SAPIENZA
UNIVERSITÀ DI ROMA

Doctoral School "Vito Volterra" in Astronomical, Chemical, Earth, Mathematical and Physical Sciences

PhD degree in Earth Sciences
Curriculum "Environment and Cultural Heritage"
CHIM/12
XXXIII Cycle

Ecosustainable and phytobased alternative methods for the conservation of biodeteriorated stone materials

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2021

To my family,

Riassunto

Il presente lavoro di tesi propone di sviluppare un metodo finalizzato al trattamento di materiali lapidei biocolonizzati, tramite l'impiego di sostanze chimiche di origine naturale (i.e. fitochimici), caratterizzati da un basso impatto ambientale, una bassa tossicità per l'uomo e che non compromettano lo stato conservativo dei beni culturali.

In questa prospettiva, sono state sfruttate le già note proprietà biocide di tre Oli Essenziali (EOs) già largamente studiati e impiegati in diversi ambiti, tra cui quello della conservazione dei beni culturali. Tali sostanze sono ottenute a partire da piante molto comuni della flora mediterranea, ed in particolare si tratta degli oli di *Origanum vulgare*, *Thymus vulgaris* e *Clinopodium nepeta*. Ogni olio essenziale ha una composizione chimica molto eterogenea, sebbene siano sempre presenti uno o due componenti chimici maggioritari, anche definiti Principi Attivi (APs), che ne definiscono il chemiotipo e sembrerebbero condizionarne l'azione biocida, sebbene non sia ancora stato sistematicamente dimostrato. In virtù di quanto detto, l'effetto biocida dei tre oli essenziali è stato confrontato con quello dei loro rispettivi principi attivi ovvero: carvacrolo per *O. vulgare*, timolo per *T. vulgaris* e pulegone per *C. nepeta*.

Per valutarne un possibile incremento dell'azione biocida, ogni olio è stato applicato singolarmente e in combinazione con gli altri due, in modo da creare diverse miscele contenenti rispettivamente, uno, due e tre composti, e lo stesso è stato fatto per i principi attivi. L'applicazione sulle superfici lapidee biocolonizzate ha previsto l'incapsulamento delle sostanze attive all'interno di una matrice idrogel (HG) di nuova formulazione, composta dalla miscela di sostanze polimeriche e tensioattivi, i cui più grandi vantaggi sono legati all'atossicità nella composizione chimica e alla capacità di formare un film omogeneo, pelabile, facile da rimuovere dalle superfici una volta asciutto. I composti sono stati testati su diversi biofilm presenti su substrati lapidei in granito e travertino. In particolare, le sperimentazioni hanno previsto l'applicazione su campioni di granito (in laboratorio) e su due superfici murarie, una in granito e una in travertino, esposte a diverse condizioni ambientali. Per la valutazione dell'azione biocida e pulente dei composti fitochimici, sono state effettuate misure non invasive di colore e di fluorescenza che potranno essere replicate anche sui beni culturali. Il sistema ottenuto dalla combinazione dell'idrogel e dei fitochimici

(phyto-HG) ha mostrato buoni risultati per quanto riguarda l'azione pulente e biocida nei confronti di differenti biofilm, anche rispetto a quelle dimostrate da un sistema classicamente utilizzato per la pulitura meccanica dei beni culturali (spazzolatura) e di un comune biocida (Preventol® RI80).

Le prove sperimentali hanno messo in evidenza l'efficacia dei principi attivi i quali, possono essere considerati, nella maggior parte dei casi, più efficienti rispetto ai corrispondenti oli essenziali. Questo risultato è molto incoraggiante per le future sperimentazioni riguardanti lo sviluppo del metodo, che potrebbe prevedere l'impiego dei soli principi attivi per la realizzazione delle formulazioni biocide, con i notevoli vantaggi legati ai minori costi di produzione e alla possibilità di ridurre i limiti legati all'impossibilità di controllare le composizioni chimiche degli estratti naturali.

L'azione ottenuta dalla combinazione di più sostanze necessita ulteriori approfondimenti, in quanto sembrerebbe che la loro azione sia condizionata dalla sinergia che si ottiene da più sostanze nei confronti di specifici microorganismi presenti.

Il monitoraggio a medio e a breve termine effettuato sui campioni sperimentali ha evidenziato un'azione prolungata delle sostanze nel tempo.

Sebbene questo non possa essere considerato sempre vantaggioso, in quanto ci si potrebbero aspettare delle reazioni secondarie che potrebbero verificarsi tra i residui e la matrice lapidea, è importante comunque sottolineare i vantaggi legati all'inibizione a lungo termine nei confronti di una possibile ricolonizzazione della superficie. Per queste ragioni, risulta importante analizzare più approfonditamente la stabilità dei fitochimici nel tempo, tramite l'osservazione di possibili modifiche delle proprietà chimiche ed estetiche dei materiali.

Abstract

The present PhD thesis aims to develop a valid strategy for the treatment of the biocolonization of stone materials, by employing natural chemical substances (i.e. phytochemicals) harmless towards ecosystems, human health, and cultural heritage materials. To achieve this goal, the well know biological properties of the Essential Oils (EOs) have been exploited, by employing three of these plant extracts that have already demonstrated inhibitory effects towards different microorganisms. These oils are obtained from Mediterranean plants of the Lamiaceae family, in particular: *Origanum vulgare*, *Thymus vulgaris* and *Clinopodium nepeta*. Each EO is characterized by the presence of one or two mayor chemical components, also defined Active Principles (APs). It seems that the biocidal action of each EO is established by the presence of specific APs, although this was not systematically proven. To demonstrate what has just been said, in this study, the effect of the EOs has been compared to the one of the respective APs, which are carvacrol for *O. vulgare*, thymol for *T. vulgaris* and pulegone for *C. nepeta*. Each EO has been applied alone and combined with the two others, in mixtures containing two and three EOs. The same process occurred with the APs. This was done to assess a possible empowerment of the biocidal action when more substances are combined. For their application on biodegraded stone surfaces, the phytochemicals were incorporated inside an innovative hydrogel (HG) matrix based on a mixture of polymeric substances and surfactants; whose main advantage is linked to its ability of forming a peelable layer when applied on surfaces. The compounds were tested on biofilms, colonizing granite and travertine surfaces. The experimentations were performed on granite samples and on two biocolonized walls, one in granite and the other in travertine. The biocidal and cleaning properties of the systems composed by the phytochemical substances and the hydrogel have been assessed through non-invasive colorimetric and fluorescence measurements. The phyto-HG systems demonstrated good cleaning and large spectrum biocidal properties against different biofilms, also compared to the ones of a commonly employed mechanical cleaning tool (brushing) and a common biocide (Preventol ® RI80).

The APs also showed their broad-spectrum biocidal action, which can be considered, in many cases, higher than the one of the respective EOs. This is an encouraging result, in

sight of possible future developments of the method, that will provide the employment of the only APs for the creation of the formulations, with notable advantages linked to the reduction of the production costs and the possibility of reducing the limits linked to the impossibility of controlling the chemical composition of the natural extracts (i.e. the EOs).

The action of the combined substances compared to biofilms needs to be further analysed, because it seems that their action is strongly linked to the synergism occurring between two substances and the specific microorganisms present.

The short-term and medium-term monitoring carried out on the experimental samples evidenced a prolonged action of the substances in time. Even though this is not always encouraged, since possible secondary reactions may occur between the chemical residuals and the mineralogical matrix, it is important to stress the advantage related to the long-term inhibition towards a secondary biological growth on the surfaces. For these reasons, it will be important to further analyse the stability of the substances in time, by observing possible modifications in the properties of the stones during the time.

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Acronyms

AgNPs	Siver Nanoparticles
APs	Active Principles
BAC	Benzalkonium Chloride
CH	Cultural Heritage
Chl a	Chlorophyll a
C. nepeta	*Clinopodium nepeta*
DGGE	Denaturating Gradient Gel Electrophoresis
EOs	Essential Oils
HSTs	Heat Shock Treatments
HG	Hydrogel
JND	Just Notable Difference
O. vulgare	*Origanum vulgare*
PAM	Pulse Amplitude Modulated
PCR	Polymerase Chain Reaction
PVA	Polyvinyl alcohol
QACs	Quaternary Ammonium Compounds
rRNA	Ribosomal RNA
SABs	Sub-Aerial Biofilms
SCE	Specular Component Excluded
SCI	Specular Component Included
T.vulgaris	*Thymus vulgaris*

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Aims

Biodeterioration is a detrimental phenomenon that involves all the physical, chemical and aesthetical damages on materials caused by microorganisms. They colonize materials, merging in a more advantageous microsystem (Sub Aerial Biofilms, SABs) that allow them to live in symbiosis and better overcome external stresses.

Due to their natural porous nature and, in many cases, their outdoor exposition, stone surfaces are a suitable substratum for the proliferation of these microorganisms.

Moreover, stones represent one of the most employed materials in the field of cultural heritage artefacts and, as such, it seems evident that they must be preserved from the biocolonization and the consequent deterioration.

Between the most frequently found biocolonizers of stones we found *algae*, *cyanobacteria, bacteria* and *fungi* that together contribute in different ways to the weathering processes for example: with the secretion of acidic substances that dissolve the inorganic material, with the penetration of adhesion structures that accelerate the internal mechanical stresses and with the production of metabolic pigments that, alter the original aspect of the work of art.

In light of the above, many physical, mechanical and chemical tools have been employed over time to avoid and to limit the biodeterioration phenomenon. However, in many cases, these tools present some important drawbacks that contribute to the worsening of the existing damage. For instance, the residues of the used biocides can become additional degradation factors on the material, not only from a chemical point of view but also from an aesthetic one, due to the chromatic alterations produced by the aging or, on the other hand, they can be substances with some kind of environmental impact which hinders the use on architectural artefacts and works exhibited outdoors in parks and gardens, as well as creating a possible health threat for the operator.

For those reasons, the community of conservation scientists is trying to find different alternatives to the traditional systems used to control biodeterioration.

In this regard, natural compounds extracted from plants are receiving great attention and, among them, is deserve to essential oils (EOs) a special mention.

Essential oils are heterogeneous mixture of phytochemical compounds, produced by plants as secondary metabolites, useful for their protection against pathogens and to attract pollinators in the way to promote and facilitate their reproduction.

Their biocidal properties are well known since antiquity and, nowadays, they are employed in many fields, including medicine, pharmacy, the food industry and personal care.

EOs can be extracted from each part of the plant, and their complex chemical composition (above 20-70 chemicals present at different concentrations) is strongly affected by the harvesting time, the growth media and the external environmental conditions.

However, the presence of one or two main components (i.e. Active Principles. APs), is assessed for each essential oil and these ones establish the specific chemotype and chemical properties. The quantification of the contribution of the APs in the biological properties of the essential oils is a topic that is receiving the attention of the researchers, in order to better understand the mechanisms involved in the biocide action of these compounds.

This research proposes to systematically study the biocidal effects of i) three different essential oils alone and combined ii) as well as their active principles, iii) in different application conditions, as natural biocides for the elimination of heterogeneous SABs using two different lithotypes as models: travertine and granite. These are among the most common building materials employed in the city of Rome, in the first case, and in the second case, in Galicia (NW Spain), where part of this research has been carried on.

The phytochemicals selected for the experimentation are three essential oils extracted from three Mediterranean Lamiaceae: *Origanum vulgare*, *Thymus vulgaris* and *Clinopodium nepeta*. The choice was made on the basis of the excellent biocidal properties already demonstrated by the previously mentioned substances in many research fields; the conservation of cultural heritage field included. On the other hand, the APs are the three terpenoids thymol, carvacrol and pulegone characterizing the composition of *T. vulgaris*, *O. vulgare* and *C. nepeta* oils respectively.

However, even though different existing studies have been performed in plate, few researches have tackled the application of these substances (both EOs and APs) on the field in real study cases, so far. For this reason, it was decided to operate directly on stone

materials by applying on them two groups of formulations containing, on one side, the EOs and, on the other side, the APs. For each group, each substance has been employed alone and mixed with one or two others, in order to assess a possible enhancement of the biological activities of two or three combined substances.

The application of the treatments was conducted combining the compounds with an innovative hydrogel matrix that easily allowed the preparation of emulsions to be applied and, at the same time, removed from the surfaces due to the filming properties of the hydrogel itself. The treatments have been applied both in laboratory (environmental controlled conditions), on biocolonized granite samples, and on site, on two outdoor exposed biocolonized surfaces: one in travertine and the other in granite.

The characterization of the microorganisms composing the biofilm and the evaluation of the cleaning and biocidal efficacy of the compounds have been always performed trying to accomplish the principles of non-invasiveness and non-destructiveness, in order to develop a strategy that could be applied in the future on real cultural heritage materials. These techniques included colorimetric characterization, fluorescence measurements and metagenomic characterization of the microorganisms.

In sum, this research aims at ideating a preliminary systematic strategy for the application of innovative cleaning and biocidal compounds for the elimination and the controlling of the biocolonization of stone materials. It has been designed by the employment of compounds and analytical methodologies harmless to the enviroment, the human health and the stone materials themselves.

The advantages related to the success of the research include i) the possibility of preparing sustainable treatments that could control the biocolonization, ii) these treatments may provide the employment of the single APs, and not only the EOs, in order to control the composition of the chemicals present and overcome the problems related to the uncontrollability of the composition of the oils and their high costs, iii) the enhancement of the cleaning of stone surfaces by employing a peelable hydrogel matrix able to lift the most superficial biofilm/dirt layers and iv) the employment of non-invasive and non-destructive analytical methods for the investigation on cultural heritage surfaces.

Being this a pioneer study and not being able to predict all the possible drawbacks related to the interaction of the substances with the substrata, the chosen experimental

surfaces, although they can be considered real samples as they belong to buildings exposed to the outdoors, are not characterized by any historical or artistic value.

The PhD thesis is divided in four chapters:

Chapter 1. General Introduction: The scientific background concerning i) the problem of the biodeterioration, ii) the advantages related to the employment of phytochemicals for the control of biodeterioration and iii) innovative soft materials for the cleaning of cultural heritage, iv) the non-invasive and non-destructive analyses for the evaluation of the biofilms and the microorganisms composing it, have been reported and commented.

Chapter 2. Materials and Experimental set-up: the materials and the experimental set-up of all the techniques employed are reported. In particular, the research has provided the set up for three different experiments, performed on biocolonized i) granite samples, ii) traverine wall, iii) granite wall. For each experiment, the same phytochemicals have been applied in different combinations. The experimental procedures adopted for each experiment have been in detail described.

Chapter 3. Results and discussion: In this section, the experimental results obtained for each performed experiment have been separately analysed. Moreover, the chapter start with the results concerning the methodology adopted for the ideation of the formulations containing the phytochemicals and the compatibility with these latter substances with the hydrogel matrix and the stone substrata.

Chapter 4. Conclusions: In this chapter, the main results obtained for each experiment have been summarized and commented. The effectiveness of the phytochemicals in the controlling of the biodeterioration of stone surfaces, also in comparison with other substances commonly employed as biocides it has been stressed.

Appendices: In this section it is possible to find the published articles which touch the same topics as the PhD thesis.

CHAPTER 1. General Introduction

1.1. The Biodeterioration of stone materials

Natural and artificial stones have always been employed in the construction of buildings and artistic artefacts, making these materials a significant part of the world cultural heritage. Stone's artefacts go through a progressive and natural decay, that can be accelerated by bad conservative conditions and by the exposition to different physical, chemical and biological agents, acting in co-association in the deterioration process [1].

Although in the past chemical and physical processes were believed to be the dominant factors of material decay, the importance of the biological effect was widely recognized [2,3], so much that it was estimated that 20-30% of stone's deterioration is the result of biological activity [4] and biological factors are considered the second cause of decay of mural paintings [5].

In light of what has been said, the presence of coexisting groups of microorganisms (it is very rare to find communities of single species [6–8]) leads to the **Biodeterioration** (Figure 1) of the rocks, which is classically defined as "*any undesirable change in the properties of a material caused by the vital activities of living organisms*"[9].

The colonization starts with the autotrophic photosynthetic microorganisms (*algae* and *cyanobacteria*), considered the pioneers in the ecological succession because they find a natural niche for their growth and proliferation in the rock's substrata. The lytic inorganic substratum, in fact, does not provide the organic material necessary for the nutrition of heterotrophic microorganisms, i.e. *fungi* and *bacteria*, or the second in the ecological succession, but it constitutes a natural source of water (mainly, the moisture retained in the porous stone matrix), which is the element necessary to allow the vital activities of autotrophic microorganisms [10–13].

Moreover, some microorganisms find in the stone surfaces and in their irregularities (i.e. pores, fissures and cracks) an adhesion site and a favourable and protected environment where to proliferate (endolithic colonization [14]), that allows them to growth away from undesirable environmental conditions, such as energetic solar radiations, temperature fluctuations, wind and desiccation [15].

Associated with the initial colonization, the microbial cells produce *extracellular polymeric substances* (EPS), such as polysaccharides, liposaccharides, proteins, glycoproteins, lipids, glycolipids, fatty acids and enzymes [1]. The EPS have different roles in the colonization: they favour the adhesion to the substrata, retain water for long periods, maintain the viability of the cells, facilitate the access to water vapour in the atmosphere, facilitate entrapment of airborne particles, aerosols, minerals and organic compounds [1,6,7]. Moreover, the EPS and, in general the accumulated photosynthetic biomass [16], provide an excellent nutrient base for the growth of heterotrophic microflora [1].

In communities of such microorganisms lichens can also be present [10,13] and then the initial microbial colonization is followed by macroscopic vegetation, which contribute to the worsening of the biodeterioration in the more advanced phases [1,7]. What has been previously described is the general mechanism of colonization. However, it is not uncommon that colonies of chemotrophic microorganisms can replace the pioneer photosynthetic microorganisms in the ecological succession: if available, they can colonize the surfaces by employing the accumulated organic material on the surface, such as airborne particles, organic vapours, the organic material naturally present in the sedimentary rocks, biomass of other microorganisms and residuals of restoration products [16]. At the same time, the early proliferation of some others halotolerant/halophilic microorganisms is favoured by the presence of soluble salts , present under the form of efflorescences or subflorescences inside the stones [2]. Among the halotolerant/halophilic species detected on salt-attacked heritage materials, there are the microorganisms belonging to *Gammaproteobacteria* (such as the genera *Idiomarina*, *Salinisphaera* and *Halomonas*) and *Firmicutes* (*Halobacillus* and *Bacillus* spp.), but also species of the phyla *Bacteroidetes* and *Actinobacteria* (as *Rubrobacter*), as well as archaea belonging to *Halococcus* and *Halobacterium*. The biodetrioration associated to this specific kind of biocolonization is characterized by the presence of rosy stains that compromise the original aspect of the substratum [2].

The whole complex system constituted by the association of the microorganisms (as said before, mainly *algae, cyanobacteria, fungi* and *bacteria*) embedded in the EPS, is defined Subaerial Biofilm (SAB), which is and ubiquitous, self-sufficient, miniature microbial ecosystem [7,17]. The main advantage of the SAB communities is that together

the microorganisms overcome environmental stresses better than any of them could do individually. This aspect concerns, for example, the resistance of this complex systems against the biocides or a better response against stressful environmental conditions [6,15].

The biodeterioration caused by microbial colonization can lead to different damages of the stones, involving aesthetical modifications of the original aspect of the artefact but, more relevantly, structural damages of the stone matrix induced by the biogeophysical and biogeochemical interactions of the SAB with the material [1,6,10].

The aesthetical damages are mainly related to the discolouration [18,19] of the substratum induced by the biogenic pigments (green chlorophyll, brownish melanin, red carotenoids) [1,12] produced by the microorganisms. Depending on the microorganisms involved, it is possible to have different changes in the original colour of the surface: i) a greening, caused by the photosynthetic pigments from algae and cyanobacteria [1,19]; ii) a blackening, caused by melanin, melanoidins produced by dematiaceous fungi and filamentous cyanobacteria and chlorophyll degradation products [1,20]; iii) a yellowing caused by carotenes and carotenoids of photosynthetic microorganisms and pigments produced by chemoorganotrophic bacteria, as well as degradation products of cyanobacteria and algae with iron enrichment [1]. Even the presence of the EPS may produce discolouration, because the airborne particles entrapped in the matrix can modify the original colour of the surface [1]. Furthermore, the differential colouration existing between the darker areas, characterized by the presence of microorganisms, and the uncolonized brighter ones, can lead to different mechanical stresses related to the temperature differences between the zones, including alterations of the thermo – hydric expansion phenomena and increasing of water retention [21]. Darker areas, can in fact, absorb more solar radiation than the brighter ones, with a consequent differential enlargements and contractions of the dark parts, that imply a mechanical stress that contribute to the physical deterioration of the surface [1,6,13], with a mechanism similar to the one occurring in the black crusts phenomenon.

In this regard, also in the black crusts formations microorganisms have a role: for example, sulphur bacteria can convert calcium carbonate in crystals of gypsum , contributing to the formation of the corroded receptive zones in which the organic and inorganic materials dispersed in the environment can accumulate. Black crusts are composed mainly by pollutants and airborne dispersed in the air, but also spores and microorganisms themselves

[13,22]. Meanwhile, the presence of the accumulated materials inside the crusts can favour the re-colonization of the surfaces in the already deteriorated areas, with a consequent worsening of the overall situation [1,16,20,23,24]. In summary, black crusts and dark discolouration can be associated, not only to undesirable aesthetical modifications of the surfaces, but more relevantly to mechanical damages [13], identified as all the modification of the surfaces related to a mechanical action, where the spontaneous movements or the growth of the colonizing living organisms also play a role, with strong consequences on the loss of coherence of the original material, detachments, increasing of the porosity and enlargement of the natural pores and fissures of the stones [11,16,25].

Particularly relevant in this sense is the penetration of the filaments employed for the adhesion of the microorganisms inside the stone matrix [1,6,14] and the endolytic colonization in the natural fissures of the stones where, the expansion of the cells, due to their hydration and dehydration depending on the water availability, exert a pressure on the stone's walls contributing to the enlargement of the natural discontinuities.

The mechanical stresses can be induced also by the only presence of the EPS, that causes the deterioration of the mineralogical structure of the stones due to shrinking and swelling cycles of the colloidal biogenic slimes inside the pore system [1]. In this regard, the studies confirmed the role of the photosynthetic microorganisms both in the physical and chemical weathering of stones because, for a long time, it was believed that the contribution of this microorganisms in the biodeterioration was only aesthetical. [14,26].

The chemical damage related to the interactions between the inorganic matrix and the products of the metabolic activity of the microorganisms also has a great importance, being one of the major causes of biodeterioration of stones [13]. The best known and studied consequence of chemical biodeterioration is the biocorrosion [1], caused by the microbial secretion of inorganic (nitric acid, sulphuric acid, carbonic acid, nitrous acid) and organic (oxalic, citric, acetic, gluconic, malic, succinic) acids [6,13]. The biocorrosion can lead to the solubilization and a weakening of the stone matrix [25]. The presence of inorganic acids (mainly HNO_2, HNO_3, H_2SO_3 and H_2SO_4 [13]) or organic ones (the ones possessing non- or weakly complexation), in presence of water, mainly contributes to the *acidolysis* phenomenon. In this case, strong acids can interact with some mineralogical components, solubilizing the inorganic material and leading to the formation of soluble salts [1,13,21,25].

Besides the loss of the original artistic value, due to the transformation of the original mineralogical components in less “noble” materials (for example, the already mentioned transformation of calcium carbonate in gypsum, caused by the reaction of $CaCO_3$ with H_2SO_4 [21]), the presence of salts (including sulfate and nitrate both as efflorescence and subflorescence) is associated to others detrimental phenomena that contribute to the worsening of the chemical and mechanical damages, through cyclic wetting and drying, capillary rise and evaporation, and hydration, especially when water is seasonally available [13]

The acidolysis may be started directly by some organisms that naturally produce acids as metabolic substances. This is the cases of *Thiobacillus*, *Thiothrix*, *Beggiatoa* and some fungi, that produce sulphur acids [27], while the nitric acid may be produced by ammonia and nitrite oxidizers, heterotrophic nitrifiers and other species of fungi [6].

However, every microorganism is potentially able to start acidolysis, simply with cellular respiration and metabolic production of carbon dioxide. In fact, the CO_2 produced by the cellular respiration reacts with the water (normally present on the surface or inside the stone matrix) and generates H_2CO_3. The carbonic acid induces the acidolysis by reacting with the minerals containing calcium and magnesium, particularly frequent in calcareous stones and mortars, to then form calcium and magnesium bicarbonates, much more soluble than the original mineral phase [21].

Another remarkable phenomenon caused by organic acid secretion is the *complexolysis*, where some metabolite acids, contained in various chemical compounds, including polyols, sugars, glycerol, polysaccharides, proteins, pigments, lipids and organic acids [13], can chelate the metal ions present inside the minerals (e.g., Ca, Al, Si, Fe, Mn and Mg), forming an unreactive organo-metal complex [25]. The most studied in this sense is the oxalic acid, mainly produced by lichens [28,29] and fungi [30], that enhances the dissolution of siliceous rocks, but it is also held responsible for the calcium oxalate patinas, both in the monohydrate (whewellite) and the biidrate (weddellite) forms [13]. The role of this patinas is not completely clear: researchers are discussing about the possibility that its presence on carbonate rocks can constitute a protective stratum, which also increase the artistic and visual value of the opera [1,29]. Other notable biogeochemical interactions

investigated on stones are caused by the presence of enzymes, the selective mobilization and accumulation of elements, cationic exchanges and alkaline reactions [21].

Figure 1 - Influence of biodeterioration processes on an angel statue at the "Peters"-Portal on the cathedral of Cologne (Germany); documented by the original object in 1880 ((a) photograph by Anselm Schmitz, Cologne) and the respective weathered statue in 1993 ((b) photograph by Dom1.2baumeister Prof. Dr. A. Wolff, Cologne)**(Picture from Warsheid and Braams 2000 [1])**

1.2. How to control the biodeterioration: traditional methods and new eco-friendly frontiers

Considering the impact of the biocolonization on cultural heritage materials, different methods have been developed and employed during the years to control this phenomenon.

An initial classification can be done, distinguish the indirect from the direct methods.

With indirect methods, also defined by Warsheid "Good House-Keeping" [12], we define all the modifications carried by external ecological factors that favour the growth of microorganisms, trying to control, for example, humidity, temperature, sun exposition etc. In controlled environmental situations, this strategy is strongly recommended, while, for outdoor exposed stone monuments, the control of the external environmental conditions is obviously very complex [6,12,13,31,32].

An indirect control of the biocolonization can also be led by exploiting the capabilities of some microorganisms themselves in the selective removal of specific detrimental compounds deriving from old interventions of restoration, varnishes, glues and also, secondary products of the metabolism of the microorganisms, such as black crusts, nitrates, sulphates and pigments [26,33–39] that can favour the colonization of the surfaces, as previously stated in section 1.1. This process, that includes the employment of microorganisms, microbial secondary metabolites and enzymes, is called 'bio-cleaning' or 'biorestoration' and is drawing the attention of the researchers for its ecological sustainability. Moreover, selective calcifying bacteria (e.g. *Bacillus* sp. and *Acinetobacter* sp.) can act as bioconsolidant, by the bio-precipitation of calcium as calcium carbonate, for on site restoration of calcareous stones, reducing stones' discontinuities and fractures that favour the water penetration and the microbial colonization [13]. On the other hand, some microorganisms (and their secondary metabolites) can act as direct controllers of the biocolonization [13], as in the cases of selective *Bacillus* species, which are able to produce antifungal peptides or biosurfactant lipopeptides for the inhibition of fungal growth on mural paintings [8,40,41] , or selective fungi (e.g. *Aspergillus allahabadii*), capable of removing microbial biofilms on stone monuments [42].

However, it is necessary to know that the employment of bacteria and enzymes for the biorestoration must be preceded by an accurate *screening* phase. All the potential drawbacks related to the presence of the microorganisms must be excluded, in particular: potential risks for human health, caused by pathogens microorganisms and the production of spores that allow the survivor of the microorganisms in a quiescent metabolic phase. Indeed, for what concerns the employment of the enzymes, it is possible to encounter difficulties in the removal of complex molecules that require the combined action of a pool of combined enzymes, that cannot be commercially available [37]. After the biorestoration a careful *soft cleaning* must be provided, in order to eliminate all the possible organic and inorganic residuals on the surfaces [37].

The direct methods concern all chemical, physical, mechanical and biological systems that allows to eliminate the already formed SAB from the stone surface. Traditional mechanical methods include brushes, scalpels, spatulas, scrapers, air abrasives, high-pressure blasting, low-pressure washing, and vacuum cleaners [43] However, the mechanical

removing of the microorganisms can be too aggressive on the substratum and so their employment is not always recommended [3]. In this scenario, the attention of the researchers is draw to the development of new physical non-aggressive methods that involve the employment of electromagnetic radiation, such as UV, lasers, microwaves, gamma rays though some side effects have been evidenced. These ones mainly concern the preservation of the operators health, given the energy of the employed radiation (UV and γ-rays), the penetration power of the radiations, the unsatisfactory complete removal of the deposits and possible secondary interactions with the materials (mostly colour changes), especially in the case of multi-material works of art (frescoes, statues, decorated stones etc.) [2,3,33]. In order to limit these potential drawbacks, new strategies have recently been studied and developed, such as methodologies that imply thermic modifications towards higher or lower temperatures (Ice-Clean system ® and Heat shock Treatments - HSTs [3]). However, the novelty of the procedures did not allow to have a complete evaluation of their potentially linked risks on the cultural heritage materials.

Since forever biocides, i.e. all the toxic chemicals able to kill microorganisms [16], represent the most common systems employed in the control of the biocolonization There is a very large number of chemicals employed as biocides, that can be applied on the surfaces in many ways (i.e. aerosol, brushes, injection, immersion or as a paste). They range from simple inorganic compounds, like sodium or calcium hypochlorite, to organometallic compounds, complex organic compounds like quaternary ammonium, aromatic compounds such as formaldehyde, phenol and its derivates, urea derivates, halogenated compounds like chlorine and iodine; pyridine derivatives, nitrogen containing compounds and isothiazol derivates. Alternative substances include metallic salts and oxides (e.g. carbonates and oxides of copper and zinc); acetic or salicylic acid; borax; p-hydroxybenzoic acid (PHB) esters and ethereal oils [16]. The chemicals can be used alone or combined with others, in order to eliminate the largest number of microorganisms, since no biocide is uniformly effective on all organisms [44,45].

However, many problems are related to a large number of these chemicals, especially concerning the biodegradability and the toxicological risks for the restorers and conservators applying the treatments [46].

Among the most employed biocides, given their well-known broad spectrum antimicrobial efficacy against a wide range of microorganisms (including bacteria, fungi and algae), are the ones based on Quaternary ammonium salts (QACs), approved for conservation of cultural heritage monuments by the European Biocide directive as relatively environmentally friendly with a relatively small hazard to the substratum [16,47,48]. The mechanism of action of these compounds is related to their ability to disrupt the microbial cytoplasmatic membrane and interfering with the proteins, killing the microorganisms. In this group, Benzalkonium Chloride (BAC) is included and represents the base for many different products available on the market (for example: Preventol ® RI50, RI80, RI90, Hyamine 3500, Cequartyl, Neo-desogen etc.) [32,49]. However, different studies have stressed out the possible side effects related to the employment of these compounds. First of all, it has emerged that they favour the re-growth of other harmful microorganisms, able to employ the residue of the biocide as a carbon source [1,2,4]. In this regard, the most notorious case of recolonization is the one of the Lascaux Cave, reported in the studies of Bastian et al. (2009) [50] Martin-Sanchez et al. (2012) [51], where the prolonged employment of Benzalkonium chloride based biocides for the elimination of *Fusarium Solani,* increased the level of organic carbon available on the surface, facilitating the recolonization by other fungi (*Ochroconis lascauxensis*) and bacteria (*Ralstonia* spp. and *Pseudomonas* spp.), highly resistant to BAC and not originally detected on the surfaces. A similar case was detected in the archeological site of S. Paul in Ephesus (Turkey), where a restoration campaign for the elimination of a massive algal and cyanobacteria biocolonization on Christians wall paintings with QACs based biocides lead to the growth of a more resistant community of microorganisms, including melanized fungi, causing severe aesthetical damages to the surfaces [2,52,53].

Moreover, it was assessed that QACs are shown to contribute to the degradation of lime mortars and hardened Portland cement [44].

In light of what has been said, it seems clear why the researchers are moving towards the investigation of new alternative solutions for the control of this problem, always taking into account the efficacy in the removal of the SABs and the minimum side effects on human health and the conservation of the ecosystems. In this aspect, a valid solution is represented by phytochemicals, as will be widely discussed in the following section.

1.2.1. Phytochemical compounds

Recently, the attention of the researchers is focused on the employment of eco-friendly natural substances for the control of the biocolonization. These methods can indeed represent a valid alternative to traditional chemical biocides, showing lower ecological impact on the ecosystems, but also lower risks for the human health [3,54,55].

Very promising results have been obtained through the employment of plant extracts and, among these essential oils are receiving great attention [31,32,35,56–60].

Essential oils (EOs) are secondary metabolites of the plants, playing different roles in their protection as antibacterial, antifungals, insecticides and in the mechanisms of attraction of pollinators. They preserve their biocidal properties when extracted by steam or hydro-distillation from all vegetal materials (i.e. roots, leaves, flowers, seeds, fruits etc.) and, for this reason, they have been employed in food preservation and in medical and personal care since ancient times [54]. These substances are very heterogeneous mixtures of **phytochemicals** (i.e. biological active chemicals produced by plants [61]), containing about 20-60 components at different concentrations. Being natural products, it is impossible to establish a univocal chemical profile characteristic of an oil extracted from a specific plant species: their composition, in fact, strongly depends from many factors, such as the harvesting time, the part of the plant employed, the growth medium and the external environmental conditions [62,63]. However, each essential oil is characterized by the presence of one or two main components (20-70% of the total concentration), which establish its chemotype, while the others can be present at lower concentrations or in traces. The complex mixture of substances contained in the oils belong mainly to two groups of different biosynthetical origin: terpenes and terpenoids and aromatic and aliphatic components [64]. Different studies have been led to assess the biological activities of essential oils and to identify the mechanisms involved in the biocidal action.

The mechanisms of antimicrobial action of EOs are multiple, changing in different ways the cell membrane permeability of the microorganisms, leading to the release of cytoplasm material and nucleic acids, and sometimes affecting the mitochondrial membrane; at times they also show insecticidal activities [31].

Moreover, it appears that the biological activity is strongly influenced by the presence of the main compounds, and that the ones containing a higher percentage of phenolic terpenes are the most effective against a large spectrum of microorganisms [47,54,65–67], including bacteria, fungi and cyanobacterial strains commonly found on cultural heritage materials. On the other hand, it seems that the antimicrobial action can be determined by the synergism of the whole phytochemicals composing the oil [66]. However, the scientific community is moving towards the investigation of the biocidal potential of the single active principles (APs, i.e. chemically defined constituents, present in the drug and responsible for biological activity [68]). For example a medical study [69] has demonstrated that carvacrol and thymol (i.e. the main components of oregano essential oil), have an inhibitory action against methicillin-susceptible and methicillin resistant staphylococci comparable to the one of the *O. vulgare* EO itself, confirming the relevant antimicrobial activity of these compounds inside the oil. A more recent study by Veneranda et al. (2018) [70] tested ten APs belonging to as many EOs (thymol from *thyme*, menthol from *mint* sp., linalool from *coriander* sp., eucalyptol from *eucalyptus* sp., cinnamaldehyde from *cinnamomum* sp., eugenol from *clove* sp., cuminaldheyde from *cumin* sp., limonene from *citric plants*, cytral from *lemongrass* sp. and citronellol from *rosae* sp.) against *Aspergillus niger* strains, a very common spoilage fungus of cultural heritage. The long-lasting antifungal activity of the substances was demonstrated, especially for thymol, eugenol and cinnamaldehyde.

Among the previously mentioned plants having biological activities the ones belonging to the Lamiaceae family deserve a special mention , that includes EOs particularly rich in volatile monoterpenes, sesquiterpenes and diterpenes [71,72]. Although Lamiaceae are widespread all over the word, they are very represented in the Mediterranean flora and some of the most studied essential oils, that demonstrated excellent biological properties against many species of pathogens, are extracted from plants coming from this area [63]. This is the case of the three essential oils of *Origanum vulgare*, *Thymus vulgaris* and *Clinopodium nepeta* (L.) Kuntze (or *Calamintha nepeta* (L) Savi [1]) [73], whose biological

[1] This plant, commonly known as lesser calamint, has been identified by many botanical synonyms. Currently, the accepted name is *Clinopodium nepeta* (L) Kuntze [164]. However, it has been named for a long time also as *Calamintha nepeta* (L) Savi. For this reason, the published papers based on this research and the papers in literature refer to this plant as *Calamintha nepeta*.

activity have been widely investigated in different research fields, including the one of conservation of stone materials. In particular, *O. vulgare* and *T. vulgaris* are among the most studied essential oils against common biodeteriogens of cultural heritage materials [31]. These compounds demonstrated excellent biological activities against fungi and bacteria isolated from stone surfaces, also compared to other essential oils. This is evidenced in the study cases lead by Mironescu et al.(2009 and 2008) [74,75] where it emerged that wild thyme and common thyme (i.e. *Thymus vulgaris* and *Thymus serpyllum*) are the most powerful essential oils against moulds isolated from cultural heritage (*Alternaria* sp., *Aureobasidium* sp. and *Penicillium* sp.) compared to other tested essential oils. These promising results lead to other evaluations, concerning the preparation of *T. vulgaris* EO based compounds to control the growth of the three previously mentioned moulds [76]. A Similar study of Stupar et al. (2014) [47] that compared the biocidal potential of *Origanum vulgare*, *Lavandula angustifolia*, *Rosmarinus officinalis* oils (Lamiaceae) against six fungal strains isolated from mural paintings (*Aspergillus niger* Tiegh, *Aspergillus ochrauceus* G.Wilh, *Penicillium* Link sp., *Thricoderma viride* Pers., *Bipolaris spicifera* (Bainier) Subram, and *Epicoccum nigrum* Link) has demonstrated that the *O. vulgare* has the higher antifungal activity. Moreover, *O. vulgare* oil has an antifungal activity against *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus versicolour*, and *Penicillium* sp. comparable to the one of a biocide (Benzalkonium chloride) commonly employed in the treatment of the biocolonization [77]. The potential of *O. vulgare* and *T. vulgaris* EOs has also been investigated against fungi isolated from paper [57,78] and wooden artworks (in combination) [46] and, also in these cases, their biological action has been strongly confirmed.

Even the antimicrobial potential of *Clinopodium nepeta* oil was investigated against four microbial strains (*Bacillus subtilis*, *Micrococcus luteus*, *Penicillium chrysogenum*, *Aspergillus* spp.) isolated from colonized artefacts and the encouraging obtained results suggest a possible future employment of this extract for the control of the biodeterioration [79]. Moreover, in the study of Panizzi et al. (1993) [63] the EOs of *Clinopodium nepeta* (L.) Savi and *Thymus vulgaris* L. demonstrated the most powerful inhibitory activity, compared to two other EOs of Lamiaceae plants (*Satureja montana* L. and *Rosmarinus officinalis* L.), against standard bacteria and mycetes strains (i.e. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and

Candida albicans). In addition, the *C. nepeta* (L.) Savi oil is the only one that affected *Pseudomonas aeuruginosa*, a very resistant bacterium, while no inhibitory activity has been registered for *T. vulgaris*.

In virtue of what emerged from the literature, these three oils have been selected in this research to be studied and applied against multispecies heterogeneous colonies of biodeteriogens of stone materials.

A recent review by Fidanza and Caneva [31] stressed out the central role of this new substances employed for the treatment of the biocolonization of artistic stone materials. Besides the advantages, the review highlights some lacks existing in this new research field. The most controversial aspects related to these compounds mainly concerns the impossibility of controlling the chemical composition of the EOs (as said before), the lack of studies *in vivo* and *in situ* on real biocolonized stone materials, the way of application of the compounds and the suitable concentrations for the elimination of the dangerous microorganisms and, at the same time, safe for the preservation of human health and the ecosystems. Considering what has been stated, this study aimed to deeply investigate these themes, by ideating a useful procedure for future application of essential oils, but more generally, phytochemical compounds, for the control of the biocolonization of artistic stone materials, in order to avoid the consequent biodeterioration. This has been accomplished first, by selecting the three previously mentioned essential oils (*O. vulgare*, *T. vulgaris*, *C. nepeta*), among the ones that demonstrated good biological activities against many microorganisms. The literature survey gave important information about these substances, especially about their chemical composition. This has allowed to identify their respective active principles that have been employed along with the essential oils in order to evidence possible differences in the biocidal action of these substances. These ones are carvacrol for *O. vulgare* (carvacrol chemotype) oil [47,80], thymol for *T. vulgaris* (thymol chemotype) oil [81] and (R)-pulegone for *C. nepeta* (pulegone chemotype) oil [66,82]. This aspect has been considered, because, if the APs demonstrate a biological activity comparable (or major) to the one of the respective EOs, the development of products containing only APs must be taken into account. This conclusion can solve the problem of the impossibility of controlling the chemical composition of the oils, allowing the production of cheaper formulations (pure high-quality essential oils are quite expensive) containing APs in chosen concentrations,

active against target microorganisms. Moreover, since pure essential oils are inapplicable on stone surfaces [31], a part of this research concerned the investigation of a product compatible with these substances, in the way to create formulations based on phytochemicals, making it possible to modulate the concentrations of the active substances present in them and easily apply the compounds on stone substrata.

1.3. Soft materials: gel-systems for the cleaning of Cultural Heritage

Cleaning is a crucial phase for the conservation of cultural heritage materials, that implies the removal of undesirable deposited materials (dust, pollutants, airborne particles, residuals of old restoration works, greasy soil etc.) or alteration products from the surfaces, in order to go back, as much as possible, the original shape of the artefact.

The “traditional” cleaning methods employed during the years mainly provided the mechanical removal of dirt deposits and/or the application of chemicals, especially organic solvents, that can have detrimental effects that may contribute to the worsening of the conservative status of the work of art [83]. Aside from the toxicological risks assessed in many cases, the release and permanence of possible residue, as well as the poor selectivity and controllability of their cleaning action, are well-known problems related to the employment of these compounds, that lead to unwanted side effects, such as: the solubilization of the original material, swelling and leaching of the blinding media, alteration of the pigments, bleaching and overcleaning and transport and redeposition of dissolved matter through the porosity of the artwork [84–86]. On the other hand, regarding the mechanical action, as already discussed for the elimination of the biofilm (section 1.2), the removal of the patina with common tools (scalpels, spatulas etc.) can produce structural alterations on the surfaces and, for this reason, their employment is not recommended.

In light of this, currently, the attention of the investigators is drawn to develop new systems, mainly based on the employment of colloids and nanomaterials, able to substitute traditional cleaning methods. These new generation compounds, including micelle, microemulsions, gels and gel-like systems, have, as main advantages, good cleaning properties, low toxicity and low environmental impact [84,87]. Gels are among the most studied and employed of these systems for the conservation of cultural heritage, being very

versatile and easily adaptable to different materials and substrata. In particular, it is possible to create combined gel-systems suitable to contain many different solvents and specific cleaning agents (enzymes, chelators, microemulsions, essential oils etc.), that can be applied on the surface to selectively remove dirt and other undesirable deposits. In this way, the slow release of the active solvent allows to control its penetration inside the substrata, with a great selectivity in the cleaning, limited to the application area [83,88].

The definition of "Gel" is still a controversial theme, because in it fall many systems (i.e. polymers used as thickeners and viscosity modifiers for organic and aqueous cleaning fluids, yielding gel-like systems [89]) that not strictly respect the requisites officially established by the International Union of Pure and Applied Chemistry (IUPAC), which defines gel as "*Non-fluid colloidal network or polymer network that is expanded throughout its whole volume by a fluid*" [90]. In general, a gel can be considered a soft-matter composed at least by two components, one of which can be a polymer forming complex chains, organic molecules or colloidal particles entrapped inside a liquid continuum phase and, as result, they are characterized by physical properties halfway between liquid and solid [85,91,92]. The chemical composition of the gels and the involved bonds in the cross-linking are criteria of classification of these substances. In general, it is possible to distinguish chemical from physical gels, depending on the nature of the cross-linking and the type of bounds between the polymeric network and the solvent. A physical gel, also defined thermoreversible, involves non-covalent and weak bonds (i.e. ionic and ion-coordination bonding, hydrogen bond, van der Waals interactions), having a low energy of interaction (1-120 kJ/mol range)[89,92] that characterize these gels for being reversible and able of turning from the solid to the gel phase due to temperature changes.

Otherwise, chemical gels are characterized by covalent bonds for the cross-linking of the 3D network, obtainable in different ways, the most common one is the free-radical cross-linking copolymerization of monomers, either in bulk or in the presence of a solvent [89]. The stronger binding energy (200 - 650 kJ/mol) [89,92] makes these gels thermally irreversible, since it is impossible to break the covalent bonds of the network with the only increment of temperature and the system is not able to reproduce the original structure by cooling down. The chemical gels can be broken only as a result of bond cleavage, and, for this reason, these networks can be unambiguously included in the gel class [89,92]. The most

relevant difference between physical and chemical gels for restoration purpose is that the first ones can be easily shaped, with the advantage of having a homogeneous interaction with the surface, while the strong cohesive force of chemical gels make this step more difficult. However, this way, the possibility of leaving residuals is very high, because physical gels need mechanical tools or solvents for their complete removal, making more advantageous the employment of chemical ones, in order to avoid this relevant drawback [85].

For the purpose of this research, and for the importance they are getting as systems for the confinement of restoration products, the attention is focused on the polymer-fluids hydrogels, in which the polymerization of the 3D network occur due to the presence of a cross-linker, and the fluid phase is constituted by water [85]. In other words, hydrogels are systems comprising of three-dimensional, physically or chemically bonded polymer networks entrapping water in intermolecular space [93]. This class of materials have been developed to confine nanofluids, in order to better control their release on the surfaces. They demonstrated to be particularly suitable for the application on cultural heritage materials due to their a-toxicity and the easiness in the complete removal from the artistic substratum [89]. A recent study by Boccalon et al. (2020) [94] has provided the testing of different biocidal compounds on biocolonized stones, embedded in a PVA-based hydrogel. Among the biocides employed, particularly interesting for the purpose of this thesis, is the choice of *Thymus vulgaris* oil, used both alone and coupled silver nanoparticles (AgNPs), in order to improve the biocidal action (EOs has a higher efficacy towards Gram-positive bacteria, while AgNPs towards Gram-negatives). The efficacy of the method was assessed [94]: it was possible to effectively remove the heterogeneous biofilm present on two biocolonized stone samples characterized by different physical properties (Carrara marble and St. Margarethen stone), by combining the biocidal action of the substances dispersed in the HG and the mechanical action in the removing of the film layer. Moreover, a slow release of these substances was obtained, making it possible to better control the application of the biocides on the works of art.

In this research, a water-based hydrogel containing Gellan gum and PVA has been employed for the realization of formulations containing the phytochemicals.

Gellan gum is a biopolymer, a linear microbial heteropolysaccharide built up by (1,3)-β-D-glucose, (1,4)-β-D-glucuronic acid, (1,4)-β-D-glucose, and (1,4)-α-L-rhamnose

units, secreted from microorganisms (*Pseudomonas spp.*) during fermentation [89,95]. Gellan gum-based hydrogels are non-toxic and biodegradable, so much that they are widely used as thickeners in food and as culture media in microbiological applications. In the field of conservation of CH, these compounds find many applications in the restoration of ancient writing media (paper and parchment), both alone as highly rigid hydrogel [96] or coupled with antimicrobial agents [97–99], including plant extracts [100]. The success of these hydrogels on highly sensitive porous supports depends on the fact that their strength and the elasticity can be easily modulated due to the presence of metallic cations (for instance, Ca^{+}), in order to create a more rigid or fluid product, according to the characteristic of the work of art on which they have to be applied [89].

Polyvinyl-alcohol (PVA) is a water-soluble and biodegradable vinyl polymer joined by only carbon-carbon linkages, which have found, in the last years, a big success in the field of cultural heritage for the development of gel and like-gels systems, especially when cross-linked with borax for the formation of peelable High Viscous Polymeric Dispersions (HVPDs) [87].

1.4. Non-invasive and non-destructive analytical techniques for the detection and characterization of the SAB

The early detection of the biocolonization is a fundamental step to avoid the previously described detrimental effects caused by the biodeterioration.

Morphological, microbiological, histochemical, biochemical, chemical and physical traditional methods have been largely employed over time, in virtue of their efficacy in the accomplishment of the purpose, but, at the same time, some disadvantages associated to their employment have been evidenced, included the lenght of the duration of the analysis and the difficulties in the interpretation of the information. However, the most relevant drawback is represented by the fact that most of these methodologies are invasive and destructive [101].

Considering the characteristics of uniqueness, irreproducibility and the intrinsic artistic and cultural values attributed to the heritage materials, during the years different analytical methods have been studied and improved, in order to make analyses and investigations that least compromise the original aspect of the work of art.

Indeed, the scientific community, dealing with the conservation of cultural heritage materials, is focusing its attention on the employment of non-invasive and non-destructive analytical methodologies.

With these terms are meant all the chemical, physical and biological applications that don't require the direct interaction with the materials, in the first case, while, in the second case, if a sampling procedure is necessary, this must be preferentially a micro-sampling that doesn't imply the destruction of the specimen.

In this scenario, even the more recent methods concerning the evaluation the biological effects on works of art are moving towards this direction. In this study, it was decided to follow this trend by employing non-invasive and non-destructive analytical techniques for the determination of the biological colonization of the studied stone materials, for the sampling of the biological material and for the characterization of the microorganisms composing the biofilm.

1.4.1. Colour and chlorophyll fluorescence measures for the detection of the biological colonization

The presence of microflora and the degree of biocolonization of a surface can be detected by the employment of optical methods, that exploit the physical intrinsic characteristics of the microorganisms. It is acknowledged that most of the biodeteriogens produce coloured pigments, often associated to photosynthetic microorganisms, producing fluorescence [102].

These characteristics allowed to develop techniques able to detect the presence of biofilms due to the colour changes of the surfaces and to the fluorescence signals produced *in vivo* by the chlorophyll-a, associated to the photosynthetic microflora.

These methods demonstrated in many circumstances their efficacy and usefulness in reaching the objective of determining the presence of biofilm, with the great advantages of being completely non-invasive (and non- destructive) and allowing the monitoring on site, being the instrumentation required portable [103].

In the first case, the employment of a portable tristimulus colorimeter or of a portable spectrophotometer are required while, in the second case, fluorescence measures are

performed with PAM – fluorometer apparatus. These two techniques are often employed in parallel in the characterization of the properties of the biofilms [104–107].

Portable colorimeter and spectrophotometer are instrumentations able to characterize the colour of the objects and transpose it in numerical data, or coordinates.

Many systems have been employed over time for the representation of the colour, but the CIELAB colour system (CIE 1986) is one of the most popular because it represents an intuitive and precise reference system for the colour characterization [106,108]. In this system, the colour is thought as a point located inside a three-dimensional plan, characterized by three cartesian coordinates: L*, a* and b*.

Each colour is determined by the means of this scalar parameter, each one contributing to the assessment of a colorimetric characteristic of the point: L* represent the variation in lightness, and can be considered as the z axis of the plan, while a* and b* are the chromaticity coordinates, or the x and y axis of the plan.

Given the directions of the coordinates, it is possible to define the location of the point inside the plan and its colour: L* ranges between 0 (absolute black) and 100 (absolute white), +a* is the red direction, -a* is the green direction; +b* is the yellow direction, -b* is the blue direction.

Alternatively, the linear coordinates can be substituted by three others angular or cylindrical parameters, where the a* and b* are replaced by the Chroma (C_{ab}*), or relative saturation, and hue (h_{ab}), while the L* value remains unchanged. The use of polar coordinates (L* C_{ab}* h_{ab}) rather than Cartesian coordinates (L*a*b*) allows a colour quantification similar to the one perceived by the human eye, i.e. by taking into account the three attributes of visual assessment typical of the human vision (hue, chroma and lightness)[102,108]. The angular parameters can be calculated with the following formulas:

$$C_{ab}{}^{*} = \sqrt{a^{*2} + b^{*2}}$$

$$h_{ab=}\ arctang(\frac{b^{*}}{a^{*}})$$

and can be converted into linear parameter by using:

a* = C* cos (h):

b* = C* sin (h)

Beside the characterization of the colour of the single point, this system allows to estimate the colorimetric difference existing between two colour points.

This can be done through the employment of the $\Delta E_{ab}*$, calculated as follows:

$$\Delta E *_{ab} = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

Where:

$\Delta a * = a *_i - a *_0$

$\Delta b * = b *_i - b *_0$

$\Delta L * = L *_i - L *_0$

Colorimetric measurements with portable colorimeter demonstrated in many cases to be a reliable method for the early detection of SABs even before the human eye perception.

This was assessed in the pioneer study of Prieto et al. (2002) [101] that, processing the idea developed by Young et al. (1995) [109] who assumed that a change in the amount of organisms colonising a substrate would give rise to a change in the colour of the substrate surface, established a direct correlation between changes in number of photosynthetic microorganisms (cyanobacteria) and changes in colorimetric parameters, concluding that colour variations are useful to characterize biofilm mass on stones. A more recent study of Prieto et al. (2004) [110] compared two employed methods for the quantification of the biomass (amount of chlorophyll a and fluorescein diacetate hydrolysis) with colour measures applied on induced biofilm of stone materials. The efficacy of colorimetric characterization was demonstrated, and the advantages related to the non-invasiveness of the methods for possible applications on cultural heritage have been stressed out. In this regard, Sanmartín et al. (2012) [19] evaluated a systematic strategy for the early detection of photosynthetic SABs colonizing a granite surface of a civil building in the city of Santiago the Compostela (Spain). On the other hand, the usefulness of the approach in the determination of the medium-term biocidal action of different compounds against an algal biofilm formed on a granite- built historical monument was also assessed [111] .

Besides the advantages already described, the instrumentation is very simple and can be used in a very easy way also by unskilled operators with minimal training to perform the measurements [103].

The PAM (Pulse Amplitude Modulated) – Fluorometry is another useful instrument to assess the vitality of microorganisms, and thus the evaluation of the degree of biocolonization of stone surfaces. This method exploits the possibility of recording the florescence signal emitted *in vivo* by chlorophyll-a, and others photosynthetic pigments, when the quiescent microorganisms after dark-adaptation (i.e. when the photosynthetic apparatus is in a quiescent state and all photosystem II (PSII) reaction centres fully open by a light source) are excited by a light source. The fluorescence signal is remitted at specific wavelengths that the system records. Due to the spectral region of the remitted fluorescence signal, it is possible to identify the photosynthetic pigment that produced it.

In general, this method allows to analyse the effectiveness of the photosynthetic system of the organisms, which is related to their physiological conditions [112,113].

The apparatus gives the minimal (F_0) fluorescence signal of the black-adapted cells and the maximal (F_m) fluorescence signal after a saturating light pulse in dark-adapted cells. These parameters are used to calculate the maximum quantum Yield, through the formula:

$Y = F_v/F_m$;

where $F_v = (F_m – F_0)$;

which is an indicator of the overall viability of the photosynthetic organisms. Moreover, it was estimated that a relation between the F_0 the concentration of chl a (a biomarker used to quantify phototropic biomass) exists: the evaluation of this parameter demonstrated its usefulness in the characterization of the biomass of biofilm presents on surfaces [103,105,114,115].

Chlorophyll fluorescence measures, together with microscopic morphological characterization, have also been recently performed with a mini-PAM portable fluorimeter to assess the inhibitory activities of natural extracts (liquorice leaves and lavender EO) against a natural biofilm grown on the walls of the Domus Aurea [116]

1.4.2. Metagenomic analysis for the characterization of the SAB

The identification of microorganisms composing the SABs is a crucial step for the determination of the scale of the damage deriving from the biocolonization. Through the knowledge of the microbial species it will be possible to establish the medium-term damage associated to the presence of some microorganisms rather than others, the symbiotic mechanisms occurring between different species, their environmental response and the possibility of limiting the damage by early targeted interventions.

The characterization of microorganisms mainly conceived traditional cultural-dependent microbiological methods, that provide the sampling of the biological material and then, the isolation and the cultivation of the microorganisms in plate. These are useful tools for the understanding of the physiological and biochemical potential of isolated microorganisms. However, various drawbacks are related to their employment, such as the involvement of different specialized scientific profiles (mycologists, phycologists, bacteriologists) for a precise characterization of the microorganisms, but, more relevantly, concerning the impossibility to characterize the whole microbial community composing the complex SABs [25]. Indeed, it was estimated that the growth on culture media allows the identification of only 0.1 up to 3.0% of the environmental bacteria and the 70% of fungi actually composing the complex communities, losing most of the real information [117,118].

For some microorganisms was assessed the impossibility of inducing their growth in plate, such as some symbiotic species, while others are still unknow, and experimentations that provided their grown in plate have never been carried on, due to the lack of appropriated methods [119].

Therefore, alternative non-culture dependent methods have been proposed and, among these, molecular methods are demonstrating to be reliable tools to investigate the diversity and the structure of cultivable and non-cultivable microorganisms [35,119].

Great attention are receiving metagenomic analyses, that refers to the set of the molecular analytical procedures that allows the study of the genetic material isolated directly from environmental sample, whereby it is possible to analyse all the microorganisms present in the sample, including the non-culturable ones, bypassing the need for isolation and lab cultivation of individual species [119,120].

The great advantage of these methods, besides the aforementioned possibility of characterizing also no-culturable microorganisms, are represented by the fact that a very small amount of biological material is required, thanks to the PCR amplification, making the sampling procedures of the biofilm non-invasive (at least micro-invasive) and thus, allowing a repetition of the sampling at different times, with the possibility of obtaining important information regarding the history of successive colonization [25,120]. The normal procedure for the metagenomic characterization provides i) the sampling of the biological material, ii) the extraction of the genetic material (nucleic acids), iii) polymerase chain reaction (PCR) amplification of ribosomal RNA (rRNA) with specific target genes; iv) fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) and/or clone library construction and sequencing [25,35,119]. Very interesting results have been obtained in different studies that provided the characterization of microbial species composing biofilms presents on many artistic supports, like paper, wood, glass stones and mural paintings [119,121,122].

CHAPTER 2. Materials and experimental set up

To evaluate the interactions between the selected phytochemicals and the biofilms present on the stone materials, three different experimental approaches

have been designed.

In this chapter are described the materials and the methods employed (section 2.1), as well as the experimental set up of each experiment (sections2.2; 2.3 and 2.4).

In the following paragraphs, the methodological approaches adopted for each experiment have been briefly summarized:

The first experiment was conducted in laboratory (controlled environmental conditions) and it included the application of the products on 12 biocolonized granite samples. The granites were previously inoculated with a heterogeneous phototropic culture sampled from the Monasterio of Sanmartiño Pinario (Santiago de Compostela, Galicia, Spain). A commercial biocide (Preventol® RI80), water coupled with brush, and hydrogel have been also tested separately to get a comparison with the phytochemical substances. An evaluation of the cleaning action of the substances was performed trough colorimetric analyses.

The second experiment included the application of the formulations containing the EOs and the APs on a biocolonized wall of the Dept. of Mineralogy at Sapienza University of Rome. The biological material was sampled using three approaches (a completely non-invasive one , a micro invasive one and an invasive one), in order to determinate which method allows to perform the best sampling of biological material with the lowest impact on the stone surface. The biological material was analysed and characterized through metagenomic analyses. The cleaning activity of the substances was evaluated by determining the colour of the surfaces before and immediately after the removal of the treatments. For the experimental surface treated with the essential oils, colour measures were repeated six weeks after the removal of the compounds, in order to evaluate a possible persistence of the products and an interference with the recolonization.

The third experiment summarizes the approaches adopted in the first and the second ones, by applying the experimental products on a granite surface exposed to the outdoors.

The selected experimental surface is a biocolonized granite wall at the Dept. of Pharmacy of the Santiago de Compostela University (USC).

The experiment required the application of fourteen treatments containing phytochemicals, one treatment made only of the hydrogel matrix, one containing a commercial biocide (Preventol® RI80) and the last one containing only water, for a total of seventeen treatments. It was decided to apply and replicate the treatments on two portions of the wall, that apparently seem characterized by a different biological colonization. This was assessed on the basis of visual observation: the selected surfaces present heterogeneous colourations, probably due to the presence of different microorganisms producing specific photosynthetic pigments. Sampling of the biological material and morphological analyses were made in order to characterize the microorganisms composing the biofilm. The application of the compounds and their removal were preceded and followed by colorimetric and fluorescence measurements, in order to assess the presence and physiological state of the microorganisms after the treatments. The whole monitoring lasted nine months.

2.1. Materials and methods

2.1.1. Stone materials

The experiments were performed on two different lithotypes: granite and travertine. These are among the most employed stone materials in the edification of civil and artistic buildings all over the word but, in particular, travertine is very representative of the city of Rome while granite constitutes almost all the entirely of the buildings of the historical centre of Santiago de Compostela (Spain). These lithotypes presents many differences in terms of chemical and mineralogical composition and physical properties.

Travertine (from latin '*lapis tiburtinus*') is a sedimentary rock derived from chemical and biochemical process of precipitation of calcium carbonate (95% of the chemical composition) under the mineralogical form of calcite (above more of the 99% of the total composition) and aragonite. It is characterized by basic pH, and a macro-porous vacuolar structure and a strong interconnection between macro and micropores [123,124]. The latter represents the discriminant property which favours the colonization by microorganisms, that found a suitable environment for the endolytic colonization and water availability [125–

128]. The granite samples and the granite wall of the Experiment 1 and Experiment 3 are monzogranites, commercially available in Galicia and sold with the general appellative of "granite". Their properties were not yet characterized in detail, but the higher homogeneity in the physical characteristics (macro and micro porosity) respect to the travertine, is evident by a preliminary naked eye observation. In general, but also in this particular case, for this lithotype, the physical factor that mostly influenced the biofilm formation is the roughness of the surface [114,129].

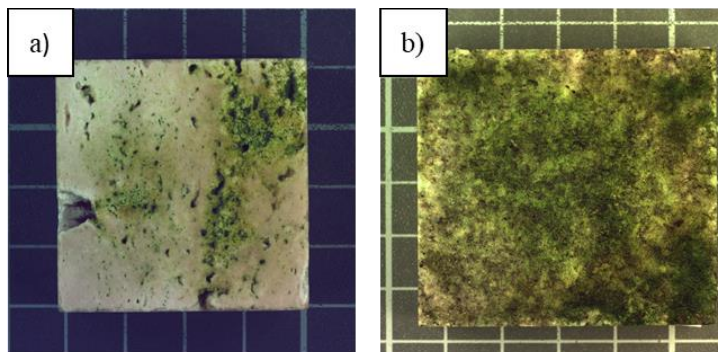

Figure 2 – Differences in the biocolonization of a travertine sample (4x4x2 cm) (a) and a granite sample (5x5x2 cm) (b) (in lab induced microbial colonization). The diffuse macro porosity of travertine favours the penetration of the microorganisms inside the pores, showing less uniformity in the biofilm layer on the surface. The granite, on the other hand, characterized by a much more uniform physical appearance, favours the colonization in the upper surface.

2.1.2. Hydrogel

An innovative chemical hydrogel has been employed in the experimentations for the development of the formulations in which the phytochemicals have been dispersed. This is a newly formulated product, conceived for a non-invasive cleaning of cultural heritage materials, by the research team of Prof. Matricardi (Dept. of Chemistry and Drugs Technologies, Sapienza University of Rome), in collaboration with Qi srl, (Via Monte D'Oro 2/A, 00071 Pomezia, Rome, Italy). The product is a water based polymeric mixture (Gelrite and PVA 87-89% hydrolyzed, Mw 85-124000, Sigma-Aldrich, St. Louis, MO, USA) enriched with a cross-linker additive (Calcium chloride) and surfactant (Acemoll CC, ACEF, Fiorenzuola D'Arda (PC), Italy), whose chemical composition is shown in detail in Table 1.

The preparation of the hydrogel provides the assembling of two solutions containing the two polymers: the solution A contains the Gelrite (0.8% in H_2O) and the solution B

contains PVA (8% in H_2O). Acemoll CC (10%) and the cross-linker (0.09%) are added to the solution B after cooling. The solutions A and B are mixed 1:1 w/w.

Table 1 – Chemical composition of the hydrogel

Hydrogel		
Components	**Chemical composition**	**[w/w] %**
Gellan Gum (Gelrite)	Deacylated Gellan gum High Mw	0.400
Polyvinyl alcohol (PVA)	PVA 87-89% hydrolyzed, Mw 85-124000	4.000
Calcium Chloride ($CaCl_2$)		0.045
Surfactant (Acemoll CC)	PEG-6 Caprylic/Capric Glycerides	5.000

2.1.3. Essential oils (EOs) and Active Principles (APs)

All the essential oils employed in the experimentation were purchased from specialized retailers of natural compounds which cultivate plants following the European directives for organic farming. The essential oil of *Clinopodium nepeta* (or *Calamintha nepeta*) was produced in Corse and sold by Huiles Essentielles Bio de Corse - Julien Fauconnier (Occhiatana, Corse, France). The essential oils of *Thymus vulgaris* and *Origanum vulgare* employed for the Experiment 1 and 3 were produced in the south of Spain, sold by Esencias Martinez Lozano (Murcia, Spain), while the ones employed in the Experiment 2 were produced in Tuscany, sold by Podere Santa Bianca (Pomarance (PI), Italy). The choice of employing high-quality commercial oils for the experimentations reflects the intention of realizing formulations that need to be reproduced in different situations, restoration sites included, when the laboratory equipment is not available. The pure thymol ($\geq$ 98.5%) carvacrol ($\geq$ 98.0%) and (R)-(+)-pulegone ($\geq$ 90.0%) compounds were purchased from Aldrich Corp. (St Louis, MO, USA).

2.1.4. General rules for the preparation of the formulations

The experiments required the realization of different mixtures of compounds, composed by the hydrogel and EOs on one side and hydrogel and APs on the other. Moreover, in order to investigate a possible empowerment of the biocidal action of these substances against microorganisms when combined together [116,130], the substances have

been employed pure and mixed with one or two other substances (always considering that the EOs were combined with other EOs and the APs with other APs).

Due to the variations in compositions of the employed EOs (see section 2.1.3 and section 2.2.2) and, in general, to the differences for each experimental set up, the formulations are not the same in the three experiments, and some differences occur, concerning both the concentrations and the chemical compositions. For this reason, in this paragraph is described the general principle adopted for the ideation of the formulation, while the specific composition of the formulations is reported with more detail in the sections 2.2.2; 2.3.1 and 2.4.1.

Formulations containing the essential oils: 2% w/w was selected as the concentration of essential oils suitable to obtain a large spectrum biocide compounds with a low ecological impact, able to form an homogeneous emulsion that preserves the rheological and filming properties of the hydrogel [131,132]. The tests realized to obtain the treatments composed by the innovative hydrogel matrix and the phytochemicals will be discussed in the Results chapter, in section 3.1

As a general rule, the formulations containing the pure essential oils dispersed in the hydrogel matrix have a 2% w/w of the single pure substance, while, the ones composed of the combination of two or three EOs, the single substances are present 1:1 w/w (the contribute of each oil is 1/2 of the total concentration) and 1:1:1 w/w (the contribute of each oil is 1/3 of the total concentration) respectively, to obtain an overall final concentration of 2% w/w.

Formulations containing the active principles: each formulation containing an essential oil (or the mixture of two or three essential oils) finds a corresponding formulation containing the respective active principle (or the mixture of two or three active principles).

The concentrations of APs contained in the formulations have been calculated in a way that reproduces the concentration of the active principle naturally present in the oil.

This means that, for example, considering an active principle X, that represents 30% of the total concentration of an oil, the concentration of X in the formulation where this compound is present in a pure state will be of 0.6% w/w, or the 30% of 2% w/w (i.e. the concentration of the pure essential oil presents in the correspondent formulation). This

approach is valid for all the developed formulations, including the ones containing two or three mixed compounds.

2.1.5. Colorimetric measurements

Colorimetric measuresments have been performed in all the experiments before and after the application of the treatments on the surfaces, to assess the colour variations associated with the presence (or absence) of the biocolonization. In fact, as reported in the introduction (section 1.4.1), colorimetric measurements have been demonstrated a reliable tool for the detection of microorganisms on stone surfaces, due to the presence of coloured pigments produced by them.

The colour data were acquired with the employment of portable colourimeter instrumentations and analysed using the CIELAB colour system (CIE 1986) (see section 1.4.1). Each colorimetric point is represented by three cartesian coordinates: L* represents lightness variations (0 = absolute black, 100 = absolute white), a* represents colour variations between red and green (a* (+) = red; a* (-) = green) and b* represents colour variations between yellow and blue (b*(+) = yellow; b*(-) = blue).

For the evaluation of the colorimetric differences existing between two colour points, partial colorimetric chromatic differences (Δa* and Δb*) and the overall colour variation (ΔE_{ab}*) were calculated with the following formulas:

$$\Delta a* = a*_i - a*_0 \quad (1)$$

$$\Delta b* = b*_i - b*_0 \quad (2)$$

$$\Delta L* = L*_i - L*_0 \quad (3)$$

$$\Delta E*_{ab} = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (4)$$

where the subscript 0 denotes the initial situation, while the subscript i denotes the colour acquisition at the time i. Positive values of Δa* indicate reddening, and negative values indicate greening. Positive values of Δb* indicate yellowing, and negative values indicate blueing. Three CIELAB units are considered the upper limit of rigorous colour tolerance. If the Δa*, Δb* or ΔE_{ab}* show values higher than 3, jus notable differences (JND) in colour can be observed [133–135].

2.2. Experiment 1: Granite samples

The effect of 8 formulations containing the three essential oils (*O. vulgare*, *T. vulgaris* and *C. nepeta*) and their main active principles (carvacrol, thymol and pulegone) on phototropic heterogeneous biofilm growth on granite samples were studied. In Figure 3 are summarized the employed compounds and their respective acronyms. Three formulations contain the pure essential oils (T1, T2, T3), three others the pure active principles (T4, T5, T6) and the two remaining the mixture of the three essential oils (T7) and their active principles (T8). To have a comparison of the efficacy of the treatments, the application also included the employment of a commercial biocide, based on Benzalkonium chloride salts, commonly used in the treatment of stone’s biodeterioration (Preventol® RI80) and pure water (T12) paired with brush removal. The Preventol® RI80 was applied on the samples both diluted in water (T10) and in the hydrogel (T11) (2% v/v). The application with water, and the following removal with a brush, is the common procedure of application of the biocide, while the employment of the hydrogel allowed to obtain a film-forming emulsion, like the others containing the essential oils. The hydrogel alone was also applied and studied (T9), in order to evaluate its ability to remove the superficial biofilm from the surface, that can be considered an additional element that improve the cleaning efficacy of the formulations. In Figure 3 are schematically represented the samples and each treatment employed, while the concentrations calculated to obtain the formulations containing the phytochemicals are described in section 2.2.3.

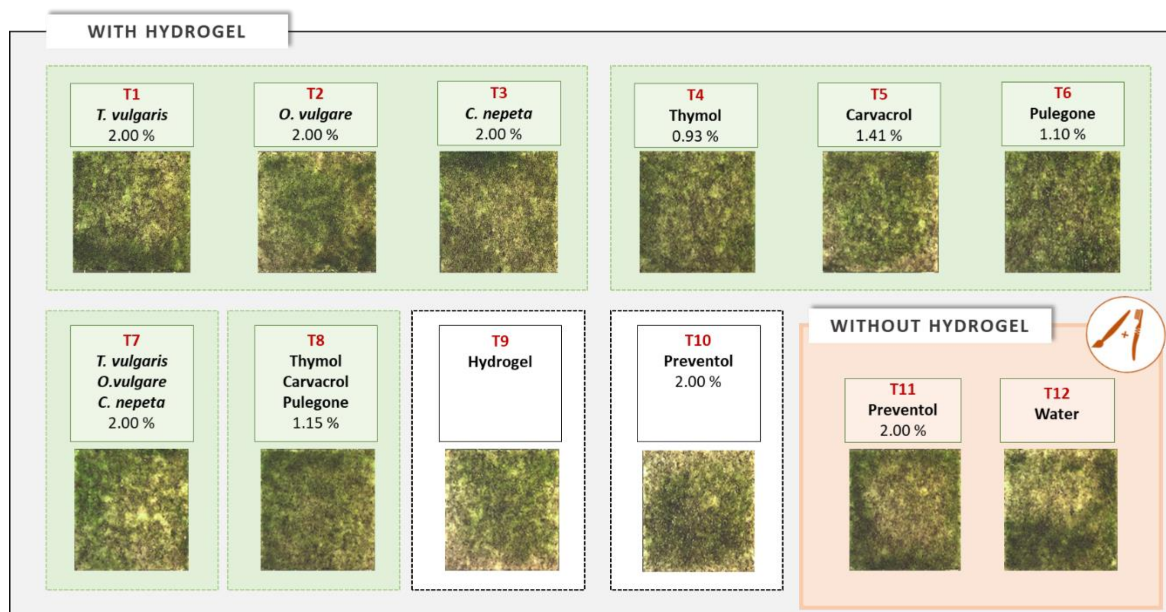

Figure 3- Schematic representation of the experimental set-up to test the phytochemical compounds, the hydrogel alone, Preventol ® RI-80 and water as cleaning agents of biocolonized granites.

2.2.1. Granite blocks inoculated with phototropic microorganisms

Each treatment (from T1 to T12) was applied on one sample. The samples (granite blocks 5 cm × 5 cm × 2 cm) were previously inoculated with 3 mL (1.19 g·L−1) a phototropic culture and maintained in controlled and stationary conditions of temperature (23 ◦C), relative humidity (80%), and light (12 h light/dark photoperiod) in a climatic chamber (SCLAB PGA-1228/2 HR) until biofilm formation, or until the samples shown the same level (or degree of presence) of biological colonization (see Figure 4). The culture is mainly composed of algae and cyanobacteria, in particular: *Bracteacoccus minor* (Schmidle ex Chodat) Petrová, *Stichococcus bacillaris* Nägeli, *Chlorella* sp., *Isocystis* sp., *Aphanocapsa* sp., *Leptolyngbya cebennensis* (Gomont) I.Umezaki and M.Watanabe [121]. The culture derived from a natural biofilm colonizing an historical granite building in Santiago the Compostela (Monastery of San Martiño Pinario, Santiago de Compostela, Galicia, Spain). This culture was selected for the inoculation because it demonstrated to be particularly suitable to reproduce a natural biofilm on granites in laboratory conditions and also because of its heterogeneous composition [106].

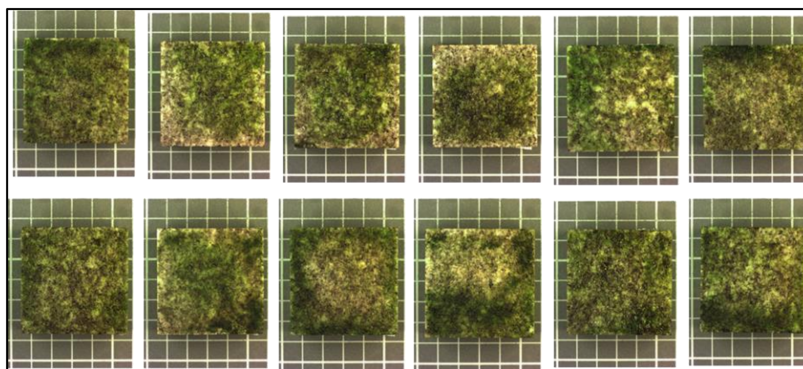

Figure 4 – Photographical representation of the biocolonized granite samples.

2.2.2. Chemical characterization of the essential oils

This section concerns the determination of the concentrations of active principles present in each essential oil used in the Experiment 1. Since it was a preliminary step necessary to calculate the concentrations of APs that have to be present inside the formulations (see section 2.1.4), the methods employed for the chemical characterization and the chemical composition of the EOs are reported below and summarized in Table 3.

In the case of O. *vulgare* and *T. vulgaris*, both the oils were acquired from the company Esencias Martínez Lozano, Murcia, Spain, which provided the chemical analyses and the relative data sheet containing the identified compounds at their respective concentrations. A total of 25 compounds have been identified in *T. vulgaris* (95.3% of the total composition) and 23 compounds in *O. vulgare* (96.7% of the total composition). The presence of the major phenolic compounds in each oil (i.e., active principles) has been confirmed, where thymol represents the 46.4% of *T. vulgaris* and carvacrol the 70.5% of *O. vulgare*. The presence of Carvacrol (4%) has been detected also in *T. vulgaris*, as well as thymol (3.8%) in *O. vulgare*.

C. nepeta oil was analysed with a gas chromatography–mass spectrometry (GC-MS) system (Shimidazu, Kyoto, Japan) equipped with a MEGA SE52 5% polydiphenyl–95% dimethylsiloxane-bonded phase column (Mega, Legnano, Italy) (dim. 30 m × 0.32 mm × 0.15 μm). The oven temperature used was initially 50 ◦C heating up to 250 ◦C at a rate of 3 ◦C/min. The operating conditions were: injection temperature 250 ◦C, carrier (helium) flow rate of 1 mL/min, electronic impact mode of 70 eV, injection in the split mode, interface at

230 ◦C, quadrupole temperature 150 ◦C, transfer line temperature 280 ◦C, SCAN acquisition mode (masses interval: 35-350 AMU). For the analyses, the oil extracts were diluted in cyclohexane (5 mg/mL). The identification of the compounds was performed by comparing the mass spectra reported in the commercial libraries and using the retention indexes compared to those of the reference libraries [136]. A total of 36 compounds have been identified in *C. nepeta* (96.8% of the total composition) and, even in this case, the presence of Pulegone (i.e. *C. nepeta* APs) has been confirmed, where this one represents the 55.2% of the total composition of the oil.

2.2.3. Chemical composition of the formulations containing the EOs and the APs

The formulations have been prepared following the methodology described in section 2.1.4. The chemical composition of the essential oils, described in section 2.2.2 has been taken into account for the evaluation of the concentration of phytochemicals to be employed in the experimentation. In Table 2 the concentrations employed to obtain the formulations have been reported while, in Figure 3 a schematic representation of the treatment and the total concentration of phytochemicals present is shown.

Table 2 – Chemical composition and relative concentrations of the treatments.

	[w/w] %					
Treatments	***T. vulgaris***	***O.vulgare***	***C. nepeta***	**Thymol**	**Carvacrol**	**Pulegone**
T1	2.00	//	//	//	//	//
T2	//	2.00	//	//	//	//
T3	//	//	2.00	//	//	//
T4	//	//	//	0.93	//	//
T5	//	//	//	//	1.41	//
T6	//	//	//	//	//	0.10
T7	0.67	0.67	0.67	//	//	//
T8	//	//	//	0.31	0.47	0.37

Table 3 - Chemical characterization and chromatographic area percentage of the compounds present in the essential oils (EOs). Minor compounds (concentration < 0.02%) have not been included. The concentrations in percentage of the active principles are indicated in bold.

Compound	*C.nepeta*	*T. vulgaris*	*O. vulgare*
α-thujene	0.1	1.3	1.3
α-pinene	1.1	0.9	0.9
camphene	0.04	1.0	0.1
sabinene	0.4		
β-pinene	1.1	0.3	0.1
3-octanone	0.1		0.3
β-mircene	1.1	1.9	1.1
3-octanol	1.7		
α-phellandrene	0.1	0.2	0.1
α-terpinene	0.3	1.6	0.7
p-cimene	0.2	15.8	6.5
limonene	9.9	0.4	0.2
1,8-cineole	0.5	0.4	0.2
cis-b-ocimene	0.2		
trans-b-ocimene	0.2		
γ-terpinene	0.5	10.2	5.9
cis-sabinene hydrate	0.1		
terpinolene	0.2	0.1	0.2
linalool	0.7	4.5	1.6
camphor	0.1	0.8	
menthone	2.1	0.2	
isomenthone	3.8		
borneol		1.2	0.2
menthol	0.1		
terpinene-4-ol	3.1	1.4	0.6
α-terpineol	0.5	0.2	0.1
verbenone		0.2	
pulegone	**55.2**		
piperitone	0.6		
thymol		**46.4**	3.8
carvacrol		4.0	**70.5**
piperitenone	10		
piperitenone oxide	0.4		
α-copaene	0.1		
β-bourbonene	0.1		
trans-β-caryophyllene	0.5	2.0	2.0
germacrene D	0.9		
α-humulene	0.1		0.2
β-bisabolene			0.3
γ-cadinene	0.3	0.1	
δ-cadinene	0.4	0.1	
carophyllene oxyde		0.2	0.2

2.2.4. Colorimetric measurements

Colorimetric measurements were performed on the granite samples to assess the effectiveness of the treatments. For this purpose, each sample was considered as a surface divided in three portions. For each portion, three colorimetric measuresments have been acquired and the mean value was considered as representative of each treatment. The measures were performed with the employment of a portable spectrophotometer (CM-700d, Konica Minolta, Tokyo, Japan) equipped with a CM-S100w software (SpectraMagicTM NX) under the following analytical conditions: D65 illuminant, 2◦ observer, target area of 8mm ø and SCI mode. Colour was measured directly on randomly selected areas of the humid colonized surfaces [137]: before cleaning, immediately after cleaning, one week after cleaning, and two weeks after cleaning. Monitorization for 14 days was carried out in order to evaluate the effectiveness and persistence of each treatment over time in terms of cleaning, and to evaluate the change of colour of the substrate over time.

2.2.5. Statistical Analyses

The data were subjected to analyses of variance (ANOVA) and Tukey's HSD post-hoc test (p-value ≤ 0.05) implemented in the SPSS statistical program (version 23.0).

2.3. Experiment 2: Travertine wall

This experiment required the application of the selected phytochemicals embedded in the hydrogel matrix on site. An evidently, homogeneously, biocolonized travertine wall located at the mineralogy build of Sapienza University of Rome (Rome, Italy) was selected for the experimentation (Figure 5). The surface was divided into two portions presenting the same degree of biocolonization: one was assigned to the application of the formulations containing the EOs (Surface A) and the other to the formulations containing the APs (Surface B). Each surface was further divided into 7 panels (dim = 12.5 cm x 13.5 cm), each of them assigned to the application of one formulation (see Figure 5), for a total of 14 different formulations. For both the EOs and the APs, the experimental set-up provided the development of 3 formulations containing the pure phytochemicals (A and B1,3,5), 3 formulations containing the mixture of two phytochemicals (A and B2,4,6) and 1 containing three phytochemicals (A7 and B7). The concentrations employed for the preparation of the formulations are reported in detail in section 2.3.1.

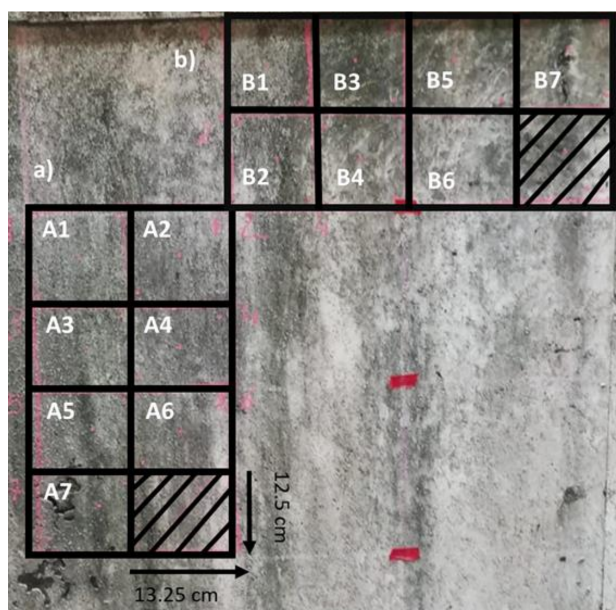

Figure 5 – The travertine biocolonized wall subjected to the treatments. a) A1= *O. vulgare*; A2 = *O. vulgare* + *T. vulgaris*; A3 = *T. vulgaris*; A4 = *T.vulgaris* + *C. nepeta*; A5 = *C. nepeta*; A6 = *O. vulgare* + *C. nepeta*; A7 = *O. vulgare* + *T. vulgaris* + *C. nepeta*. b) Surfaces treated with the formulations containing the terpenic active components B1 = carvacrol; B2 = carvacrol + thymol; B3 = thymol; B4 = thymol + pulegone; B5 = pulegone; B6 = carvacrol + pulegone; B7 = carvacrol + thymol + pulegone.

2.3.1. Chemical composition of the formulations

Always considering the procedure described in the section 2.1.4, for the experimental procedure it was decided to employ as concentration for the development of the formulations containing the APs, a representative average of the values of APs inside the correspondent oil, as reported in literature.

In particular: for the *C. nepeta* oil the R-pulegone ≈ 40% [82,138,139], for *T. vulgaris* oil the thymol ≈ 50% [140–142] and for *O. vulgare* oil carvacrol ≈ 15% [80,143].

The compositions and the concentrations of the formulations are reported in Table 4.

Table 4 - Composition of the treatments and concentrations of the substances employed.

EOs [w/w] %				**APs [w/w] %**			
N °	*O. vulgare*	*T. vulgaris*	*C. nepeta*	N°	Carvacrol	Thymol	Pulegone
A1	2.00	//	//	B1	0.3	//	//
A2	1.00	1.00	//	B2	0.15	0.50	//
A3	//	2.00	//	B3	//	1.00	//
A4	//	1.00	1.00	B4	//	0.50	0.40
A5	//	//	2 .00	B5	//	//	0.80
A6	1.00	//	1.00	B6	0.15	//	0.40
A7	0.67	0.67	0.67	B7	0.10	0.33	0.24

2.3.2. Colorimetric measurements

To assess the efficacy of the treatments, colorimetric measuresments were performed on each treated panel: before the application of the treatments and immediately after their removal.

In the case of the surface treated with the EOs the colorimetric measuresments have been performed also six weeks after the removal of the treatments.

A total of 3 readings on 3 selected points of each panel were obtained and the average values were calculated, in order to estimate the general colour of each one. For the analyses, a portable spectrophotometer (CM-2600d, Konica Minolta) was used under the following analytical conditions: illuminant D65, observer 10°, diameter of observation 8 mm, wavelength range 360–740 nm. As in the Experiment 1 (section 2.2) all the colorimetric data were processed and expressed in the CIELAB colour coordinates system. The colour points

have been plotted in the bidimensional graph of a*b* and in the monodimensional space of L*. The partial and the total colour differences (Δa^*, Δb^*, ΔL^* and ΔE^*_{ab}) were calculated through the formulas (1), (2), (3), (4) reported in the section 2.1.5.

2.3.3. Sampling of the biological material

The biological material was collected from a biocolonized area adjacent to the experimental surfaces, in order to characterize the species of microorganisms composing the SAB. It was decided to employ three different sampling methods: swab (non-invasive), adhesive tape (micro-invasive) and scalpel (invasive) (see Figure 6).

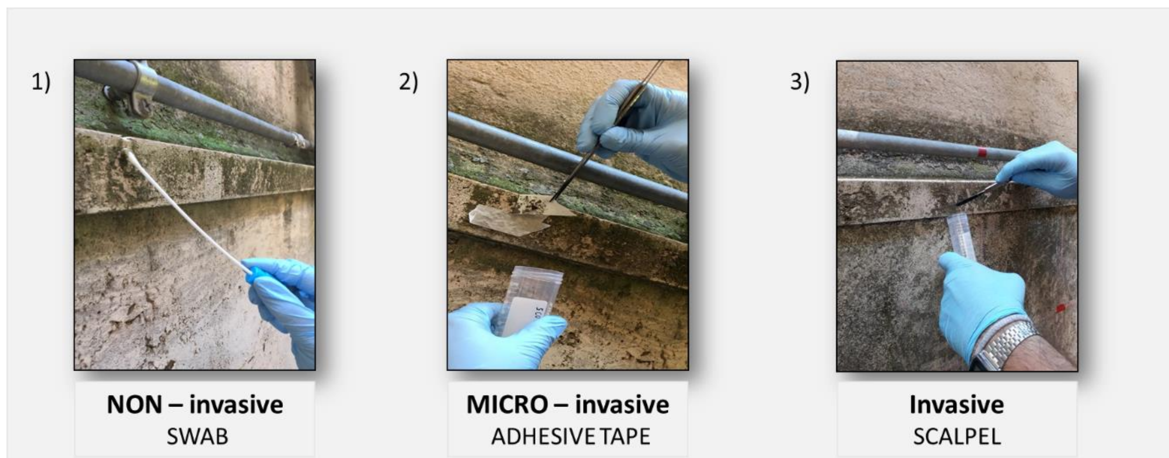

Figure 6 – Sampling procedures for the collection of the biological material present on the surfaces. 1) Swab sampling (non-invasive); 2) Adhesive tape (micro-invasive); 3) Scalpel (invasive).

For each methodology three sampling on three different surface portions (sized 5 × 5 cm) were performed, for a total of 9 samples of biological material. The samples have been preserved in sterile tubes at -20°C until the DNA extraction procedures. The biological material was then analysed and characterized by employing metagenomic methods.

2.3.4. DNA amplification and quantification

DNA extraction quality was evaluated by spectrophotometric analyses using NanoDrop 2000c (ThermoScientific, USA) spectrophotometer, in order to evaluate DNA concentration, and 260/280 absorbance ratio. This is a useful parameter to estimate the pureness of the extracted DNA: if the ratios are ≈ 1.8 the DNA is generally accepted as "pure" [144]. In order to detect the presence of microorganism's DNA in the total extracted

DNA, such as bacteria, fungi and plants kingdoms, PCR analyses were performed. 50 ng of genomic DNA were used using specific primers for 16S [145,146], ITS [147], and 18S regions respectively. The specific primes are summed in Table 5. The amplification was performed with Taq Polimerase (Bioline) and the amplification products were verified through Agarose gel (1%) electrophoresis.

Table 5 - Summary of the primers used for PCR amplification of sequences belonging to fungi (ITS1, ITS4), plants (EukA, EukB) and bacteria (27For, 1495Rev) kingdoms. Primer sequence, annealing temperature and expected amplicon length are also given.

Kingdom	Primer	Primer sequence (5'-3')	T	Length
Fungi	ITS1	TCCGTAGGTGAACCTGCGG	57	400-800
	ITS4	TCCTCCGCTTATTGATATGC		
Plantae	EukA	ACCCTGGTTGATCCTGCCA	60	1500-2000
	EukB	TGATCCTTCTGCAGGTTCACCTAC		
Bacteria	27For	GAGATTTGATCCTGGCTCAG	54	1000-1500
	1495Rev	CTACGGCTACCTTGTTACGA		

2.4. Experiment 3. Granite wall

The action of the phytochemicals was evaluated on two biocolonized portions of a wall located at the faculty of pharmacy of the Campus Sur of University of Santiago de Compostela (Santiago de Compostela, Galicia, Spain).

The wall was selected because of the evident presence of heterogeneous microflora differentially colonizing some areas of the wall. Indeed, the two portions seemed to present a very heterogeneous biocolonization, where the two SABs are apparently characterized by the presence of various photosynthetic microorganisms. This was hypothesized since, given by a preliminary observation of the biofilm, differences in colour it can be noticed: the wall on the left (α) presents a coloured SABs with a dark-green chromatic predominance, while the wall on the right (β) is characterized by a stronger red component. This could be considered an indicator of the biodiversity of the colonizer microorganisms, that produce different photosynthetic pigments that contribute to the colour variations of the surfaces (Figure 7).

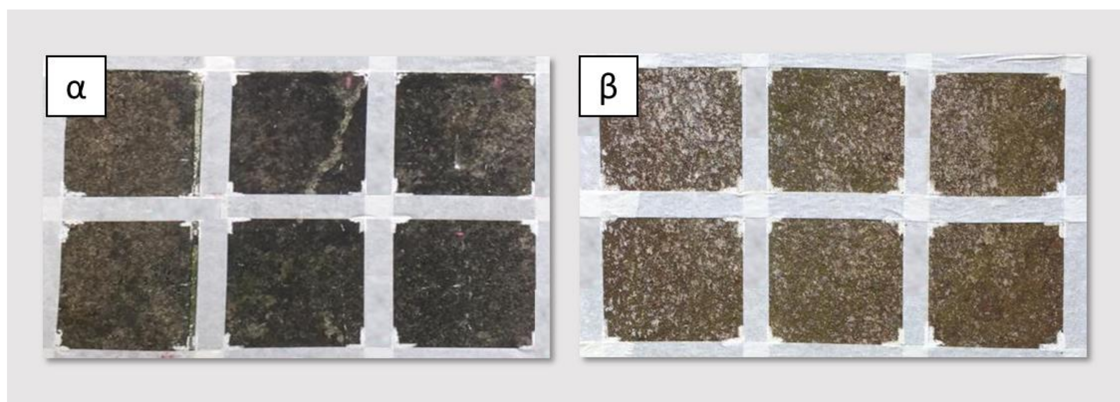

Figure 7 – Differences in the visual appearance of the SABs presents on the two selected surfaces: particular of the two surfaces selected for the experiments. **Surface α** - predominance of dark-green colour components; **Surface β** - predominance of red-green colour components.

Both surfaces have been divided in 17 sections (dim = 10 cm x 10 cm, each panel), each one assigned to the application of one treatment (Figure 8). The treatments provided the ideation of 14 formulations containing the phytochemicals (from T1 to T14), the hydrogel alone (T15), Preventol® RI80 (T16) (2% v/v) and distilled water (T17)[2].

[2] The name of the treatments is the same both for the surface α and the surface β. For this reason, in the following sections, the treatments can be distinguished by the Greek letter following the name of the treatment.

The formulations containing the phytochemicals included the employment of the EOs (T1- T3 and T7 - T10) and the APs (T4- T6 and T11-T14). These ones included the phytochemicals pure (T1-T6), two phytochemicals (T7-T9 and T11-T13) and three phytochemicals (T10 and T14) coupled together. In Figure 8 the way of application of the substances and their specific compositions are summarized. For what concerns the ideation of the formulations and the concentrations employed, please refer to the section 2.4.1

All the treatments were applied on the surfaces with a paint brush and left on the surface for one month, in order to allow i) the complete drying of the hydrogel matrix (above 2 weeks; the time of drying is strongly influenced by the external environmental conditions, in particular rainfalls and humidity), ii) the interaction between the biocides and the microorganisms. The treatments composed of the hydrogel matrix have provided the peeling of the superficial film with the help of water and a spatula. Otherwise, the treatments providing the employment of Preventol ® RI-80 (T16) and Water (T17) only required the mechanical removal with a toothbrush.

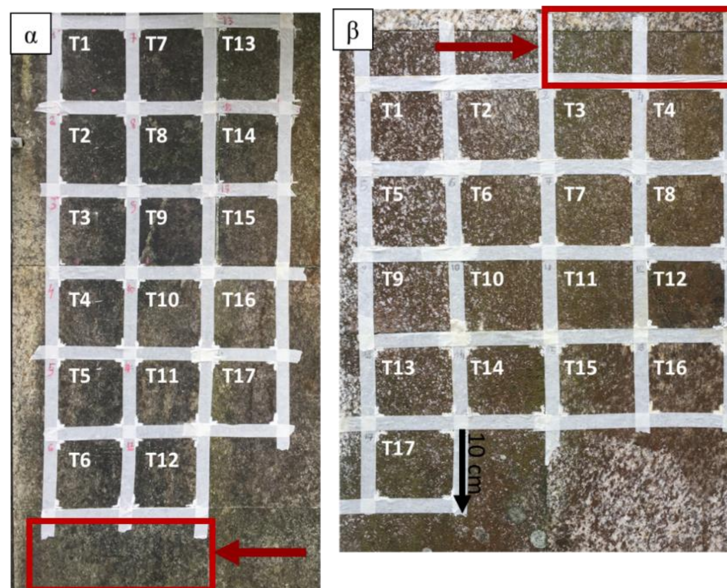

Figure 8 - The granite biocolonized walls subjected to the treatments. α) Prevalence of dark-green patina; β) Prevalence of red-green patina. Composition of each treatment: T1 = *O. vulgare*; T2 = *T. vulgaris*; T3 = *C. nepeta*; T4 = Carvacrol; T5 = Thymol; T6 = Pulegone; T7 = *O. vulgare* + *T. vulgaris*; T8 = *O. vulgare* + *C. nepeta*; T9 = *C. nepeta* + *T. vulgaris*; T10 = *O. vulgare* + *T. vulgaris* + *C. nepeta*; T11 = Carvacrol + Thymol; T12 = Carvacrol + Pulegone; T13 = Pulegone + Thymol; T14 = Carvacrol + Thymol + Pulegone; T15 = Hydrogel; T16 = Preventol® RI80; T17 = Water. The red rectangles and the arrows individuate the zones where the sampling of the biological material has been performed.

2.4.1. Chemical composition of the formulations containing the EOs and the APs

The ideation of the treatments employed for the experimentation included the same procedures described for the Experiment 1 and Experiment 2. The concentration employed to prepare the formulations containing the pure APs and the three paired APs reflect ones of the Experiment 1, having used the same EOs bought from the same retailers.

Anyway, in the following experiment, six more treatments have been employed, three containing the mixture of two EOs and three others the two APs, as in Experiment 2. In Table 6 all the concentrations and the composition of the single treatments containing the phytochemicals have been reported.

Table 6 – Composition of the treatments containing the phytochemicals and concentrations of the substances employed for their preparation applied on the biocolonized wall of the faculty of Pharmacy of the USC.

	[w/w] %					
Treatments	*O. vulgare*	*T. vulgaris*	*C. nepeta*	Carvacrol	Thymol	Pulegone
T1	2.00	//	//	//	//	//
T2	//	2.00	//	//	//	//
T3	//	//	2.00	//	//	//
T4	//	//	//	1.41	//	//
T5	//	//	//	//	0.93	//
T6	//	//	//	//	//	1.10
T7	1.00	1.00	//	//	//	//
T8	1.00	//	1.00	//	//	//
T9	//	1.0	1.00	//	//	//
T10	0.67	0.67	0.67	//	//	//
T11	//	//	//	0.70	0.46	//
T12	//	//	//	0.70	//	0.55
T13	//	//	//	//	0.46	0.55
T14	//	//	//	0.47	0.31	0.37

2.4.2. Sampling of the biological material

The biofilm was collected from two portions adjacent to the two selected biocolonized surfaces, in order to characterize the microbial species present. The sampling areas are indicated by a red rectangle in Figure 8. For each surface, three sampling of

biological material have been collected. The sampling required the employment of sterile swabs that, after the procedure, were preserved in sterile tubes containing a buffer solution 5% of NaCl. Afterwards, the biological material was characterized, as reported in the following section.

2.4.3. Characterization of the biofilm

The microorganisms of the subaerial biofilm present in the samples were examined under light microscopy (LM) with a Nikon Eclipse E600 equipped with an E-Plan 40x objective (N.A. 0.65) and differential interference contrast (Nomarski) optics. LM photographs were taken with an AxioCam ICc5 Zeiss digital camera.

Species identification and the nomenclature used was mainly based on [148] and [149] for the identification of green algae (Chlorophyceae); [150] for cyanobacteria (Cyanoprokariota); and [151] for diatoms (Bacillariophyceae).

The samples have been homogenized and the taxa cells quantitatively counted in a Utermohl sedimentation chamber [152], the abundance of the taxa was expressed in percentage from a total count of 1000 cells (Table 10, section 3.4.1).

2.4.4. Colorimetric and Fluorescence measures for the characterization of the cleaning and biocidal action

The monitoring lasted eight months from the application of the treatments, in order to have a medium-term determination of the action and the lasting potential biocidal efficacy of the compounds. The measurements were performed on the biocolonized surfaces (t_0), immediately after the removal of the treatments (t_1), six months after the removal (t_2) and eight months after the removal (t_3). The cleaning effectiveness of the product was assessed at t_1 while, to have an evaluation of the medium-term biocidal efficacy of the products, the considered data are the ones acquired at the end of the monitoring (t_3).

Colorimetric measurements were performed on each single quadrant, for a total of 3 random acquisition per each treated surface. The average values have been calculated and employed for the data elaborations.

As reported in sections 2.2 and 2.3, the data were analysed using the CIELAB colour system [153], and the ΔE^*, Δa^* and Δb^* were calculated using the equations (1), (2) and (4).

The apparatus employed is a Konica Minolta portable spectrophotometer equipped with a measure head with 50-mm-diameter viewing area (CR-310, Konica Minolta, Japan). Illuminant D65, 2° observer, Specular Component Included (SCI) mode were employed as measurements conditions.

Chlorophyll-a fluorescence measurements were also performed by using a Pulse Amplitude Modulated (PAM) fluorometry apparatus, to assess the biological activity of the microorganisms. Fluorescence signals were acquired with a Phyto-PAM (Heinz Walz GmbH) equipped with a fibreoptic emitter-detector unit Phyto-EDF.

In this study, the emitted F_0 (minimal 'in vivo' fluorescence, or the fluorescence signal of dark-adapted cells) was obtained at 665 nm. This parameter quantify the chll a content, and it is considered and indicator of the presence of photosynthetic microorganisms on the surfaces [103,114,129].

The relative differences (ΔF_0) were calculated between t_1 and t_0 and t_3 and t_0 for the assessment of the cleaning and biocidal action.

The apparatus requires darkness conditions to perform the measurements, in order to guarantee an ample dark-adaptation time to allow all the PSII reaction centres to open. For this reason, the fluorescence measurements were performed under a black plastic cover, in hours of the day where the surfaces were not directly exposed to the sunlight [111].

For each panel, 5 readings have been acquired on 5 randomly selected points and then the average value, representative of one panel, was calculated.

CHAPTER 3. Results and Discussion

3.1. Assessment of the compatibility of the hydrogel with the stone materials and the phytochemicals

When each concentration has been established (sections 2.1.4; 2.2.3; 2.3.1 and 2.4.1), the preparation of the formulations required only the mixing of the needed quantity of compound with the hydrogel. The substances were mixed for 15 minutes, with the help of a magnetic stirrer, and homogeneous emulsions in which the phytochemicals are dispersed have been obtained.

The employment of this hydrogel presents many advantages for the success of the current experimental study: i) it allows to create formulations in which the active substances can be dispersed at certain established concentrations, in a way that prevents them from being applied directly on the surfaces since this is not a recommended practice ; ii) the preparation of the formulations, as the way of application on the surfaces, require a very essential laboratory equipment (mainly: a precision balance ±0.01, a magnetic stirrer, water, spatula and paint brush) and in a very short amount of time; iii) the film-forming properties of the hydrogel allow to simply peel off the superficial film, leaving no residue on the surfaces; iv) its adhesive properties contribute to the detachment of the biofilm, and other particles, from the surface, with an enhancement of the overall cleaning action, combined with the biocidal one of the phytochemicals, v) its chemical composition is in line with the principle of creating products with low environmental impact.

To assemble the formulations, experimental testes were led to assess the compatibility of the hydrogel with the phytochemicals and the stone materials.

As expected, it emerged that the needed quantity of hydrogel for the formation of a uniform film layer on the stone surface after its drying depends on the intrinsic porosity of the stones [154]. In fact, for travertine, which presents a diffuse macro porosity, the needed quantity of hydrogel (0.5 g/cm2) is double compared to the amount for granite (0.25 g/cm2), a lithotype characterized by a more uniform and compact structure.

The hydrogel is easily applicable on the stone surface with a brush and, after a relatively short period, depending on the external environmental conditions and the humidity present (from 7 days in controlled condition to 14 days on site, in presence of precipitations

and relatively high humidity), the gel layer solidifies forming a compact homogeneous film that was easily peeled off from the surface, leaving no residue.

Once the compatibility of the material with the stones was established, the hydrogel was combined with the EOs and the APs. The preparation only requires mixing the hydrogel with the phytochemicals under stirring motion (above 15 minutes) at the chosen concentrations. The obtained products preserve almost the totality of the physico-chemical properties of the original matrix, except for a decrease of the viscosity for high concentrations of the solute, with possible consequences on the application on site, where the product may fall down from a vertical surface before its complete drying.

In Figure 9 an example of application of the emulsions prepared with the hydrogel and one essential oil is shown.

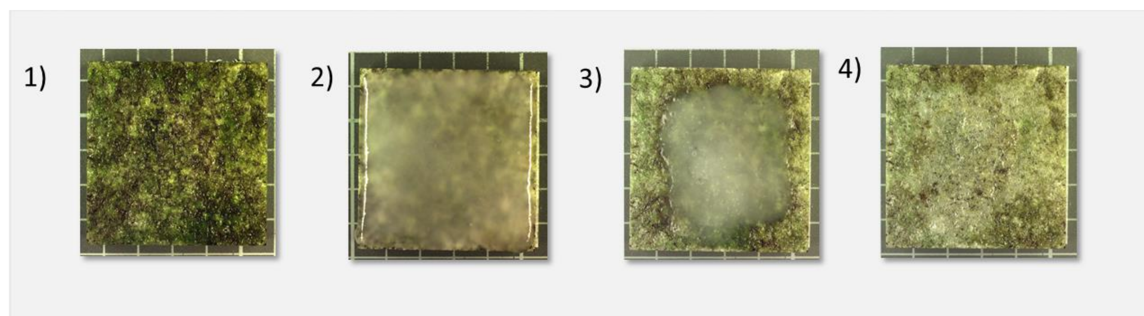

Figure 9 – Photographical illustration of how the new formulated products appear when applied on stones (granite samples). 1) Untreated biocolonized granite; 2) Immediately after the application of the treatments; 3) Two days after the first application; 4) Dry product (one week).

At the end, the experimental tests allowed to select 2% w/w as the suitable concentration for the preparation of the formulations, taking into account both the biocidal effect and the easiness of application (see also section 2.1.4).

The application on site followed the laboratory experimental tests, demonstrating once again the suitability of the methodology, as shown in Figure 10.

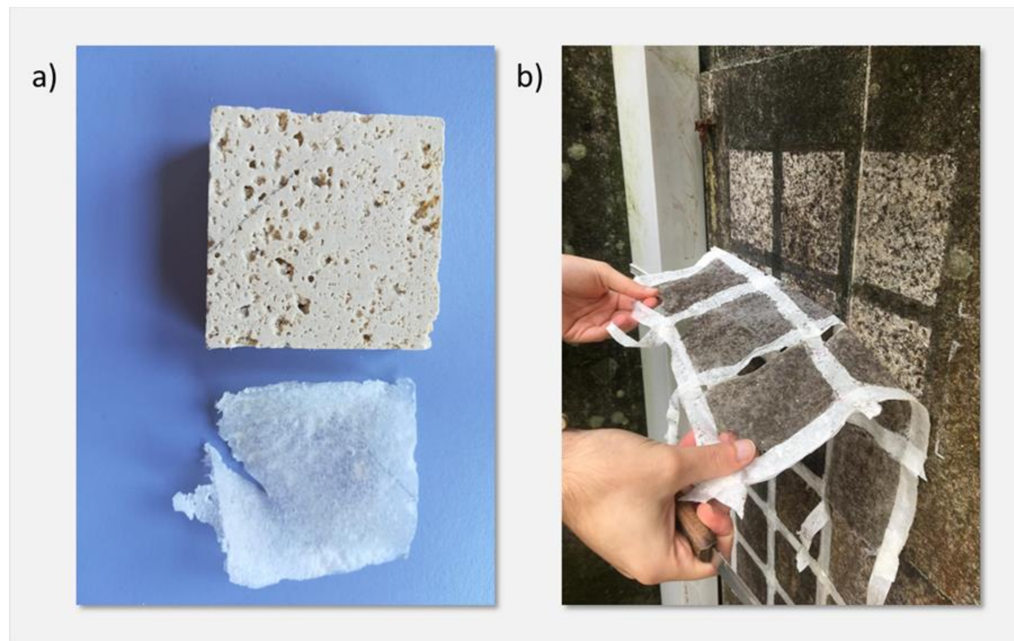

Figure 10 – Removal of the treatment after complete drying. The hydrogel matrix forms a uniform film layer that can be easily detached from the stone by peeling. **a)** Travertine sample (5 cm x 5 cm x 2 cm); **b)** The experimental biocolonized granite surface (Dept. of Farmacia, Universidade de Santiago de Compostela, USC).

3.2. Experiment 1

In all cases, a single application of the treatment successfully removed most of the subaerial biofilm, as assessed by naked eye observation (Figure 11). After cleaning, main visual changes were observed in T3, T6, T9, T11 and T12; two weeks after cleaning T4 and T7 were added to the list (Figure 11).

Before cleaning, chromatic colour data from all samples were included in a small colour gamut, ranging between -5.0 and -3.0 CIELAB units for a*, and between 18.1 and 15.3 CIELAB units for b* (Figure 12). They can be considered similar starting points according to colorimetric criteria and considering the upper limit of rigorous colour tolerance or noticeable change in colour of three CIELAB units [133–135].

As seen in Figure 12 and Table 7, after cleaning, all treatments led to colour changes towards red (marked by an increase in the coordinate a* and positive values of Δa*) and blue (marked by a decrease in the coordinate b* and negative values of Δb*) components.

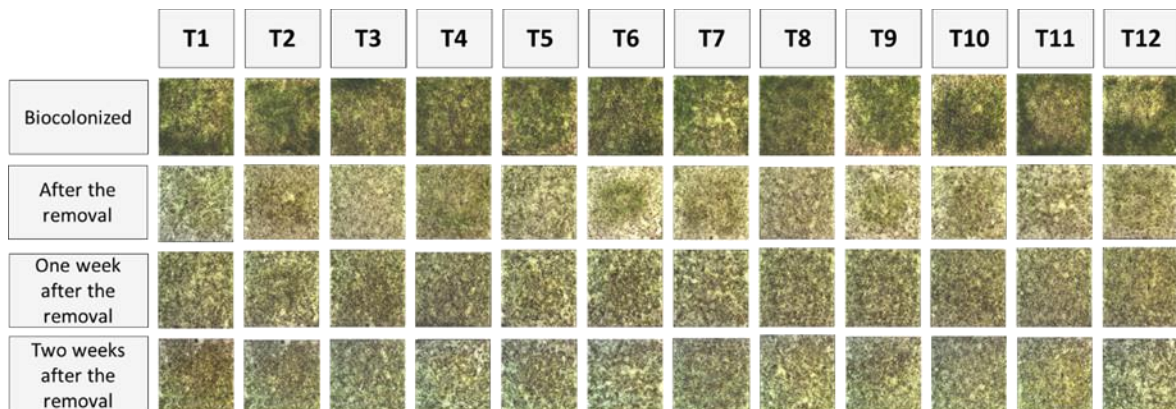

Figure 11 - Macroscopic appearance of the samples studied throughout the experimental period.

This trend continued until the end of the experiment, and it increased with time. Thus, two weeks after cleaning the chromatic values are close to the reference value indicated by an uncolonized-clean granite sample (Figure 12). It demonstrated that the changes were associated with the effective cleaning process of the experiment and not with a change in colour of the granite substrate. In this regard, when observing the more consistent variations of Δb*, it seems that this coordinate was more informative for the purpose of the study, in line with previous studies of phototrophic biofilms on granite rocks [19].

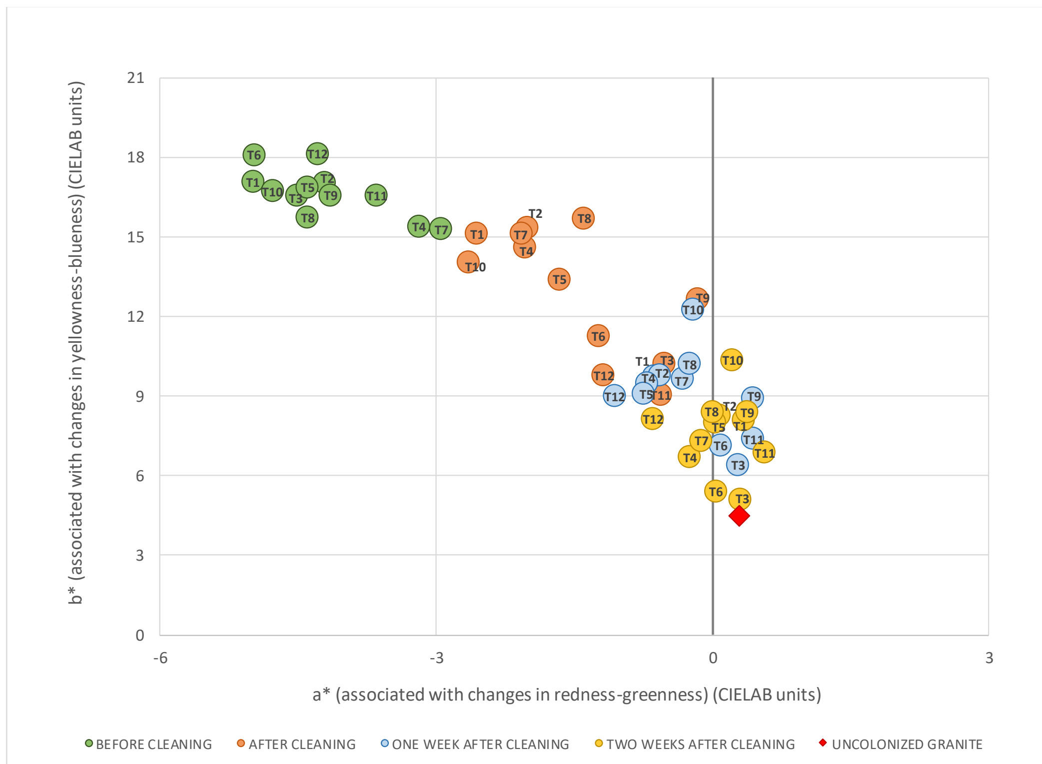

Figure 12 - Colour changes of the samples studied throughout the experimental period in the b* versus a * diagram. Each symbol is also identified by a sub-legend showing the sample's treatment (codes are shown in Figure 3, section 2.2).

Taking into account both chromatic coordinates, the treatments that provoked significant colour changes after their application were T3, T5, T6, T9, T10, T11 and T12, while those that exceeded the threshold of 3 CIELAB units in both partial differences Δa*and Δb* were T3, T6, T9, T11 and T12 (Table 7). These results are consistent with those reported by naked eye observation (Figure 11).

In T6, T9 and T11 there were significant changes in values of Δa* (increasing) and Δb* (decreasing) since the moment immediately after treatment and one week after, and in the case of T3 it was only in Δb* (Table 7). It shows that after cleaning, these four treatments left behind some alive organisms over the surface of the stone, whose colour after one week turned from pale green to yellow, and then bleached, due to the senescence and death of the cells, and the concomitant degradation of chlorophyll-a content [19,137,155].

Table 7 - Changes in the green-red colour component (Δa^*) and blue-yellow colour component (Δb^*) in the treated samples throughout the study period. Different superscript letters in each row indicate significant differences ($p \leq 0.05$) in relation to the different stages of the sample's treatment for a given partial colour difference[3].

		Δa* (CIELAB units)			**Δb* (CIELAB units)**		
		After cleaning	**One week after cleaning**	**Two weeks after cleaning**	**After cleaning**	**One week after cleaning**	**Two weeks after cleaning**
T1	*T. vulgaris*	2.4^{A}	4.4^{B}	5.3^{B}	-2.0^{A}	-7.3^{B}	-9.0^{B}
T2	*O. vulgare*	2.2^{A}	3.6^{B}	4.3^{C}	-1.7^{A}	-7.3^{B}	-8.8^{B}
T3	*C. nepeta*	4.0^{A}	4.8^{A}	4.8^{A}	-6.3^{A}	-10.1^{B}	-11.5^{B}
T4	Thymol	1.2^{A}	2.5^{B}	2.9^{B}	-0.8^{A}	-5.9^{B}	-8.7^{B}
T5	Carvacrol	2.7^{A}	3.6^{AB}	4.4^{B}	-3.5^{A}	-7.8^{B}	-8.9^{B}
T6	Pulegone	3.7^{A}	5.1^{B}	5.0^{B}	-6.8^{A}	-10.9^{B}	-12.7^{B}
T7	All EOs	0.9^{A}	2.6^{B}	2.8^{B}	-0.2^{A}	-5.6^{B}	-8.0^{C}
T8	All APs	3.0^{A}	4.1^{B}	4.4^{B}	0.0^{A}	-5.5^{B}	-7.3^{C}
T9	Hydrogel	4.0^{A}	4.6^{B}	4.5^{B}	-3.9^{A}	-7.6^{B}	-8.2^{B}
T10	Preventol + Hydrogel	2.1^{A}	4.6^{B}	5.0^{B}	-2.7^{A}	-4.4^{AB}	-6.3^{B}
T11	Preventol	3.1^{A}	4.1^{B}	4.2^{B}	-7.5^{A}	-9.2^{B}	-9.7^{B}
T12	Water	3.1^{A}	3.2^{A}	3.6^{A}	-8.4^{A}	-9.1^{A}	-10.0^{A}

At the end of the experiment, all treatments achieved values of a* ranged between -0.7 and 0.6 CIELAB units, very close to the reference a* value of the uncolonized granite of 0.3 CIELAB units (Figure 11). In the case of b*, the values ranged between 10.4 and 5.1 CIELAB units, with a reference value of 5.2 CIELAB units (Figure 11), allowing to use this coordinate to evidence the differences between the treatments success rate in line with what has been mentioned above. Accordingly, T3 and T6 obtained a value practically identical to that of the reference, with values of b* of 5.1 and 5.4 CIELAB units respectively, yielded therefore the best results. T4, T11, T7, T5 and T1 produced results of b* away from reference value in 1.5, 1.7, 2.1, 2.8 and 2.9 CIELAB units, all below the previously indicated visual threshold of 3 CIELAB units. The other treatments, i.e. T12, T2, T8 and T9, with b* values ranged between 8.2 and 8.4 CELAB units, barely exceeded this threshold; whereas T10 reached a partial difference of b* compared to the reference of 5.2 CIELAB units (> 5

[3] For example: considering the treatment T1, significative differences (i.e. p-value ≤ 0.05) occur between the Δa* after cleaning and Δa* one week after cleaning, being these values characterized by two different superscript letters (A and B), while no significative differences occur between the Δa* one week after cleaning and Δa* two weeks after cleaning, being these points characterized by the same superscript letter (B). This is valid for all the other points.

CIELAB units, the normal limit of perception in industrial or technical applications [133,156]).

Pulegone (T6) is the main component of *C. nepeta* oil (T3), so the greatest effectiveness of both treatments in the cleaning should be attributed to this terpenic ketone, which influences the biocidal properties of the oil itself. For visual comparative purposes, these results can also be observed in Figure 13.

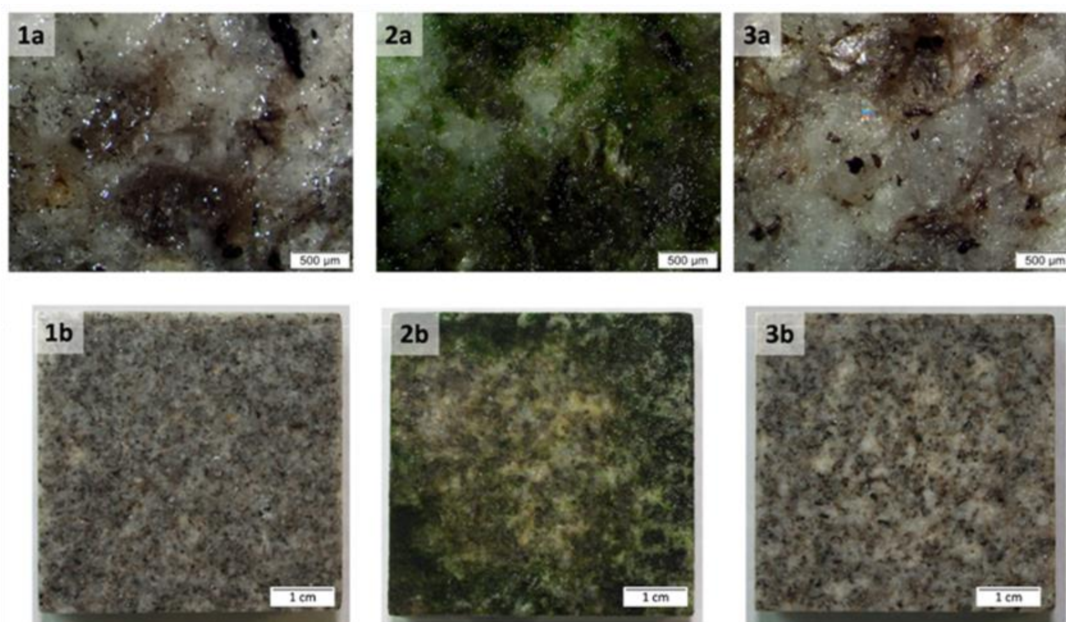

Figure 13 - Granite samples (a) examined under a stereoscopic microscope Nikon Eclipse E600, Tokyo, Japan. Scale bar is 500 µm. (b) photographed with a macro lens Tamron SP 90mm F/2.8 Di MACRO 1:1. Scale bar is 1 cm. (1) Uncolonized, (2) colonized, (3) cleaned with *C. Nepeta* (T3).

A previous study where the essential oil active components (EO-ACs): limonene, menthone, pulegone and menthol were tested against the bacteria *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella veneziana*, *S. paratyphi* B and *S. typhimurium*, and the fungi *Fusarium moniliforme*, *Botrytis cinerea*, *Aspergillus niger* and *Pyricularia oryzae*, using the agar diffusion technique, also showed that only pulegone had an effective response regarding the antimicrobial activity, particularly against the *Salmonella* species [82]. Preventol RI-80® embedded in a hydrogel matrix (T10), on the contrary, it was the least effective cleaning treatment of the twelve tested, but not its two components separately, Preventol RI-80® applied with a brush (T11) and hydrogel (T9). This could be due to an incompatibility of both compounds when mixed, which impedes the effective action. Similarly, the treatments

where the three essential oils were combined (T7) and, to a greater extent, where the three active compounds were combined (T8) yielded rather poor results, similar to *T. vulgaris* (T1) and *O. vulgare* (T2) oils separately, and their active components Thymol (T4) and Carvacrol (T5) also separately, which indicated that the effect of *C. nepeta* (T3) and Pulegone (T6) in the mixture, is either neutralized or turns out to be in an excessively low percentage to achieve a successful effect. However, in a previous study of Bruno et al. (2019) [130] the application of a combination of essential oils from *L. angustifolia* and *T. vulgaris* was effective in kill phototrophic biofilms even at low concentrations. Also, *O. vulgare* proved, in a previous study [47], to be the most effective, compared to *L. angustifolia* and *R. offocinalis*, with regards to antifungal properties, using *Epicoccum nigrum* and *Bipolaris spicifera* as test species, with comparable results to those obtained with the commercial biocide QACs derivate benzalkonium chloride.

It is well-known that essential oils are widely used in various industries. However, it should be remembered that the chemical composition of the EOs and the content of bioactive compounds are variable, even when they come from the same plants. Differences in composition of the EOs may affect their effectiveness and may be variable. Also, it is possible to assume that the efficacy of essential oils is closely related to the ratio of the active components in their composition, whereas the efficacy of essential oils and essential oil active components are related to the targeted microorganisms. As an example, *C. nepeta* of autochthonous aromatic plants from Alentejo (Portugal) demonstrated high toxicity against Artemia salina shrimp larvae and presented higher content in oxygenated monoterpenes,with 1,8-cineole as the main component [157] ,while in our study 1,8-cineole was found at a concentration of barely 0.5% (Table 1).

3.3. Experiment 2

3.3.1. Quantification of the extracted biological material

In Table 8 are reported the results of the spectrophotometric analyses, for the DNA extracts obtained from the non-invasive sampling procedure with swabs (Sw 1, Sw 2, Sw 3), from the micro-invasive sampling procedure with adhesive tape (AT 1, AT 2, AT 3) and for the invasive sampling procedure with a scalpel (Sc 1, Sc 2, Sc 3). As expected, the sampling method that produced the best results is the one provided by the invasive procedure (Sc 1, Sc 2, Sc 3) The concentration and the quality of the DNA extracted is lower in the samples obtained with the non-invasive swab compared to the adhesive tape procedure. These variations can be associated to a very low concentration of genomic material or to the presence of different contaminants associated to the extraction protocol, such as phenol or other reagents [158]. Anyway, it can be assumed that few adjustments to the microinvasive sampling technique and the DNA extraction procedure can lead to optimal results ($A_{260/280}$ in the optimal 1.8-2.0 range).

Table 8 - Concentration and pureness values of the extracted DNA from the Swab (Sw1, Sw2, Sw3) and the Adhesive Tape (AT1, AT2, AT3) and Scalpel (Sc 1, Sc 2, Sc 3) procedures.

Samples	**[DNA] (ng/µL)**	**A_{260}/A_{280} (ng/µL)**
Sw 1	35.3	0.70
Sw 2	29.8	0.63
Sw 3	68.9	0.94
AT 1	125.4	1.21
AT 2	87.3	1.08
AT 3	264.4	1.51
Sc 1	829.7	1.78
Sc 2	558.4	1.72
Sc 3	336.7	1.60

3.3.2. Biofilm genomic characterization

The amplification of the rDNA of the extracted biological material with PCR allowed to have a preliminary characterization of the biofilm. The results of the agarose gel

electrophoresis, shown in Figure 14, shown the amplification of the DNA of the samples for each selected rDNA sequences (16S, ITS, 18S), confirming the presence of microorganisms belonging to the selected kingdoms: bacteria (Figure 14 a,d,g), fungi (Figure 14 b,e,h) and plants (Figure 14 c,f,i). This can be stated comparing the unknown samples with the positives samples (Figure 14 a,b,c,d,e,f,g,h,i): 1550 bp (Escherichia coli), 1808 bp (Arabidopsis thaliana), 550 bp (Fusarium verticillioides) for 16S, ITS and 18S amplicons respectively.

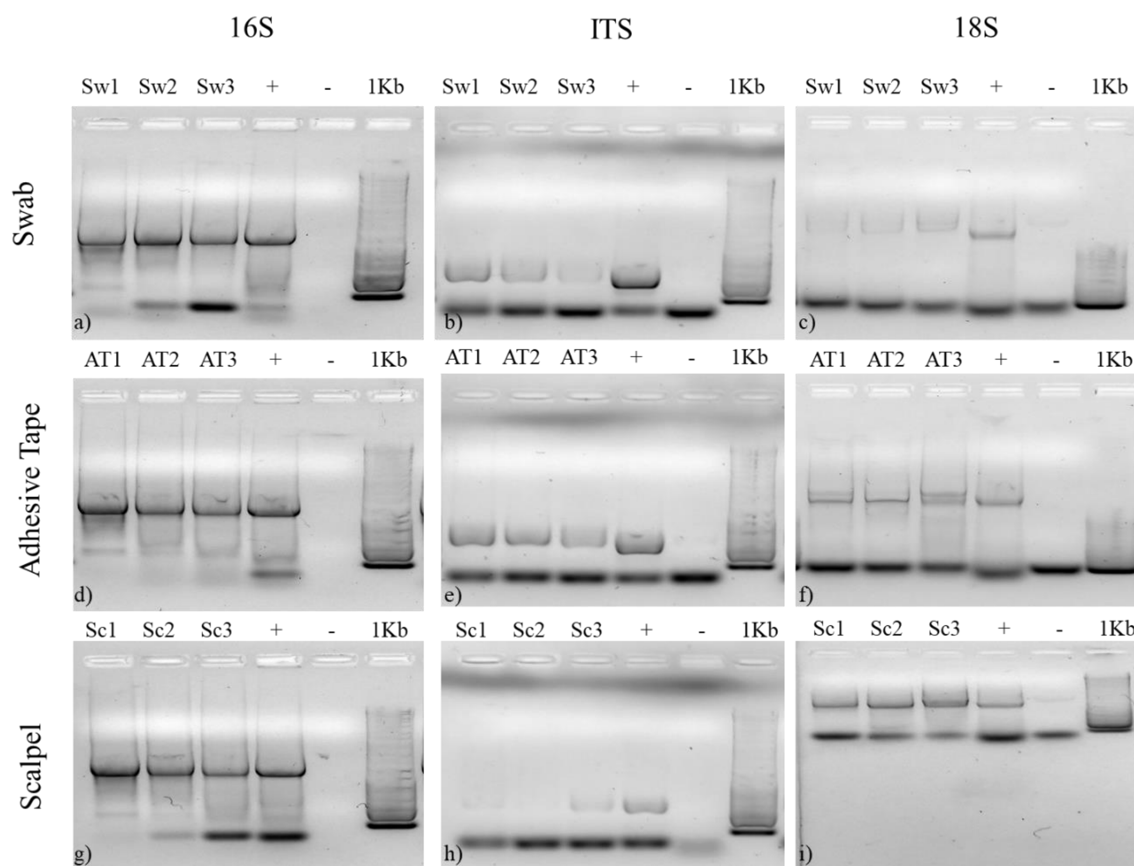

Figure 14 - Agarose gel electrophoresis of 16S (a,d,g), ITS (b,e,h) and 18S (c, f,i) regions, amplified from DNA extracted from biological material sampled with swab (a, b, c) adhesive tape (d, e, f) and Scalpel (g,h,i).

3.3.3. Colour characterization

Colour measurements were carried out on the biocolonized travertine surface B before (time t_0) and after (time t_1) the application of the emulsions containing the essential oils (surfaces A) and the phenolic compounds (surfaces B) (see Figure 5). In the case of the

surfaces treated with the essential oils, the colour measurements were replicated six weeks after the cleaning procedure (t_2) to verify eventual chromatic variations[4].

To evidence the existing colour differences existing, in Figure 15 are shown four graphical representations of the colorimetric results In Figure 15a and Figure 15c the data have been plotted in a mono-dimensional graph to evidence their displacement along the L* axis, in Figure 15b and Figure 15d the points have been plotted in a bidimensional cartesian coordinates system, in which a* is the abscissa and b* the ordinate. Observing the representation reported in Figure 15a and Figure 15b, where the colour points of the surfaces treated with the essential oils and the phenolic compounds at t_0 and t_1 have been compared, a shift emerges towards positive values of a* for most of the points (excepted A5 and B4) and a significant increasing in L* in each panel at t_1. This general behaviour is also confirmed in the graphs represented in Figure 15c and d, where the comparison of the colour spots treated with the essential oils at t_0, t_1, t_2 occurs. However in this case too each point shows an increase of L*, the most remarkable aspect concerns the progressive shift of all the points towards higher values of a* that at t_2 are all situated in the quadrant of the positive a* and b*, in which the green component is not present. In general, these results suggest: i) a possible disappearance of green photosynthetic pigments produced by microorganisms commonly detected in cultural heritage biofilm linked to the decrease of the green component in colour, ii) the variation of the colour surface towards white, (i.e. the colour of travertine), iii) a prolonged action of the products over time, as confirmed by the colorimetric variations six weeks after the end of the cleaning procedure. All these elements suggest a possible return to the original colour of the stone, although it was impossible to determine it since all the surrounding surfaces presented colour alteration and a sample of the original quarry material was not available.

In light of the above, it is possible to state the biocidal potential of the phytochemicals against a multispecies biofilm, composed by microorganisms belonging to the bacteria, fungi and plants kingdoms, as evidenced by the genomic characterization. This seems to confirm the efficacy of phytochemicals also against microorganisms belonging to the plant kingdom, such as algae, frequent colonizers of stone artistic surfaces. In fact, the biocidal properties

[4] Unfortunately, for reasons of force majeure, it was impossible to reproduce the same measures also for the surfaces treated with the APs.

of the phytochemicals against fungi and bacteria have been demonstrated in different studies, while little is known about their activity against photosynthetic microorganisms.

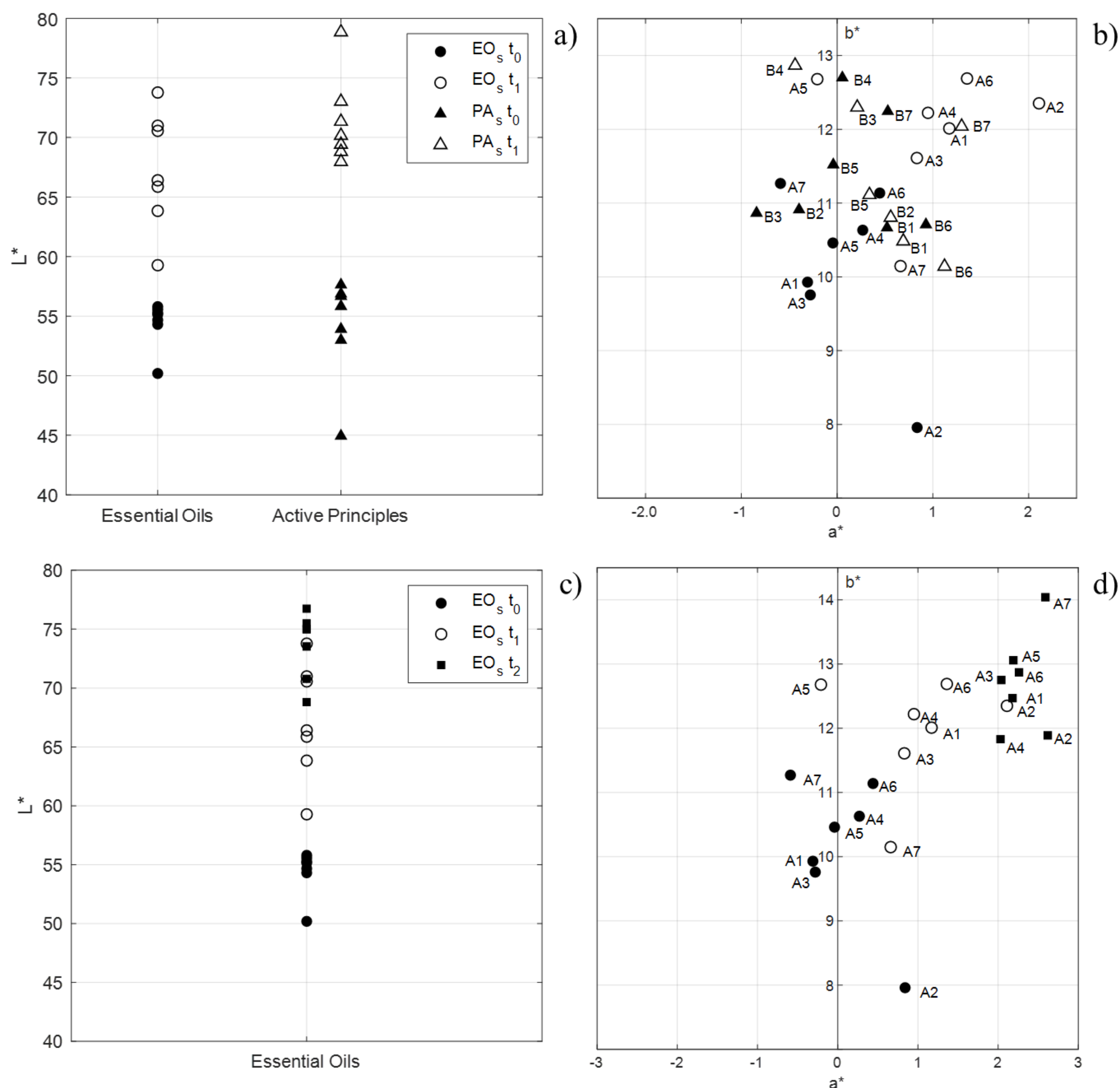

Figure 15 - Representation of the displacement of the colour points inside the monodimensional L* (a, c) and bidimensional a*b* (b, d) colour spaces. Due to the difficulties in the representation of each treatment inside the graphs, in a) and c) only the shift of the colour points along the L* is represented, in order to evidence the increase of the Lightness during the time, regardless of the specific treatment. In a) and b) the colours obtained from the cleaning of the surfaces with the essential oils and the phenolic components at t_0 and t_1 are compared. In b) and c) the colours obtained from the cleaning of the surface with the essential oils at t_0, t_1 and t_2 are compared.

Considering what has been said about the impossibility of identifying the original colour of the surface, the ΔE^*_{ab} parameter was considered to evidence the efficacy the treatments and the differences existing between them, since this parameter quantifies the overall colour difference between the initial and the final situation for each colour point, which corresponds to a specific treatment. The ΔE^*_{ab} have been calculated (Table 9) and showed in Figure 16.

Table 9 - Colorimetric variations (ΔL^*, Δa^*, Δb^*, ΔE^*_{ab}) before (t_0) and after (t_1) and after 6 weeks (t_2) from the cleaning procedure

	t_1-t_0				**t_2-t_1**			
	ΔL*	**Δa***	**Δb***	**ΔE^*_{ab}**	**ΔL***	**Δa***	**Δb***	**ΔE^*_{ab}**
A1	10.86	1.48	2.08	11.22	7.11	1.01	0.45	7.33
A2	17.98	1.27	4.39	18.60	1.24	0.52	0.46	2.77
A3	9.54	1.11	1.86	10.04	4.95	1.21	1.13	5.71
A4	15.72	0.68	1.59	15.82	4.51	1.08	0.40	7.57
A5	11.19	0.16	2.22	11.55	9.10	2.39	0.38	9.47
A6	15.41	0.91	1.55	15.54	6.18	0.91	0.18	6.41
A7	9.09	1.25	1.12	9.70	11.49	1.93	3.89	12.59
B1	15.39	0.17	0.18	15.51	//	//	//	//
B2	14.97	0.96	0.11	15.04	//	//	//	//
B3	24.47	1.05	1.43	24.56	//	//	//	//
B4	11.94	0.49	0.17	12.01	//	//	//	//
B5	15.51	0.38	0.41	15.57	//	//	//	//
B6	22.18	0.19	0.57	22.24	//	//	//	//
B7	16.25	0.77	0.21	16.40	//	//	//	//

In Figure 16a the comparison of the ΔE^*_{ab} of the essential oils (EOs) and the active principles (APs) between t_0 and t_1 are represented, while in 4Figure 16b it is possible to compare the colour variations of essential oils at t_1 and t_2. Observing the graph (a) it appears evident that the spots treated with the products containing the active principles demonstrated a better efficacy related to the colour variations. This is true for all cases except for B2 and B4, containing respectively the mixtures of carvacrol-thymol and thymol-pulegone. It is interesting to notice that the bigger chromatic variation is associated to the emulsion containing pure thymol (B3). In light of the above, it appears that the action of the pure thymol decreases in presence of other compounds, as also confirmed when comparing B3 with the value obtained for B7. This statement is not always true for pulegone and carvacrol,

the combination of which (B6) has proved to be second in terms of effectiveness. Comparing the results, correlations between the emulsions containing the essential oils with the respective active components do not emerge. In the cases of the surfaces treated with EOs, the combined action of two compounds (A2, A4, A6) gives better results compared to the pure substances (A1, A3, A5), though this in not true for the mixture containing the three oils together (A7) which is overall the one with the lower value of ΔE^*_{ab}.

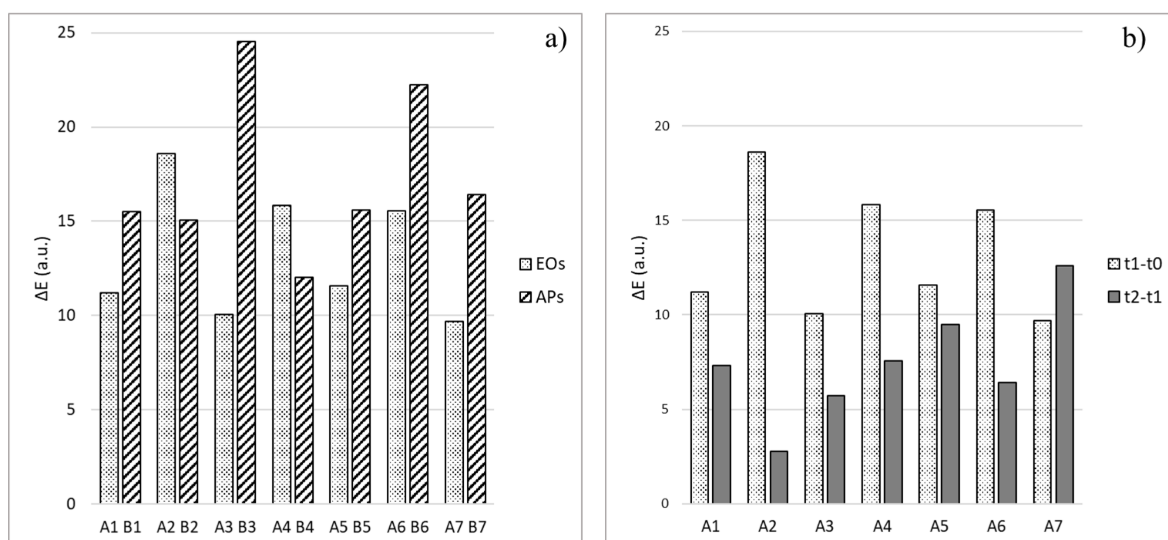

Figure 16 - Total colour differences (ΔE*ab) of the surfaces subjected to the cleaning procedures. a) Comparison of the ΔE*ab (t_1-t_0) of the formulations containing the essential oils and the phenolic components. b) Comparison of the ΔE*ab (t_1-t_0) and ΔE*ab (t_2-t_1) of the formulations containing the essential oils.

These synergic or antagonistic effects provoked by the combined action of two essential oils have been underlined in other studies. In some cases, two essential oils combined together demonstrated an evident increase of the biocidal action also against resistant microorganisms immune to the effect of the single substances [159]. This can be explained because in essential oils many chemical compounds coexist together, each of which is characterized by a specific role in the defence against microorganisms. For example in *Thymus vulgaris* oil, the main biocidal action is attributed to carvacrol and thymol as discussed in this research, but the presence of p-cymene seems to favour the swelling of microorganisms' cell membrane, contributing to the biocidal action [160].

However, this cannot be assumed as always true, because opposite mechanisms have been demonstrated in other circumstances [72]. In light of the above, it is very difficult to

univocally determine the mechanisms involved in the biological activity of essential oils and to create a selective product against a specific population of microorganisms, because of the heterogeneity in the composition of the natural substances. For this reason, even if the essential oils seem to be the elective product in the removal of the biofilm, the isolated phenolic compounds can be considered a valid alternative demonstrating good results. The ΔE^*_{ab} results reported in Figure 16b confirm what was discussed previously: the colour of the surfaces continues to change in every treated panel, even six weeks after the removal of the products. In particular, in the case of A7 it is significant how the colour difference is bigger at t_2 than at t_1, suggesting the penetration of the oils in the porous structure of the stone and a prolonged biocidal action over time.

In light of these results it is possible to highlight the following: both the essential oils and their active principles have proven to be active in tackling the problem of biodeterioration of the stone material, as expected. However, both positive and negative synergistic effects are observed when mixed formulations of oils or active principles are created: for example some active ingredients are more effective than the corresponding oils that contain them and when they are mixed with other principles their effectiveness is reduced, contrary to what happens instead with oils where the presence of minority components evidently plays a role in rendering the corresponding principles more active even if present at a lower concentration.

3.4. Experiment 3

3.4.1. Biofilm characterization

The biodiversity of the two SABs was confirmed by taxonomical and morphological analyses. The taxa composition (Figure 17) and relative abundances data of the subaerial biofilm are summarized in Table 10. Both SABs are mainly composed of green algae. Cyanobacteria have been also detected but are much more abundant in the samples collected from the **surface α**, which is also the one presenting the most heterogeneous microbial composition. This one, indeed, shows a co-dominance of green algae (48.1 – 61.9 %) and cyanobacteria (37.1 – 50.9 %), where dominating the taxa *Apatococcus lobatus* (Chodat) J.B.Petersen, *Desmococcus olivaceus* (Persoon ex Acharius) J.R.Laundon, *Nostoc* sp. Vaucher ex Bornet & Flahault, and *Gloeocapsa punctata* Nägeli (Table 10).

While the samples collected from the **surface β** are mainly composed of green algae (88.6 - 99.0%), especially dominating the *Trentepohlia aurea* (Linnaeus) C. Martius biomass. The presence of this alga inside the biofilm confirm the colour differences assessed by naked eye observation existing between the two surfaces: in fact all *Trenteopholia* species produce large amount of carotenoids, especially β-carotene (followed by zeaxanthin, neoxanthin, lutein, ascorbic acid, and α-tocopherol) which protect them from the energetic UVa and UVb solar radiations. The presence of these photosynthetic pigments gives to this terrestrial algae a typical yellow-orange and red colouration, in accordance with was has been observed in **surface β** [161].

On the other side, the abundance of the dark-green colouration of the **surface α** is attributable to the presence of the other microorganisms detected. In fact, is known that *A. lobatus*, a frequently found colonizer of stone materials, forms pale-green coatings over stone walls [162], while the dark coloration was assessed in biofilms containing *Nostoc* sp and *Gloeocapsa* sp.[163].

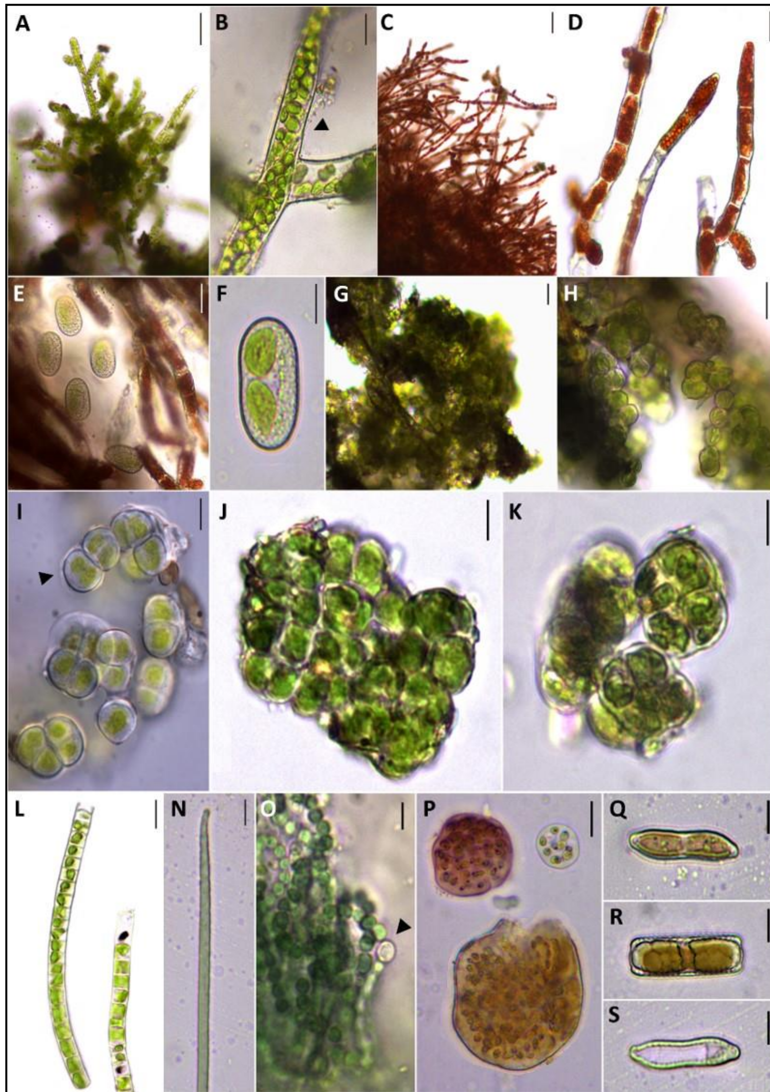

Figure 17 - Taxa identified in subaerial biofilms developed on the walls of the Faculty of Pharmacy of the University of Santaigo de Compostela, including bryophytes (A,B), green algae (C-L), cyanobacteria (N-P) and diatoms (Q-S): A-B) Protonema of bryophyta, general view (A) and philamentous with detail of the oblique walls indicated by an arrow (B); C-D) *Trentepohlia aurea* **(Linnaeus) C.Martius**, general view (C) and detail of the filaments with thick-walled cells and the numerous small discoid chloroplasts indicated by an arrow (D); E-F) *Mesotaenium macrococcum* **(Kützing ex Kützing) J.Roy & Bisset**, cells embedded in mucilage under the ramifications of *Trentepohlia* (E) and detail of a cell ovid with truncate ápices with a plate like chloroplast (F); G-I) *Apatococcus lobatus* **(Chodat) J.B.Petersen**, general view of irregular cellular aggregates (G,H) and detail of the cells with bilobate chloroplast without pyrenoid indicate by arrow (I); J-K) *Desmococcus olivaceus* **(Persoon ex Acharius) J.R.Laundon**, general view of irregular cell packets (J) and detail of cells with a sinlge parietal massive chloroplast (K); L) *Klebsormidium flaccidum* **(Kützing) P.C.Silva, K.R.Mattox & W.H.Blackwell**, philamentous with cylindrical-shaped cells and parietal chloroplast; N) *Oscillatoria formosa* **Bory ex Gomont**, trichome straight, flexible, gradually tapering at end, end cell blunt-conical without calyptra; O) *Nostoc* sp. **Vaucher ex Bornet & Flahault**, trichomes without mucilage formed by subspherical cells of 2.0-4.5 µm in diameter and heterocytes indicated by an arrow; P) *Gloeocapsa punctata* **Nägeli**, colonies with subspherical cells of 1.3-3.2 µm in diameter with individual or group mucilaginous envelopes, the mucilage of the colony is translucent in early stages and coloured in mature colonies from yellowish-brown to reddish; Q-S) *Hantzschia amphioxys* **(Ehrenberg) Grunow**, living cell in valvular vision (Q) and pleural vision (R), and frustule in valvar vision (S). Scale bar = 50 µm (A, C, G); 20 µm (E, H); 10 µm (B, D); 5 µm (F, I, K, L-S).

Table 10 - Composition and relative abundance of the subaerial biofilm taxa studied in the surface α (α1, α2, α3) and β (β1, β2, β3) samples.
(Bryo. = Bryophyta; Chloro. = Chlorophyceae; Cyano. = Cyanoprokariota; Bac. = Bacillariophyceae).

Taxa		Phylum/ Class	α 1 (%)	α 2 (%)	α 3 (%)	β 1 (%)	β 2 (%)	β 3 (%)
Bryophyte (protonema)		Bryo.	0	0	0	8.1	0	5.6
Trentepohlia aurea	(Linnaeus) C.Martius	Chloro.	0	0	0	73.7	75.2	71.6
Mesotaenium macrococcum	(Kützing ex Kützing) J.Roy & Bisset	Chloro.	0	2.5	0	5.8	12.8	9.7
Apatococcus lobatus	(Chodat) J.B.Petersen	Chloro.	51.6	37.8	36.2	6.4	9.2	8.5
Desmococcus olivaceus	(Persoon ex Acharius) J.R.Laundon	Chloro.	8.5	7.0	9.2	2.7	1.8	3.6
Klebsormidium flaccidum	(Kützing) P.C.Silva, K.R.Mattox & W.H.Blackwell	Chloro.	1.8	2.5	2.7	0	0	0
Oscillatoria Formosa	Bory ex Gomont	Cyano.	0	1	0	1	1	1
Nostoc sp.	Vaucher ex Bornet & Flahault	Cyano.	12.5	9.5	10.1	0	0	0
Gloeocapsa punctata	Nägeli	Cyano.	24.6	38.7	40.8	2.3	0	0
Hantzschia amphioxys	(Ehrenberg) Grunow	Bac.	1	1	1	0	0	0
		Bryo.	0	0	0	8.1	0	5.6
		Chloro.	61.9	49.8	48.1	88.6	99.0	93.4
		Cyano.	37.1	49.1	50.9	3.3	1	1
		Bac.	1	1	1	0	0	0

3.4.2. Preliminary characterization of the surfaces colour

The colorimetric results confirm what has been evaluated by naked eye observations and by the biofilm characterization. Visual differences in colour, due to the different colonization, are noticeable by observing the colorimetric data obtained from the experimental areas before the application of the treatments, or the removal of the biopatina.

In Figure 18 are represented the values reported in Table 11 of a*b* and L* inside the bidimensional (a) and monodimensional (b) colour spaces.

Table 11 - L*a*b* colour coordinates of the treated surfaces at time t_0 (biocolonized surfaces), for the **surface α** and the **surface β**.

	Surface α			**Surface β**		
Treatments	**L***	**a***	**b***	**L***	**a***	**b***
T1	25.9	-2.1	6.9	33.1	0.3	12.1
T2	23.8	-1.9	6.3	36.8	-0.9	11.7
T3	27.7	-1.6	7.1	33.3	-2.0	13.6
T4	28.9	-1.7	6.9	33.7	-0.7	14.9
T5	27.4	-1.7	6.8	35.7	-0.4	13.0
T6	26.3	-1.7	7.5	31.8	-1.4	13.1
T7	25.4	-2.1	6.7	31.9	-2.4	14.2
T8	25.0	-2.2	5.7	30.5	-1.6	14.7
T9	27.4	-1.2	5.3	31.8	-0.6	11.1
T10	28.5	-2.0	7.3	31.1	-1.7	13.3
T11	30.1	-1.5	8.2	29.6	-2.5	13.2
T12	27.3	-1.3	7.2	26.0	-1.1	11.4
T13	30.7	-3.6	9.4	29.8	-1.9	11.0
T14	31.4	-2.1	8.6	28.3	-1.8	13.3
T15	29.4	-1.6	8.5	32.1	-1.6	9.1
T16	33.1	-1.7	10.9	33.8	0.8	13.0
T17	29.0	-1.6	8.6	32.0	-3.7	12.3

A greater uniformity of the colours characterizing the **surface α**, which is the one presenting a darker-greenish patina is noticeable. In fact, except for T13α (a* = -3.6; b* = 9.4) and T16b (a* = -1.7; b* = 10.9) that can be considered outliers, all the data are included in a small colour gamut ranging between -2.2 and -1.2 for a* and 5.3 and 8.6 for b*. These data, can be considered similar starting points according to colorimetric criteria and considering the upper limit of rigorous colour tolerance or noticeable change in colours of three CIELAB colour units [133–135].

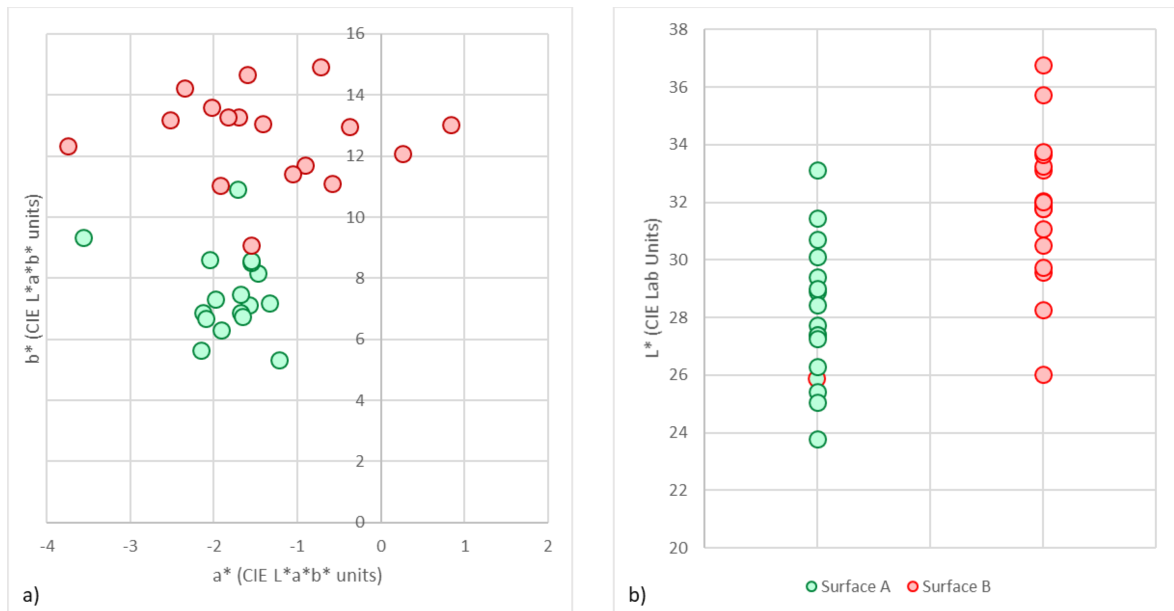

Figure 18 – Displacement of the colour data at the beginning of the experimentation inside the bidimensional (a) and monodimensional (b) colorimetric plans of a*b* and L*.

Moreover, the presence of the green component, attributable to the photosynthetic pigments produced by the microorganisms, is confirmed, because all the data (from T1α to T17α), at the beginning of the experimentation, are included in the part of the colorimetric space characterized by positive values of b* (predominance of yellow on the blue component) and negative values of a* (predominance in green on the red component). Otherwise the colour points obtained for **surface β**, apparently showing the presence of red photosynthetic pigments, are widely distributed inside the colour space, in an interval ranging from -3.7 to 0.8 for a* and from 9.1 to 14.9 for b*. However, compared to the points belonging to **α**, a major predominance in the red component is evident, especially for what concerns T16β (a* = 0.8 and b*= 13.0) and T1β (a* = 0.3 and b* = 13.0), located inside the part of the colour space of the positive a* and b* (predominance of red and yellow). T2β, T4β, T5β, T9β and T12β deviate from the more uniform colour range included between -2.0 and -1.4 for a* and 13.6 and 14.7 for b* (T3β, T6β, T7β, T8β, T10β, T11β, T13β, T14β, T15β), with a visible shift towards positive values of a*, and thus a lower contribute of the green colour component. T17β is the most shifted point towards the negative a*, with a predominance of the green component on the red one, probably indicating a significant presence of green photosynthetic pigments.

For what concerns the differences in lightness (Figure 18b), as expected from the visual observations, the colour points belonging to **β** are shifted towards bigger values of L*, that indicate a lighter colouration than the **surface α**.

3.4.3. Assessment of the colour changes of the surfaces after the application of the treatments

The peeling and the mechanical brushing (only in the cases of T16α and T16β, T17α and T17β) of the treatments after a single application, successfully removed the superficial biofilm present on the surfaces, as shown in Figure 19, where it is possible to observe the evident differences between the treated (lighter) and the untreated (darker) parts.

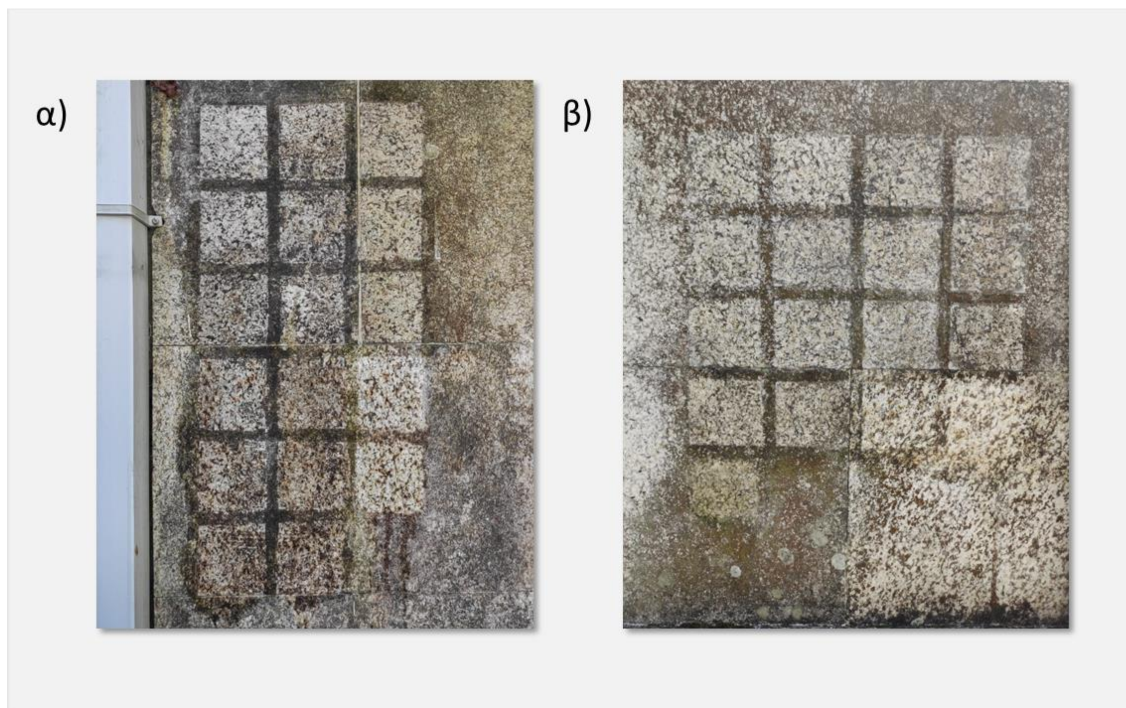

Figure 19 – Photographic illustration of how the treated surfaces appear after the removal of the products. It is possible to notice the visual differences in colour of the treated (brighter) and the untreated (darker) parts.

However, by naked eye observations, some treated parts appear lighter than others.

In **surface α**, this is found for the experimental areas treated with Preventol® RI80 and water, paired with the mechanical brushing (T16 and T17), while in the **surface β** this occurs for the surfaces treated with the hydrogel alone (T15) and Preventol ® RI80 (T16). In this last case, the mechanical brushing with water seems to produce the worst result.

In the other cases, the hydrogel combined with the phytochemicals apparently seems to not allow for the complete detachment of the more adhered biofilm, although this last assertation is denied by the colorimetric results. The colorimetric data obtained immediately after the removal of the compounds from the surfaces, in fact, proved the effectiveness in removing the biofilm, especially in the case of the surfaces treated with the HG-phyto systems, which showed the best results, as will be discussed as follows.

The overall colour variation (ΔE_{ab}*) that compares the colour data at t_1 (after the removal) to the ones at t_0 (biocolonized surface), evidences a significant colour variations of each treated surface (both in **surface α** and **β**), greatly exciding the three CIELAB colour units (upper limit of rigorous colour tolerance) (Figure 20 and Table 11).

The colorimetric data found a partial correspondence to what has been assessed by the naked eye observations: i) the mechanical removal with water produced better results on **surface α**, rather than on **surface β**, where it represent the worst treatment in terms of colour difference; ii) Preventol® RI80 (T16) demonstrate to effectively remove the biofilm, giving results comparable in both the walls (Table 12). However, the best results, for both the surfaces at t_1, have been obtained by the application of the treatments T1 (*O. vulgare*) and T2 (*T.vulgaris*), although this didn't emerged from the observations *in visu*, as previously discussed. A reduction in the removal of the biofilm is noticeable when these two substances are mixed together (T7) or in presence of the other EO (T8, T9 and T10).

At the same time, the APs alone seem to work worse than the corresponding essential oils, except in the case of pulegone (T6), that shows a strong correspondence to the respective EO (*C. nepeta*) (Table 12). The hydrogel alone (T15) effectively removes the superficial biofilm, enhancing the properties of the single substances.

For what concerns the comparison of the results at t_1 obtained from the two surfaces, no relevant differences (the difference between the values of $\Delta E^*_{ab} < 3$ CIE units)[5] can be observed for nine points out of seventeen between **surfaces α** and **β**. This indicates that the treatments, in the cleaning phase (t_1), have had a similar effect on both the surfaces.

[5] In this case we meant the difference existing between $|\alpha\Delta E^*_{ab} - \beta\Delta E^*_{ab}|$. These data are not shown, but it is possible to evaluate the differences by observing the data reported in Table 12 and in Figure 20.

Table 12 – Comparison of the total colour difference (ΔE*) calculated at t_1-t_0, for all the treatments on both the surfaces (**surface α** and **surface β**).

		Treatments																
		T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**	**T13**	**T14**	**T15**	**T16**	**T17**
ΔE*	**α**	26.3	26.9	16.0	18.1	14.6	14.3	19.5	18.0	13.6	11.9	11.5	12.0	14.9	16.8	17.8	19.3	21.6
	β	20.7	20.4	15.8	13.1	15.8	15.6	14.7	15.4	17.0	16.6	12.4	15.4	16.1	15.3	17.8	18.2	10.5

This is the case of the treatments T3 (*C. nepeta*), T5 (thymol), T6 (pulegone), T8 (*O.vulgare* + *C. nepeta*), T11 (carvacrol + thymol), T13 (pulegone + thymol), T14 (carvacrol + thymol + pulegone), T15 (hydrogel) and T16 (Preventol ® RI 80), where for T3, T15 and T11 the differences are very small (< 1 CIE units). In the other cases, T1 (*O. vulgare*), T2 (*T. vulgaris*), T4 (carvacrol), T7 (*O. vulgare* + *T. vulgaris*), T17 (water) demonstrated higher values of ΔE* for **surface α** and T9 (*C. nepeta* + *T. vulgaris*), T10 (*O. vulgare* + *T. vulgaris* + *C. nepeta*) and T12 (Carvacrol + Pulegone) for **surface β** (Table 12).

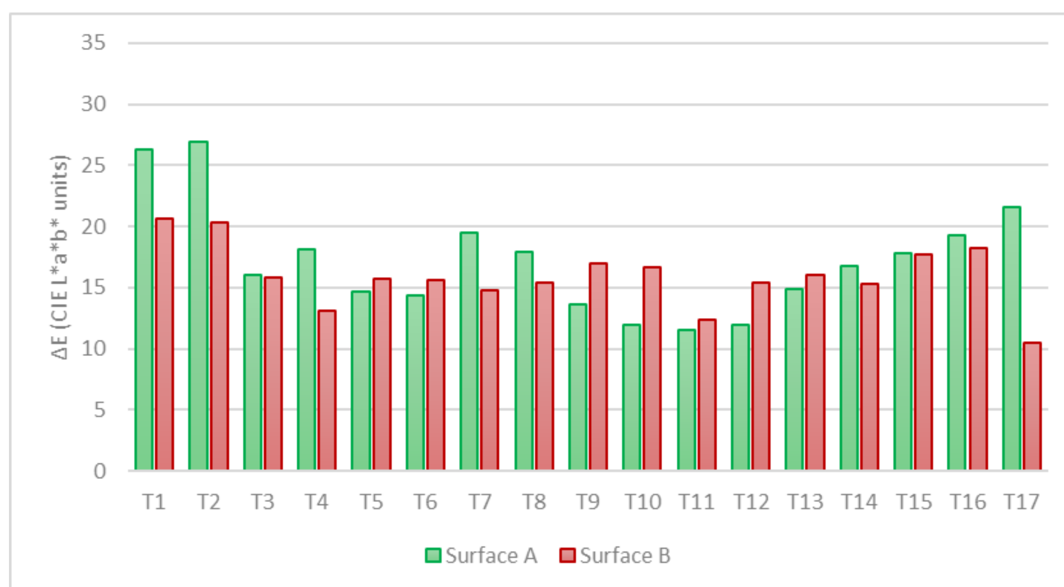

Figure 20 – Graphical representation of the ΔE_{ab}* values, representing the overall colour difference of the treated surfaces between t_1 (after the removal of the compounds) and t_0 (biocolonized surface).

However, some observations are useful to give correct interpretation of the colorimetric results. The ΔE_{ab}* represents a useful tool in the evaluation of cleaning efficacy, which is linked to the removal of the most superficial layer of biopatina/dirt. This is due to the fact that the ΔE_{ab}* derives from an equation (eq.4 section 2.1.5) that takes into account

the differences of obtained for all the colorimetric parameters calculated between an initial and a final situation. For this reason, the result of the equation indicates the magnitude of the difference in colour, which can be considered proportional to the amount of biofilm present (or removed) from the surfaces. This is even more useful since the colour of the original granite (as in the Experiment 2) was not available and thus, it was impossible to establish by the only a*b* and L* parameters which point is closer to the original uncolonized lithotype.

However, it is important to stress the fact that one, between the three colorimetric parameters, may alter the result of the evaluation. Even though the usefulness of ΔE_{ab}* in the assessment of the degree of biocolonization has been demonstrated in other studies [114], in this case it was decided to not consider the values of ΔE_{ab}* as an index of medium-term biocidal efficacy (t_2 and t_3), since it was assessed that the L* parameter exceedingly alter the colour evaluation (data not shown). This depends by the fact that the colorimetric data have been acquired outdoor, in a range of time that included all seasons of the year (February = winter, March = spring, July = summer, October = autumn). In light of the above, the experimental surface was affected by all the environmental factors related to seasonal changes, including the rainfalls and external humidity. For example, the L* in spring/summer is higher than in fall/winter, due to the minor presence of moisture inside the wall (the surface appears brighter, data not shown).

On the other side, it was considered in this dissertation to evaluate the immediate effect produced by the removal of the compounds (t_1), that was assembled in a time sufficiently close to the first application of the products (February – March), whereby seasonal environmental changes can be considered negligible as well as the systematic errors related to the L* influence.

However, in other studies [111], it was demonstrated the suitability of the chromatic parameters (i.e. a* associated with changes in redness-greenness and b* associated to changes in yellowness-blueness) in the evaluation of the efficacy of the treatments in medium-term monitoring, related to the presence (or absence) of the photosynthetic biofilm.

For these reasons, the a* and the b*, and their partial differences (Δa* and Δb*) have been evaluated to assess the degree of colonization of the surfaces and the efficacy of the medium-term biocidal action of the treatments.

The chromatic changes, associated to the partial colour differences Δa^* and Δb^*, for the whole monitoring have been reported in Figure 21a and b.

Figure 21 – Representation of the partial colour differences (Δa^* and Δb^*) during the monitoring calculated at t_1 (immediately after the removal of the treatments), at t_2 (6 months after the removal of the treatments) and at t_3 (8 months after the removal of the treatments).

Observing the graphical representations, it is possible to assess that the greater colorimetric differences are associated to the b* component (blue-yellow), while the a* (red-green) component variate more randomly. This is in accordance to what has been demonstrated in the study of Sanmartín et al. (2012) [19] that emphasize the role of b* in the early detection of phototropic colonization, because it variates most over time and represents the most important parameter in determining the colour changes.

The evolution of Δb^* over time suggests an evident difference in the response of the surfaces to the treatments, probably attributable to differences in the biocolonization.

At the end of the monitoring (t_3) an overall yellowing of the treated areas belonging to **surface α** is evidenced by positive values of Δb^*, obtained for all the colour points.

On the contrary, on **surface β** a general bluening is noticeable due to the negative values of Δb*. This is true for almost all the colour points, excluded T14, T15, T16 and T17.

The results described by the partial colour differences reported in Figure 21 can be visualized also in the bidimensional graph a*b* in Figure 22, where it is possible to appreciate the displacement of the colour points after the end of the monitoring.

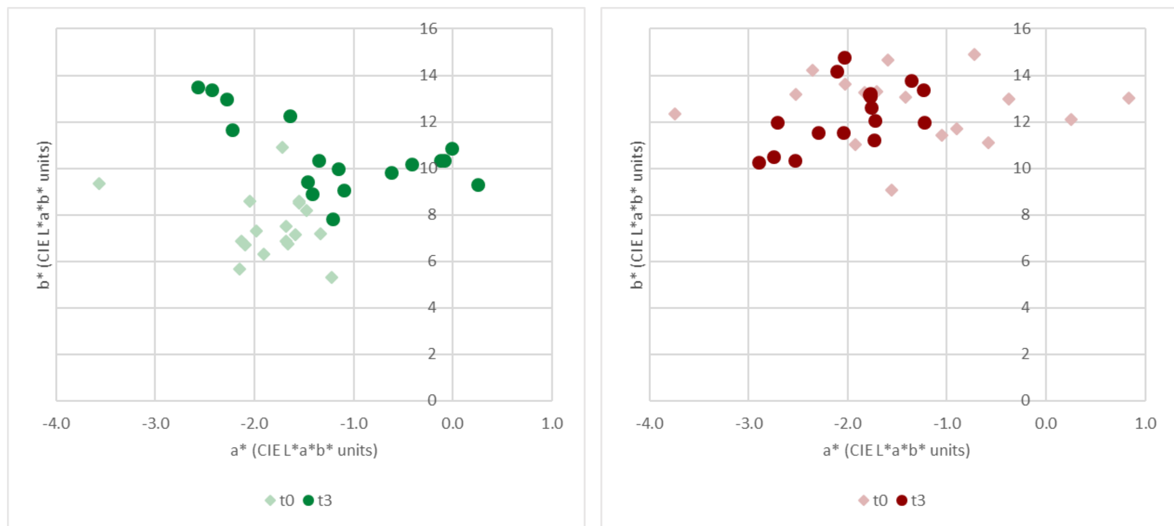

Figure 22 – Displacement of the colour points inside the bidimensional a*b* colorimetric plan at t0 and t3. The figure a) represent the treated areas of the **surface α**, the figure b) the treated areas of the **surface β**.

By comparing the colour data at the beginning of the experimentation (t_0, biocolonized surface) with the final step of the monitoring (t_3), two different situations can be observed.

On **surface α**, the one visually dominated by the green component at the beginning of the monitoring, the colour data at t_3 appear less uniformly distributed inside the colour space compared to the initial situation. A general shifting of the points towards the positive b* is evident for all the colorimetric data: an increase of the yellow chromatic component compared to the blue one, confirming what has been previously assessed by the observation of the Δb*. This is partially confirmed also for the a* values: in fact, a shifting of some colour points towards the positive a* is reported, indicating a loss of the green component. For **surface β**, the general trend of the colour points after the removal of the products seems opposite to the one observed in the **surface α**. In fact, at t_3, the colour points are more

uniformly grouped compared to the original situation, when the surface was characterized by a red-green biocolonization. Moreover, it is noticeable that all the colour points, are distributed inside the plan of the negative a* and positive b*, indicating a loss of the red component compared to the initial situation.

Considering what has been discussed, and by observing the graphs (Figure 22 a and b), it appears evident that the biocidal action is related to changes in the b* components.

However, these changes are opposite for the two separate cases: in α, the effectiveness of the treatments seems related to an increase of the b* while, on the contrary, in β it seems related to a decrease of the same parameter.

Indeed, at the end of the monitoring, the differences occurring between the b* values for the two surfaces, are remarkably reduced compared to the initial situation.

It seems that the points tend to occupy a smaller delimited space, in which the original colour of the uncolonized granite is probably located.

Concluding, it can be assumed that the bluening or the yellowing depends by the elimination of the specific photosynthetic microorganisms, characterized by differences in colour determined by the respective produced pigments. More specifically, the bluening (and the loss of the yellow component) seems associated to the reduction of carotenoids produced by the photosynthetic microorganisms of **surface β,** and the opposite situation seems to occur for the pigments produced by the photosynthetic pigments produced by the microorganisms of **surface α.**

3.4.4. Evaluation of the cleaning and biocidal action of the compounds through the chlorophyll a fluorescence signal

The cleaning and the biocidal effects of the treatments have also been evaluated by monitoring the minimal chlorophyll-a fluorescence signal (F_0) of dark-adapted cells, already valued as an efficient estimator of the biomass of photosynthetic microorganisms present in SABs [105].

Fluorescence data have been acquired during the whole monitoring (t_0, t_1, t_2 and t_3) but the most significant results have been obtained at t_1, for the assessment of the cleaning action and at t_3 for the biocidal one. For this reason, the data acquired at t_2 are not shown.

The cleaning effect and biocidal effects were evaluated by calculating the difference between the F_0 signals produced by the microorganism at t_0 and t_1 (immediately after the removal of the treatments from the surfaces) in the first case, and t_0 and t_3 in the second one.

Interesting considerations can be done on the basis of the results resumed in the bar graph represented in Figure 23.

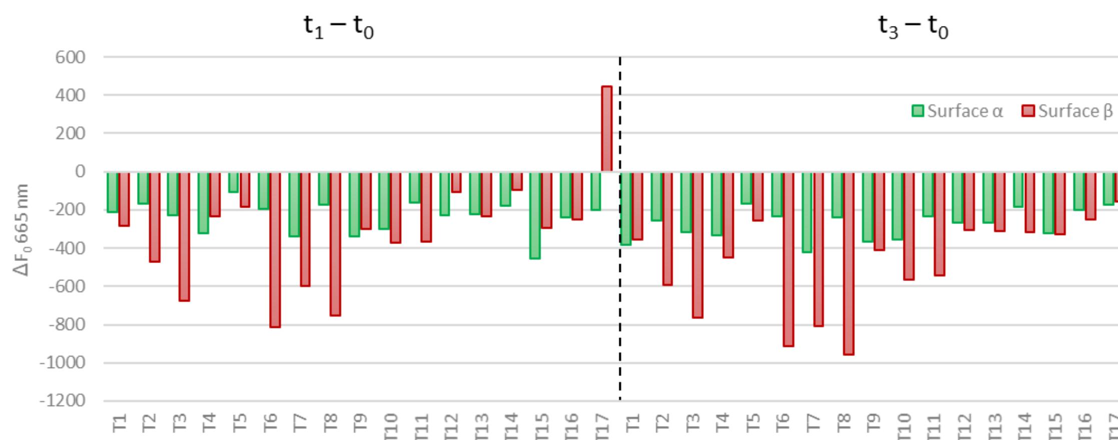

Figure 23 – Representation of the difference (relative units) of the minimal fluorescence signal (ΔF_0) at 665 nm (chl a), obtained between the untreated colonized surfaces (t_0) and the surfaces immediately after the treatments (t_1) and at the end of the monitoring (t_3).

In general, all treatments, except for water at t_1 (T17β) on **surface β**, produced a decrease of the fluorescence signal, indicating an overall biocidal effect of all the treatments, except for the T17β, in virtue of the negative values obtained from the relative differences ΔF_0 ($t_1 - t_0$) and ΔF_0 ($t_3 - t_0$).

By comparing the two biocolonized surfaces, the treatments were more effective on **surface β**, for what concerns both the cleaning and the biocidal effects.In general, the results of **surface β** seem more coherent, and an interesting discussion can arise from the observation of the results produced by each single treatment in this study case.

For what concerns the single essential oils, the compound that demonstrated the best cleaning action is the *Clinopodium nepeta* (T3β), as well as its active principle (pulegone, T6β) which also demonstrated the best results compared to all the other treatments.

It follows *Thymus vulgaris* (T2β) and *Origanum vulgare* (T1β). By combining two products, good results have been obtained with the compositions containing *O. vulgare* paired with both the other two EOs (i.e. *T. vulgaris* in T7b and *C. nepeta* in T8b), while the

one combining *C. nepeta* with *T. vulgaris* (T9β) shows a lower efficacy. This would seem caused by the presence of the *T. vulgaris* that, indeed seems to reduce the cleaning action of the two other essential oils (this is true, considering that the absolute difference for T8β is higher than for T7β).

From the combination of the three EOs does not emerge an enhancement neither of the biocidal or the cleaning action compared to the single substances. For what concerns the APs, apart for pulegone (T6β), scarce results can be observed both when employed alone (T4β ad T5β) and combined (T11β, T12β, T13β and T14β). The worst cleaning result was obtained in case of T17β, that included the mechanical removal of the biofilm with water paired with brushing.

The classical biocide (Preventol® RI80 = T16) demonstrated a lower cleaning and biocidal action compared to the other treatments, in particular, the absolute value of ΔF_0 ($t_1 - t_0$) for T16 β is lower than the one of T1β, T2β, T3β, T6β, T7β, T8β, T9β, T10β, T11β, T12β and T15β, and comparable with the one of T4β. It should be added that the only action of the hydrogel (T15β) provided a better cleaning action than the one of the mechanical brushing. The ΔF_0 ($t_3 - t_0$) demonstrated that, for all the surfaces treated with the phytochemicals, a significant increase of the absolute difference is evident, while in the cases of T15b and T16b, a small reduction in the difference can be observed, thus confirming the efficacy of phytochemicals as biocidal agents.

A different situation occurs in the case of **surface α**, where the cleaning action of the phytochemicals is lower (T5) or comparable (in the other cases) to the one of the mechanical brushing.

The hydrogel alone demonstrated, during the cleaning phase, the best results in terms of removal of the superficial biofilm. This result cannot be confirmed in the last phase of the monitoring, where an increase of the fluorescence signal can be observed because of the decreasing of the absolute value of the ΔF_0.

This is an expected result, in accordance to the fact that the hydrogel alone is characterized by good filming and adhesive properties, that contribute to the cleaning action trough the detachment of the biofilm. However, this one alone does not contain biocides in its composition, and so a regrowth of the microorganisms during the time is expected.

It should be added that the results of the hydrogel at t_3 are very close in both the surfaces.

3.4.5. Comparison of the colorimetric and fluorescence results in the determination of the biocidal action of the treatments

In Figure 24, the two parameter Δb^* and ΔF_0, assessed as good indicators of the biocidal action of the compounds, have been correlated inside a bidimensional graph, where the ΔF_0 is the abscissa and Δb^* the ordinate. The points considered are the ones acquired at t_3. The first element that can be noticed is that the points corresponding to the treatments applied on **surface α** are grouped in a small cluster inside the plan, limited by T7α (ΔF_0 = -423.02) and T5α (ΔF_0 = -169.67) on the x axis and T13α (Δb^* = 1.89) and T8α (Δb^* =7.44) on the y axis, demonstrating a higher correspondence between each other's, compared to the treatments of **surface β**, that are displaced in a more random way inside the plan.

The differences between the surfaces are associated to the general trends recorded for **surface β** in the decreasing of the fluorescence signal and the shifting towards negative values of Δb^* (shifting towards blue). On the contrary, as already assessed in the previous sections, **surface α** is characterized by an overall increase of the Δb^* values, and thus, a shifting towards a more yellow coloration.

Concerning the fluorescence signal, T1β, T12β, T5β, T16β, T14β, T13β and T3β, are included inside the space delimited by T7α and T5α, while T17β and T9β are excluded from the limits because of very small differences.

If negative values of ΔF_0 and Δb^* are considered indicators of the good biocidal action of the compounds towards the microorganisms characterizing **surface β,** the product that demonstrate the best results is the one that provided the combination of the *O. vulgare* and *C. nepeta* EOs (T8). This doesn't find a correlation to the corresponding mixture of APs (T12 = carvacrol + pulegone) that demonstrate lower absolute values of both ΔF_0 (mainly) and Δb^*. The other treatment containing *O. vulgare,* combined with *T. vulgaris* (T7) also seems to give good results, confirming the fact that the biocidal action of the *O. vulgare* essential oil is enhanced when combined to another phytochemical. *T. vulgaris* (T2), Cavacrol + Thymol (T11) and the combination of the three EOs (T10) shows comparable results, as well as pulegone + thymol (T13) and the three APs paired (T14) with each other.

More generally it seems that great differences in the fluorescence signal cannot be recorded for the points included between T1 and T5 (ΔF_0 T1= -356.3; ΔF_0 T5 -253.6), or T1 (O. vulgare), T5 (thymol), T12 (carvacrol + pulegone), T13 (pulegone + thymol), T14 (3 APs) and T16 (Preventol ® RI80). T16 (ΔF_0 = -251.0) and T5 (ΔF_0 = -253.6) presents similar results in terms of the decreasing of fluorescence signal.

In general, the treatment composed by the commercial biocide (Preventol ® RI80 = T16), demonstrated lower efficacy compared to the phytochemicals, except for T5 (= thymol), which also represents the worst treatment among these last few.

C. nepeta (T3) is the oil that demonstrated the highest correlation compared to its corresponding AP (pulegone = T6), also showing good biocidal properties in relation to the obtained results for ΔF_0 and Δb^*. In general *C. nepeta* and pulegone demonstrated to be the best EO and AP compared to the others.

Considering the EOs alone *O. vulgare* (T1) represents the worst, and *T. vulgaris* (T2) the intermedium treatment.

An opposite situation occurs for the respective APs, where thymol (T5) demonstrates worse results compared to carvacrol (T4).

Water paired with brushing removal (T17) is the compound that demonstrated the worse efficacy in terms of fluorescence, while the Hydrogel alone (T15) is the worse in terms of colour changes.

For **surface α** it is more difficult to make a similar evaluation given, as stated before, that the points are much closer inside the plan. However, it can be assumed that: i) Water (T17) can be compared to the same corresponding treatment applied on the surface β in terms of fluorescence signal, ii) T5 (thymol), T16 (Preventol ® RI 80) and T14 (three APs) are very closer inside the plan and give the worse results in terms of fluorescence, iii) this is in accordance with the results obtained on **surface β**, where it was stated that T5 is the worst phytochemical and its fluorescence signal is comparable to the one of T16 and T14 does not differ much from these values, iv) the best effect, weighing the values of ΔF_0 and Δb^*, is assessed for the treatment containing *O. vulgaris* + *T. vulgaris* (T7), even though v) the one composed by *O. vulgaris* + *C.nepeta* (T8) is the best in terms of colour variations, confirming the result obtained for **surface β**; vi) T3 and T6 (C. nepeta and pulegone) are confirmed the EO and the AP with higher correspondence in the biological activity.

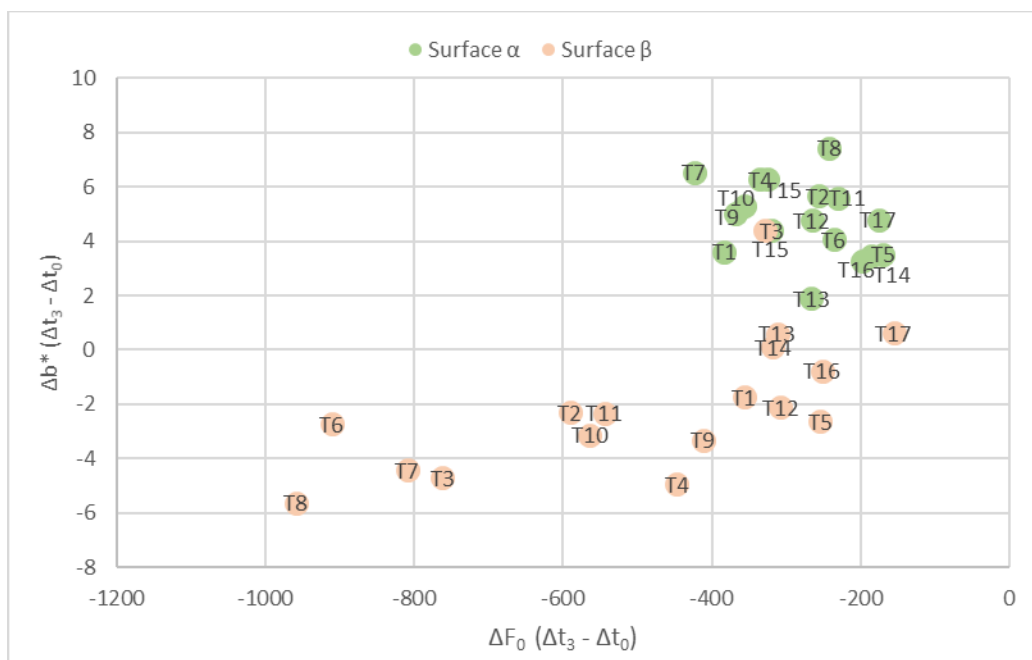

Figure 24 – Graphical representation of the correlations existing between the ΔF_0 (abscissa) and Δb^* (ordinate) of the analysed colour points. The codes individuate the corresponding treatment. The points belonging to the surface α are represented in green, the points belonging to the surface β are represented in green.

In conclusion: both the cleaning and the biocidal action of seventeen treatments containing phytochemicals (seven composed by EOs and seven APs), a polymeric film forming hydrogel matrix, a common biocide (Preventol ® RI-80) and water (these latter combined with mechanical brushing) have been assessed trough measurements of colour and fluorescence. The treatments have been applied to two surfaces, differently biocolonized by photosynthetic microorganisms. This was assessed by naked-eye observation, colorimetric characterization and morphological characterization of the microorganisms.

The first surface (called **surface α)** is characterized by a more heterogeneous microbial composition, dominated by the presence of green algae and cyanobacteria, producing green and dark photosynthetic pigments, while the other presents a more homogeneous composition, represented by green algae mainly producing carotenoids, for this reason it was possible to observe a reddish coloration of the surface.

The cleaning action of the treatments was recorded immediately after the removal of the compound, while the biocidal action conceives the medium-term effect of the products

on the recolonization of the surfaces and, for this reason, it was evaluated in function to the results of the last monitoring, performed 8 months after the end of the cleaning procedure.

For what concerns the cleaning action, the results of the overall colour difference (ΔEab^*) immediately demonstrated the efficacy of the treatments, as possible to observe also by simple naked eye observations. This last assertion was confirmed also by the fluorescence results, which evidenced a decrease of the fluorescence signal after the removal of the products.

However, to assess the medium-term biocidal effect of the treatments, in the colour evaluation, only the chromatic parameter Δb^* (variations between yellow (+) and blue (-)) , already used as a good indicator of biofilm formation, have been employed, in virtue of the more coherent results demonstrated and the fact that it is not affected by external factors.

The surface characterized by the dark-green patina demonstrated that the increase in the Δb^* (or the neutralization of the green component) during the whole monitoring is an indicator of the biocidal effect of the products. The opposite mechanisms occur for the surface characterize by the brighter-red colouration, that demonstrated a general reduction of Δb^* (bigger negative values of Δb^*). This is surely associated to the different original colouration of the microorganisms: in fact, at the end of the monitoring, the differences between the b^* values of the two surfaces are reduced compared to the beginning of the monitoring. This probably means that all the colour points belonging to the surface α and β are moving towards the original colour of the uncolonized granite, even though it has not yet been characterized.

The biocidal efficacy was confirmed also by the fluorescence measurements, that demonstrated an overall decrease of the fluorescence signal over the time.

It is very difficult to relate the effects of the single biocidal among the surfaces, because of the differences in the colonization of these last ones.

However, as a general consideration, the treatments containing the phytochemicals demonstrated, in almost all the cases, the best results, when compared to the common biocide (Preventol ® RI 80).

Also in this case, as observed in Experiment 1, weighing all the considered parameters, the EOs and their respective APs, that demonstrated the best biocidal action, are *C. nepeta* and Pulegone.

Again, it appears unpredictable to assess the empowerment of the efficacy of the phytochemicals when combined, because in some cases they demonstrate an enhancement of the biocidal action given by the presence of other compounds (this is the case of *O. vulgare*), and in other the single substances seem more effective.

Differently from other experimental studies, in this case the action of the EOs don't always seem comparable to the one of their respective APs, where the first ones (alone and combined) generally showed better results. In any case, the biocidal action of the APs is not negligible, except for the case of thymol that, in both the cases, demonstrated results comparable to the ones of Preventol ® RI80.

The removal with mechanical brushing demonstrated to be less effective than the one of the hydrogel. The latter, in fact, shows good cleaning properties, but scarce biocidal effects when not paired with other substances.

CHAPTER 4. CONCLUSIONS

The current research proposes to ideate a methodological approach suitable for the future applications of biocidal compounds, based on phytochemical substances, for the treatment of biocolonized stone materials, in order to eliminate the SABs and prevent the biodeterioration and a possible recolonization of the surfaces.

The formulations have been created in such a way that allows an easy preparation of the products, also for the restorers working in field, which include the employment of an innovative hydrogel matrix for the cleaning of cultural heritage materials that incorporates the biocidal substances, i.e. the phytochemicals. These ones have been selected on the basis of the good biocidal properties already shown in other studies and also because they can be considered less dangerous for the preservation of eco-systems and human health than other biocidal products normally employed in the elimination of biodeteriogens; there are three essential oils of *Origanum vulgare*, *Thymus vulgaris* and *Clinopodium nepeta*, extracted from common plants species (commonly known as origanum, thymus and lesser calamint) , typically present in the Mediterranean area Moreover, their main active principles, carvacrol for *O. vulgare*, thymol for *T. vulgaris* and pulegone for *C. nepeta*, have been employed for the evaluation of their specific contribution to the biocide properties inside the EOs.

On the other hand, the hydrogel is a newly formulated product based on gellan gum crosslinked with calcium chloride ($CaCl_2$) where polyvinyl-alchol (PVA) and a surfactant were also included, that allowed to create emulsions where phytochemicals have been dispersed at different concentrations and combinations. The employment of this hydrogel presents many advantages related to the a-toxicity of its chemical composition, the vehiculation of phytochemicals on the stone's surfaces and the possibility of peeling off the treatments once the hydrogel is dry. In fact, the possibility of confining the biocides inside a peelable matrix, prevents from the use of a not recommended direct application of the substances on the surface and allows a complete removal of the chemicals, to avoid the drawbacks related to a possible permanence of the biocides themselves on the substrata, which may lead to secondary chemical reactions between the reagents and the mineralogical components of the stone matrix.

Whereas the products are created to be applied on stone materials of artistic interest, all the experimental adopted procedures concerned the employment of non-invasive and non-destructive methods for the characterization of the biofilm and for the evaluation of the efficacy of the treatments, before and after the cleaning procedures.

To accomplish this goal, three different experiments were performed, and these ones aimed to compare the biological activities of six selected phytochemical substances, pure and combined with others, when applied on biocolonized sample's surfaces in granite and travertine.

The first experimental approach concerned the application of the treatments composed by the phytochemicals embedded in the hydrogel matrix on granite samples, the latter evenly colonized by a SAB mainly composed by phototrophic algae and cyanobacteria. Their biological activity was also compared to the one of a biocide based on BACs (Preventol ® RI80) and water.

The hydrogel was also applied separately, besides its utilization in combination with the EOs, the APs and the Preventol ® RI 80. The three EOs were used alone and mixed together, in a combination of three essential oils, as well as the APs. Water and pure Preventol ® RI80 diluted in water were removed from the surfaces with a mechanical action (brushing). The cleaning effectiveness associated to the removal of the biopatina was established by colorimetric evaluations, that were performed immediately after the removal of the treatments as well as two weeks after.

The second experimental approach was conceived to recreate a similar application of the phytochemicals on a travertine surface exposed to the outdoors. The experimental surface presented a severe biocolonization. The microbial species forming the biofilm were genetically characterized, and the presence of species belonging to plants, fungi and bacteria kingdoms was assessed. The treatments included the employment of the same formulations containing the phytochemicals employed in the first experimental approach with the addition of another six formulations each containing a compound paired with another. This is valid both for the EOs and the APs.

The last experimental approach was conceived to include all the treatments employed in the other two experiments, applied on two portions of an external wall of the university of Santiago de Compostela, characterized by two distinct colonization as stated by

morphological analyses that evidenced the differences in the microbial composition. The treatments included the formulations containing phytochemicals, Preventol ® RI-80 and water. In this case, the monitoring lasted 8 months after the removal of the treatments and fluorescence measurements have been performed, together with the colorimetric ones, to assess the effectiveness of the treatments.

In all cases, as possible to assess by a preliminary observation of the photographical report (available for the granite samples and granite wall in Figure 10, Figure 13 and Figure 19), a single application of the treatments visibly changed the appearance of the treated surfaces, by removing the superficial patina present on the samples. This finds a correspondence in the colorimetric results, that shown, in all the experiments, strong chromatic variations before and after the application of the treatments, associated to their cleaning properties.

As evidenced by the colorimetric results obtained in Experiment 1 and 3, the pure hydrogel demonstrated to be a reliable tool for the detachment of the more superficial layers composed by dirt and microorganisms, due to the combination of its surfactant and peeling actions, more effective than the only mechanical action performed with a brush.

However, as expected, the pure hydrogel (as well as the treatments composed of pure water) lacks the biocidal properties and seems to not be enough to prevent a possible recolonization of the surfaces, as confirmed by the measurements repeated two weeks after the cleaning in the Experiment 1 and 8 months after the cleaning procedure in Experiment 3. Indeed, compared to the other treatments the chromatic variations over the time are inferior than the ones associated to the formulations containing a biocidal agent. This is also confirmed by the fluorescence measurements performed in Experiment 3 where, after 8 months from the application of the treatments, an increase of the fluorescence signal was registered. This can be associated to the unpausing of the vital activity of the survived microorganisms or to a recolonization of the surface.

Nevertheless, the obtained results are very encouraging for the ideation of newly formulated compounds, that will combine biological properties of biocides with the peeling action of the hydrogel. Moreover, a great advantage related to the application of the hydrogel in its liquid phase allows the penetration of the biocides in the natural pores of the stone, making possible the interactions between the chemicals and the microorganisms present in

them, usually hardy to eliminate with traditional methods, unable to penetrate deep into the pores. This aspect is particularly useful for all lithotypes characterized by a high diffuse macroporosity, as in the case of travertine.

For what concerns the treatments containing biocides (i.e. the phytochemicals and Preventol® RI-80), the biological activities of the substances have been assessed in all the Experiments, both immediately after the removal of the products and over time (the medium term and the long term monitoring performed in the three experiments). One of the most interesting result is related to the comparison of the Benzalkonium chloride-based biocide to the treatments containing the phytochemicals: in both cases the growth of microorganisms and the recolonization of the surfaces were inhibited by the treatments, confirming that the biocidal activity of the phytochemicals is comparable to the one of a commonly employed biocide. Moreover, in some cases, the phytochemicals showed better results in terms of chromatic changes over time. This is particularly evident in the second Experiment, where, the samples treated with *C. nepeta* and pulegone (respectively the EO and its AP) are the ones that, two weeks after the cleaning procedure (i.e. at the end of the short-term monitoring), are closer to the original colour of an uncolonized granite, differently from the samples treated with Preventol ® RI-80 that, in addition, didn't show relevant colorimetric differences between the second and the third measurement. What has been said is also confirmed in the Experiment 3, where the indicators used to assess the biocidal action of the substances (i.e. Δb^* and ΔF_0) highlighted that Preventol ® RI-80 is characterized by a lower efficacy compared to the phytochemicals (except for thymol, the biocide that showed the worst results).

In general, both the EOs and the APs can be considered good biocides in the elimination of biofilms characterized by a very heterogeneous microbial composition, as evidenced by the morphological characterization of SABs colonizing the granite samples and the granite walls and by the metagenomic characterization of the biofilm colonizing the travertine wall. Regarding this last methodological approach, the quantification of the biological material obtained with three different sampling methods (non-invasive, micro-invasive and invasive) showed that the one performed with adhesive tape made it possible to collect a sufficient amount of biofilm from artistic materials in a micro-invasive way, enabling the identification of the microorganisms present within them. Established this, an

improvement of the extractive process of the DNA is required and it will allow the employment of next generations sequencing methods for the identification of microorganisms, with the advantages of sparing time and money when generating high quality reads which need to be processed in customised bioinformatic tools exploiting the existence of, for instance, 16S and ITS databases.

However, it is important to stress that the presence of photosynthetic microorganisms have been detected in all the four biofilms studied, confirming the effectiveness of the phytochemicals also against these species, and not only against fungi and bacteria, which represent a research area that has not been well explored.

Concerning the comparison between the efficacy of the EOs and the APs, the latter showed, in most cases, better (or comparable) results to the ones obtained for the EOs, except for the last experiments where the EOs have been more effective.

Taking into account that the formulations have been created to contain the APs at the same concentrations present inside the respective EOs, this result confirms that the presence of the active principles strongly influences the biocidal properties of the essential oils themselves. This can be considered a great achievement of the research, representing a starting point for future developments of biocidal treatments based on the only presence of phenolic compounds. This will be advantageous in the treatment of biocolonization both for the lower production costs (the pure phenolic substances are cheaper than high quality essential oils) and for the possibility of ideating formulations containing substances at suitable concentrations for the elimination of target microorganisms composing the biofilms.

In this regard, the microbial composition of the biofilm seems to be a discriminating element for the assessment of the biocidal action of the single phytochemicals or combination of phytochemicals, that showed different results depending on the study case. This is particularly evident by observing the results obtained for the third Experiment where, in the long term, the same treatments applied on two biofilms characterized by a different microbial composition, have produced different effects.

Considering all the experiments and the assembled formulations, it seems difficult to establish which product is characterized by the largest-spectrum biocidal action, although some observations can be made.

Among the phytochemicals, *C. nepeta* and pulegone demonstrate to be the most effectives in the long/medium-term in two study cases and are the ones that showed the best correlation EOs/APs. The possible enhancement of the biocidal properties of the substances paired together seems also to depend to the microbial composition of the biofilm, since in some circumstances they demonstrated to be more effective by themselves alone and vice versa. The three oils combined didn't produced good experimental results, although, in the Experiment 2, it was observed that in the short-term, the greater enhancement in the colour variation was registered for this formulation.

In light of this, it seems necessary to deeply investigate the action of single treatments, containing both pure phytochemicals and combined together , when they are applied on target microorganisms, possibly in plate, in order to accomplish the previously mentioned idea of creating specific formulations effective against biofilms with a certain biological composition.

However, the suitability of the method on real outdoor exposed surfaces has been demonstrated, as well as the prolonged effect of the phytochemicals in the inhibition of the recolonization of the surfaces after the removal of the treatments. Although this may represent an advantage, on the other hand, the possible drawbacks and secondary chemical reactions produced by the remaining substances must be excluded, and a deeper investigation of this aspect must be considered.

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Appendices

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Phytochemical Compounds as Cleaning Agents on Granite Colonized by Phototrophic Subaerial Biofilms

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Received: 20 February 2020; Accepted: 19 March 2020; Published: 22 March 2020

Abstract: The society has become increasingly interested in using natural products over chemicals for cleaning activities. In this study, the cleaning potential of formulations embedded in a hydrogel matrix and composed respectively of essential oils (EOs) of *Origanum vulgare*, *Thymus vulgaris*, and *Calamintha nepeta*, and their respective main active components (EO-ACs), viz., Carvacrol, Thymol, and Pulegone, on a phototropic biofilm growing on granite was investigated. In addition, and for comparative purposes, analysis with the combination of the three EOs, the combination of the three EO-ACs, and Preventol RI-80® (one of the most effective commercial cleaning agents based on quaternary ammonium salts) in all three cases embedded in a hydrogel matrix, as well as only the hydrogel matrix, distilled water, and Preventol RI-80®, in both latter cases applied with brush, were also studied. The cleaning effect of the treatments was assessed immediately after the treatment and after one and two weeks by color spectrophotometry, a reliable tool to evaluate the presence and vitality of the phototrophs and the cleaning effectiveness in granite. *C. nepeta* and its active component Pulegone proved to be the most effective and yielded similar results, comparable to those of uncolonized granite, and better than those obtained with Preventol RI-80® applied with brush (most common way), especially at the end of the experiment. These promising first results support the suitable use of the phytochemical compounds used on phototrophs field where there are still few published studies and encourage further investigation toward the evaluation of their exhibited biocidal activity.

Keywords: essential oils (EOs); essential oil active components (EO-ACs); biodeterioration; granite; non-destructive techniques; algae; antibiofilm; green methods

1. Introduction

Because it has been estimated that from 20% to 30% of stone deterioration is a result of biological activity [1], it is clear that formation of subaerial biofilms, shortened to SABs and defined as "microbial communities that grow on solid surfaces exposed to the atmosphere" [2], represent an important topic for cultural conservation researchers. Many different methods, such as mechanical (brushing and rubbing, washing and steaming, wet and dry abrasives, etc.), physical (UV radiation, laser, etc.), chemical (alkaline and acidic treatments, organic solvents, etc.), and biological (viable bacterial cells, enzymes, etc.) have been employed to eliminate SABs-forming colonizing microorganisms from stone

Coatings **2020**, *10*, 295; doi:10.3390/coatings10030295 www.mdpi.com/journal/coatings

surfaces [3]. Cleaning of SABs is a necessary operation for conserving historical stone buildings, which generally is a very complicated procedure and involves a significant financial outlay. Chemical products are currently most practical and are employed for this purpose [4–6], although their use is not always encouraged on the basis of possible secondary implications on the environment and human health [7].

Furthermore, in the case of poultices with quaternary ammonium-based compounds (QACs), chemicals are largely employed in different fields because of their wide action for cleaning purposes (since, besides being antimicrobial agents, secondarily are used as surfactants) [8–10]. They can promote (as main side-effect) the recolonization of the surfaces by phototropic and heterotrophic microorganisms able to use the organic residuals of the QACs as carbon and nitrogen sources [1,11]. Indeed, in the study case of the Cave of Lascaux (France) it was demonstrated that the prolonged employment of a QACs derivate (benzalkonium chloride) to eliminate the fungus *Fusarium solani* from a subterranean mural painting increased the level of organic carbon content in the cave, with a consequent spread of other fungi (*Ochroconis lascauxensis*) and bacteria (*Ralstonia* spp. and *Pseudomonas* spp.) highly resistant to benzalkonium chloride and not originally detected on the surfaces [12,13]. Similarly, more recently, Urzì et al. [14] evidenced a recolonization by bacteria, with a drastic increase in their diversity, after a treatment with a mixture of quaternary ammonium compounds and octylisothiazolone (OIT) on hypogea environment.

A possible alternative to chemicals is represented by the use of essential oils (EOs): secondary metabolites produced by plants for the defense against pathogenic microorganisms and predators [15]. The increased interest in the use of these natural substances is partly attributable to their proved cleaning and biocidal properties at concentrations low enough to be harmless for the environment and human health [16]. The cleaning efficacy and biocide properties of some EOs on fungi and bacteria naturally present on stone materials have been established, also in comparison with some QACs based biocides. Stupar et al. [17] compared the minimal inhibitory concentration (MIC) of benzalkonium chloride and the essential oil of *Origanum vulgare* against four strains of fungi isolated from a mural painting (*Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus versicolor,* and *Penicillium* sp.). Essential oils rich in phenolic compounds such as carvacrol were reported to possess high levels of antimicrobial activity [18]. Other study of Stupar et al. [9] provided a similar test, employing three essential oils belonging to the Lamiaceae family (*Lavandula angustifolia*, *Rosmarinus officinalis,* and *Origanum vulgare*) and benzalkonium chloride in different concentrations against six fungi strains (*Aspergillus niger* Tiegh, *Aspergillus ochrauceus* G.Wilh, *Penicillium* Link sp., *Thricoderma viride* Pers., *Bipolaris spicifera* (Bainier) Subram, and *Epicoccum nigrum* Link). The lower MICs have been detected for *O. vulgare* and the QACs based biocide while *L. angustifolia* and *R. officinalis* showed a significantly lower antifungal activity. According to the authors [9], the different efficacies may be attributed to the composition of the EOs. Each EO was constituted by a mixture of 20 to 60 compounds but one or two of these, in greater proportion (20%–70% of the total composition), are the ones that establish the chemotype [15].

Phenolic compounds are one of the most important compounds in the EOs and are natural plant metabolites with different functions, among which their antimicrobial action stands out. The cleaning and biocidal efficacy of EOs with a predominantly phenolic composition have been demonstrated in the study of Mironescu et al. [19], where the fungicidal action of *Thymus vulgaris*, *Thymus serpyllum* and *Foeniculum vulgare*, mainly composed respectively by the monoterpenoids carvacrol, thymol, and estragol, was significantly greater than that attained by three EOs characterized by a predominant concentration of hydrocarbons.

Even if the bactericidal and fungicidal action of EOs has been demonstrated, few research deals with their potential use on photoautotrophic microorganisms (cyanobacteria and algae), i.e., pioneer colonizer of stone substrata [20]. Among those few studies, recently Bruno et al. [21] tested the efficacy of two EOs (*Lavandula angustifolia* and *Thymus vulgaris*), mixed together, against three commonly found cyanobacterial strains of subaerial biofilms detected in catacombs (*Scytonema julianum*, *Oculatella subterranean*, and *Leptolyngbya* sp.). The results showed that both concentrations

used (1% and 10%) inhibited the photosynthetic activities of the cyanobacteria, even if the mixture at 1% required two applications to make the biological activity undetectable.

In the present study, the cleaning potential of formulations embedded in a hydrogel matrix and composed respectively by *Origanum vulgare*, *Thymus vulgaris*, and *Calamintha nepeta* essential oils (EOs) and their respective main active components (EO-ACs), viz., Carvacrol, Thymol, and Pulegone were assessed on a phototropic biofilm growing on granite stone. The comparison of EOs versus EO-ACs, such as terpenic phenols (thymol and carvacrol) and terpenic ketone (pulegone) is very valuable to know if the effect of the latter is increased by being combined with other compounds present in the commercial oils. In addition, and for comparative purposes, analysis with the combination of the three EOs, the combination of the three EO-ACs, and Preventol RI-80® in all three cases embedded in a hydrogel matrix, as well as only the hydrogel matrix, distilled water and Preventol RI-80®, in both latter cases applied with brush, were also analyzed. The cleaning effect of the treatments was assessed immediately after treatment and after one, and two weeks by color spectrophotometry.

In the context of this article, these treatments denote the cleaning effectiveness, i.e., removal of biofouling from granite surface, only removal not considering the microbial abatement related to biocide effect of treatment, intended to destroy, deter, or exert a controlling effect on organisms by chemical or biological means. In this sense, color spectrophotometry, the technique used in the study, allows to reliably evaluate the cleaning effectiveness in granite [3,8].

2. Materials and Methods

2.1. Granite Blocks Inoculated with Phototrophic Microorganisms

The culture selected for the experimentation was already described in the study of Vázquez-Nion et al. [22]. This latter is mainly characterized by the presence of phototrophic microorganisms (green algae and cyanobacteria), in particular: *Bracteacoccus minor* (Schmidle ex Chodat) Petrová, *Stichococcus bacillaris* Nägeli, *Chlorella* sp., *Isocystis* sp., *Aphanocapsa* sp., *Leptolyngbya cebennensis* (Gomont) I.Umezaki and M.Watanabe. The culture was derived from a natural biofilm growing on a historic granitic building in Santiago de Compostela (Monastery of San Martiño Pinario, Santiago de Compostela, Spain) and demonstrated to be particularly suitable to reproduce a natural biofilm on granite stones in laboratory conditions, as the study of Vázquez-Nion et al. evidenced [23].

2.2. Essential Oils and Their Main Active Compounds

Three EOs of *Origanum vulgare*, *Thymus vulgaris*, and *Calamintha nepeta* were purchased from specialized retailers of natural phytochemical products. Oils of *Origanum vulgare* and *Thymus vulgaris* were purchased from Esencias Martínez Lozano (Murcia, Spain) and the oil of *Calamintha nepeta* from Joulienne Fauconnier (Corse, France). On the basis of their composition (Table 1), carvacrol, thymol, and pulegone were selected as the "active principles" (respectively carvacrol for *O. vulgare*, thymol for *T. vulgaris*, and pulegone for *C. nepeta*). The pure thymol (≥98.5%) carvacrol (≥98%) and (R)-(+)-pulegone (≥90%) compounds were purchased from Aldrich Corp. (St Louis, MO, USA).

Table 1. Chemical characterization and chromatographic area percentage of the compounds present in the essential oils (EOs). Minor compounds (concentration < 0.02%) have not been included. The concentrations in percentage of the active principles are indicated in bold.

Compound.	*C. nepeta*	*T. vulgaris* [1]	*O. vulgare* [1]
α-thujene	0.1	1.3	1.3
α-pinene	1.1	0.9	0.9
Camphene	0.04	1.0	0.1
Sabinene	0.4	–	–
β-pinene	1.1	0.3	0.1
3-octanone	0.1	–	0.3
β-mircene	1.1	1.9	1.1
3-octanol	1.7	–	–
α-phellandrene	0.1	0.2	0.1
α-terpinene	0.3	1.6	0.7
p-cimene	0.2	15.8	6.5
Limonene	9.9	0.4	0.2
1,8-cineole	0.5	0.4	0.2
cis-b-ocimene	0.2	–	–
trans-b-ocimene	0.2	–	–
γ-terpinene	0.5	10.2	5.9
cis-sabinene hydrate	0.1	–	–
Terpinolene	0.2	0.1	0.2
Linalool	0.7	4.5	1.6
Camphor	0.1	0.8	–
Menthone	2.1	0.2	–
Isomenthone	3.8	–	–
Borneol	–	1.2	0.2
Menthol	0.1	–	–
terpinene-4-ol	3.1	1.4	0.6
α-terpineol	0.5	0.2	0.1
Verbenone	–	0.2	–
Pulegone	**55.2**	–	–
Piperitone	0.6	–	–
Thymol	–	**46.4**	3.8
Carvacrol	–	4.0	**70.5**
Piperitenone	10	–	–
piperitenone oxide	0.4	–	–
α-copaene	0.1	–	–
β-bourbonene	0.1	–	–
trans-β-caryophyllene	0.5	2.0	2.0
germacrene D	0.9	–	–
α-humulene	0.1	–	0.2
β-bisabolene	–	–	0.3
γ-cadinene	0.3	0.1	–
δ-cadinene	0.4	0.1	–
carophyllene oxyde	–	0.2	0.2

[1] Data provided by the producer (Esencias Martínez Lozano, Murcia, Spain).

2.3. Experimental Setup

The capacity of the essential oils (*O. vulgare*, *T. vulgaris* and *C. nepeta*) and their active principles (Carvacrol, Thymol, and Pulegone) as cleaning agents on phototrophs colonizing granite blocks was studied. For this purpose, twelve granite blocks (5 cm × 5 cm × 2 cm) were inoculated with 3 mL (1.19 $g \cdot L^{-1}$) of the previously described phototropic culture (Section 2.1) and maintained in controlled and stationary conditions of temperature (23 °C), relative humidity (80%), and light (12 h light/dark photoperiod) in a climatic chamber (SCLAB PGA-1228/2 HR) until biofilm formation (Figure 1).

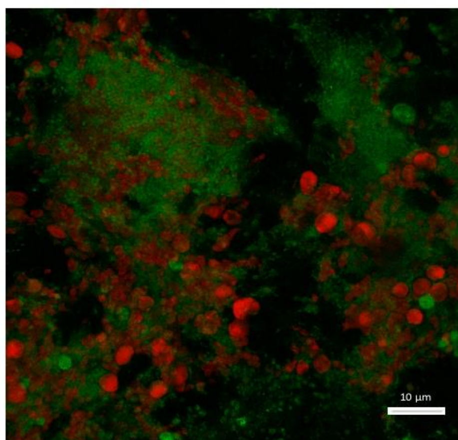

Figure 1. Confocal laser scanning microscopy (CLSM) imaging of the biofilm thrived on the granite surface. The red is the autofluorescence from the chlorophyll of the phototrophs, the green indicates the lipids stained with Lipidtox Green, and the grey is the reflection of the granite stone. Scale bar is 10 µm. A Leica TCS SP5 X CLSM (Leica Microsystems, Wetzlar, Germany) equipped with a white light laser and a 63X objective (NA 1.4, glycerol) was used to obtain the image.

When the granite samples showed the same level (or degree of presence) of biological colonization, each sample surface was divided into three replicate areas, each of ca. 1.7 cm × 5 cm, and different treatments were applied (Figure 2).

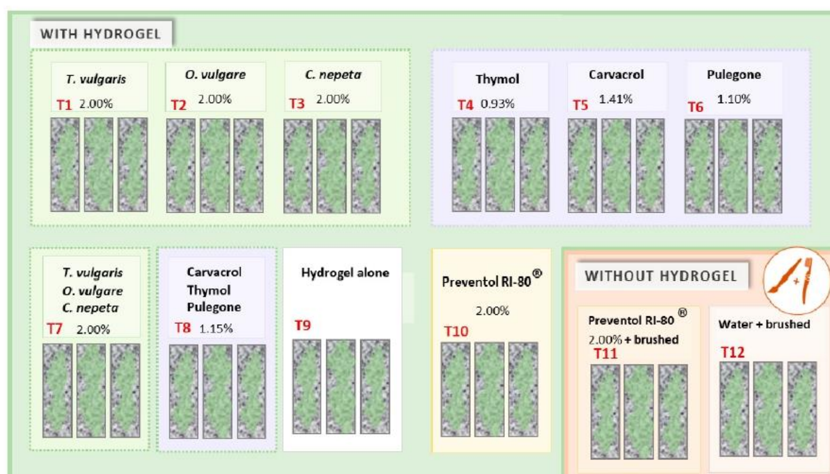

Figure 2. Schematic diagram of the experimental setup for testing the phytochemical compounds as cleaning agents.

The surfaces were treated with three commercial essential oils of the aromatic plants *O. vulgare* (T1), *T. vulgaris* (T2), and *C. nepeta* (T3), and the active principles present on the aforementioned plants: Carvacrol (T4), Thymol (T5), and Pulegone (T6). Furthermore, to evaluate an eventual synergic action

between the substances and a consequent empowerment of their respective biocides properties, two additional emulsions were prepared mixing together the three essential oils (T7) and the three active components or phenolic compounds (T8). For the application, the products were incorporated inside an innovative hydrogel matrix conceived for the cleaning of cultural heritage materials, based on a mixture of surfactants and polymeric substances (0.4% *w*/*w* Gelrite (Sigma-Aldrich, St. Louis, MO, USA), 4% *w*/*w* PVA (Sigma-Aldrich), 0.045% *w*/*w* $CaCl_2$, and 5% *w*/*w* Acemoll CC (ACEF, Fiorenzuola D'Arda (PC), Italy) dissolved in distilled water). The hydrogel matrix was also applied alone (T9) to determinate its own cleaning efficacy, enabling it to make a comparison when it is used alone or in combination with other substances. Finally, treatments with commercial biocide Preventol RI-80®, a well-known QAC that has given suitable results in cleaning of phototrophic biofilms [24], incorporated in the hydrogel matrix (T10) and applied with a brush (T11), and distilled water applied with a brush (T12) were included, for a total of 12 different treatments (Figure 2). An uncolonized clean granite sample was also incorporated as reference.

The concentration employed in the treatment emulsions containing the essential oils, alone (T1–T3) and combined (T7), and those containing Preventol RI-80® (T10 and T11), was 2% (*w*/*w*) and 2% (*v*/*v*) for T10 [25–28]. As already mentioned, the essential oils used in the study have as principal active component various phenolic compounds at certain concentrations. To investigate and establish the potential contribution of phenols in the biocidal action of oils, it was decided to use the same concentration of phenolic substances presents in the essential oils in the hydrogel emulsions (results reported in Table 1). For example, considering that 70.5% is the percentage of Carvacrol in *O. vulgaris*, the final concentration of the active substance in the emulsion with the hydrogel was 1.41% (T5 in Figure 2). The same was applied in the other cases: T4, T6, and T8 (Figure 2).

All treatments with hydrogel (from T1 to T10) were applied to the colonized surfaces with a paintbrush, left for a week until the hydrogel matrix dried completely, and then removed by peeling it off. In the cases without hydrogel, i.e., Preventol RI-80® (in T11) and distilled water (T12) both treatments were applied with a paintbrush, allowed to dry, and then removed brushing the surface with a soft brush.

2.4. Chemical Characterization of the Commercial Essential Oils (EOs)

In the case of *O. vulgare* and *T. vulgaris*, both oils were acquired from the company Esencias Martínez Lozano, Murcia, Spain, which provided the chemical analyses and the relative data sheet containing the identified compounds at the respective concentrations (Table 1). *C. nepeta* oil was analyzed with a gas chromatography–mass spectrometry (GC-MS) system (Shimidazu, Kyoto, Japan) equipped with a MEGA SE52 5% polydiphenyl–95% dimethylsiloxane-bonded phase column (Mega, Legnano, Italy) (dim. 30 m × 0.32 mm × 0.15 µm). The oven temperature used was initially 50 °C heating to 250 °C at a rate of 3 °C/min. The operating conditions were: injection temperature 250 °C, carrier (helium) flow rate of 1 mL/min, electronic impact mode of 70 eV, injection in the split mode, interface at 230 °C, quadrupole temperature 150 °C, transfer line temperature 280 °C, SCAN acquisition mode (masses interval: 35-350 AMU). For the analyses, the oil extracts were diluted in cyclohexane (5 mg/mL). The identification of the compounds was realized by comparing the mass spectra reported in the commercial libraries and using the retention indexes compared with those of the reference libraries [29].

2.5. Evaluation of Cleaning Effectiveness by Using Color Spectrophotometry Analysis

The cleaning effect of the treatments was assessed immediately after treatment, and after one and two weeks, by color spectrophotometry, a reliable tool to evaluate the presence and vitality of phototrophs [30–35] and the cleaning effectiveness in granite [8,24].

A portable spectrophotometer (CM-700d, Konica Minolta, Tokyo, Japan) equipped with a CM-S100w software (SpectraMagic™ NX) was used for instrumental color measurements, under the

following analytical conditions: D65 illuminant, 2° observer, target area of 8mm ø and SCI mode. Three measurements were made in each replicate area, i.e., nine measurements by each treatment.

Color was measured directly on randomly selected areas of the humid colonized surfaces [36]: before cleaning, immediately after cleaning, one week after cleaning, and two weeks after cleaning. Monitorization for 14 days was carried out in order to evaluate the effectiveness and persistence of each treatment over time in terms of cleaning, and to evaluate the change of color of the substrate over time. The data were analyzed using the CIELAB color system [37], where of the three parameters (L*, a* and b*) that define it, only the chromatic parameters, i.e., a* (associated with changes in redness-greenness) and b* (associated with changes in yellowness-blueness) proved relevant for evaluating the efficacy of the treatments, as in Sanmartín et al. [8,24]. Thus, partial differences in these parameters were determined using the following equations:

$$\Delta a^* = a^*_i - a^*_0 \quad (1)$$

$$\Delta b^* = b^*_i - b^*_0 \quad (2)$$

where the subscript i denotes the average value of the parameter immediately after, one or two weeks after the cleaning procedure, and the subscript 0 denotes the average value of parameter before the application of the treatments. Positive values of Δa* indicate reddening, and negative values indicate greening. Positive values of Δb* indicate yellowing, and negative values indicate blueing.

2.6. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) and Tukey's HSD post-hoc test (p-value ≤ 0.05) implemented in the SPSS statistical program (version 23.0).

3. Results and Discussion

3.1. Essential Oils (EOs) Content and Chemical Composition

The chemical compositions of the essential oils (*C. nepeta*, *T. vulgaris*, *O. vulgare*) are detailed in Table 1. A total of 36 compounds have been identified in *C. nepeta* (96.8% of the total composition), 25 compounds in *T. vulgaris* (95.3% of the total composition) and 23 compounds in *O. vulgare* (96.7% of the total composition). The presence of the major phenolic compounds in each oil (i.e., active principles) have been confirmed, where Pulegone represents the 55.2% of the total composition of *C. nepeta*, thymol the 46.4% of *T. vulgaris* and carvacrol the 70.5% of *O. vulgare*. The presence of Carvacrol (4%) has been detected also in *T. vulgaris*, as well as thymol (3.8%) in *O. vulgare*.

3.2. Cleaning Effectiveness of the Treatments

In all cases, a single application of the treatment successfully removed much of the subaerial biofilm, as assessed by naked eye observation (Figure 3). After cleaning, main visual changes were observed in T3, T6, T9, T11, and T12; two weeks after cleaning T4 and T7 were added to the list (Figure 3).

Before cleaning, chromatic color data from all samples were included in a small color gamut, ranging between −5.0 and −3.0 CIELAB units for a*, and between 18.1 and 15.3 CIELAB units for b* (Figure 4). They can be considered similar starting points according to colorimetric criteria and considering the upper limit of rigorous color tolerance or noticeable change in color of three CIELAB units [38,39].

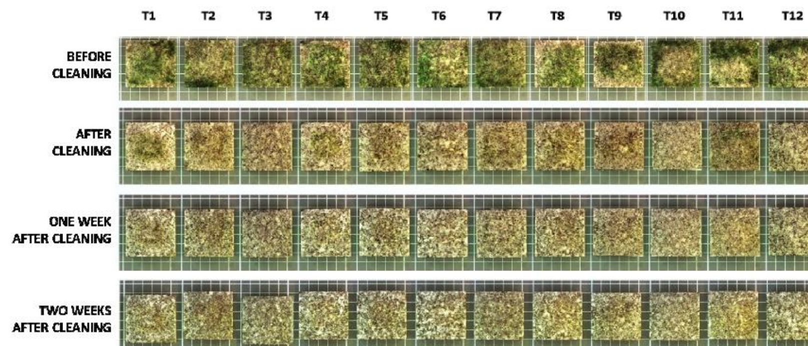

Figure 3. Macroscopic appearance of the samples studied throughout the experimental period.

As seen in Figure 4 and Table 2, after cleaning all treatments led to changes toward red (marked by an increase in the coordinate a* and positive values of Δa*) and blue (marked by a decrease in the coordinate b* and negative values of Δb*) components. This trend continued until the end of the experiment, and increased with time. Thus, two weeks after cleaning the chromatic values are close to the reference value indicated by an uncolonized clean granite sample (Figure 4). It demonstrated that the changes were associated with the effective cleaning during the experiment and not with a change in color of the granite substrate. In this regard, it also indicates that observing the more consistent variations of Δb*, it seems that this coordinate was more informative for the purpose of the study, in line with previous studies of phototrophic biofilms on granite rocks [33].

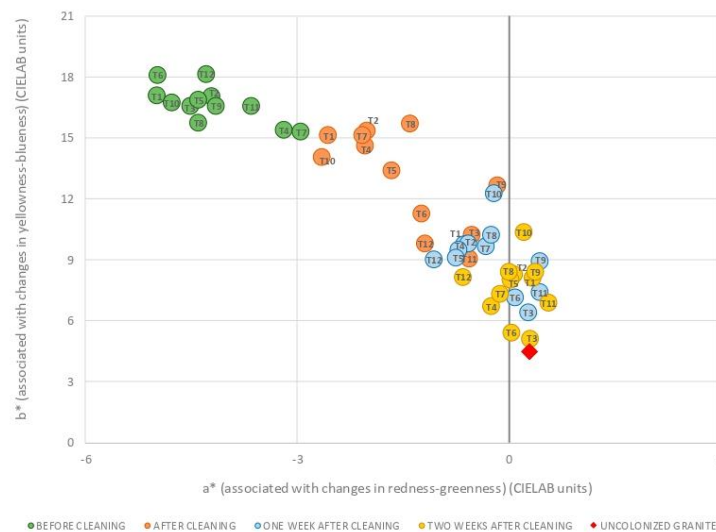

Figure 4. Color changes of the samples studied throughout the experimental period in the b* versus a* diagram. Each symbol is also identified by a sub-legend showing the sample's treatment (codes are shown in Figure 2).

Table 2. Changes in the green-red color component (Δa*) and blue-yellow color component (Δb*) in the treated samples throughout the study period. Different superscript letters in each row indicate significant differences ($p \leq 0.05$) in relation to the different stages of the sample's treatment for a given partial color difference.

Treatment	Components	Δa* (CIELAB Units)			Δb* (CIELAB Units)		
		After Cleaning	One Week after Cleaning	Two Weeks after Cleaning	After Cleaning	One Week after Cleaning	Two Weeks after Cleaning
T1	*T. vulgaris*	2.4^{A}	4.4^{B}	5.3^{B}	-2.0^{A}	-7.3^{B}	-9.0^{B}
T2	*O. vulgare*	2.2^{A}	3.6^{B}	4.3^{C}	-1.7^{A}	-7.3^{B}	-8.8^{B}
T3	*C. nepeta*	4.0^{A}	4.8^{A}	4.8^{A}	-6.3^{A}	-10.1^{B}	-11.5^{B}
T4	Thymol	1.2^{A}	2.5^{B}	2.9^{B}	-0.8^{A}	-5.9^{B}	-8.7^{B}
T5	Carvacrol	2.7^{A}	3.6^{AB}	4.4^{B}	-3.5^{A}	-7.8^{B}	-8.9^{B}
T6	Pulegone	3.7^{A}	5.1^{B}	5.0^{B}	-6.8^{A}	-10.9^{B}	-12.7^{B}
T7	All EOs	0.9^{A}	2.6^{B}	2.8^{B}	-0.2^{A}	-5.6^{B}	-8.0^{C}
T8	All APs	3.0^{A}	4.1^{B}	4.4^{B}	0.0^{A}	-5.5^{B}	-7.3^{C}
T9	Hydrogel	4.0^{A}	4.6^{B}	4.5^{B}	-3.9^{A}	-7.6^{B}	-8.2^{B}
T10	Preventol + Hydrogel	2.1^{A}	4.6^{B}	5.0^{B}	-2.7^{A}	-4.4^{AB}	-6.3^{B}
T11	Preventol	3.1^{A}	4.1^{B}	4.2^{B}	-7.5^{A}	-9.2^{B}	-9.7^{B}
T12	Water	3.1^{A}	3.2^{A}	3.6^{A}	-8.4^{A}	-9.1^{A}	-10.0^{A}

Taking into account both chromatic coordinates, the treatments that provoked significant color changes after their application were T3, T5, T6, T9, T10, T11, and T12 (data not shown), while those that exceeded the threshold of 3 CIELAB units in both partial differences Δa* and Δb* were T3, T6, T9, T11, and T12 (Table 2). These results are consistent with those reported by naked eye observation (Figure 3).

In T6, T9, and T11 there were significant changes in values of Δa* (increasing) and Δb* (decreasing) between immediately after treatment and one week later, and in the case of T3 it was only in Δb* (Table 2). It shows that after cleaning, these four treatments left some organism alive over the surface of the stone, whose color after one week turned from pale green to yellow and then bleached due to the senescence and death of the cells and the concomitant degradation of chlorophyll-a content [32,33,36].

At the end of the experiment, all treatments achieved values of a* ranging between −0.7 and 0.6 CIELAB units, very close to the reference a* value of the uncolonized granite of 0.3 CIELAB units (Figure 4). In the case of b*, the values ranged between 10.4 and 5.1 CIELAB units, with a reference value of 5.2 CIELAB units (Figure 4), allowing to use this coordinate to make differences between the treatments' success, in line with the above mentioned. Accordingly, T3 and T6 obtained a value practically identical to that of the reference, with values of b* of 5.1 and 5.4 CIELAB units respectively, yielded therefore the best results. This resulted in the end of study, not continuing it over time. T4, T11, T7, T5, and T1 produced results of b* away from reference value in 1.5, 1.7, 2.1, 2.8, and 2.9 CIELAB units, all below the previously indicated visual threshold of 3 CIELAB units. The other treatments, i.e., T12, T2, T8, and T9, with b* values ranged between 8.2 and 8.4 CELAB units, barely exceeding this threshold; whereas T10 reached a partial difference of b* with respect to the reference of 5.2 CIELAB units (> 5 CIELAB units, the normal limit of perception in industrial or technical applications [38,40]).

Pulegone (T6) is the main component of *C. nepeta* oil (T3), so the most effectiveness of both treatments in the cleaning should be attributed to this terpenic ketone, which seems to be effective either applied individually or incorporated in an oil product. For visual comparative purposes, these results can also be observed in Figure 5. A previous study where the essential oil active components (EO-ACs): limonene, menthone, pulegone, and menthol were tested against the bacteria *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella veneziana*, *S. paratyphi* B, and *S. typhimurium*, and the fungi *Fusarium moniliforme*, *Botrytis ÿinereal*, *Aspergillus niger*, and *Pyricularia oryzae*, using the agar diffusion technique, showed also that only pulegone had an effective response regarding the antimicrobial activity, particularly against the *Salmonella* species [39]. Preventol RI-80® embedded in a hydrogel matrix (T10), on the contrary, was the least effective cleaning treatment of the twelve tested, but not its two components separately, Preventol RI-80® applied with a brush (T11) and hydrogel (T9).

It could be due to an incompatibility of both compounds when mixed, i.e., they are more effective separately than together, which impedes the effective action. Similarly, the treatments where the three essential oils were combined (T7) and, to a greater extent, where the three active compounds were combined (T8) yielded rather poor results, similar to *T. vulgaris* (T1) and *O. vulgare* (T2) oils separately, and their active components Thymol (T4) and Carvacrol (T5) also separately, which indicated that the effect of *C. nepeta* (T3) and Pulegone (T6) in the mixture, is either neutralized or turns out to be in an excessively low percentage to achieve a successful effect. However, in a previous study by Bruno et al. [21] the application of a combination of essential oils from *L. angustifolia* and *T. vulgaris* was effective in killing phototrophic biofilms also at low concentrations. Also, *O. vulgare* proved, in a previous study [9], to be the most effective, compared to *L. angustifolia* and *R. offocinalis*, with regards to antifungal properties, using *Epicoccum nigrum* and *Bipolaris spicifera* as test species, with comparable results to those obtained with the commercial biocide QACs derivate benzalkonium chloride.

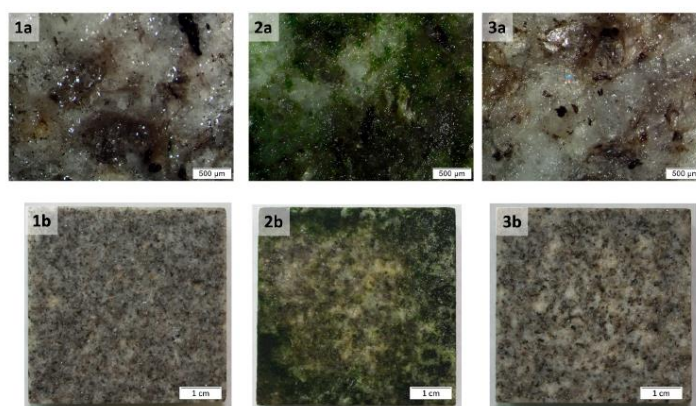

Figure 5. Granite samples (**a**) examined under a stereoscopic microscope Nikon Eclipse E600, Tokyo, Japan. Scale bar is 500 µm. (**b**) photographed with a macro lens Tamron SP 90mm F/2.8 Di MACRO 1:1. Scale bar is 1 cm. (**1**) Uncolonized, (**2**) colonized, (**3**) cleaned with *C. Nepeta* (T3).

It is well-known that essential oils are widely used in various industries. However, it should be remembered that the chemical composition of the EOs and the content of bioactive compounds are variable, even when they come from the same plants. Differences in composition of the EOs may affect its effectiveness and may be variable. Also, it is possible to suppose that the efficacy of essential oils is closely related with the ratio of the active components in their composition, whereas the efficacy of essential oils and essential oil active components are related with the target microorganisms. As an example, *C. nepeta* of autochthonous aromatic plants from Alentejo (Portugal) demonstrated high toxicity against *Artemia salina* shrimp larvae and presented higher content in oxygenated monoterpenes, with 1,8-cineole as main component [41], while in our study 1,8-cineole was found at a concentration of barely 0.5% (Table 1).

4. Conclusions

In general, the results obtained for the essentials oils (both alone and combined) are comparable with those of their respective major terpenic compounds. This confirms the high contribution in the biocidal action of the major compound (i.e., active principle) in the heterogeneous composition of the essential oil. This result is encouraging in view of possible future experimentations that will provide the employment of the active principles alone, which presents advantages as: (i) formulation of *ad hoc* treatments with simpler product combinations and (ii) lower treatment costs.

C. nepeta and its active component Pulegone yielded similar results, comparable to those of uncolonized-clean granite, proving to be the most effective treatment, even better than application of Preventol RI-80® plus brushing (most common treatment at present).

The efficacy of the phytochemicals on the target microorganism seems to depend on their chemical composition. This matter must be investigated deeply in order to create treatments containing single or mixtures of products against a specific, characterized biofilm.

To investigate the biocidal properties of these EOs, and not only the cleaning ones, future studies should involve re-inoculating the treated samples with the same phototropic culture to determine if the presence of the eventual residual compounds may influence the growth of the microorganisms.

Finally, it is worth indicating that it is the first study where phytochemical compounds are embedded in a hydrogel matrix and applied on phototrophic subaerial biofilms. This innovative mode of application, which uses harmless gel with high adhesive properties, avoids the deleterious effect of brushing on surface stone.

Author Contributions: Conceptualization, C.G., G.F., and B.P.; methodology, C.G., E.F., and B.P.; validation, C.G., E.F., P.S., and B.P.; laboratory work, C.G. and E.F.; resources, B.P.; writing—original draft preparation, C.G., E.F., and P.S.; writing—review and editing, C.G., E.F., P.S., G.F., and B.P.; supervision, B.P.; project administration, G.F. and B.P.; funding acquisition, G.F. and B.P. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partly funded by projects ED431C 2018/32 and CGL2016-79778-R AEI/FEDER, UE. E. Fuentes is supported by a PhD Fellowship-Contract MICINN-FPI (BES-2017-079927).

Acknowledgments: The authors are grateful to P. Matricardi (Sapienza University of Rome, Rome, Italy) and C. Cencetti (QI technologies s.r.l, Pomezia (RM), Italy) for the ideation of the hydrogel and QI technologies s.r.l. for making available the hydrogel itself and the materials necessary for its realization. Thanks to Paula Castiñeiras for assistance in the laboratory and other activities, and Carlo Bicchi and Barbara Sgorbini (Università degli Studi di Torino, Turin, Italy) for kindly providing data of the chemical characterization of *C. nepeta* essential oil.

Conflicts of Interest: The authors declare no conflict of interest.

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Microchemical Journal 156 (2020) 104918

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

An integrated approach to the recovery of travertine biodegradation by combining phyto-cleaning with genomic characterization

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ARTICLE INFO

Keywords:
Essential oils
Phytochemicals
Biodeterioration
Colorimetry
Genomics

ABSTRACT

Travertine is a sedimentary carbonate rock widely used in Roman arts and frequently subjected to biodeterioration. In this preliminary multi-analytical study, three essential oils (EOs) from *T. vulgaris, O. vulgare, C. nepeta* and their respective phenolic major components (thymol, carvacrol, pulegone) were selected to evaluate their biocidal potential against a multi-species biofilm grown on a travertine test wall located inside Sapienza University Campus in Rome. A preliminary characterization of the biofilm occurred through the employment of genomic methodologies. It has been confirmed the presence of microorganisms belonging to fungi, plant and bacteria kingdoms amplifying the rDNA in the characteristic regions (ITS, 18S, 16S) by performing PCR amplification with specific primers. For this purpose, two different biofilm sampling procedures, one completely non-invasive (swab in physiologic saline solution) and one micro-invasive (adhesive tape) have been employed: the adhesive tape performed better than swab in relation to DNA purity and quantity. After the biofilm characterization, the application of the phytochemicals occurred. For the realization of formulations, the EOs and their major components have been incorporated inside an innovative hydrogel matrix conceived for the cleaning of cultural heritage. The biocidal and cleaning action was evaluated with colorimetric measurements before and after the application of the products and, in the case of the surfaces treated with the essential oils, these measurements have been repeated 6 weeks after the end of the treatment. It emerged that the formulations containing the active phenolic compounds have, in most cases, a better efficacy against the multispecies biofilm. Anyway, it was verified that the biocidal action of the essential oils lasts in time, since colour variation towards the original colour of the surface have been determined 6 weeks after the end of the procedure.

1. Introduction

One of the most relevant problems of the outdoor-preserved stone materials is Biodeterioration, defined by Hueck [1] as “any undesirable change in the properties of a material caused by the vital activity of living organisms”. The most important and studied joint venture in biodeterioration is given by microorganisms (mainly algae, bacteria, cyanobacteria and fungi) that merge in complex symbiotic communities, forming on stones the so-called Sub-Aerial Biofilm (SAB) [2], which causes irreversible aesthetical and structural damages on the colonized surface [3]. Some lithotypes seem to be more prone than others to the colonization, because of their bioreceptivity, defined by Guillitte [4] as “the ability of a material to be colonized by living organisms”, which is directly linked to some intrinsic characteristics of the stones themselves, in particular to the physical ones. In fact, it has been demonstrated that high values of capillarity and superficial porosity promote the primary bioreceptivity [5] and thus, the proliferation of microorganisms inside the natural pores of the stones, that hold the water necessary for the growth and survival of microorganisms and constitute a natural niche that assure them the protection against the dangerous environmental conditions [6]. These properties are common in different lithotypes, among which travertine [7], a sedimentary carbonate rock widespread near Rome, known as “the Stone of Romans” for its large employment in roman arts since ancient times [8].

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https://doi.org/10.1016/j.microc.2020.104918
Received 12 September 2019; Accepted 10 April 2020
Available online 03 May 2020
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Considering the relevance of the phenomenon, different synthetic biocidal products have been used to preserve biodegraded cultural heritage but, in most cases, they present risks to the environment and human health [9,10]. Following the new trend in science of employing eco-friendly and eco-sustainable products [11–13], this study proposes an innovative approach for the cleaning of a biodegraded travertine surface replacing the classical methods using phytochemical compounds. In particular, the biocidal properties of *Calamintha nepeta, Origanum vulgare, Thymus vulgaris* oils, belonging to the Lamiaceae family and commonly present in the Mediterranean flora, are exploited: their antibacterial, antifungal and antioxidant activities have been demonstrated in different research fields, included the conservation of cultural heritage one [14–17], although few studies have provided the application of these products on real study cases. In recent times, scientific investigations have been aroused trying to determine the chemical mechanisms responsible of essential oils biological activities. One of the most interesting points concerns their heterogeneous chemical composition (20-60 components), subjected to many variations depending on different factors, such as: the growth area of the plants, the part of the plant employed, and the harvesting time [18]. However, they are always characterized by one or two major components (20–70% composition) that establish the chemotype of the oil [19]. At present, the contribution of the major components is still not well known, however it was demonstrated that the oils having higher concentration in phenols, as in the case of the ones extracted from Lamiaceae, have better biological activities, also against biological species isolated from monuments [20]. In light of the above, in this research the evaluation of the activity of the isolated phenolic major constituents to determine if their action is comparable to the corresponding essential oil has been attempted, with the intention of replicating the biocidal products by using the isolated phytochemical phenols, thus respecting the initial idea of employing natural substances produced by plants. The compounds are: pulegone for *C. nepeta* [21], thymol for *T. vulgaris* [22] and carvacrol for *O. vulgare* [23]. The application consisted in blending the chosen phytochemicals in different compositions and concentrations inside an innovative polymeric and surfactants-based matrix especially conceived for the cleaning of cultural heritage [24]. For the monitoring of the biofilm and the cleaning efficacy of the products, non-invasive and non-destructive colorimetric analysis was performed, in order to find a good systematic approach that could be applied in future, also on different lithotypes [25,26]. Considering that one of the most interesting aspects of the work is the experimentation *in situ*, it was necessary to characterize the microorganisms in order to determinate the efficacy of the products on a heterogeneous biofilm, overpassing the classical *in vitro* assays which tested the phytochemicals on isolated colonies of fungi and bacteria. For the collection of the biological material, two sampling methods have been employed: swab in sterile solution [27] and adhesive tape [28]. These are consolidated non-invasive (or micro-invasive) sampling techniques for cultural heritage, because allow to collect enough biological material for the cultivation and isolation of microorganisms in plate, without affecting the integrity of the artistic artefact. However, these traditional cultural methodologies limit the characterization of the whole biofilm because only the 1-3% of the microorganisms constituents the real sample are able to proliferate on the culture media at the same growth conditions [29]. On the contrary, these limitations are overcome by the employment of molecular methods which can support the traditional approach for the characterization of the biofilm [30]. Through the analysis and sequencing of the rDNA it is possible to obtain specific information about the microbial communities i) directly in their natural environment; ii) sampling a small quantity of biological material; iii) repeating the procedure easily in the way to obtain information about the history of successive colonisations [31].

In this study, it has been decided to compare the two sampling methods in order to determinate which one is the best in terms of quantity and quality of collected biological material. The set-up of a robust detection method could allow standardising the procedure for the cleaning-up of artistic artefact by using an innovative bio-based method.

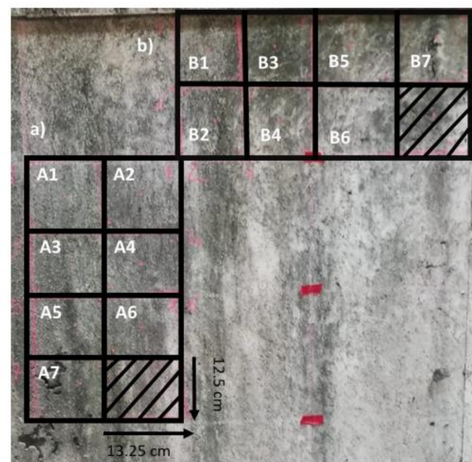

Fig. 1. The travertine biodegraded surface subjected to the cleaning procedures. a) Surfaces treated with the formulations containing the essential oils (A1 = *O. vulgare*; A2 = *O. vulgare* + *T. vulgaris*; A3 = *T. vulgaris*; A4 = *T.vulgaris* + *C. nepeta*; A5 = *C. nepeta*; A6 = *O. vulgare* + *C. nepeta*; A7 = *O. vulgare* + *T. vulgaris* + *C. nepeta*). b) Surfaces treated with the formulations containing the terpenic active components (B1 = carvacrol; B2 = carvacrol + thymol; B3 = thymol; B4 = thymol + pulegone; B5 = pulegone; B6 = carvacrol + pulegone; B7 = carvacrol + thymol + pulegone).

2. Experimental

2.1. Application on the stone surface

An evidently biodegraded travertine wall, located in the mineralogy building of Sapienza – University of Rome, was chosen as the test surface for performing the experiment. Two adjacent portions of the wall were selected for the application of the products: one assigned to the formulations containing the essential oils and the other to the ones containing the respective active principles. Then, each portion was divided in seven panels (a total of 14, dim: 165 cm^2) each of them assigned to the application of a specific formulation. In Fig. 1 a picture of the wall and its subdivision in the different cleaning panels is shown. Each letter, associated with a number, represents the formulation used in the specific panel (resumed in Table 2). The emulsions were applied on the surface with a brush and, after seven days, when they dried completely and formed a homogeneous film, were peeled off with the help of a spatula and water, as to allow an initial detachment of the film adherent to the surface.

2.2. Sampling of biological material

The biological material was collected from a biocolonized area adjacent to the one where the cleaning procedures have been realized (see section 2.1). The two sampling methodologies adopted provided the employment of sterile swab (non-invasive) and scotch tape (micro-invasive). For each methodology three sampling on three different surface portions (sized 5 × 5 cm) were performed, for a total of 6 samples of biological material. The samples have been preserved in sterile tubes at

-20°C until the DNA extraction procedures

2.3. DNA extraction

The DNA extraction was performed following the CTAB method as described by Scala et al. 2017 [32] with some modifications of the first step which provided the addiction of 1.5 mL of C-TAB I, 1.5 mL of SOL2A, 15 μL of Proteinase K (20 mg/mL) per the samples, preserved in sterile tubes of 50 mL, and the incubation overnight (55°C, 250 rpm). The next steps have been realized according to the cited protocol.

2.4. DNA quantification and amplification

DNA extraction quality was evaluated by spectrophotometric analyses using NanoDrop™ 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA), in order to evaluate DNA concentration, and 260/280 absorbance ratio. This is a useful parameter to estimate the pureness of the extracted DNA: if the ratios are ≈ 1.8 the DNA is generally accepted as "pure" [33]. In order to detect the presence of microorganism's DNA in the total extracted DNA, such as bacteria, fungi and plants kingdoms, PCR analyses were performed. 50 ng of genomic DNA were used using specific primers for 16S [34,35], ITS [36], and 18S regions respectively. The specific primes are summed in Table 1. The amplification was performed with Taq Polymerase (Bioline, London, UK) and the amplification products were verified through Agarose gel (1%) electrophoresis.

2.5. Selection of the phytochemicals

The three oils of *Thymus vulgaris, Origanum vulgare* and *Calamintha nepeta,* used for the experimentation, have been purchased from specialized retailers of natural phytochemical products, which cultivate plants following the european directives for organinc farming. The oils of *Thymus vulgaris* and *Origanum vulgare* were obtained from plants grown and harvested in Tuscany (Podere Santa Bianca, Pomarance (PI), Italy) while the oil of *Calamintha nepeta* was obtained from plants grown and harvested in Corse (Huiles Essentielles Bio de Corse - Julien Fauconnier, Occhiatana, Corse, France). The choice of leading the experimentation with commercial oils reflects the necessity of i) ideating formulations very easy to reproduce in different situations, especially when laboratory products and equipment are not available, ii) employing products used in every day's life. Despite it is difficult to individuate the suitable concentration of essential oils to be employed against a multispecies biofilm, the 2% is the one used in this research because, according to Hammer et al [37], it can be individuated as the concentration at which different essential oils demonstrated biological activities against many microorganisms. The literature survey also allowed to select the phenolic major constituent of each oil and it was evidenced that the composition and concentration of the chemicals inside the oils are very variable depending on the chemotype and other environmental factors. For the experimental procedure, the concentration employed is a representative average of the values reported in literature, in particular: for the *C. nepeta* oil the pulegone ≈ 40% [38–40], for *T. vulgaris* oil the thymol ≈ 50% [22,41,42] and for *O.vulgare* oil carvacrol ≈ 15% [23,43]. The pure thymol (≥ 98.5%) carvacrol (≥ 98.0%) and (R)-(+)-pulegone (≥ 90.0%) compounds were purchased from Aldrich Corp. (St Louis, MO, USA).

2.6. Preparation of the solutions

To apply the phytochemicals on the travertine wall, it was necessary to find a medium in which the substances can be incorporated, applied and then easily removed from the surface. In order to individuate a method that can be easily reproduced by restorers when the laboratory equipment is not available, the medium have to satisfy the following requirements: non-toxicity, compatibility with the phytochemicals, practicality in preparing the solutions. For this purpose, an innovative film-forming hydrogel produced by the research team of Professor P. Matricardi (Dept. of Chemistry and Drug Technologies, Sapienza University of Rome), especially conceived for the cleaning of stone artistic materials, was considered. This is composed by 0.4% w/w Gelrite (Sigma-Aldrich, St. Louis, MO,USA), 4% w/w Poly (vinyl acetate) (87-89% hydrolyzed, Mw 85-124000, Sigma-Aldrich, St Louis, MO, USA), 0.045% w/w $CaCl_2$, and 5% w/w Acemoll CC (ACEF, Fiorenzuola D'Arda (PC), Italy) dissolved in distilled water . Preliminary laboratory tests were realized on travertine specimens sized 5 cm x 5 cm x 2 cm and 0.50 g/cm^2 was individuated as the optimal quantity of hydrogel needed for the formation of a homogeneous layer on the stone surface, very easy to peel off when dried. The compatibility between the products was already verified in the study of Genova et al. [24]: mixing together for 15 minutes with a magnetic stirrer the phytochemicals with the hydrogel, it is possible to obtain a homogeneous emulsion that preserve the adhesive and film-forming properties of the original hydrogel matrix. To achieve the objective of the work of comparing the biocidal action of the essential oils with the respective active components, two groups of emulsions were prepared: one containing the essential oils (alone or mixed together) and the other one containing the active principles.

For each substance, 4 different emulsions were made: one contains one phytochemical pure, two contain two phytochemicals mixed together and the last contains three phytochemicals, for a total of fourteen different emulsions (seven with essential oils and seven with active components). The total concentration of essential oils present in each emulsion is 2% (see section 2.5). This means that the emulsions in which the pure essential oil is present is concentrated at 2%. Conversely, in the emulsions in which two or three products are present, the contribute of each essential oil is respectively 1/2 (1.00%) and 1/3 (0.67%). On the other hand, the concentrations of the pure carvacrol, thymol and pulegone in the formulations correspond to the percentage of the substance naturally present in the essential oils (see section 2.5), reported at 2%. In the emulsions containing the mixture of two or three products, the same principles of the respective formulations containing the essential oils have been applied.

The specific composition of each emulsion is reported in Table 2.

2.7. Colorimetric measurements

Colour measurements were performed on each panel before and

Table 1
Summary of the primers used for PCR amplification of sequences belonging to fungi (ITS1, ITS4), plants (EukA, EukB) and bacteria (27For, 1495Rev) kingdoms. Primer sequence, annealing temperature and expected amplicon length are also given.

Kingdom	Primer name	Primer sequence (5'-3')	T Annealing (°C)	Length (bp)
Fungi	ITS1	TCCGTAGGTGAACCTGCGG	57	400-800
	ITS4	TCCTCCGCTTATTGATATGC		
Plantae	EukA	ACCCTGGTTGATCCTGCCA	60	1500-2000
	EukB	TGATCCTTCTGCAGGTTCACCTAC		
Bacteria	27For	GAGATTTGATCCTGGCTCAG	54	1000-1500
	1495Rev	CTACGGCTACCTTGTTACGA		

Table 2
Composition and concentration of the formulations.

Essential oils [w/w] %					Active principles [w/w] %				
N °	*O. vulgare*	*T. vulgaris*	*C. nepeta*	Tot	N°	Carvacrol	Thymol	Pulegone	Tot
A1	2.00	//	//	2.00	B1	0.30	//	//	0.30
A2	1.00	1.00	//	2.00	B2	0.15	0.50	//	0.65
A3	//	2.00	//	2.00	B3	//	1.00	//	1.00
A4	//	1.00	1.00	2.00	B4	//	0.50	0.40	0.90
A5	//	//	2 .00	2.00	B5	//	//	0.80	0.80
A6	1.00	//	1.00	2.00	B6	0.15	//	0.40	0.55
A7	0.67	0.67	0.67	2.00	B7	0.10	0.33	0.24	0.67

after the application of the products: colour variations represent an index of the cleaning action of the products and thus an indirect estimator of their biocidal properties. A total of 3 readings on 3 selected points of each panel were obtained and the average values were calculated, in order to estimate the general colour of each one. For the analyses, a portable spectrophotometer (CM-2600d, Konica Minolta, Tokyo, Japan) was used under the following analytical conditions: illuminant D65, observer 10°, diameter of observation 8 mm, wavelength range 360–740 nm. All the colorimetric data are expressed in the CIE-L*a*b* colour coordinates system where: L* represents lightness variations, a* coordinate represents colour variations between red and green (a* (+) = red; a* (-) = green) and b* coordinate represents colour variations between yellow and blue (b*(+) = yellow; b*(-) = blue). To quantify the colour variations before and after the superficial treatments, the ΔE^*_{ab} values were determinate using the following formula [44]:

$$\Delta E^*_{ab} = \sqrt{(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})}$$
$$\Delta L^* = L^*_2 - L^*_1$$
$$\Delta a^* = a^*_2 - a^*_1$$
$$\Delta b^* = b^*_2 - b^*_1$$

3. Results and Discussion

3.1. Quantification of the extracted biological material

In Table 3 the results of the spectrophotometric analyses are reported, both for the DNA extracts obtained from the non-invasive sampling procedure with swab (Sw1, Sw2, Sw3) and from the micro-invasive sampling procedure with the adhesive tape (AT1, AT2, AT3). The concentration and the quality of the DNA extracted is lower in the samples obtained with the non-invasive swab compared to the adhesive tape procedure. These variations can be associated to a very low concentration of genomic material or to the presence of different contaminants associated with the extraction protocol, such as phenol or other reagents [45]. Anyway, it can be assumed that few adjustments of the microinvasive sampling technique and the DNA extraction procedure can lead to optimal results ($A_{260/280}$ in the optimal 1.8-2.0 range).

Table 3
Concentration and pureness values of the extracted DNA from the Swab (Sw1, Sw2, Sw3) and the Adhesive Tape (AT1, AT2, AT3) procedures.

Samples	[DNA] (ng/μL)	A_{260}/A_{280} (ng/μL)
Sw1	35.3	0.70
Sw2	29.8	0.63
Sw3	68.9	0.94
AT1	125.4	1.21
AT2	87.3	1.08
AT3	264.4	1.51

3.2. Biofilm genomic characterization

The PCR amplification of rDNA regions from the extracted DNA, using both swab and tape biological material sampling methods, allowed to have a preliminary characterization of the biofilm. The results of the agarose gel electrophoresis (in Fig. 2) shown the rDNA amplification for each selected region (16S, ITS, 18S), confirming the presence of microorganisms belonging to the selected kingdoms: bacteria (Fig. 2 a,d), fungi (Fig. 2 b,e) and plants (Fig. 2 c,f). This can be stated comparing the unknown samples with the positives samples (Fig. 2 a,b,c,d,e,f): 1550 bp (*Escherichia coli*), 1808 bp (*Arabidopsis thaliana*), 550 bp (*Fusarium verticillioides*) for 16S, ITS and 18S amplicons respectively.

3.3. Colour characterization

Colour measurements were carried on the biocolonized travertine surface before (time t_0) and after (time t_1) the application of the emulsions containing the essential oils (surfaces A) and the phenolic compounds (surfaces B) (see Fig. 1). In the case of the surfaces treated with the essential oils, the colour measurements were replicated six weeks after the cleaning procedure (t_2) to verify eventual chromatic variations. To evidence the colour differences existing, in Fig. 3 four graphical representations of the colorimetric results are shown. In Fig. 3a and 3c the data have been plotted in a mono-dimensional graph to evidence their displacement along the L* axis, in Fig. 3b and 3d the points have been plotted in a bidimensional cartesian coordinates system, in which a* is the abscissa and b* the ordinate. Observing the representation reported in Fig. 3a and Fig. 3b, where the colour points of the surfaces treated with the essential oils and the phenolic compounds at t_0 and t_1 have been compared, a shift emerges towards positive values of a* for most of the points (excepted A5 and B4) and a significant increasing in L* in each panel at t_1. This general behaviour is also confirmed in the graphs represented in Fig. 3c and 3d, where the comparison of the colour spots treated with the essential oils at t_0, t_1, t_2 occurs. Although also in this case each point shows an increasing of L*, the most remarkable aspect concerns the progressive shift of all the points towards higher values of a* that at t_2 are all situated in the quadrant of the positive a* and b*, in which the green component is not present. In general, these results suggest: i) a possible disappearance of green photosynthetic pigments produced by microorganisms commonly detected in cultural heritage biofilm, linked to the decrease of the green component in colours, ii) the variation of the colour surface towards white, i.e. the original colour of the travertine, iii) a prolonged action of the products over time, as confirmed by the colorimetric variations six weeks after the end of the cleaning procedure. In light of the above, it is possible to state indirectly the biocidal potential of the phytochemicals against a multispecies biofilm, composed by microorganisms belonging to the bacteria, fungi and plants kingdoms, as evidenced by the genomic characterization. This seems to confirm the efficacy of phytochemicals also against microorganisms belonging to the plant kingdom, such as algae, frequent colonizers of stone artistic surfaces [24]. In fact, the biocidal properties of the phytochemicals against fungi and bacteria

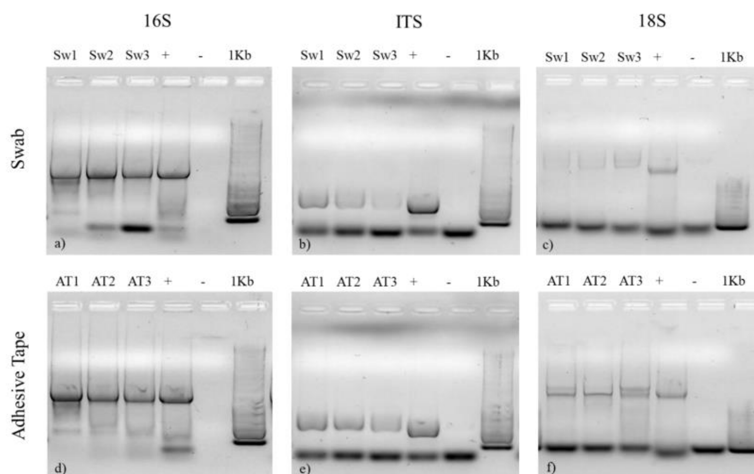

Fig. 2. Agarose gel electrophoresis of 16S (a,d), ITS (b,e) and 18S (c, f) regions, amplified from DNA extracted from biological material sampled with swab (a, b, c) and adhesive tape (d, e, f).

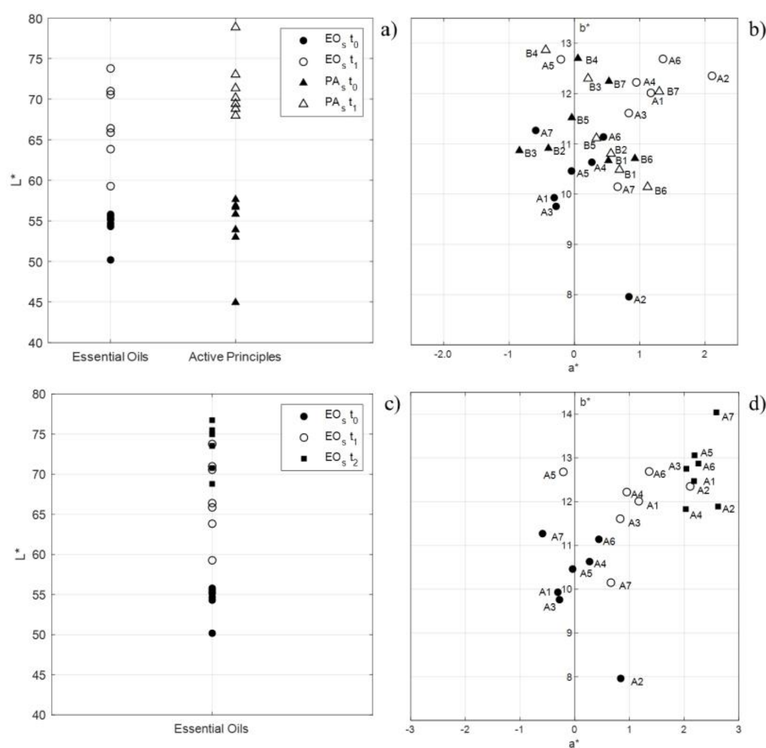

Fig. 3. Representation of the displacement of the colour points inside the monodimensional L* (a, c) and bidimensional a*b* (b, d) colour spaces. In a) and b) the colours obtained from the cleaning of the surfaces with the essential oils and the phenolic components at t_0 and t_1 are compared. In b) and c) the colours obtained from the cleaning of the surface with the essential oils at t_0, t_1 and t_2 are compared.

Table 4
Colorimetric variations (ΔL^*, Δa^*, Δb^*, ΔE^*_{ab}) before (t_0) and after (t_1) and after 6 weeks (t_2) from the cleaning procedure.

	t_1-t_0 ΔL^*	Δa^*	Δb^*	ΔE^*_{ab}	t_2-t_1 ΔL^*	Δa^*	Δb^*	ΔE^*_{ab}
A1	10.86	1.48	2.08	11.22	7.11	1.01	0.45	7.33
A2	17.98	1.27	4.39	18.60	1.24	0.52	0.46	2.77
A3	9.54	1.11	1.86	10.04	4.95	1.21	1.13	5.71
A4	15.72	0.68	1.59	15.82	4.51	1.08	0.40	7.57
A5	11.19	0.16	2.22	11.55	9.10	2.39	0.38	9.47
A6	15.41	0.91	1.55	15.54	6.18	0.91	0.18	6.41
A7	9.09	1.25	1.12	9.70	11.49	1.93	3.89	12.59
B1	15.39	0.17	0.18	15.51	//	//	//	//
B2	14.97	0.96	0.11	15.04	//	//	//	//
B3	24.47	1.05	1.43	24.56	//	//	//	//
B4	11.94	0.49	0.17	12.01	//	//	//	//
B5	15.51	0.38	0.41	15.57	//	//	//	//
B6	22.18	0.19	0.57	22.24	//	//	//	//
B7	16.25	0.77	0.21	16.40	//	//	//	//

have been demonstrated in different studies, while little is known about their activity against photosynthetic microorganisms.

However, to better interpret the colorimetric results and evidence the differences between the products, the ΔE^*_{ab} total colour differences have been calculated (Table 4) and showed in Fig 4. In Fig. 4a the comparison of the ΔE^*_{ab} of the essential oils (EOs) and the active principles (APs) between t_0 and t_1 are represented, instead in Fig. 4b it is possible to compare the colour variations of essential oils at t_1 and t_2. Observing the graph in Fig. 4a appears evident that the spots treated with the products containing the active principles demonstrated a better efficacy relating to the colour variations. This is true except for the cases of B2 and B4, containing respectively the mixtures of carvacrol-thymol and thymol-pulegone. It is interesting to notice that the bigger chromatic variation is associated to the emulsion containing pure thymol (B3). In light of the above, seems that the action of the pure thymol decreases in presence of other compounds, as also confirmed comparing B3 with the value obtained for B7. This statement is not always true for pulegone and carvacrol, the combination of which (B6) has proved to be the second in terms of effectiveness. Comparing the results, correlations between the emulsions containing the essential oils with the respective active components do not emerge. In the cases of the surfaces treated with EOs, the combined action of two compounds (A2, A4, A6) gives better results compared to the pure substances (A1, A3, A5), though this in not true for the mixture containing the three oils together (A7) which is in absolute the one with the lower value of ΔE^*_{ab}.

These synergic or antagonistic effects provoked by the combined action of two essential oils have been underlined in other studies. In some cases, two essential oils combined together demonstrated an evident increasing of the biocidal action also against resistant microorganisms immune to the effect of the single substances [46]. This can be explained because in the essential oils coexist together many chemical compounds, each of them characterized by a specific role in the defence against microorganisms. For example in *Thymus vulgaris* oil, the main biocidal action is attributed to carvacrol and thymol as discussed in this research, but the presence of p-cymene seems to favour the swelling of microorganisms' cell membrane, contributing to the biocidal action [47].

However, this cannot be assumed as always true, because the opposite mechanisms have been demonstrated in other circumstances [48]. In light of the above, it is very difficult to univocally determine the mechanisms involved in the biological activity of the essential oils and create a selective product against a specific population of microorganisms, because of the heterogeneity in the composition of the natural substances. For this reason, even if the essential oils seem to be the elective product in the removal of the biofilm, the isolated phenolic compounds can be considered a valid alternative demonstrating good results. The ΔE^*_{ab} results reported in Fig. 4b confirm what was discussed previously: the colour of the surfaces continues to change in every treated panel, even six weeks after the removal of the products. In particular, in the case of A7, it is significant how the colour difference is bigger at t_2 than at t_1, suggesting the penetration of the oils in the porous structure of the stone and a prolonged biocidal action over time.

In light of these results, it is possible to highlight the following: both the essential oils and their active principles have proven to be active in tackling the problem of biodeterioration of the stone material, as expected. However, both positive and negative synergistic effects are observed when mixed formulations of oils or active principles are created: for example some active ingredients are more effective than the corresponding oils that contain them and, when they are mixed with other principles, their effectiveness is reduced to the contrary of what happens with oils, where the presence of minority components evidently plays a role in rendering the corresponding principles more active even if present at a lower concentration. This suggests the need to address the problem according to a complex experimental approach where the present work represents the first systematic step in this direction.

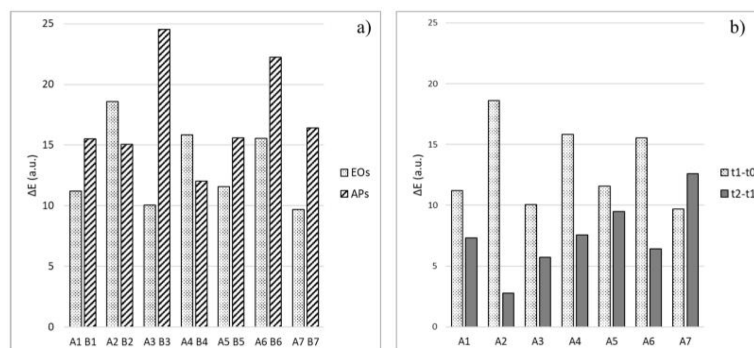

Fig. 4. Total colour differences (ΔE^*_{ab}) of the surfaces subjected to the cleaning procedures. a) Comparison of the ΔE^*_{ab} (t_1-t_0) of the formulations containing the essential oils and the phenolic components. b) Comparison of the ΔE^*_{ab} (t_1-t_0) and ΔE^*_{ab} (t_2-t_1) of the formulations containing the essential oils.

4. Conclusions

The aim of this work was to evaluate *in situ* the efficacy of some innovative chemical and biological methodologies in order to ideate a systematic strategy for the future biofilm's characterization and removal from biodegraded artistic stone materials, considering the intrinsic values of the cultural heritage and the preservation of the surrounding environment and human health. The employment of phytochemical substances combined with an experimental hydrogel matrix for the cleaning of the travertine surface provided interesting results. First of all, it was confirmed the easiness of realizing the emulsions, in the way to obtain a product that works also when applied on outdoor exposed stone materials, that allows to drive the phytochemicals and to control the processes of application and removal of the substances from the stone surface directly in *situ*. In addition, the suitability of a micro-invasive sampling method was evidenced: the improvement of the extractive process of DNA from scotch tape makes it a very suitable methodology for the collection of biopatina from artistic materials. This preliminary result allowed us to define a strategy for building-up a workflow: microinvasive sampling procedure→DNA extraction and rDNA amplification→ amplicon sequencing→ Bioinformatic customised tool for the metagenomic analyses, which allows to have more detailed information about the microorganisms. The possible use of the next generations sequencing methods would allow sparing time and money for generating high quality reads to be processed in customised bioinformatic tools exploiting the existence of, for instance, 16S and ITS databases.

The non-invasive colorimetric analyses evidenced significant chromatic variations on the surface after the removal of the phytochemical compounds, demonstrating their cleaning action and the persistence of the biocidal action over time, since colour variations have been recorded six weeks after the procedure. Even if the biocidal properties of the selected essential oils have been demonstrated in different studies, little is known about the contribute and the biocidal potential of their major constituents. In this research emerged that the formulations containing the active principles, in the same concentrations in which they are naturally contained in the oils, have shown a greater efficacy in bringing back the travertine surface to the original colour even if not always the synergic action of two or more products gives better results against the biological patina. Anyway, the phytochemicals have proved effective against a heterogeneous biological patina constituted by different microorganisms belonging to the plants, bacteria and fungi kingdoms, according with the genomic characterization of the biofilm.

Acknowledgments

The authors are grateful to Qi srl for making available the hydrogel and the materials necessary for its realization. The authors are also deeply grateful to Prof. Massimo Reverberi (Dept. of Environmental Biology, Sapienza University of Rome) for his precious contributes and suggestions. Thanks also to Tiziano Busti, Eleonora Cerafogli, Laura Giuliani, Giulia Rinaldi and Ylenia Vassallo for contributing in the laboratory activities.

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Acknowledgments

There are many people I would like to thank for their indirect ad direct support in the achievement of my PhD research.

First of all, I would like to express all my gratitude to my PhD supervisor, Professor Gabriele Favero, that made the accomplishment of this project possible but, more relevantly, he demonstrated to be a real teacher, giving me all his intellectual and human support during these important, and sometimes, very particular years

I am very grateful to the reviewers of the thesis, Prof. Heather Viles and Prof Beatriz Prieto Lamas, that contributed to the improvement of the scientific value of the work, with their precious suggestions.

Moreover, Prof. Prieto gave me the opportunity to develop a significant part of the project, by hosting me in her laboratories at Santiago de Compostela University where I have had the possibility to meet extraordinary people that gave me all their support and sympathy, especially during the darkest moments. For this reason, I would like to thank Professor Prieto herself and the GEMAP group: Dr. Javier Cancelo Gonzales, Dr. Paula Castiñeras, and Dr. Daniel Vazquez Nion, and a special thought goes to Dr. Elsa Fuentes Alonso and Dr. Patricia Sanmartín for their precious friendship.

A special thanks goes to all the people that helped me in the development of the scientific analyses and methodologies, included Professor Pietro Matricardi for providing the hydrogel, Prof Massimo Reverberi and his group for the metagenomic analyses and Dr. Rafael Carballeira for the morphological characterization of the biofilm.

Thanks to my laboratory fellows, but first of all, friends Rosaceleste Zumpano, Cristina Tortolini, Francesca Polli, Laura Lambertini, Giulia Fuoco and Leonardo Ciogli.

I would like to mention my lifelong friends, Marco, Silvia and Livia that contributed to the achievement of the thesis not only by supporting me as they always do, but also for helping me with the English revisions.

Thanks to my sweet Spanish friends, Minia and Nerea , along with Elsa, who strongly contributed to make my permanence in Santiago an unforgettable experience.

However, all my accomplishments would not have been possible without the continuous support and love of my parents and my beloved grandparents, to whom this work is dedicated.

Last but not the least, my gratitude goes to Michele, that in these last months has given me all the necessary strength to carry on and reach my objectives.