Chapter

Biotechnology and Cultural Heritage Conservation

Franco Palla

Abstract

The deterioration of cultural asset is induced by biological, chemical, and physical factors, influenced by anthropogenic activity and environmental conditions. In this study, the contribution of biotechnology is emphasized to define the conservation strategy, for a marble Fountain (Two Dragons, XV century) located in Palermo city center, based on an integrated approach and eco-friendly procedures. Biotechnological protocols are preliminarily applied as an integrated approach, based on microscopy observation, *in vitro* culture and genomic DNA analysis to recognize and characterize microbial communities. Several biological systems have been identified: green algae (*Chlorella*) and cyanobacteria (*Cyanobium, Oscillatoria*); bacteria (*Arthrobacter*, *Bacillus, Micrococcus, Paracoccus*); fungi (*Alternaria*, *Aspergillus, Penicillium, Phoma*, *Fusarium*, *Cladosporium*). In order to address biological colonization, the commercial Tea Tree Oil (*Melaleuca alternifolia*) and laboratory-distilled (*Calamintha nepeta* and *Allium sativum*) EOs, have been assayed by *in vitro Agar disc diffusion*, *Well-plates diffusion,* and *Micro-dilution* methods; the result allows to define the most appropriate EOs concentration to use. In a *green conservation* prospective, this study highlighted that EOs can potentially replace the traditional biocides, but the activity must be preliminary evaluated by centring the choose specifically on each microbial taxon identified.

Keywords: stonework deterioration, integrated approach, biocides, essential oil, green conservation.

1. Introduction

The biological colonization of stone artifact is basically related to the mineral components and bio-receptivity of the constitutive material, the presence of particulate on the surface, the environmental condition, and the availability of nutrients [1–3]. Generally, for outdoor Fountains, the biodeterioration is mainly induced by microalgae and cyanobacteria [4], but other biological agents such as bacteria, fungi, mosses, and lichens were frequently revealed [5, 6]. Moreover, the biological colonization is enhanced by the occurrence of water that cooperate in deterioration processes [7], acting mechanically and chemically, producing visible effects on stonework surface (cracking, detachment, crusts formation, and chromatic alterations) allowing to structural damage and loss of material [8–10].

Fungi (such as *Alternaria*, *Cladosporium*, *Epicoccum*, *Aureobasidium*, *Phoma*) have a significant biodeteriorative action and may penetrate into the stone surface, causing the bio-pitting; fungi colonies can be in close association with lichens [11, 12].

Autotrophic (photolithotrophs and chemolithotrophs) and heterotrophic bacteria have also been isolated from stonework and since many of these microorganisms contain pigments (β-carotene, α-bacterioruberin, and derivatives) and salinixanthin in their cell membranes, their proliferation can produce typical rosy stains on the stone surface [10, 13, 14].

Furthermore, the deterioration is also the direct result of atmospheric pollution due to soot, grease, dust, etc., implying the deposition of suspended particles on the stonework surface, enhancing the SO_2 deposition, a very reactive compound with a significant corrosive effect on marble surface [15, 16]; especially for outdoor monument, anthropogenic factors must be also considered [17].

To control biodeteriogen growth of powerful biocides, as well as water-repellents, with a broad spectrum of action are usually utilized against green and brown algae, bacteria, yeasts, lichens, molds, and micro-fungi [18–21].

In the last decades, integrated approaches (based on microscopy, *in vitro* culture and molecular biology analysis) have been applied to reveal and identify the greater number of microorganisms involved in the deterioration processes of cultural assets [22–31].

In this case study, in order to define adequate conservative strategies, the identification and evaluation of biological colonization of the Two Dragons fountain (sculptured by Nunzio La Mattina, XV century) were carried out, providing needful information to choose the appropriate biocide both for active compound and concentration.

Recently, non-toxic natural compounds (essential oils, EOs), in order to replace the chemical compounds, have been utilized to control artworks biological colonization and to inhibit re-colonization events [32–37].

The aim of this work has been the revealing of microbial communities on the stonework surface, evaluating the antimicrobial activity of traditional (Benzalkonium chloride) and green biocides (*Melaleuca alternifolia* – TTOil, *Calamintha nepeta* and *Allium sativum* EOs) *vs* the identified microbial taxa [38–41].

The results of *in vitro* assays and controlled step by step application on stonework samples, prompt us to hypothesize the EOs as valid alternative to traditional biocides, in respecting human health and environment, according to modern restoration procedures.

2. Material and methods

2.1 Sampling

Samples were collected from different Fountain areas, affected by chromatic alterations, deposits, exfoliations, incrustation, or biological patinas, by sterile swabs moistened with NaCl-Tween solution (0.9% Sodium Chloride, 0.02% Tween-80, Polyoxyethylene sorbitan monooleate) or sterile scalpel **Figure 1**.

2.2 *In vitro* microbial culture

Nutritive media specific for bacteria or fungi colonies (Nutrient or Sabouraud agar, *Difco*) were inoculated by the swab collected samples, incubating at 30°C for 18–48 hours.

2.3 Morphological analysis

Morphological profiles of algae and bryophytes were revealed by stereomicroscope (Wild Heerbrugg) and digital microscope (DinoLite) observations. After

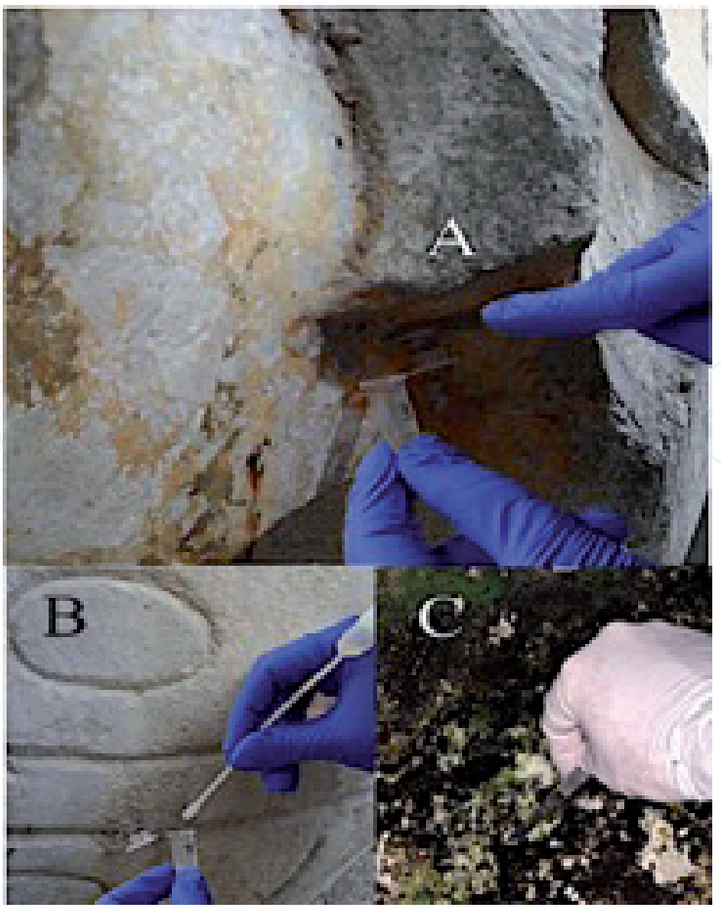

Figure 1.
Stonework altered areas, sampling performed by sterile swab o scalpel: (A) dark-rust red area; (B) light – green calcareous deposit; (C) dark-green area.

Lugol's iodine staining, the reproductive structures of isolated fungal colonies were also distinguished by Optical Microscope (Leica). Coccoid bacteria have also been noticed by Scanning Electronic Microscope (Leica Cambridge – Leo 400), after coating (Agar-Auto-Sputter – Coater B7341) by gold particles (13 nm).

2.4 Molecular biology investigation

Patina sample of approximately 200 mg, collected by sterile scalpel, undergone to three freezing (−80°C) and thawing (+ 55°C) cycles, in presence of 500 μl – 1X TE Buffer (10 mM Tris-HCl pH 8.0/1 mM EDTA), to achieve the lysis of microbial cells; genomic DNA was extracted by *QI Amp DNA stool Kit* (Qiagen), partially modified (+ Proteinase K (5 mg/ml) and incubation at 65°C for 4 hours). Instead, from *in vitro* isolated microbial colony, the *Genomic DNA Purification Kit* (Fermentas) has been appropriate.

Genomic DNAs were utilized as template molecules in Polymerase Chain Reaction (PCR), in order to amplify bacterial or fungal target sequences, specifically, the Internal Transcribed Sequences (ITS) 16-23S rRNA for bacterial and ITS 18-26S rRNA for fungal species [25, 26, 40]. Each PCR reaction solution consisted of: microbial Genomic DNA as template; 10 μM Primer Forward; 10 μM Primer Reverse; 3.0 mM dNTP mix; 1X Reaction Buffer including $MgCl_2$; 0.5Us Taq DNA polymerase (Sigma).

PCR products were resolved by electrophoresis on 2.5% agarose gels (1X TAE – Tris-HCl/Acetate/EDTA, in 1X SYBER-safe DNA gel stain) and related aliquots were sequenced by Eurofins MWG-Operon sequencing service (Germany).

Referring to genomic databases (EMBL-Germany, NIH-USA), the sequences were analyzed (percentage of similarity) by BLAST analyzer [42].

2.5 Commercial (CB) and natural (EOs) biocides

The antimicrobial activity of: (i) commercial EOs, *Melaleuca alternifolia* (Maiden and Betche) Cheel -Tea Tree Oil; (ii) laboratory distilled EOs (*Calamintha nepeta* (L.) Savi, *Allium sativum* L.; (iii) B*enzalkonium chloride* commercial biocides (CB), was tested by outlined *in vitro* assays [36–38].

The microbial taxa were *Bacillus subtilis*, *Micrococcus luteus*, *Penicillium chrysogenum*, *Aspergillus* spp.

2.6 Antimicrobial activity assays

Three *in vitro* methods, *Agar disc diffusion*, *Well-plates diffusion*, and *Micro-dilution* in micro-titer plates [38, 43, 44] were performed:

- *Agar disc diffusion*: paper disc (4 mm in diameter) was placed onto the surface of Nutrient or Sabouraud agar (90 mm Petri dish), previously wetted with 10 μl of CBs (25, 50%) or EOs (12.5, 25, 50, 100%) The agar surface has been previously seeded by microbial cells (bacterial cells = 1×10^6 CFU/ml or fungal suspension = 1×10^4 conidia/ml) and incubated for 18–48 h at 30 ± 1°C. Confluent microbial growth was observed and the diameter (mm) of growth-inhibition-halo measured (> 6 mm = sensible; < 6 mm = resistant); CB was Benzalkonium chloride (25, 50%). Each test was performed in triplicate.

- *Well plate diffusion*: the microbial inoculum was uniformly spread on Nutrient or Sabouraud agar surface, then holes of 4 mm in diameter were punched aseptically [38] and 10 μl aliquots (12.5, 25, 50, 100%) of each essential oil solutions loaded. After 18/48 h of incubation at 30 ± 1°C, the diameter (mm) of growth inhibition halos were measured. Each test was performed in triplicate.

- *Micro-dilution*: was performed in 96-wells micro-titer, in order to define the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), distinguishing between biocide or biostatic action [39]. In each well, 30 μl of plant extracts (6.25, 12.5, 25, 50, 100%)/ liquid nutritive medium and an equal volume of microbial suspension were added; to facilitate the dispersion of the oil in the medium solution, 1% of Tween 80 (not toxic for microbial cells) was added. Benzalkonium chloride (0.2%, vol/vol) was utilized as CB. Microbial growth, after 18 h of incubation at 30°C was evaluated by estimating the optical density at 500–600 nm. The MIC value was measured as the lowest concentration corresponding to any visible microbial growth, after incubation at 30°C. The MBC and MFC were determined as the lowest concentration of antimicrobial agent able to kill the 99.5% of the original inoculum, evaluating on antimicrobial-free sub-culture [45].

3. Results

Green algae as *Chlorella* (**Figure 2**) and cyanobacteria as *Cyanobium and Oscillatoria* genera were revealed in fountain samples, classified as biodeteriogen and also as first pioneering of stone substrates colonization. Particularly, algae can

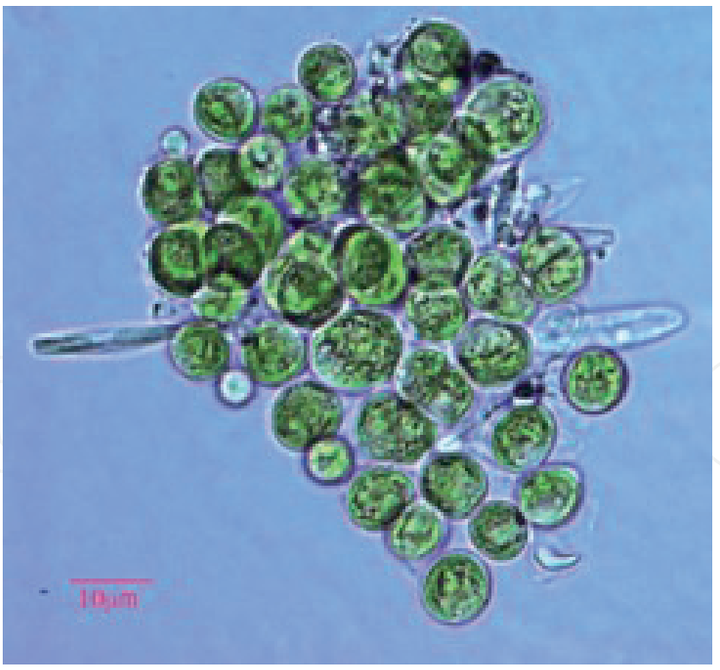

Figure 2.
Chlorella *green algae, optical microscope images; bar = 10 micromillimeters.*

induce carbonate precipitation on stone substrates and their metabolic processes also generate organic acids (aspartic, citric, glutamic, glycolic, oxalic, and uric) promoting the dissolution of same minerals [3, 46, 47]. Cyanobacteria, algae, and lichens contribute to the weathering of stone in humid as well as in semiarid and arid environments [48–50]. Furthermore, cell compounds such as chlorophyll, carotenoid, and melanin may generate chromatic alteration from yellow, orange, and red to brown [10, 13, 51].

Bacterial and fungal diversity was also distinguished, bacteria or fungi genera mainly belonging to *Arthrobacter, Bacillus*, *Micrococcus* or *Alternaria, Fusarium*, *Cladosporium*, *Penicillium*, and *Aspergillus*, respectively (**Figures 3**–**5**). Moreover, bacteria of the *Bacillus* genus are able to produce crystalline aggregates and precipitates (carbonate and phosphate), which can form insoluble complexes with pigments, producing different spots on stonework surface [52, 53]. Fungi, in relationship to their metabolic activities, are able to produce efflorescence and patina, breaking and cracking processes, contributing to chemical-physical alteration of the constitutive materials [54, 55]. Fungi also represents an important group of deteriogen systems for stonework exposed to the environment, due to the release of acids compounds during hyphae development or in the apical growth zones, able to penetrate inside the stone surface [56, 57].

Finally, biological systems referable to *Mosses* [58] were revealed in a green patina, **Figure 1C**, with a detrimental action related to the keeping of moisture, the production of carbonic acid and, after their death, the indirect damages by enriching and increasing the humus content of stone surfaces, supporting the consequent growth of plant species [59].

In order to inhibit biological colonization, traditional (benzalkonium chloride) or green (*Melaleuca alternifolia*, *Calamintha nepeta*, and *Allium sativum* EOs) biocides have been tested.

In **Figure 6**, the inhibition activity of *Melaleuca alternifolia* (TTOil) *vs. Bacillus subtilis* (A) or *Micrococcus luteus* (B) has been evaluated by the *Well plate diffusion* method; the size of inhibition halos is related to the essential oil concentration.

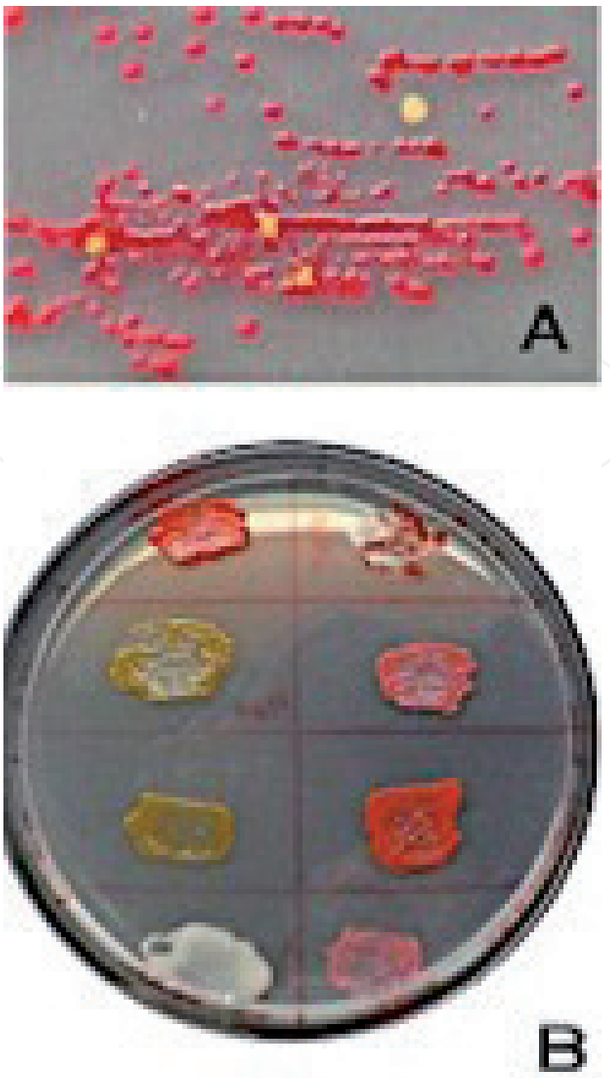

Figure 3.
Morphological profile of pigmented bacterial cells isolated from the sampled areas on nutrient agar: (A) Micrococcus *sp. colonies; (B) different bacterial colonies;* Bacillus *sp. colonies agar; plates incubated at 30°C for 18 h.*

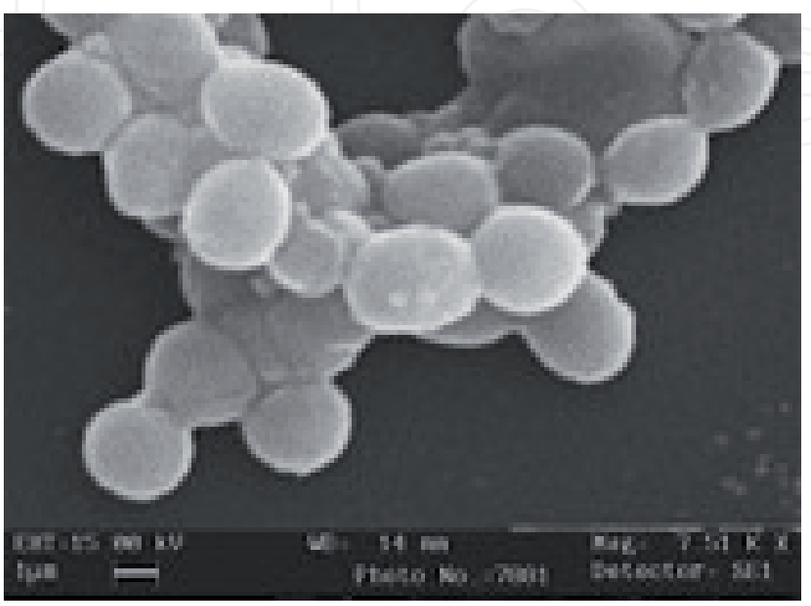

Figure 4.
SEM micrograph of Coccoid bacterial cell; bar = 1 micromillimeter.

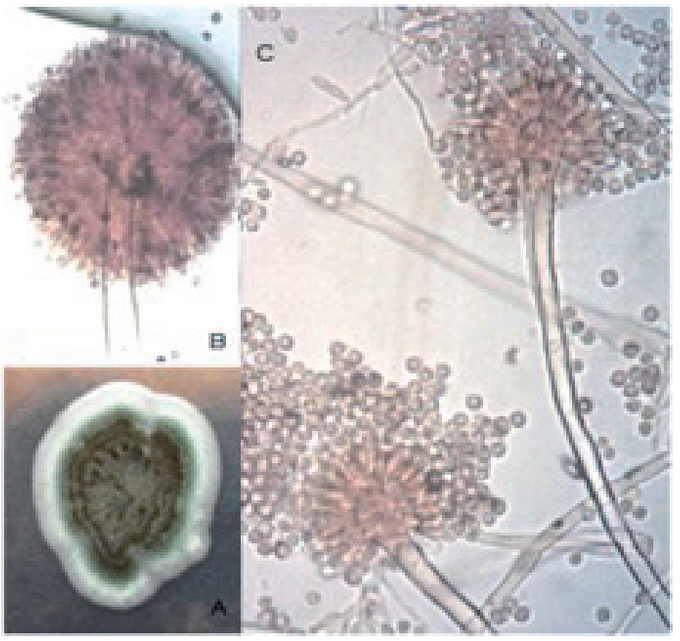

Figure 5.
Morphological profile of Aspergillus *sp. colony isolated on Sabouraud agar (A), related fungal spore and reproductive structure stained by Lugol's iodine reactive (B–C); optical microscopy (40× magnification).*

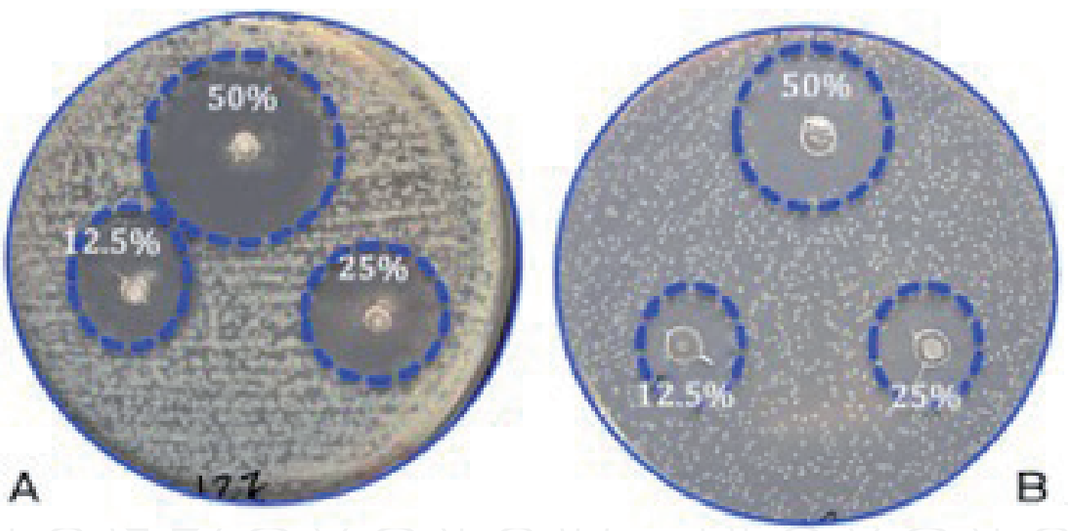

Figure 6.
Well plate diffusion *method. Antimicrobial activity of* Melaleuca alternifolia *(TTOil)* vs. Bacillus subtilis *(A) or* Micrococcus luteus *(B). The inhibition halos show a different antimicrobial activity related to the EO concentration.*

The antimicrobial activity has been also performed using the three EOs or CB at different concentration (12.5, 25.0, and 50.0%) *vs* microbial taxa identified in the stonework colonized areas; the results have been summarized in **Table 1**. Particularly, a relevant inhibition on bacterial growth was performed by *M. alternifolia* and *A. sativum* EOs against *B. subtilis* and *M. roseus*, so strong that the halo inhibition was equal to the petri dish diameter.

Minimum Inhibitory Concentration (MIC) *vs* bacterial colonies has been evaluated by the *Microdilution method*. Particularly, biocidal activity *vs M. luteus* and *B. subtilis* has been showed by *M. alternifolia* and *C. nepeta* EOs; while *A. sativum* EO showed both biocidal and biostatic activity *vs M. luteus* and biocidal activity against *B. subtilis* (**Table 2**); the MIC related to benzalkonium chloride was also performed.

Microbial taxa		Essential oils (EOs)			Classical biocide (CB)
	(%)	*Melaleuca alternifolia*	*Calamintha nepeta*	*Allium sativum*	*Benzalkonium chloride*
Bacillus subtilis	50.0	*	7.0	*	9.2
	25.0	8.4	6.5	9.2	7.0
	12.5	5.0	3	5.5	4.0
Micrococcus roseus	50.00	*	8	*	9.0
	25.0	8.0	6	9.0	7.0
	12.5	2	2	4	4.0
Penicillium chrysogenum	50.0	8.2	5.0	10	4.0
	25.0	6.5	3.8	7.0	3.0
	12.5	5.0	2.5	4.2	≥1
Aspergillus spp.	50.0	6.8	5.0	10	3.0
	25.0	6.0	3.0	6.9	2.0
	12.5	3.8	2.5	4.0	≥1

**Total inhibition of microbial growth.*

Table 1.
Well plates diffusion *method: Measurement of microbial growth inhibition as halo diameter (mm): Diameter ≥ 9 mm. (sensible strain); 6–9 mm. (relative sensible strain); ≤ 6 mm (resistant strain).*

EOs or CB	*Micrococcus luteus* (%)	Bacillus subtilis (%)
Tea tree oil	0.6	0.6
Calamintha nepeta	1.56	1.56
Allium sativum	100	100
Benzalkonium chloride	0.0031	0.0031

Table 2.
Minimum inhibitory concentration (MIC) %, of EOs and CB vs. bacterial taxa.

4. Conclusions

The results showed that the fountains are differently colonized by several biological systems (**Table 3**).

Particularly for the dark-greenish area, *Mosses* [58] were also revealed, enhancing the bio-detrimental action due to the keeping of moisture, the production of carbonic acid and, after their death, enriching and increasing the humus content helping a following growth of plants on the stonework surface.

The identified colonizers were utilized to test the antimicrobial activity of three EOs *Melaleuca alternifolia*, *Calamintha nepeta*, and *Allium sativum*, in order to test natural product as alternative biocide. In **Figure 7**, the growth inhibition activity, measured by both *Agar disc* and *Well plate* diffusion methods of the three EOs was performed in parallel to a commercial biocide benzalkonium chloride.

The results of this study confirm the need of a fuller identification of microbial colonizers in order to perform an adequate biocidal treatment, focalizing the attention on *green alternatives.*

The innocuousness of essential oils in respecting of human health and environment protection, prompt us to hypothesize the use of these plant products as

Area	Algae	Bacteria	Fungi	Mosses
A. Dark-rust red	*Chlorella* sp. *Cyanobium* sp.	*Micrococcus* sp.	*Aspergillus* sp. *Cladosporium* sp. *Fusarium* sp.	–
B. Light-green calcareous deposit	*Chlorella* sp.	*Bacillus* sp. *Arthrobacter* sp.	–	–
C. Dark-greenish	*Chlorella* sp. *Oscillatoria* sp. *Cyanobium* sp.	Bacillus sp. Micrococcus sp.	*Alternaria* sp. *Aspergillus* sp. *Penicillium* sp	*Bryophyta* class Bryopsida

Table 3.
Microbial taxa colonizing stonework areas showed in **Figure 1**.

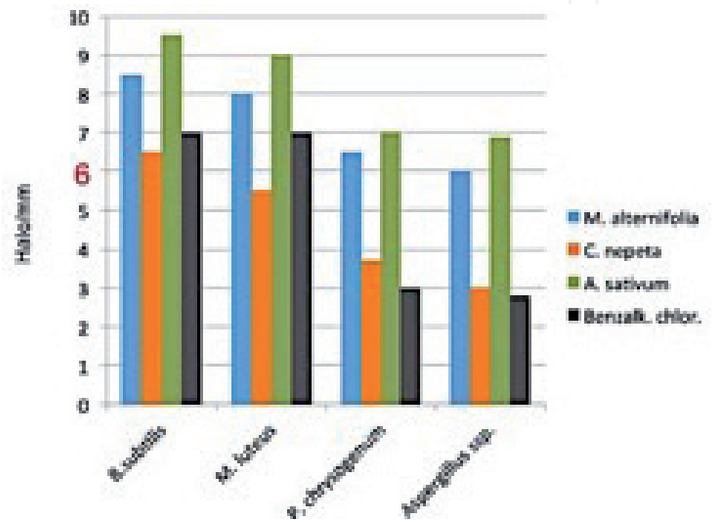

Figure 7.
Evaluation of the growth inhibition activity of the three EOs and the CB, against two identified bacterial and fungal taxa. Histograms represent the medium value obtained performing both Agar disc *and* Well plate diffusion *methods for each sample, in triplicate*.

natural biocides, although more studies on permanence and durability on artifacts surfaces are needed.

The antimicrobial efficiency of these and other vegetal biocompatible extracts is on-going in our laboratory in order to set up *green strategies* to control the biodeteriogen growth and colonization on cultural assets.

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Conflict of interest

The authors declare no conflict of interest.

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