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# Solunto archaeological park in Sicily: life under mosaic tesserae*

**Abstract**

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Biodeterioration is a complex process induced by the growing and metabolic activity of a wide range of macro and microorganisms, becoming a revelling problem also for the mosaic tesserae of "Casa di Leda" in the Greco - Roman site of Solunto in Sicily.
In this case-study, a thick biofilm inducing a deep alteration of mortar and consequently the mosaic tesserae detachment has been highlighted during the restoration plan.
The biofilm microbial consortium has been investigated by an integrate approach based on Microscopy analysis (O.M., C.L.S.M.), in vitro culture (Nutrien and Saboraud media) and molecular biology investigation (DNA target sequence amplification, sequencing, sequence analysis).
A microbial diversity has been revealed belonging to bacteria (*Bacillus*) and fungi (*Alternaria*, *Aspergillus*), besides cyanobacteria (*Chroococcus*) and green algae (*Chlorella*).
In order to control the biofilm colonization two essential oils (EO), *Thymus vulgaris* and *Origanum vulgare*, have been utilized and their antimicrobial activity, preliminarily in vitro (agar disc diffusion methods) and after ex situ and in situ evaluated. This experimentation is aimed at identifying and implementing green biocides for the control of microbial colonization, a promising technology with a reduced impact on human health and environment, able to replace traditional biocide action.

*Key words*: biodeterioration, microsopy analysis, in vitro colture, molecular biology.

## Introduction

Stone artworks biodeterioration is related to the combination of biological colonization and environmental factors, as happen in archaeological areas, where complex *biofilm* have been frequently revealed (Thomas & Demas 2013; Marzano & Métraux 2018).

Concerning inorganic specimens, the first colonizers are represented by pioneered photoautotrophic organisms, such as cyanobacteria, algae and lichens, but also oligotrophic or poichilotrophic microbial groups, such as some fungi and chemo-lithotrophic bacteria (i.e. using as oxidation inorganic compounds) have been identified; converting the substrate

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and helping the colonization of other heterotrophic organisms (Tiano & al. 1995; Warscheid & Braams 2000; Caneva & al. 2007).

Moreover, the association as *biofilm* confers a character of resistance to the biocenoses involved (Flemming 1993), becoming problematic to remove (Chorianopoulos 2008).

In this case-study, biofilm was not straightaway seen, since it was spread under several tesserae of the *impluvium*, located in the centre of the "Casa di Leda" *peristylo*, in Solunto (Palermo, Italy) one of the archaeological-park in Mediterranean basin (Marzano & Métraux 2018). Combining biological and biotechnological approaches (Palla & al. 2010, 2013; Di Carlo & al. 2016; Palla & Barresi 2017) microbial taxa such as bacteria, fungi, cyanobacteria and green algae have been identified.

Since the extensive microbial spreading affects both the constitutive materials and the legibility of the artworks (Barranguet & al. 2005), the control of biodeterioration phenomena represents a significant phase in conservation project. In routine practice, microbial colonization is addressed by chemical biocides, which toxicity and persistence in the environment is well known (De La Paz & al. 2006; Guiamet & al. 2006).

In the last decade, in order to develop green conservation strategies, plant products have been applied in prevention and treatment of microbial contaminants (Guiamet & al. 2008; Afifi 2012; Sasso & al. 2013; Fierascu & al. 2014; Stupar & al. 2014; Borrego & al. 2016; Rotolo & al. 2016).

In this study, the antimicrobial activity of two commercial essential oils, *Origanum vulgare* and *Thymus vulgaris*, have been evaluated against microbial species isolated from *biofilms* of floor mosaic tesserae of "Casa di Leda" in the *Greco - Roman site of Solunto* in Sicily (Fig. 1), to control the biodeterioration phenomena, applying novel protocols safe for humans and environment (Rotolo & al. 2017).

## Materials and methods

*Essential oils (EO)*– The *Origanum vulgare* and *Thymus vulgaris* essential oils have been used as pure essence (100%) or diluted solutions (50, 25, 12.5, 6.5 %) in 70% Ethanol.

Fig. 1. Mosaic floor around the *impluvium* in the *peristyle* of Leda's house, in archaeological park - Solunto, Sicily.

*Origanum vulgare*, is an aromatic hemicryptophyte, belonging to *Lamiaceae*, native to Europe and in particular in the Mediterranean basin, but today cultivated all over the world. The plant is rich in carvacrol, a phenolic monoterpene with recognized anti-inflammatory and antitumor properties (Burts & al. 2007; McCann & al. 2014).

*Thymus vulgaris*, is a chamaephyte belonging to *Lamiaceae*, including 250-350 evergreen taxa, native to southern Europe, North Africa and Asia.

The aromatic active molecules have a strong antiseptic and antibacterial properties, they also have digestive, warming, spasmolytic, carminative, diuretic and disinfectant action of the urinary tract. In addition they contain phenolic monoterpenes such as thymol (30-70%) and carvacrol (3-15%), oxides such as cineol and thymol methilesters, alcohols such as borneol, geraniol, linalool, esters such as acetate and linalyl, hydrocarbons such as cymene and terpinen (Mitsch & al. 2004; Bolukbasi & al. 2007).

*Sampling*.– Biofilm aliquots were collected by sterile scalpel or swab, and directly observed by optical microscopy or utilized for *in vitro* culture and molecular investigations.

*Microscopy observation*.– Cyanobacteria and microalgae, as well as the morphological profile of bacterial and fungal isolated colonies were analysed by Stereo (Wild-M1B, 14X) or Fluorescent ( DMR-Leica, 40X) microscopes. Conidiophores and conidia fungal structures were observed by Optical Microscope (Leica, 40X), after Lugol's iodine staining (Di Carlo & al. 2016).

*In vitro culture*.– Sabouraud Dextrose Agar + Chloramphenicol (CAF) and Nutrient Agar plates, seeded by biofilm aliquots, were incubated at 30°C (Palla & al. 2006; Pasquarella & al. 2015); after 24/48 h fungi and bacteria colonies

*Microbial DNA extraction*.– Each isolated bacterial and fungal colony was lysed at 94°C per 2 min in 20 µl of 1X T.E. (10 mM TRIS-HCl pH 7,5 / 1 mM EDTA), extracting the genomic DNA by the *Genomic DNA purification* and *Gene JET Genomic DNA purification* kits (Fermentas).

*Polymerase Chain Reaction (PCR)* – Genomic DNA molecules were utilized as template for *in vitro* amplification (Polymerase Chain Reaction) of target ribosomal-DNA sequences: 16S-rDNA gene or Internal Transcribed Sequence (ITS – rDNA), specific for bacteria or fungi.

ITS-PCR reactions were carried out by the following oligonucleotides as primers, bacteria: ITS1= 5'-GTCGTAACAAGGTAGCCGTA-3' and ITS2= 5'-GCCAAGGCATCCACC-3'; fungi: ITS4 = 5' - TCCTCCGCTTATTGATATGC-3' e ITS1 = 5' –CTTGGTCATTTAGAGGAAGTAA - 3' (Cardinale & al. 2004).

PCR products were analysed by 2% Agarose gel electrophoresis, nucleotide composition determined by MWG Operon Sequencing Service and sequences comparison performed by BLAST platform (Altschul & al. 1997; Palla & al. 2010, 2013; Palla 2012; Palla & Barresi 2017).

*Evaluation of antimicrobial activity*.– *In vitro* assay: *agar disc diffusion* method was performed utilizing paper disc (6 mm in diameter), wetted with 10µl of each EO at differ-

ent concentrations (100%, 50%, 25%, 12.5%, 6,25%) and placed onto the surface of Nutrient or Sabouraud agar (90 mm Petri dish), previously seeded by $1x10^6$ CFU/mL - bacterial cells or $1x10^4$ conidia/mL - conidia suspension (Borrego & al. 2012).

Control assays were performed wetting the paper disc with 70% Ethanol or 0.2% (vol/vol) Benzalkonium chloride + chlorhexidine (BC, one of the frequently used commercial biocides).

After incubation at 30°C for 24/48 h, confluent microbial growth was observed and the diameter (mm) of growth inhibition areas was measured (Balouiri & al. 2016; Rotolo & al. 2016); each test was performed in triplicate.

*Ex situ* assay: the antimicrobial activity of the two essential oils was also evaluated exposing the biofilm to the volatile compounds. Particularly, eleven colonized tesserae, gathered from specific areas of *impluvium* were placed on paper discs (60 mm, Whatman) into equal in diameter sterile Petri dishes, soaked with: *i*) *Oregano* (100, 50, 15%) EO; *ii*) *Thyme* (100, 50, 15%) EO; *iii*) Biotin-R (100, 50, 15%); *iv*) Ethanol (70%); Petri dishes were sealed by Parafilm membrane (Heathrow Scientific) to prevent evaporation phenomena. The effect of EOs was evaluated after 72h and up to 7 days, analysing both biofilm green pigmentation and auto-fluorescence (MD Fluorescent Microscope, Leica).

*In situ assay*: biocide activity was also directly evaluated on selected (eleven) *peristyle mosaic* areas. As performed in laboratory assays, differently concentrated Essential Oils (100, 50, 15%) and Controls (100, 50, 15% Biotin-R or 70% Ethanol) solutions were injected (by sterile needle/syringe) through interstitial mortar tesserae.

## Results

In order to characterize the microbial species constituting the pigmented *biofilm* revealed below the mosaic tesserae (Fig. 2), an integrated approach was applied (Palla & Barresi 2017). Particularly, a complex microbial community with prevalence of cyanobacteria, belonging to the genus *Chroococcus* and green algae such as *Chlorella* (Fig. 3), was observed by Optical Microscopy. Through *in vitro* culture and molecular investigations, the presence of bacteria, *Bacillus* sp. pl. (Fig. 4) and fungi, *Alternaria* (Fig. 5) sp. and *Aspergillus* sp. (Fig. 6) have been also identified.

The characterization of microbial consortium components is strictly related to the control of microbial spreading, accordingly to the biocide product that will be applied. The aim of this study was also the set-up of alternative biocides, as plant essential oils, less invasive/dangerous than commercial chemical compounds (Guiamet & al. 2008; Afifi 2012; Sasso & al. 2013; Fierascu & al. 2014; Stupar & al. 2014; Borrego & al. 2016; Rotolo & al. 2016).

Performing *in vitro* agar diffusion disc method, different antimicrobial effects of EOs and Controls solutions were defined against *Bacillus* sp. p.l, *Alternaria* sp. and *Aspergillus* sp. colonies, basing on inhibition-halo diameter (Tab.1)

Particularly, *in vitro* assays highlight a stronger antimicrobial activity EOs, as showed in Fig.7a, per *Oregano* solutions (50, 25. 12,5%) *versus Bacillus* sp., compared to the activity of commercial biocide Benzalkonium Chloride solutions (100, 6.25%), showed in fig. 7b.

Fig. 2. Pigmented *biofilm* revealed below the mosaic tesserae in some area of the Leda's house *peristyle*.

Similar strength is showed by *Oregano* solutions (100, 12.5%) vs *Aspergillus* sp. (Fig. 8a) and *Alternaria* sp. (Fig. 8b) colonies, significantly greater comparing the inhibition halo produced by 12.5% Benzalkonium Chloride solution against *Aspergillus* sp. (Fig. 9a) and *Alternaria* sp. (Fig. 9b).

The *ex situ* assays were performed exposing the mosaic-colonized tesserae to the volatile compounds of *Oregano* or *Thyme* EOs, analysing the biofilm after 7 days (will be assessed up one year). As showed in Fig. 10, a different pigmentation can be distinguished between control (D = no-biocidal compound) and treated samples (A= Biotin-R; B= Thyme-EO=; C= Oregano-EO), allowing us to hypothesize that the lack of green pigmentation is related to the reducing in biofilm photosynthetic activity; related Fluorescent Microscope observations performed by Zeiss-Axioskop 2-Plus, are showed in Fig. 11. It is evident only a background of fluorescence in treated samples A, B, C, while defined biological structures are evident in no-treated sample D.

Finally, *in situ* tests were performed injecting below the tesserae, *Oregano* or *Thyme* EOs solutions (100, 50, 15%), through the interstitial space. As showed in Fig. 12, a clear effect on biofilm was revealed for the 15% *Thyme* EO solution, concentrically diffused

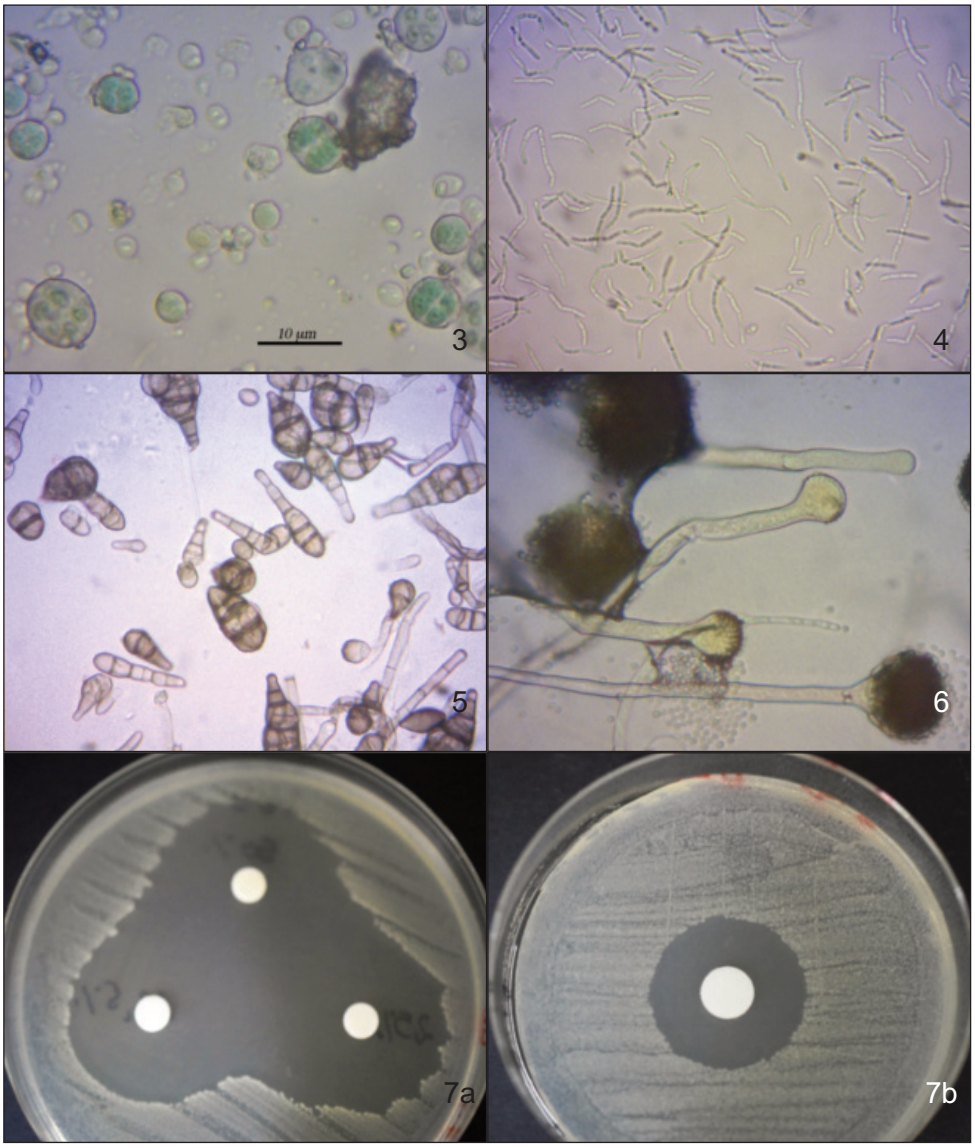


Fig. 3. *Chlorella* sp. colonies observed by Optical Microscope (Leica, 40× magnification).
Fig. 4. *Bacillus* sp. bacterial cells observed by Optical Microscope (Leica, 40× magnification).
Fig. 5. *Alternaria* sp. spore structure, observed by Optical Microscope (Leica, 40× magnification), after Lugol's staining.
Fig. 6. *Aspergillus* sp. conidiophores and spore structures, observed by OM (Leica, 40× magnification), after Lugol's staining.
Fig. 7a. Antimicrobial activity of *Oregano* solutions (50.0, 25.0, 12.5%) *versus Bacillus* sp., the confluent inhibition halos prove a strong activity of EO.
Fig.7b. Antimicrobial activity of the commercial biocide, Benzalkonium Chloride (6.25%) solution, a confluent inhibition halo is show.

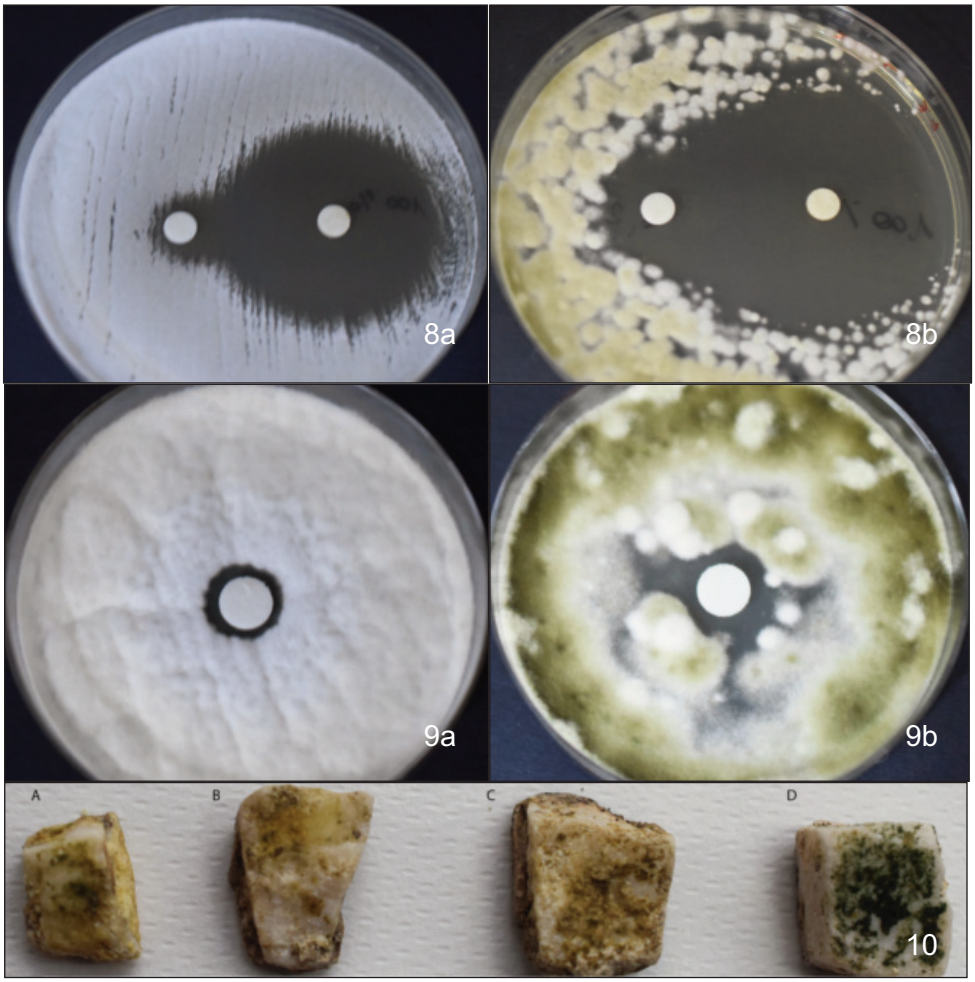


Fig. 8a. Inhibition halo produced by *Oregano* solutions (100, 6.25%) vs *Aspergillus* sp.
Fig. 8b. Inhibition halo produced by *Oregano* solutions (100, 6.25%) vs *Alternaria* sp.
Fig. 9a. Inhibition halo produced by 6.25% Benzalkonium Chloride solution against *Aspergillus* sp.
Fig. 9b. Inhibition halo produced by 6.25% Benzalkonium Chloride solution against *Alternaria* sp.
Fig. 10. *Ex situ* assays. Effect of volatile compounds of Oregano or Thyme EOs, after 7 days of exposure, on the biofilm of mosaic-colonized tesserae: A= Biotin-R (commercial biocide); B= Thyme-EO; C= Oregano-EO; D= no-biocidal compound.

with respect to the injection point (highlighted as red line). Particularly, the biofilm came in contact with the EO solution showed a loss in pigmentation, while a vital green colour is still present in the portion not reached by the oil solution (pointed by a red asterisk). Instead, the 15% *Oregano* OE treatment does not show similar effect, may be related to the presence of inhibitory substances in the completely degraded bedding mortar.

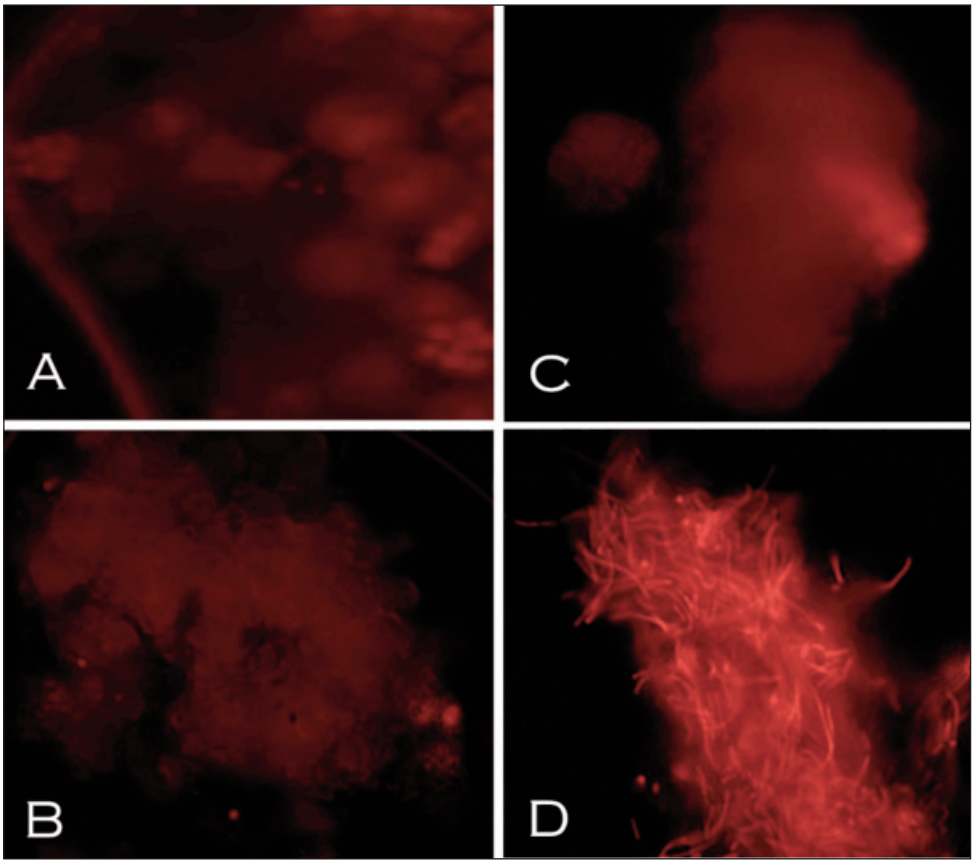


Fig. 11. *Ex situ* assays. The lack of biofilm photosynthetic activity, observed by Zeiss-Axioskop 2-Plus Fluorescent Microscope observations. Corresponding to Fig.10 samples: A= Biotin-R (commercial biocide); B= Thyme-EO; C= Oregano-EO; D= no-biocidal compound (40× magnification).

**Discussions and Conclusions**

The rich distribution of archaeological assets in the Mediterranean basin, their cultural, artistic, religious significance and high social impact, highlight the requirement of dedicated conservation strategies. The identification of the different factors (biological, chemical, physical) able to induce structural and compositional changes (Warscheid 2000) is of fundamental importance in defining suitable fruition, maintenance and conservation policies.

The role played by micro-organisms in the processes of cultural heritage deterioration (biodeterioration) has been widely demonstrated: growth, development and metabolic activities can bring physical-chemical and aesthetic damage to works of art, inducing negative consequences for their conservation (Fort & al. 2006).

Many species of microorganisms (bacteria, fungi, unicellular algae) can find favourable conditions for their development both on statues and monumental works and on archaeo-

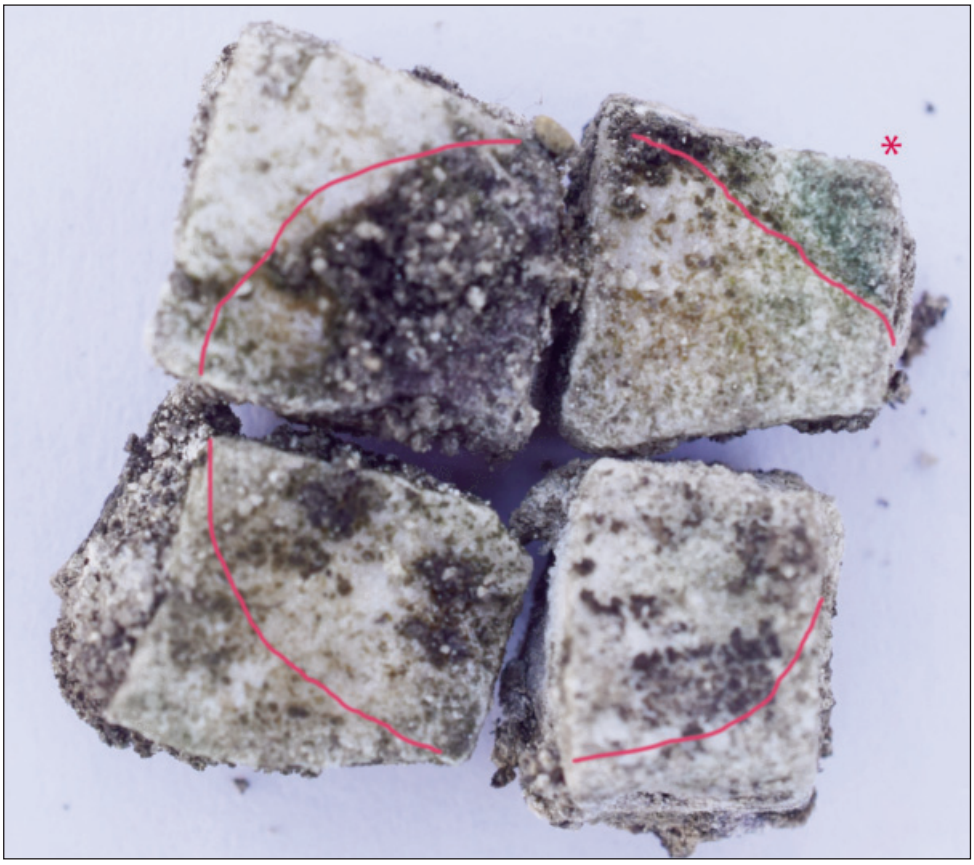

Fig. 12. *In situ* assays. Mosaic tesserae after treatment with 15% *Thyme* solution, concentrically diffused with respect to the injection point (highlighted as red line), a lost in pigmentation is clearly recognizable. A vital green colour is still present in the portion not reached (pointed by an asterisk).

logical remains (Kovacik 2000), as the mosaic tesserae of "Casa di Leda" - Greco - Roman Solunto archaeological site park, Sicily.

Particularly, a thick *biofilm* inducing a deep alteration of mortar and consequently the mosaic tesserae detachment has been identified during the restoration activities.

The *biofilm* has been characterized through optical microscopy, *in vitro* culture and molecular biology techniques, allowing the identification of microbial *taxa* as *Bacillus* sp., *Alternaria* sp., *Aspergillus* sp., as well as cyanobacteria, *Chroococcus* sp. and green algae, *Chlorella* sp. These microorganisms are considered biodeteriogens, able to induce precipitation of mineral crystals or promoting pH changes, causing damage on stonework surface (Sanchez-Moral & al. 2003; Albertano & al. 2003).

In this study new biocompatible products to control *biofilm* growth have been also assessed, evaluating the antimicrobial activity of two commercial essential oils, *Origanum vulgare* and *Thymus vulgari*s.

Table 1. *Agar disc diffusion* method. Average inhibition halo diameter (mm) of EOs (*O. vulgare*, *T. vulgaris*) Antimicrobial activity: Positive ≥ 9 mm; Moderate: 6 - 9 mm; Negative ≤ 6mm; *total growth inhibition. Benzalkonium chloride + chlorhexidine and Ethanol solutions were the controls.

| Essential olis | Conc. (%) | *Bacillus* sp. pl. | *Alternaria* sp. | *Aspergillus* sp. |
|---|---|---|---|---|
| ***Origanum vulgare*** | 100 | * | 60 | 50 |
| | 50.0 | 42 | 49 | 45 |
| | 25.0 | 40 | 48 | 46 |
| | 12.5 | 39 | 38 | 35 |
| | 6.25 | 28 | 16 | 16 |
| ***Thymus vulgaris*** | 100 | * | * | * |
| | 50.0 | * | * | * |
| | 25.0 | * | * | * |
| | 12.5 | 48 | * | * |
| | 6.25 | 30 | 36 | 32 |
| ***Benzalkonium chloride + chloroexidine*** | 0.2% (vol/vol) | 12 | 6 | 8 |
| ***Ethanol*** | 70% | 3 | 1 | 1 |

The results of *agar disc diffusion* assays are summarized in Table 1, showing a strong antimicrobial activity of both essential oils against bacterial and fungal colonies, consistent with the dedicated literature (Reichling & al. 2009; Stupar & al. 2014; Casiglia & al. 2015), although little is known about the permanence and application methods of EOs (Salem & al. 2014; Noshyutta & al. 2016).

In this case study *Origanum vulgare* and *Thymus vulgari*s EOs solution (100, 50, 15 %) have been tested *in situ*, on mosaic tesserae of "Casa di Leda", after seven days of application.

Particularly, *Thymus vulgari*s 15% EO solution has proven to be the best diffused, strongly influencing the *biofilm* liveliness (Fig. 12).

Although further studies are needed to set up a standard protocol, according to these and previously result (Rotolo & al. 2016, 2017), we hypothesize the use of OEs to contrast microbial colonization, representing valid alternatives to traditional biocides, without negative environmental impacts and respecting the human health, in accordance with modern restoration strategies.

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