only traces of these mural paintings remain, hence it is difficult to discern the original figures [52,63,66].

Tomb No. 1 was constructed between the late 6th and 7th centuries. It is considered the King and the Royal Family's tomb and the outer shape consists of a circular tumulus. It is a corridor-style stone chamber tomb dug from the ground with the east, west, and north walls made of well-trimmed stone slabs [52,67–69]. Four types of rock were used for main chamber: "(1) augen gneiss: on the northern and eastern wall, (2) two-mica granite: on the western wall, (3) hornblende schist: on the floor, and (4) granodiorite: on the ceiling" [70,71]. Four guardian deities are painted on the four walls of the burial chamber inside the tomb, with lotuses and clouds engraved on the ceiling. It was excavated in 1916 and the layout of the murals was clear at that time [61]. After excavation, it was opened to the public, but the murals gradually faded leaving only a faint trace, hence it was closed in 1971 to preserve the wall paintings.

Republic of Korea is geographically located in the mid-latitude temperate climate zone with four distinctive seasons (spring, summer, autumn, and winter: the annual average temperature is 10–15 °C, the highest temperature ranges 23–26 °C, and the lowest temperature of minus 3–6 °C, with an annual relative humidity of 60–75% [72]. The indoor temperature of the two tombs differed from the outdoor temperature, as there was little heat transfer due to the tumuli [73,74]. The indoor temperature of Tomb No. 6 ranged from 13.6 to 20.6 °C, and the annual average temperature was 17.0 °C, with the lowest values in April, and the highest in September and October (environmental data from April 2018 to March 2019) [75]. The indoor temperature of Tomb No. 1 ranged from 12.9 to 18.0 °C and the annual average temperature was 15 °C, with the lowest values in May and the highest in November (environmental date from January to December 2019) [34]. The indoor relative humidity of the two ancient tombs was maintained at approximately 100% throughout the year. The shape of the tombs was reassembled when repair and restoration work took place [76–79]. Moreover, these two tombs were passively controlled without a specific environmental control system.

3. Results

Shared data on main BPs found in both countries, such as the phenomenology of biodeterioration and species found, arising from 23 Etruscan tombs in Italy and from two tombs in Republic of Korea are listed in Tables 1 and 2.

Table 1. Phototrophic and bacterial deteriogens associated to biodeterioration patterns found in Italian [25,26,28,32,37–48,80–82] and South Korean tombs [33,34,55–60,62,73,83–87].

			Biodeterioration Patterns						
			Green	White Patina	White Fluffy	Whitish-Gray	Gray	Rose/ Purple	Brown-Blackish
Phototrophs		Phototrophs	+++						
	Algae	Diatoms	+/+						
		Apatococcus sp.	+						
		Chlorella sp.	+						
		Chlorella vulgaris	+						
		Muriella terrestris	+						
		Stichococcus sp.	+						
		Pseudococcomyxa simplex	+						
		Trebouxia sp.	+						
	Cyanobacteria	*Chroococcus* sp.	+						
		Gloeocapsa sp.	+						
		Leptolyngbya sp.	+						
		Lyngbya sp.	++						
Actinobacteria		*Actinobacteria*		++/++	+	+		+	
		Arthrobacter sp.		+					
		Cellulosimicrobium sp.		+					
		Dermacoccus sp.		+					
		Isoptericola sp.		+					
		Microbacterium sp.		+					
		Micrococcus sp.		+					
		Mycobacterium sp.		+					
		Nocardia sp.		+					
		Pseudonocardia sp.		+					
		Rhodococcus sp.		++/++					
		Sinomonas sp.		+					
		Streptomyces sp.	+	++/+		++			

Table 1. *Cont.*

		Biodeterioration Patterns						
		Green	White Patina	White Fluffy	Whitish-Gray	Gray	Rose/Purple	Brown-Blackish
Other bacteria		Other Bacteria	++		++	++	+		
	Firmicutes	*Firmicutes*		++					
		Bacillus sp.		++/++					
		Bacillus cereus		+					
		Bacillus megaterium		+					
		Bacillus simplex		+					
		Paenibacillus sp.		++/++					
	Proteobacteria	*Proteobacteria*		+++					
		Alphaproteobacteria		+					
		Rhizobiales		++					
		Bradyrhizobium sp.		+/+					
		Hyphomicrobium sp.		++					
		Pedomicrobium sp.		+					
		Phyllobacterium sp.		+					
		Prosthemicrobium sp.		+					
		Ochrobactrum sp.		+/+					
		Betaproteobacteria							
		Crenobacter sp.		+					
		Cupriavidus sp.		+					
		Burkholderia sp.		+					
		Variovorax sp.		+					
		Gammaproteobacteria		+					
		Dyella sp.				+			
		Lysobacter sp.			+				
		Pseudomonas sp.				+	+		
		Serratia sp.		+					
		Stenotrophomonas sp.		+/+					
		Acidobacteria		+					

(+) Italian and (+) South Korean records. Frequencies are expressed as + = cited; ++ = recurrent; +++ = very common. Heterotrophic bacteria are grouped in four phyla (left). In *Proteobacteria* were highlighted also classes (bold underlined) and orders (bold).

Table 2. Fungal deteriogens associated to biodeterioration patterns found in Italian [25,26,28,32,37–48, 80–82] and South Korean tombs [33,34,55–60,62,73,83–87].

		Biodeterioration Patterns					
		Green	White Patina	White Fluffy	Whitish-Gray	Gray	Brown-Blackish
Mucoromycota	*Mortierella* sp.			++/+			
	Mortierella alpina				+		
	Mortierella ramanniana			+			
	Mucor sp.			+/+	+		
	Mucor racemosus			++	+		
	Rhizomucor sp.					+	
	Umbelopsis sp.				+		

Table 2. *Cont.*

| | | Biodeterioration Patterns | | | | | |
		Green	White Patina	White Fluffy	Whitish-Gray	Gray	Brown-Blackish
Ascomycota	*Sordariomycetes*	*Acremonium* sp.	+			+		
		Acremonium-like fungi						+
		Chaetomium sp.					+	
		Doratomyces sp.					++	
		Engyodontium sp.				+		
		Engyodontium album				+		
		Fusarium sp.			++	++		
		Fusarium oxysporum				+		
		Fusarium solani				+		
		Glyomastix cerealis					+++	
		Hypocrea sp.				+		
		Isaria farinosa			+			
		Lecanicillium sp.			+			
		Microascus sp.					+	
		Purpureocillium sp.				+		
		Scopulariopsis sp.		+		+		
		Scopulariopsis brevicaulis				+		
		Torrubiella sp.		+				
		Trichocladium asperum				+		
		Trichoderma sp.	+				+	+
		Verticillium sp.				+		
		Verticillium bulbillosum			+++		+	
	Eurotiomycetes	*Aspergillus* sp.				+		
		Aspergillus creber				+		
		Aspergillus flavus						+
		Aspergillus versicolor					+	
		Eurotium sp.		+				
		Eurotium herbariorum						+
		Exophiala sp.						+
		Exophiala angulospora						+
		Paecilomyces sp.			+	+	++	
		Penicillium sp.			+	+/+	++	+
		Penicillium brevicumpactum				+		
		Penicillium communis				+		
		Penicillium miczynszii				+		
		Penicillium rugulosum				+		
	Dothideomycetes	*Cladosporium* sp.					+	++
		Cladosporium cladosporioides					+	+
		Paraphaeosphaeria sp.						+
		Preussia terricola						+
		Epicoccum sp.						+
		Leotiomycetes						
		Geomyces pannorum		+				
		Saccharomycetes						
		Geotrichum sp.				+		

(+) Italian and (+) South Korean records. Frequencies are expressed as + = cited; ++ = recurrent; +++ = very common. Fungi are organised in two divisions (-mycota) and *Ascomycota* in five classes (-mycetes).

3.1. The Etruscan Tombs

3.1.1. Recurrent Biodeterioration Phenomena

Research which commenced in the 1980s indicate that the Etruscan tombs are mainly populated by *Actinobacteria* (belonging mainly to the order Actinomycetales) which give rise to biodeterioration phenomena of the painted layers. Firmicutes, in particular Bacillaceae, and Alphaproteobacteria (order Rhizobiales) also seem to play an important role.

Modern biomolecular tools utilised for the Tarquinia and Chiusi tombs have permitted a more precise identification of these microbiomes, even if some species do not seem to play a part in the biodeterioration processes. In the case of the Chiusi tombs, microbial colonisation was observed on both painted and unpainted wall surfaces, and a total of 456 non-chimeric bacterial sequences were obtained from different areas and pigments, mostly belonging to the Alphaproteobacteria (order Rhizobiales) and Firmicutes groups, such as *Rhodococcus* sp., *Streptomyces* spp., *Bacillus* spp., *Paenibacillus* sp., and also of *Nocardia* and *Pseudonocardia* genera [46–48]. In the Mercareccia tombs (Tarquinia), 142 different heterotrophic colony morphotypes were identified, 16% of which had not been described in relation to artistic heritage up to now. The most representative bacteria belonged to Actinomycetales and Bacillales, and the most common genera were *Bacillus* (24 strains) and *Streptomyces* (18 strains), together with *Rhodoccoccus* (seven strains), *Paenibacillus* (two strains) and *Pseudomonas* (one strain) [39]. In one Tarquinia tomb (Shields) [42], were also detected moonmilk deposits, which consist of very fine crystals of calcium carbonate, made up of calcite needle fibres (1–2 µm) and nanofibres (less than 1 µm). This phenomenology arises from the progressive accumulation of calcite which also traps microorganisms in the crystal matrix, until they are completely encompassed in the crystals [88,89]. The patina is similar to talcum or chalk powder when dry, and the white layers having different consistencies. One is softer while the other is thick and hard, thus more difficult to remove. The microbial community was made up of Proteobacteria, representing approximately 50% of the population, followed by the phyla *Acidobacteria* and Actinobacteria [42]. More precisely, the class Alphaproteobacteria predominated the community (25% of the population), followed by Betaproteobacteria, Gammaproteobacteria and Actinobacteria. The most representative genera were *Pseudonocardia* and *Hyphomicrobium*. In this case, the high affinity in composition highlighted how representative they were of microbiota contained in the tomb [42].

A comparison of the taxa using biomolecular techniques often shows that microbial communities can vary greatly, in fact, the two tombs of Chiusi have very few in common [47,48].

The presence of fungi on the mural paintings displayed a variable, though not negligible, aspect. In the Chiusi tombs, amplification of archaea and fungi was unsuccessful from all samples taken from the whitish alterations [47,48]. On the contrary, in the Tarquinia, Cerveteri and Veio tombs, a certain amount of fungi was recorded. For example, in the Mercareccia Tomb (Monterozzi Necropolis), fungi occurred in both the dromos and inner chamber. The taxa identified were Sordariomycetes, Eurotiomycetes, and Dothideomycetes, along with several species of *Penicillium* (*P. rugulosum*, *P. communis*, *P. brevicompactum*) and *Aspergillus* (*A. candidum*, *A. versicolor*, *A. flavus*). Other species that were detected included *Cladosporium cladosporioides*, *Preussia terricola*, *Trichocladium asperum*, *Scopulariopsis brevicaulis*, *Engyodontium album*, *Eurotium herbariorum*, and *Isaria farinosa* [39]. Our present study on the Moretti and Blue Demons Tombs in Tarquinia highlighted melanised fungi as responsible for the dark pattern deterioration found in both tombs (Figure 3). *Acremonium*-like fungi (three strains), *Cladosporium* sp. (one strain), and *Exophiala angulospora* (two strains) were identified from the Moretti Tomb, while *Cladosporium* sp. (two strains) and *Exophiala* sp. (one strain) were isolated the Blue Demons Tomb [32]. Tracks of rhizomorphs arising from Basidiomycetes, which utilized the remains of dead wooden roots, were also conjectured for some Tarquinia Tombs [80].
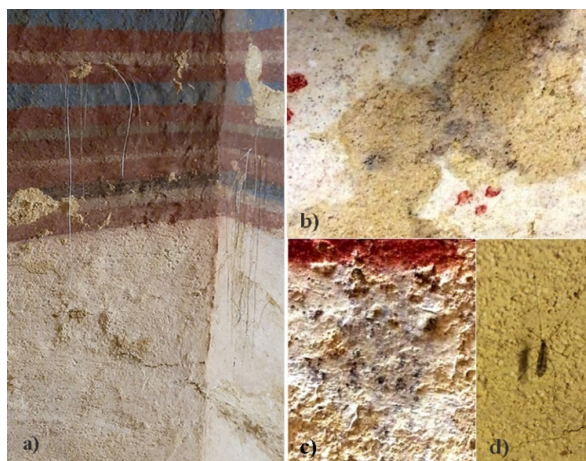
Figure 3. Biodegradation patterns in the Italian tombs from the Monterozzi Necropolis in Tarquinia. (**a**) Roots—Lotus flower Tomb; (**b**,**c**) fungal dark discoloration—Moretti Tomb; (**d**) *Limonia* cf. *nubeculosa*—Blue Demons Tomb.

Phototrophic microbial groups, such as algae and cyanobacteria are very rarely reported in Etruscan tombs. Few data available on the Tarquinia tombs (Leonesse, Bartoccini) indicate that they occur inside the tombs where a certain amount of sunlight is present, such as around the lighting systems or close to the entrance [80]. The occurring species were cyanobacteria (*Leptolynbya*), green algae (*Muriella terrestris)*, and to a lesser extent *Pseudococcomyxa simplex* and *Chlorella vulgaris*, and Diatoms [80].

As observed in several tombs in Cerveteri and Tarquinia [41,51], the roots of wooden and herbaceous plants were also common deteriorative agents in many Etruscan sites. In the case of herbaceous roots in hypogea (Figure 3a), a precise identification of the occurring species has not yet been carried out [80].

Moreover, insects, slugs, and miriapods (*Scutigera coleoptrata*) were detected in such sites, though their presence did not merit particular attention, despite their potential role in spore dissemination and in utilising and releasing organic materials on surfaces [81]. The occurrence of termites was also detected in the Giustiniani Tomb (Tarquinia), where typical tunnels of *Reticulitermes lucifugus* colonisation were observed [81].

3.1.2. Conservation Treatments

Variations in temperature and humidity inside the burial chambers of Etruscan tombs over lengthy periods of time have created significant deterioration [2,23,82]. Indirect control methods were also carried out, stabilising the microclimatic conditions after a partial reconstruction of overlaying tumuli and the creation of a system of double doors to close off the burial chambers, hence preventing entrance to visitors [51]. Glass barrier protection systems were put in place to prevent condensation at the tomb entrance and to allow for good visibility of the interior [23]. A cold light illumination system that does not modify the internal temperature of the chamber was also installed to prevent microclimatic changes and growth of photoautotrophic organisms [51]. In the case of Tarquinia, tests on the possible use of new vegetation cover to reduce the effects of summer heat are still underway [51].

To keep biodeterioration phenomena in check, the most common biocide compounds utilised were quaternary ammonium salts. The product that was initially used was Desogen, and then Neodesogen (both based on benzalkonium chloride), and more recently Preventol R80 (Bayer) Preventol® RI 80 (Lanxess) (dodecyl dimethyl dichlorobenzyl ammonium chloride) [25,47,81]. However, the disinfection of tombs with these biocidal compounds occasionally gave rise to a certain extent of interference with materials and to unexpected problems. In fact, after treatment with Desogen (10%) in the Cerveteri tombs (in particular, a painted relief in the Banditaccia Necropolis), a huge growth of a white fluffy mycelium

was observed due to the development of *Mortierella alpina*, *Mucor racemosus*, *Verticillium bulbillosum*, *Mortierella ramamniana*, *Penicillium* sp., and *Gliomastix cerealis.* This last mentioned was darker than others and the most difficult to eradicate [25]. Instead, species belonging to *Acremonium, Geotrichum* and the *Penicillium* genera were found in the Tomb of the Triclinium at the same necropolis, as a recolonisation effect following this kind of treatment [27]. Similarly, in the Tomb of the Ducks (Veio), the newly formed white mycelium was associated to *Penicillium miczynskii*, *Fusarium* sp., and *Paecilomyces* sp. [27]. Such a phenomenon was interpreted as the consequence of treatment in limiting the competition of these fungi with more sensitive bacteria. After several tests, only treatment with orto-phenylphenol was effective in blocking their growth [25]. In tests sites, the use of Metatin 58/I0 (Acima Chemicals) a broad-spectrum biocide, resulted as the most effective method of eliminating all microorganism species definitively [27]. The authors suggested to improve tests and not removing the residuals of biocides as usual methods during restoration [27].

In fact, in several further ISCR reports [80,81], treatment with benzalkonium chlorides compounds was suggested since the secondary effect of provoking further colonisation were not observed in several cases. Restorers also consider such treatment as a common practice in case of microbiological attacks, using them at doses of 2% [51]. Some recent studies also looked into identifying microorganisms capable of surviving biocide treatment with ammonium quaternary salts (Preventol RI80) by producing biofilms and/or spores [40]. An analysis carried out one month from the biocide treatment in various places of the tomb showed that it was effective on most bacteria, but not so on species capable of producing resistant forms of life like spore-forming bacteria, such as *Bacillus* sp. and moulds which were still alive [40].

Furthermore, microwave heating treatment was also applied as a new methodology to control biodeterioration phenomena [46]. The prototype system used a 2.45 GHz microwave generator with an adjustable output power of up to 1 kW and an optimal application dose of 65 °C for 3 min. The authors indicate that they checked the possible interference of microwaves on substratum and pigments with a micro photogrammetric system. The efficiency of the treatment, which was evaluated with the Plant Cell Viability Kit on the collected biological samples and on a white spotted area, gave some positive results [46].

In the case of root development, biocide treatments directly on the occurring roots were also carried out in the past, using glyphosate (Rodeo Gold) and hexazinone (Velpar L, Dupont) as herbicides [80].

3.2. The Ancient Tombs of the Baekje Dynasty

3.2.1. Recurrent Biodeterioration Phenomena

Considerable biodeterioration phenomena took place in both of the analysed tombs with changes occurring after the opening and with further intervention procedures.

In the conservation history of Tomb No. 6, heavy rainfall in Gongju in 1995 led to leakage, which caused blue-green algae, such as *Lyngbya* spp. and *Gloeocapsa* spp. which spread throughout the tomb [83,84]. Since blue-green algae causes colouration and erosion of the structure, they were removed and subsequently no secondary damage caused by the algae was confirmed by a detailed ecosystem study after its removal [90]. In 1997, the Ancient Tombs in Songsan-ri, Gongju were permanently closed to preserve the original state of the mural paintings. Research on microbial distribution of Tomb No. 6 was first conducted in 2010 and twelve species of fungi were identified (e.g., *Acremonium* spp., *Aspergillus* spp., *Epicoccum* spp., *Cladosporium* spp., *Penicillium* spp., *Fusarium* spp., were found in the main chamber [55]. Bacteria belonged to Actinobacteria (three strains), Firmicutes (seven strains) and Proteobacteria (two strains). *Bacillus* sp. resulted as the dominant strain and *B. simplex* was also discovered on the wall surface. After some negative results were produced with microclimatic conditioning, studies were conducted to monitor the microbial distribution of the ancient tombs. Before entering the main chamber of Tomb No. 6, where the wall paintings are present, a person (i.e., visitor, operator) must pass through three buffer zones. The microorganism distribution tended

to decrease upon entering the main chamber, due to the buffer activity of such areas caused by the inflow of outdoor air. *Aspergillus*, *Cladosporium*, *Penicillium*, *Bacillus*, and *Pseudomonas* genera were isolated in the air and *Aspergillus* was also identified on the bricks and walls. Soil microorganisms of the genus *Bacillus*, which seem to have flowed inside the tomb from the soil environment, were the dominant bacteria [56]. In a further study, strains collected in the air (*Doratomyces*, *Engyodontium*, *Fusarium*, *Aspergillus* genera) were identified in all four cardinal points of the walls [85].

Since 2008, numerous in-depth studies have been conducted on Tomb No. 1 which was closed in 1971 to preserve the wall paintings, along with an analysis of the biological distribution focusing on discoloured areas on the walls of the main chamber as well as the soil. As a result, 16 bacteria species were identified, including *Actinomycetes* and a total of 15 fungal species (four species of *Penicillium*, three species of *Aspergillus*, one species of *Fusarium*, one species of *Trichoderma*, and six unidentified species) were found. The green specimens collected at the entrance of Tomb No. 1 were microalgae (*Apatococcus*, *Chlorella*, *Trebouxia*, and *Stichococcus*), and cyanobacteria (*Chroococcus*) [86]. Results of microbial distribution monitoring performed from 2016 to 2018 highlighted a decrease in microbial numbers upon entering the ancient tomb (entrance > outside > antechamber > corridor > main chamber). The highest airborne microbial distribution was found to be at the entrance as insects inhabiting the space and the air inflow while entering the tomb stationed there for a long time. *Cladosporium*, *Penicillium*, and *Bacillus* were dominant airborne microorganisms and *Bacillus*, *Cupriavidus*, and *Streptomyces* were identified on all the walls of the main chamber. *B. cereus*, *B. megaterium*, *Dermacoccus nishinomiyaensis*, *Mucor* sp., *Rhizomucor* sp., and *Penicillium* sp., identified on the walls were equally represented in the air. Accordingly, it was concluded that microorganisms in the air can harm the wall paintings when they settle and grow on the walls [59,60].

Patinas presumed to be microorganisms were observed on the walls of the main chamber in Tomb No. 1 (Figure 4). They were distributed in the middle and bottom sections of the eastern and northern walls and, based on colour, were classified either white or ivory, with white patinas first being mentioned in 2008. At the time, they were thought to be Actinomycetes [86]. Later, studies in 2016 and 2018 confirmed that the patinas continued to exist in the same location. They were presumed to be Actinomycetes based on SEM-EDS analysis, but accurate determination was difficult [60]. Metagenomic sequencing analysis was attempted to accurately identify these patinas, and further studies are currently underway.
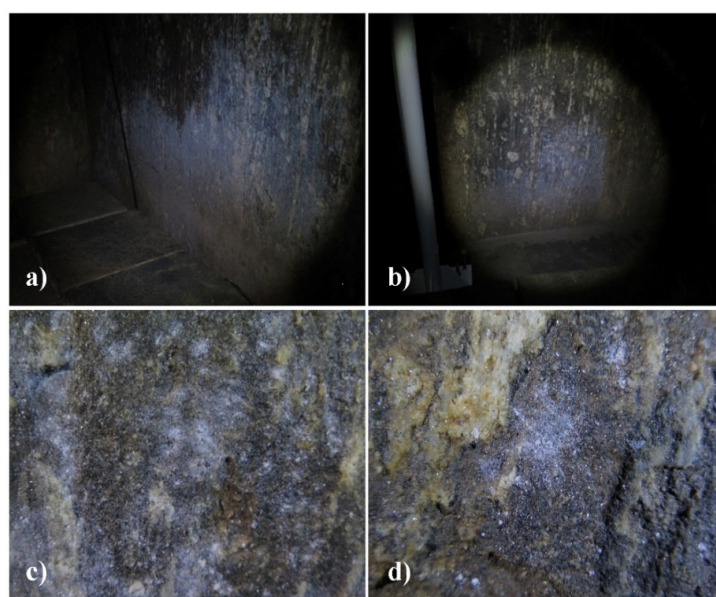

Figure 4. Biodeterioration patterns (whitish-grey patinas) on the wall in the main chamber of the Ancient Tombs in Neungsan-ri, Buyeo; (**a**,**b**) general view of the walls, (**c**,**d**) close view of biological patinas.

As regards to roots, photographs and reports showed that only Tomb No. 6 had such a problem. In the early phases of excavation, tree roots were found growing between gaps in the brick work [91]. After maintenance procedures were carried out, no further events have been recorded. Despite the wide distribution of pitch pine (*Pinus rigida* Mill.) around the tumuli, no microbial activity nor root intrusion has been reported for Tomb No. 1 [92].

3.2.2. Conservation Treatments

In Republic of Korea, indirect methods have been used to control the environment to prevent problems caused by microorganisms in ancient tombs. Since 2003, the indoor temperature and humidity in Tomb No. 6 has been maintained with an air conditioning system at 18 °C and 90% humidity on average. However, air flow from the system dried the air too much creating problems for the wall paintings hence the system was shut down in 2011. An analysis of the microclimatic conditions was carried out and buffer zones were set up to assist prolonged conservation of the hypogea. Research was also conducted on the biochemical characteristics and environment influences (temperature, moisture, and nutrients) of microorganisms over the seasons [58]. On the basis of previous investigations, internal microclimatic conditions, and thermal preferences of isolates, three risk periods for microorganism occurrence in ancient tombs were classified [56] as follows: (1) Safe: $T < 18 \,^\circ C$, (2) Warning: $T = 18–22 \,^\circ C$, (3) Risk: $T > 22 \,^\circ C$ [6]. In addition, the risk periods for microorganism occurrence in the two tombs were respectively: "safe period for microorganism occurrence" from January to July for Tomb No. 6 and from January to October for Tomb No. 1 while the "warning period for microorganism occurrence" was from August to December for Tomb No. 6 and from November to December for Tomb No. 1. Such differences in the risk periods for microorganism occurrence in the two ancient tombs is due to the different structures and environments.

Further research was also conducted to select biocides to remove the blue-green algae (*Gloeocapsa* spp. and *Lyngbia* spp. [83,84] that occurred in Tomb No. 6 and the surroundings tombs. Reagent K101 was selected (composition: EDTA, $(NH_4)_2CO_2$, Na_2CO_3, sodium hypochlorite) which was developed by differentiating the composition of AC322 [93] and B57 solutions [83]. After spraying the biocides to the walls, poultices were applied, and ultraviolet rays were subsequently irradiated for 24 h [83,85]. The treatment did not give rise to recolonisation phenomena and secondary damage [90]. A thermo-hygrostat was installed in 2003 to maintain stable environmental conditions but unfortunately it caused the drying of murals and walls, so that its use has been stopped in the Tomb No. 6 since 2011. Then, a treatment was conducted to prevent possible damage being fungal growth started on the facing when the thermo-hygrostat was stopped. Benzalkonium chloride ($C_{22}H_{40}CN$) 1% was used to remove the fungi, and the rayon paper that was applied was later removed using tepid distilled water [94]. After that, there have been no cases in which biocides have been used directly inside ancient tombs and on the wall paintings.

Since heterotrophic microorganisms and algae may grow if there are suitable nutritional and environmental conditions, the development and application of efficient eco-friendly biocides that do not damage the murals is necessary [87]. Several studies have been conducted to select and field test substances from medicinal plants and tree extracts capable of managing microorganisms that occur on such sites [95–101]. Research has also been conducted on ingredients and extracts of herbal medicine to remove biofilms from external stone cultural heritage elements [102]. Such studies have led to selecting a biocide (Stone Keeper) based on eugenol with an eco-friendly emulsifier [103]. This biocide was applied to fungi taken from ancient tombs on the basis of a study showing that the natural substances anethole and eugenol have antibacterial and antifungal properties [87]. The mixture of anethole and eugenol (1:2) significantly inhibited the growth of *Aspergillus creber* and *Aspergillus versicolor*. A simulation also tested the effects of natural ingredients, thereby verifying their potential to inhibit and kill microorganisms that occurred on ancient tomb murals [85,87].

4. Discussion

4.1. Biodeteriogenic Taxa, Phenomenology of Alteration and Biodeteriogenic Processes

As shown in Tables 1 and 2, in both Italian and Korean sites, a great number of bacterial and fungal species were found in the analysed tombs. The influence of different bioclimatic conditions, such as of the use of different traditional materials (stone, mortar, brick and pigment) explain the significant number of taxa involved. In Italian sites, hundreds of bacterial species (mainly Actinobacteria, but also Firmicutes (Bacillaceae family) and Alphaproteobacteria) and fungi (principally Sordariomycetes and Eurotiomycetes) were detected by molecular methods [39,40,42,46,48]. In Korean sites, *Actinomycetes* and *Bacillus* sp., which seem to have entered the tomb via the soil were the dominant bacteria [56]. Fungal species of different genera (*Aspergillus*, *Cladosporium*, *Doratomyces*, *Engyodontium*, *Fusarium*, *Geomyces*) were also widespread in the air and on the walls of the various tombs [85]. *Geomyces pannorum* and *Fusarium solani* were also identified, and the latter has also been described on the Lascaux cave paintings in France as well as in the Kitora Tomb and Takamatsuzuka Tomb in Japan [19,104]. The presence of some fungal species (e.g., *A. niger and A. flavus*) can also be dangerous to humans due to their production of mycotoxin and allergens [104]. A peculiar situation occurred in the case of burial remains inside the tombs, which determined a specific selection of microbial colonisation [17].

New studies on old Chinese tombs confirmed the important role of bacterial and fungal colonisation in deterioration processes. In particular, bacteria of the genera *Bacillus*, *Massilia* and *Brevibacillus* were identified in the bricks and were considered possible contributors to brick weathering [20]. Such sites contain previously unknown species; new bacterial species of (*Paenibacillus tumbae* sp. nov.) were isolated from the Tomb of the Emperor Yang of the Sui Dynasty [20]. Fungi were also found in great number, the most common genera being *Cordyceps, Fusarium, Harpochytrium, Emericellopsis, Volutella, Cladosporium, Stachybotrys, Trichoderma, Cochlonema* [105,106]. Further studies of interesting and unknown fungal species are underway in Etruscan tombs [32].

In both Italian and Korean sites, algae and cyanobacteria were observed only in conditions of high levels of humidity and sufficient lighting. Similar genera were identified, principally being *Apatococcus*, *Chlorella*, *Trebouxia*, *Stichococcus*, *Pseudococcomyxa*, and *Muriella* for green algae, and *Chroococcus* and *Leptolynbya* for cyanobacteria. It is possible that vicariant species most likely occur in both countries though further in-depth taxonomical studies are needed. The development of roots was sometimes a frequent occurrence as study results illustrated at the Italian and Korean sites, even if the importance of such phenomenon seems to vary between the two countries, probably due to the different management procedures of the areas. The maintenance of herbaceous cover and regular cutting can reduce the risk of damage [43], even if data from Tarquinia reported depth penetration from non-wooden plants. Recent studies have addressed root identification utilising molecular tools. Insects, Arthropoda, and slugs were also detected, in both countries, even if they have not been sufficiently investigated.

The role that some identified species may have needs to be carefully evaluated via biomolecular analysis, since many species may simply be part of the soil ecosystem, without being responsible for causing damage. On the contrary, other bacterial and fungal species can be more problematic, since their potential growth produces staining and chemical and physical interference with the materials. As observed for the Mogao Grottoes (Dunhuang, China), biodeterioration of wall paintings suggests that only a small fraction of the rich microbial community may in effect grow and cause damage, whereas most of these microorganisms are dormant or metabolically slow [105]. Hence it is of the utmost importance to study the metabolic traits of isolates to associate them to a risk. For example, moonmilk which is present in Etruscan tombs, as in karst caverns or in other hypogeal environments [107] does not seem to greatly detrimental, and *Streptomyces* species may actually exert a protective effect, since restorers have reported that under the patina the wall paintings were well preserved [42].

The most evident effect of microbial colonisation are the different chromatic alterations on the surfaces, often producing whitish or grayish patinas and spots which resemble salt efflorescence or carbonate deposits [26,38,80]. As shown in Tables 1 and 2, the same taxa can at times be associated with

quite different biodeterioration patterns, probably due to the complexity of the microbial communities and subterranean trophic networks as recorded for example in *Acremonium* sp. found as a minority component of a green phototrophic patina as well as in a whitish-gray patina (Table 2). Moreover, as observed in other subterranean environments, apparently similar alterations which were often attributed to different species of *Streptomyces*, which arise from the growth of different kinds of bacteria [12]. At times, purple pigmented patinas also due to Actinobacteria colonisation have been detected in the tombs of Tarquinia as well as in other Roman tombs [26,108]. The phenomena of dark spots caused by various fungal colonisations have also been documented [19,32,104,105]. Microbial patinas may not be easily observable at times, probably due to their growth in underground layers, though their potential aggressiveness should not be neglected.

The physico-chemical interaction of the identified species is often underestimated, despite the fact that some species can give rise to biomineralisation phenomena as well as acid metabolite production, such as chelating compounds which can extract ions from the substrata and produce carbonate dissolution, penetrating inside crystal matrices [4,13,32].

Several authors also stress the role of microorganisms in biodeterioration processes, since different actinobacteria are involved in the bio-precipitation of minerals. In the Mercareccia Tomb, a relevant number (71) of bacterial strains capable of precipitating carbonates have been detected [39]. Mechanical interaction, such as the capacity of several fungi to dissolve minerals and mobilise metals, is well known. When colonising rock substrata, fungal hyphae can induce a chemical deterioration secreting siderophore-like compounds, whereas the greatest damage is due to mechanic activity. In fact, hyphae of some black fungi demonstrated their ability to penetrate silicate and carbonate rocks exerting strong mechanical pressure up to 12.39 bar gouging cavities at depths ranging from few hundreds of microns to several millimeters and producing pulverisation of the substrate through chemical processes [109,110]. Recent studies showed that dark spots occurring in the Tomb of Tutankhamun were caused by *Penicillium chrysogenum* colonisation, which would suggest their detrimental nature, due to the ability to produce malic acid [111]. On the light of some interesting metabolic features showed by soil microorganisms, the use of subterranean strains for biotechnological applications could be a possibility, but further analyses are required.

In Etruscan tombs, a fair amount of mechanical damage was caused by root penetration and its development, even up to several meters in depth, which has led to the disintegration of the plaster and painted layers. In some cases, roots systems of different sizes and aggressiveness were highly developed. In the case of herbaceous species, distances of more than 3 m in depth were observed, also for small-sized herbaceous species. Such data were also confirmed in other studies carried out in several hypogeal archaeological sites in Rome, such as the *Domus Aurea* [112], where the root system of an old specimen of *Pinus roxbourgii* was found to be 15 m in depth and 25 m wide. Another example can be found at the Jewish catacombs of Villa Torlonia, where *Ficus carica* was able to grow underground for more than 50 m [113]. In any case, roots should be considered detrimental since they favour water penetration and have been shown to play a conditioning role on the microbial community [47]. In the South Korean ancient tombs, conservation activities included the removal of previously overlying trees. Then, surrounding landscape has been maintained, and burial mounds have been restored after excavation and maintenance activities. Such new situation resulted safe for avoiding root damages [67].

Finally, with respect to insects and slugs, several observations indicate their negative impact due to the mechanical damage they cause, creating micro tunnels under the painted surface and staining light-coloured surfaces [51,80], or supporting the presence and growth of entomophilous/entomopathogenic fungi, such as for example *Isaria farinosa* and *Engyodontium album* (Table 2). These issues have yet to be sufficiently analysed.

4.2. Environmental and Biotic Influences

In both countries, microorganisms that had entered the ancient tombs and microorganisms that originally inhabited the tombs were able to grow in particular environmental conditions. The microbial

composition varies in relation to environmental changes, which in turn increase the risk of a biological attack as observed from the very first studies conducted [28]. The microbial colonisation of the hypogeal surfaces is the result of selective, competitive and inhibitory dynamics among different microorganisms from rocks and from the air under diverse and changing environmental conditions. It is also the result of human activity and intervention which form part of the "history of the site". Furthermore, every process of colonisation is different from another, since each community is conditioned by the pioneer species which will in turn influence further processes [12]. The study conducted at Mogao Grottoes also showed some interesting results in that the diversity index of the fungal community was positively correlated with the building period of the caves, which had a greater impact than temperature and relative humidity of the caves [105]. Without disregarding the role of environmental factors, it seems possible that the opening time of the tombs to outdoor exchanges and human presence has a significant influence on its biodiversity.

In fact, several studies which show the great impact that environmental and edaphic factors have in facilitating different taxa are discussed and summarised in Figure 5.
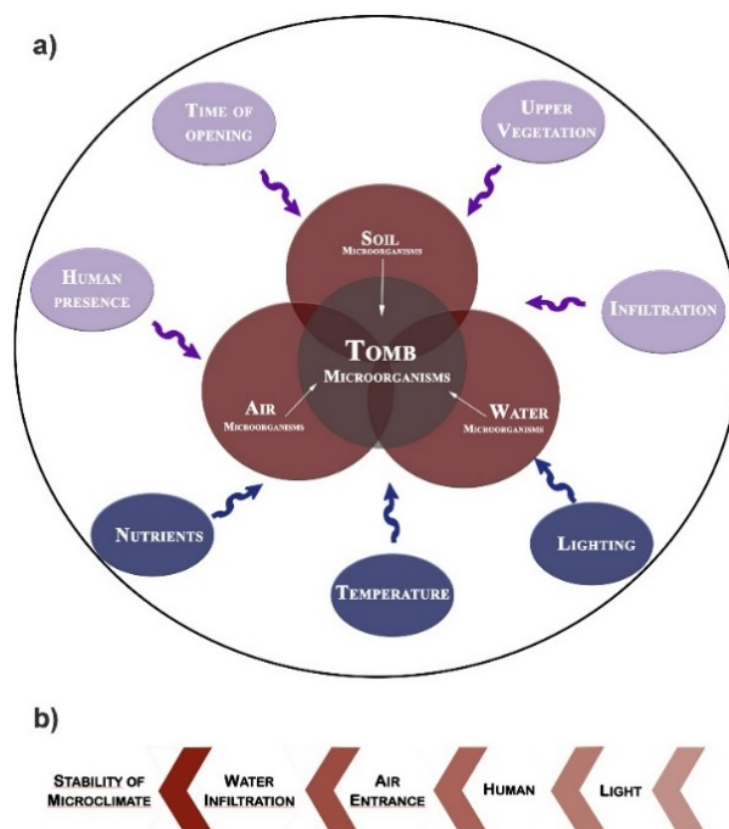
Figure 5. Microorganisms occurring in hypogeal tombs and factors influencing their qualitative and quantitative composition (**a**); main factors affecting the microclimate stability, this last is the main goal to be achieved (**b**). (Original elaboration by the authors).

Water originating from percolation, infiltration and capillarity, and at times from human presence along with flooding was found to be the most important factor. The level of humidity greatly affects the various microbial communities as in any archaeological context [30,114]. Such an influence was also recently confirmed by studies conducted in Chinese tombs (Emperor Yang, 1600 years old), where humidity showed the highest degree of microbiological variance (19.2%) than all other environmental factors, followed by illumination (18.3%) and height (12.8%) [106]. In general, fungi were found to be more resistant to dryness, with respect to bacteria.

Lighting conditions were also found to be a very important factor, especially for the Korean sites because, in such humid conditions, the development of phototrophic organisms arises if the amount of radiation exceeds <2 μmoll photons m^{-2} s^{-1}, which is approximately three orders of magnitude less than full sunlight [1]. In the areas adjacent to the opening or near artificial sources of light, photoautotrophs grow as they are also aided by the high concentration of CO_2, (sometimes ten times higher than those in the atmosphere) [8]. Data from other hypogea, such as catacombs, show that the spectral composition of the light selects different taxonomic groups. Due to their different photosynthetic pattern, green algae and diatoms are favoured by emissions peaking in the blue and red parts of the spectrum, while cyanobacteria are favoured by the emissions in the green and orange-red ones [115].

Furthermore, temperature also greatly influenced the biological risk and for this reason, a risk assessment of microbial colonisation in the Korean sites suggested establishing three ranges of temperature and the risk conditions that occur when temperatures are above 22 °C [6]. In the Italian sites, we observed a higher risk at the end of summer, when the thermal heating reaches below ground, but no precise evaluations have been made. Inside a hypogean, the various walls can be at different risks of biological colonisation. When analysing Korean Tomb No. 1, the interval between the highest and lowest monthly temperature was found to be in the passageway, followed by antechamber and the main chamber [34], due to heat transfer into the tomb. In this case, whitish-grey patinas were observed in the middle and bottom sections of the eastern and northern walls of main chamber, possibly caused by dew and moisture condensation that permeates the tomb.

The edaphic conditions and the porous nature of the substrata were also found to be the primary causes of the development and proliferation of bacteria and fungi, because for these heterotrophic communities, an increase in organic nutrients can change the oligotrophic conditions of mural paintings. The nature of bedrock and its different absorbance potential for organic macromolecules and porosity play an important role. In particular, in one of the Chiusi tombs (Tomb of the Monkey), the abundant actinobacterial colonisation was bolstered by the presence of organic matter and clays on the walls, along with iron oxide that represents an additional factor in promoting growth [47]. Organic matter is also influenced by vegetation cover and root penetration, as observed in the Chiusi tombs [48]. The differences among the microbial communities in the two tombs could not be explained simply by considering the bedrock or bioclimatic conditions, but by considering the influence of differential root penetration as well [48].

Roots from different plant cover were considered responsible for specific microbial communities in response to nutrient availability in the rhizosphere, and in the case of the Tomb of the Hill, there was an abundance of Rhizobiales. Instead, the most common Actinobacteriales were found to be consistent with data on nitrogen-fixing bacteria and rhizosphere studies [48]. The authors suggested that roots had a role in introducing microorganisms into the tomb as well as in influencing organic carbon through root litter and root exudates. Even a small variability of organic substances from biodegraded lignin and humic substances seems to have an important influence on subterranean microbial communities and tomb colonisation by *Nocardia* and *Pseudonocardia* connected to agricultural and/or livestock activities, among other macromolecules on the topsoil [48].

The nature of the pigments utilised in the tombs themselves produced variable effects. In one tomb in Chiusi, the red pigment hematite utilised for the wall paintings played a discriminatory role, since the identified *Nocardia* need iron oxides for the optimal growth [47]. However, in the case of other tombs in Tarquinia, a direct effect of the pigmented minerals displayed no influence on the selection of communities, as indicated by the similarity of the bacterial phyla identified in samples with red, black and ochre pigments. Moreover, in the case of moonmilk, the patina that covered the entire surface of the pictorial layer and its variable thickness showed no relation to the underlying pigments [42].

High values in airborne spore concentration were found to be a risk factor, and preliminary data collected in two Etruscan tombs of Veio on the relationship between biodeterioration of wall frescoes and microbial concentration of the air reported the absence of a qualitative or quantitative parallelism

between the microorganisms isolated from the walls and the ones in the air [45]. Ventilation and the consequent risk of contamination/dissemination is also an important factor to take into consideration, hence aerobiological studies were also carried out to assess which areas could be more sensitive [28,116]. Furthermore, the importance of paying great attention during restoration activities was also stressed, as the normal cleaning operations can transfer microorganisms from the altered surfaces to the air, giving rise to further colonisation [38]. The materials used in restoration can sometimes be colonised by several microorganisms as shown in data from the Koguryo Tomb in China with cases of hypocrealean fungi forming "white mould spots" on the acrylic varnish coatings of the murals [22].

Inside the tomb ecosystem, insects and other animals have an additional detrimental effect because they can carry microorganisms which spread inside the tomb, as well as leave behind organic residue as already reported for caves and catacombs [117]. In fact, the highest microbial distribution ascertained at the entrance and the reduced distribution of microorganisms upon entering the ancient Korean tomb may be due to the fact that contamination levels increased as the insects inhabiting the space and the air at the entrance of the tomb had long remained stationary.

4.3. Indirect Control Methods and Biocide Treatments

The maintenance of the stability of microclimatic conditions reducing and setting times for the lighting systems, through a decrease in the indoor temperature, and avoiding condensation are goals to be achieved for the tombs conservation [23,28,55,118,119], as shown in Figure 5b. For Etruscan tombs, the presence of a dromos appears to have a positive effect in reducing the risk of microbial development because it helps to stabilise the microclimatic conditions [28]. Further control methods included the reconstruction of overlaying tumuli over the tombs that had been destroyed, and water infiltration prevention by maintaining an herbaceous vegetation layer capable of absorbing humidity and limiting overheating in the hottest seasons [51,58,120]. Conversely, the use of air conditioning systems was found to be counterproductive also because they stabilise the thermo-hygrometric conditions and the air flow can lead to loss of moisture of the wall paintings, hence passive systems are considered preferable. The construction of draught-proof doors and buffer zones inside the hypogea to prevent uncontrolled air circulation was found to be the most important and effective indirect control method [55,56,58,90]. Being human presence a disturbing factor, visits to these hypogea should be planned, the entrance to visitors should be greatly limited, and protective clothing and shoes should be worn (as usually worn by restorers. See Figure 6).

When a biological attack occurs, direct control methods are undoubtedly necessary. The practical use of microwaves as suggested by preliminary testing [46] seems very critical in these fragile environments. The use of biocides firstly needs a careful evaluation of the BPs and the risks entailed [121], and their effective use implies a thorough knowledge of the chemical features of the products as well as the spectrum of efficacy on the resident community [31], this last acting sometimes as a limiting factor for fungal spreading [27,121,122]. With this approach, harmless colonisations could be maintained [42], while the most dangerous ones can be treated. Resistance to certain biocide treatments by spore-forming bacteria, such as *Bacillus* sp. and fungi, also needs to be carefully evaluated. We do not concord with the conclusion that biocide treatments are useless and even detrimental because particular bacteria or fungi can still proliferate on the frescoes [40]. We believe that if the colonisation and the potential microbiomes are well known, efficient treatments can be found with indirect control methods carried out to remove the causes of the proliferation.

In fact, testing new biocides with low toxicological values that do not interfere with materials is still needed. Therefore, it is necessary to develop and apply effective eco-friendly biocides that do not damage the murals and that are harmless for humans and the environment [85,123,124]. In the field of cultural heritage conservation science in South Korea and Italy, studies conducted on such compounds and the efficiency of mixed eugenol with an eco-friendly emulsifier seem promising [125], such as the potential of allelopathic substances derived from lichens [126].

Figure 6. Restoration activities carried out in Korean (**a**–**c**) and Italian sites (**d**–**f**) respectively. (**d**–**f** Courtesy of Amici delle Tombe di Tarquinia).

5. Conclusions

The collected data show the complexity of the microbial communities in hypogea, as the result of variable environmental values and edaphic conditions. Events that have occurred over time regarding the tombs (opening, flooding, upper vegetation, entrance of visitors, conservation treatments) also play an important role in the biodeterioration processes. Considering the absence of a standardization of the detected sampling methods, and the complexity of the influencing factors, this study shows some limitations in the prevision of the potential occurring biodeterioration phenomena. However, we can observe, as in very humid conditions, cyanobacteria and algae prevail, though only if lighting is sufficient, whereas fungi and bacteria play the greatest role. A possible transition of colonisation among different taxa of fungi and bacteria (Actinomycetes) has been observed, also in consideration of the nutrients and temperatures values. The similarity of phenomena observed in the two countries indicate the importance of these studies. Calibrating indirect control methods and developing eco-friendly biocides are needed.

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