Science of the Total Environment 877 (2023) 162913

Contents lists available at ScienceDirect

Science of the Total Environment

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

Essential oils to contrast biodeterioration of the external marble of Florence Cathedral

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HIGHLIGHTS

- Essential oils (EOs) do not affect colour and water absorption of marble samples.
- EOs applied as sustainable biocides on the external marble of Florence Cathedral
- EOs biocidal efficacy on marble microbiota similar to that of a traditional biocide
- Good correspondence between microbial viable titer and ATP determination
- EOs effect on total colour change similar to that that of a traditional biocide

GRAPHICAL ABSTRACT

ARTICLE INFO

Guest Editor: Patricia Sanmartín

Keywords:
Marble biodeterioration
Microbial communities
Essential oils
In situ stone treatment
Biocides in cultural heritage
Evaluation of biocide treatment

ABSTRACT

The search for more sustainable strategies to contrast biodeterioration of stone cultural heritage has been developing in recent years to find alternatives to synthetic biocides, since their toxicity and potential impact on the environment and health. In this study, the application of the oregano and thyme essential oils (EOs) was tested to control microbial growth on the external marble of Florence Cathedral affected by extended darkening. Before *in situ* application, preliminary tests were carried out to evaluate the interference of the EOs with marble (colorimetric and water absorption assays on marble specimens) and their efficacy in inhibiting marble microbiota (sensitivity test on nutrient media). EOs inhibited the whole cultivable microbiota sampled from the Cathedral marble at a very low concentration, while they did not interfere with colour and water absorption capability of uncolonised marble samples when applied as a 2 % solution. Then the two EOs and the commercial biocide Biotin T were used in *in situ* trials on marble in two outdoor study sites of Florence Cathedral. The effectiveness of the treatments was assessed through short- and mid-term evaluation by multidisciplinary *in situ* non-invasive (colorimetric and ATP assays, microscopy) and *ex situ* (microbial viable titer) tests. Concerning results, we found a good correspondence between parameters for evaluation of viability (bacterial and fungi viable titer) and activity (ATP determination) and some correspondence among these and microscopy and colorimetry. Considering the whole data, treatments with oregano and thyme EOs were effective against microbial community, in more cases comparably to the commercial biocide. Some differences found, particularly by viable titer,

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http://dx.doi.org/10.1016/j.scitotenv.2023.162913
Received 22 December 2022; Received in revised form 11 March 2023; Accepted 13 March 2023
Available online 16 March 2023

in the two study sites or in bacterial and fungal components of the microbiota can be partly attributed to differences in structure and colonization pattern of the microbial community due to the peculiar climatic conditions of the differently exposed study areas.

1. Introduction

Stone cultural heritage material, especially in outdoor conditions, is quickly colonized by complex microbial communities often growing as biofilms which, due to their vital activities, cause over time not only aesthetical, but also chemical and mechanical deterioration (Pinna, 2017). Cleaning techniques to prevent or control microbial growth and its negative effects on stone and building materials have been developed over centuries and their advantages and drawbacks have been recently reviewed (Pinna, 2017; Cappitelli et al., 2020; Romani et al., 2022). The most widely used strategy to contrast biodeterioration on cultural heritage so far has been the application of synthetic biocides (Pinna, 2017). However, because of their intrinsic properties, commercial large-spectrum and aggressive biocides are toxic and potentially have a high environmental and health impact (Pinna, 2017; Cappitelli and Villa, 2021), even if the risk they pose depends on their toxicity level and the exposure conditions (Pinna, 2022). Many are poorly biodegradable and lead to long-term soil and water pollution (Romani et al., 2022). Moreover, biocides harm non-target populations of the surrounding environment and they carry a risk of the development of resistance to themselves as well as cross-resistance to antibiotics (Cappitelli and Villa, 2021). The ecotoxicity of biocides is a significant factor associated with their use on outdoor building materials and, according to Romani et al. (2022), the costs of the fight against biodeterioration will include in the future "also the costs associated with their application in safe conditions for customers and likely the costs associated with their environmental impact".

In recent years, the search for more sustainable strategies has been developing and new cleaning procedures using less harmful molecules have been tested in cultural heritage conservation (Romani et al., 2022). Among these, biocides of natural source have been extensively considered against biodeterioration of stone cultural heritage (reviewed in Fidanza and Caneva, 2019) and, among natural biocides, essential oils (EOs) appear to be highly preferred, due to their well-recognized antimicrobial activity investigated in several fields (Fidanza and Caneva, 2019). EOs are complex mixtures of substances (mainly aromatic compounds) produced by plants as secondary metabolites with high chemical variability and broad-spectrum activities (Saad et al., 2013; Algburi et al., 2017; Sezen et al., 2019). EOs have been employed for centuries for their natural antimicrobial properties in folk medicine. Today EOs are readily available commercially and are commonly used in the pharmacology, aromatherapy, food, agriculture, and cosmetics (Palla et al., 2020; Pinna, 2022). The antimicrobial activity of several EOs as well as of some their single components are well known, particularly on bacteria against which has been mainly experimented (Sezen et al., 2019). Some EOs are very effective against bacteria, algae, and fungi at low concentrations (Romani et al., 2022) and show synergistic interactions with antibiotics reducing drug resistance (Owen and Laird, 2018). They can produce various effects on microbial cell that lead to the inhibition of its growth, *i.e.*, inducing the deterioration of the cytoplasmic membrane, regulating intermediary metabolism, activating or inhibiting enzymatic reactions, or affecting the enzyme synthesis (Saad et al., 2013). For instance, the oregano EO affects cell membrane permeability, respiratory metabolism, energy metabolism, and gene expression of methicillin-resistant *Staphylococcus aureus* (MRSA) (Cui et al., 2019). Some of these effects can be imputable to single components of EOs; for instance, carvacrol and thymol are able to permeabilize and depolarize the cytoplasmic membrane of *Escherichia coli* cells (Xu et al., 2008).

However, their action mechanisms remain poorly understood also due to their high chemical variability and broad-spectrum activities (Algburi et al., 2017; Owen and Laird, 2018).

To date most of the studies with EOs in conservation have been carried out *in vitro* on microbial strains often isolated from cultural heritage objects (*e.g.*, Stupar et al., 2014; Bruno et al., 2019; Ilieș et al., 2021; Macedo-Arantes et al., 2021; Ranaldi et al., 2022). Among the few studies on EOs applications on stone specimens or monuments, oregano and thyme EOs have been the most widely selected to be tested (Devreux et al., 2015; Bartolini and Pietrini, 2016; Genova et al., 2020a; Romano et al., 2020; Genova et al., 2020b; Boccalon et al., 2021; Spada et al., 2021a, 2021b; Genova et al., 2023) due to their previous demonstrated efficacy *in vitro*. Beyond the experimental set-ups and the evaluation tests used, these two EOs seem to inhibit biofilm growth on stone, with oregano EO giving sometimes the best results both in single and in mixture (Devreux et al., 2015; Spada et al., 2021a, 2021b). However, results are in some cases controversial (Bartolini and Pietrini, 2016) and further *in situ* studies are required to demonstrate the effectiveness of simple or mixtures of EOs on lithobiontic communities in environmental conditions and to explore the possible interference of EOs with building materials before considering their usage at larger scales (Fidanza and Caneva, 2019; Pinna, 2022; Romani et al., 2022).

The Cathedral of Santa Maria del Fiore (SMFC; Fig. 1) is one of the greatest artistic and architectural masterpieces of the world. It belongs to the UNESCO World Heritage Site of the Historic Centre of Florence, Italy, and its conservation is a main issue of worldwide concern. The exterior of the Cathedral is mainly covered by Apuan white marble and shows extended forms of decay, macroscopically visible, mainly consisting of deposits, discolourations, patinas, and crusts (Santo et al., 2020). In recent years, the Opera di Santa Maria del Fiore (OSMF)— the institution actively engaged in the protection of the monuments of the complex of Santa Maria del Fiore—started a maintenance program for the cleaning and restoring of all the external façades of the Cathedral. The OSMF expressed also interest in testing innovative methods to remove biological patinas through on-site trials on selected external areas, not accessible to visitors, that did not undergo recent restoration interventions. We agreed with OSMF to carry out an experimentation focused on the use of EOS on the SMFC external white marbles in two study sites of the Cathedral, northwest (NW) and southeast (SE) exposed respectively. In these areas, marble surfaces showed extended dark discolourations as the main deterioration phenomenon. In a previous work, we first investigated the cause of the darkening of marble in these two areas and demonstrated that it was mainly due to the growth of black fungi and cyanobacteria (Santo et al., 2021). We also investigated the biodiversity of the lithobiontic community of lichens, algae, cyanobacteria, bacteria, and fungi inhabiting the darkened marble, assessed through microscopy, cultivation and metataxonomic approaches, as well as the deterioration and metabolic potential of the community (Santo et al., 2021; Checcucci et al., 2022).

In this work we report the results of the trials performed with oregano and thyme EOs on the SMFC marble. We first carried out preliminary tests to assess the efficacy of the EOs against the resident cultivable microbial communities and the absence of undesirable effects by EOs on marble. Then we applied the EOs and a commercial biocide to the darkened external marble in the two study sites. The effectiveness of the treatments was assessed through short- and mid-term evaluation by multidisciplinary *in situ* and *ex situ* tests.

2. Materials and methods

2.1. Essential oils and biocide used

Commercial EOs used in this study were *Thymus vulgaris* L. (thymol chemotype) and *Origanum vulgare* from ERBAMEA S.r.l. (Italy).

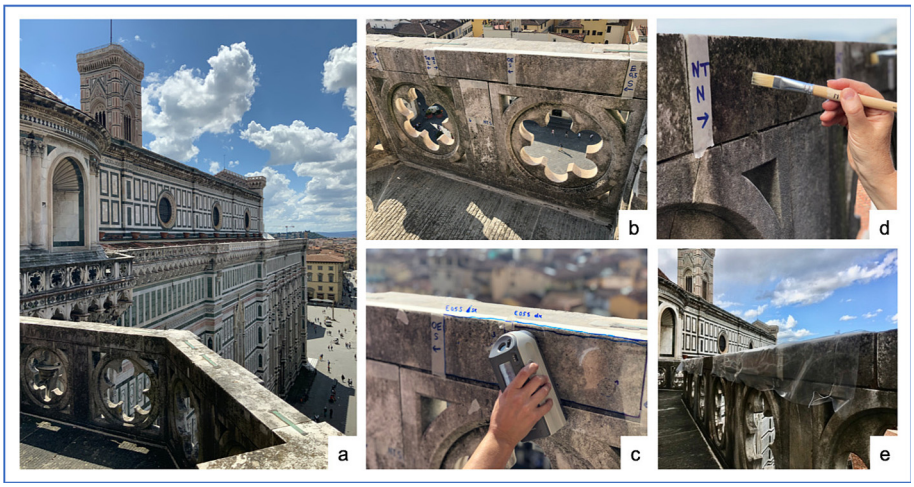

Fig. 1. The study sites for EOs treatment of marble of the Cathedral of Santa Maria del Fiore. (a) The external gallery running around the apses of the NW exposed façade; (b) a detail of the marble surface of the inner face of the openwork parapet of the SE exposed gallery; (c) colorimetric measurements on one selected SE area after application of the corresponding transparent sheet of acetate; (d) application of the reference solution (NT) by brush; (e) covering of the NW areas after treatment with transparent nylon sheets.

The commercial biocide used was Biotin T (C.T.S. S.r.l., Italy) composed of n-octyl isothiazolinone and an ammonium quaternary salt as active ingredients.

2.2. Chemical analysis of EOs

1 μl of essential oil was diluted with 1 ml of heptane in a glass vial sealed with a Teflon septum and crimped with an aluminum cap and then vortex-mixed for 5 min at 25 °C.

An Agilent 7820 Gas Chromatograph system equipped with a 5977E MSD with EI ionization was employed, all from Agilent Tech. (Palo Alto, AC, USA). A Gerstel MPS2 XL autosampler equipped with liquid option was used to inject 1 μl of samples. The chromatographic settings were as follows: injector in the splitless mode set at 260 °C, HP INNOWax 50 m, 0.20 mm i.d., 0.4 μm film (Agilent J&W GC columns); oven temperature program: initial temperature 40 °C for 1 min, then 5 °C min-1 until 200 °C, then 10 °C min-1 until 220 °C, then 30 °C min-1 until 260 °C, hold time 3 min. The mass spectrometer was operating with an electron ionization of 70 eV, in scan mode in the *m/z* range 29–330, at three scans sec^{-1}. Data were acquired and analyzed using the Agilent MassHunter software. The deconvoluted peak spectra, obtained by Agilent Masshunter software, were matched against NIST 11 spectral library for tentative identification. Kovats' retention indices were calculated for further compound confirmation and compared with those reported in the literature for the chromatographic column used. When available, authentic standards were injected to obtain a positive identification. Each terpene was expressed as a percentage of the total terpene content.

2.3. Sensitivity of cultivable bacteria and fungi sampled from marble to commercial EOs and biocide

To test the sensitivity of the microbial community inhabiting the SMFC marble, marble powder was preliminarily sampled from the two differently exposed study areas (Fig. 1) as already described in Santo et al. (2021). Superficial particulate was gently scraped from marble with a sterile spatula and collected into sterile tubes. Three samples of about a few tens of mg were taken from a surface of 15 cm^2 each at both the NW and SE sites. Samples were immediately brought to the laboratory and processed. The three samples from each area were mixed and 10 mg of the mixed marble particulate were suspended in 1 ml of Phosphate Buffered Saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, g/l 0.24 KH_2PO_4, pH 7.4) added with 0.001 % Tween 80 and vortexed at 1200 rpm for 1 h.

To cultivate bacteria, the NW suspension was diluted 10^{-1} in PBS + 0.001 % (*v*/v) Tween 80, then 0.1 ml of the undiluted and diluted suspensions were plated each in duplicate on R2A agar (VWR International) (Santo et al., 2020) without and with EOs or Biotin T at the concentrations of 0.025 % (*v*/v). The starting stock solutions of EOs were 25 % (v/v) in dimethyl sulfoxide (DMSO); the stock solution of Biotin T was 25 % (v/v) in demineralized sterile water. The possible inhibitory action of DMSO was tested by adding to R2A the same DMSO volume as that in the plates with the 0.025 % EOs concentration.

To cultivate fungi, 0.1 ml of the undiluted SE suspension was plated in duplicate on MEA (Oxoid) without and with EOs or Biotin T at the concentration of 0.025 % (*v*/v).

The MEA and R2A plates were incubated at 30 °C for at least seven days. Viable titer was calculated as the mean value of the number of Colony Formant Units (CFUs) per gram of marble particulate.

2.4. Treatment solutions

Tween 20 was used as an emulsifier to favour the dispersion of EOs in water. To test the appropriate concentration of Tween 20, it was added at concentration of 0 (control), 0.01, 0.05, 0.1, 0.5, and 2.0 % (*v*/v) in a 4 % (v/v) *Origanum* solution in demineralized water. Solutions were shaken for 1 h, then kept static for 24 h; the dispersion was evaluated by the naked eye. The minimum concentration of Tween 20 giving a visible emulsifying effect was 0.5 %, thus it was chosen for the treatment solutions. The concentration of EOs working solutions was 2 % (*v*/v) as previously reported by other authors (Devreux et al., 2015). Biotin T was used at 2 % (v/v) as well, according to the manufacturer's instructions. Moreover, a solution of Tween 20 at a concentration of 2 % (v/v), as that of EOs and Biotin T, was tested for its possible inhibition effect on the microbial community. As reference solutions we used demineralized water (NT) and 0.5 % Tween 20 (E0.5).

All the treatment solutions used for *in situ* and *ex situ* tests were in demineralized water and are described in Table 1.

2.5. Evaluation of EOs treatment on the appearance and properties of marble specimens

To evaluate the effect of EOs on marble surfaces, preliminary colorimetric and water absorption measurements were conducted in laboratory on fresh cut specimens of white Carrara marble (Gioia quarry, Tuscany) showing no signs of microbial colonization. Sixteen marble slabs (5 × 5 × 2 cm) were treated with the solutions listed in Table 1. Each treatment was applied twice on the surface of two different marble slabs.

2.5.1. Colorimetry

To test the impact of the treatment on the colour of marble, colorimetric measurements were carried out by means of a Konica Minolta CM-2600d

Table 1
Solutions for marble treatment.

Name	Composition
NT	Demineralised water
E0.5	0.5 % Tween 20
OE	0.5 % Tween 20 + 2 % oregano EO
TE	0.5 % Tween 20 + 2 % thyme EO
OT	2 % oregano EO + 2 % thyme EO
OTE	0.5 % Tween 20 + 2 % oregano EO + 2 % thyme EO
BIOT	2 % Biotin T
E2	2 % Tween 20

NT = Not Treated, E = Emulsifier (Tween 20), O = Oregano, T = Thyme, BIOT = Biotin T.

(Osaka, Japan) spectrophotometer using the CIELAB colour space system described by a three-dimensional coordinate system L* a* b*, according to the UNI EN 15886:2010 norm. The a*-axis is defined as the green-red axis; the b*-axis runs perpendicular to the a*-axis and represents the blue and yellow colours; the L*-axis is perpendicular to this plane and represents the lightness from black to white. All measurements were made in D65 illuminant and 10-degree observer conditions with SCI mirror-like components included. The investigated area had a SAV (small aperture size) of 5 mm. To obtain representative values of colorimetric coordinates, four measurements were made on each slab using a mask with four holes as large as the instrument spot size. The mask allowed measuring the colour at the same points. The results, consisting of a mean of eight measurements from two marble slabs, both before and after each treatment, are expressed as variations of L*(lightness), a* (red/green coordinate), and b* (yellow/blue coordinate) parameters computed as ΔL*, Δa*, and Δb*, respectively. ΔE* reflects the total colour variation and is obtained by the Eq. (1):

$$\Delta E^* = \sqrt{\left(L_f^* - L_i^*\right)^2 + \left(a_f^* - a_i^*\right)^2 + \left(b_f^* - b_i^*\right)^2} \quad (1)$$

where subscripts *f* and *i* indicate the colour coordinates recorded after and before the treatment, respectively.

The colorimetric changes ΔE* were evaluated before and up to 2 years after the treatments of the marble specimens keeping them in outdoor conditions.

2.5.2. Water absorption

To test the impact of the treatment on marble porosity, the water absorption content of the specimens was established by the contact sponge (about 5 cm in diameter) test method (UNI EN 11432:2011); the test provides information related to the first layers of a porous material (Vandevoorde et al., 2009). By using the Eq. (2), it is also possible to obtain the capillarity absorption factor (W_a):

$$W_a = \frac{(P_i - P_f)}{23.76 * t} \ (g/cm^2 * \text{min}) \quad (2)$$

where P_i is the starting weight and P_f the final weight of the sponge, 23.76 is the sponge area in cm^2 and t is the contact time in minutes.

The test was performed one month after the treatments of the marble specimens with the solutions listed in Table 1.

2.6. In situ marble treatments

The treatments were performed in two study sites, NW and SE exposed, of the external gallery running around the apses on the upper part of SMFC at a height of 36 m above ground (Fig. 1a). The treatments were carried out on the darkened marble of the vertical inner surface of the gallery's openwork parapet (Fig. 1b). At each study site seven areas were chosen, each with a surface of 500 cm^2, and treated with the seven solutions described in Table 1. An eighth area was treated only with demineralised water and considered as a reference area (not treated, NT, Table 1). For each area, the perimeter was delimited with masking tape and a transparent sheet of acetate was used to correctly identify the subareas and points where performing sampling, observations, and measurements before and after the treatment (Fig. 1c). The solutions were applied on the corresponding area twice by brush (Fig. 1d), a few minutes away from each other. After solution absorption, the eight areas were covered with transparent nylon sheets to avoid the interference of some environmental factors (*e.g.* wind, rain, birds) with the biocide activity during incubation (Fig. 1e). The whole treatment consisted of three applications, as above described, a week apart from each other (from September to October 2019).

2.7. Evaluation of in situ treatments

2.7.1. Determination of bacterial and fungal viable titer

Marble sampling was carried out 5 days after the treatment (*i.e.*, 5 days after the last application of the treatment). For each area, superficial powder of a few tens of mg was gently scraped with a sterile scalpel from three random subareas with a surface each of 12 cm^2 (for a total of 36 cm^2) and collected into a sterile tube. The preparation of marble suspension in PBS and cultivation of bacteria and fungi to determine viable titer were carried out without biocides as described in 2.3, except that undiluted and diluted suspensions were plated in quadruplicate.

Bacterial CFUs data were analyzed by one-way ANOVA. Statistically significant differences among treatments and NT values were evaluated by Tukey's pairwise.

2.7.2. Determination of microbial vitality on marble surface

The ATP (adenosine triphosphate) amount was measured with the help of a 3M™ Clean-Trace™ Bioluminometer and its dedicated Surface ATP test swabs. The ATP amount is measured in RLU (relative light units) on cm^2 according to the provider's instructions; the higher these values the higher the biological activity on the tested areas (about 16 cm^2 each), according to the provider's instructions. Before the treatment, some untreated extra references (not like NT treated with demineralised water as described in 2.6), were considered on differently colonized types of marbles, *i.e.* (i) one on the old and naturally colonized marble by black patinas similar to the ones on which the experiments were performed (positive reference, called NT+), (ii) one on the old marble without a visible black patina (negative reference, called NT-); and (iii) one on a recently substituted piece of marble located on the NW side with no visible microbial colonization (negative reference, called NT–). The images of these reference areas are shown in Fig. S1. On each treated area three measurements were performed on three different subareas five days and four months after the treatment. The obtained data were analyzed by one-way ANOVA. Statistically significant differences among treatments and NT values were evaluated by Tukey's pairwise.

2.7.3. Microscopical observations

A microscope Scalar DG-2A equipped with optical zoom 25-200× was used for *in situ* observations of the morphological aspect of the investigated areas. For each NW and SE area, three subareas were chosen to perform the microscopical observations before and five days after the treatments.

2.7.4. Colorimetric measurements

Colorimetric measurements were performed *in situ* on the surface of the same three subareas (for each treated area) used for the microscopic observations (2.7.3) before and five days and four months after the treatments. The same instrument conditions as described in 2.5.1 were used. For each sub-area, three different measurements of the chromatic parameters were carried out, for a total of nine measurements for each area.

3. Results

3.1. Chemical analysis of EOs

The chemical composition of EOs is shown in Table 2. *T. vulgaris* EO was characterised by p-cymene (39.4 %), thymol (34.3.0 %) and linalool

Table 2
Relative content of terpenes in the essential oils of *Origanum vulgare* and *Thymus vulgaris* used in this study.

	Oregano (%)	Thyme (%)
a-Pinene	0.3	3.6
Camphene	0.2	1.1
b-Pinene	0.4	0.4
Myrcene	0.3	1.7
a-Terpinene	0.2	0.2
Limonene	0.6	1.4
b-Phellandrene	0	0.1
Cineolo	2	0.8
g-Terpinene	1.1	0.2
p-Cymene	10.3	39.4
Terpinolene	0	0
Linalool oxide	0.1	0
Linalool	4.5	8.8
Camphor	1.4	0.7
4-ol-terpinen	1.1	0.2
b-Caryophillene	2.2	2.2
Isoborneol	0	0.7
a-Humulene	0.4	0.2
a-Terpineol	1.6	1.1
(−)-borneol	2	1.4
b-Bisabolene	0.3	0
p-Cymene-8-ol	0	0.1
Caryophillene-oxide	2.5	0.5
Thymol	7.8	34.3
Carvacrol	60.5	1.1

(8.8 %) as main components, while carvacrol (60.5 %), p-cymene (10.3 %) and thymol (7.8 %) were the major terpenes detected in *O. vulgare* EO. Minor terpenes of *T. vulgaris* EO, which ranged in relative content from 2.2 to 1.1 %, were β-caryophyllene, myrcene, limonene, (−)-borneol, camphene, α-terpineol and carvacrol, whereas for *O. vulgare* EO, minor terpenes were caryophyllene-oxide, β-caryophyllene, cineole, (−)-borneol, α-terpineol, camphor, γ-terpinene, 4-ol-terpinen (ranging from 2.5 to 1.1 %). All the other terpenes detected in both EOs showed a relative content <1 %.

3.2. Sensitivity of cultivable microbiota isolated from marble to commercial EOs and biocide

In laboratory conditions, biocides were tested on the cultivable bacteria and fungi of the NW and SE sites, respectively, at concentrations very much lower than that used for *in situ* treatment, based on our previous tests on microorganisms isolated from other sources.

The viable titer of bacteria cultivated from the NW marble (2.3) was $1.1 \times 10^7 \pm 7.0 \times 10^5$ CFU/g. An almost identical value was obtained from bacteria grown on DMSO, $1.1 \times 10^7 \pm 2.6 \times 10^6$, indicating no bacterial inhibition by DMSO at the concentration used.

No bacterial colonies grew on thyme EO, oregano EO and Biotin T tested at the concentration of 0.025 %.

The viable titer of fungi cultivated from the SE marble was 8.7×10^3 CFU/g. No fungal colonies grew on thyme EO, oregano EO and Biotin T tested at the concentration of 0.025 %.

3.3. Effects of EOs treatment on marble specimens

To evaluate the interference of EOs treatment with marble before applying it *in situ*, preliminary tests on colour and porosity changes were carried out in laboratory on fresh cut uncolonized Carrara marble specimens.

The results of the colorimetric measurements, carried out after two years, are shown in Fig. S2. To assess if the colour changes were significant, a threshold of $\Delta E^* = 3$ was chosen as the upper limit of just-noticeable differences as proposed by some authors (Prieto et al., 2010; Sbardella et al., 2020), although in the field of cultural heritage other authors consider a value of $\Delta E^* < 5$ as a fully acceptable total colour change (García and Malaga, 2012; Gherardi et al., 2016; Becherini et al., 2017). After the application of treatments, almost all samples displayed a value of the total colour difference (ΔE*) lesser than 3. TE and OTE treatments gave the highest colour difference close to 3. Analyzing the behavior of the colour coordinates, it can be concluded that this colour change is mainly due to the decrease of b* parameter; at the same time the L* coordinate of TE and OTE increases more than the others indicating a slight whitening effect. However, despite these variations, the ΔE* values do not indicate significant colour changes in the white marble specimens after the treatments (Fig. S2).

Concerning water absorption, the obtained results gave similar values for treated and untreated (NT) specimens; the values were very low, by an average of $W_a = 0.0008$ g/cm^2 min (Table S1). The tested treatments, therefore, did not lead to significant changes in the porosity of marble specimens.

3.4. Evaluation of in situ treatments

3.4.1. Bacterial and fungal viable titer

Bacterial and fungal viable titer values determined 5 days after the treatment of the NW and SE areas are reported in Table 3.

The treatments showing a significant reduction in bacterial viability (p value <0.05) respect to the NT areas were (in order of efficacy) BIOT, E2, OTE and OT at NW, and BIOT, OE, TE, OTE, OT, and E2 at SE (Fig. S3). The reduction was about 1–2 orders of magnitude at NW and 2–3 orders of magnitude at SE.

The most effective treatment was BIOT at both NW and SE (reduction of about 3 orders of magnitude), with a greater effectiveness at SE. Among EOs, the most effective treatments were oregano (OE) and thyme (TE) at SE with a reduction of bacterial viability comparable to that of BIOT (about 3 orders of magnitude). On the other hand, the EOs combination (OT and OTE) resulted less effective than when applied in single at SE, with a reduction, however, of about 2 orders of magnitude. On the contrary, at NW the treatments with EOs in combination (OT and OTE) resulted effective (reduction of at least 1 order of magnitude) while the treatments with EOs in single did not.

Concerning fungi, the best treatments to inhibit their growth were OE, OTE and BIOT at NW, with a reduction of viability of at least 3 orders of magnitude, followed by OT and TE, and, with a lower reduction, by E2 (Table 3). BIOT showed the same effect at SE, confirming it as the most effective biocide, while the effect of EOs, observed in all cases, was lower at SE with reduction up to 1 order of magnitude in the case of OT.

3.4.2. In situ determination of microbial vitality on marble surface

As expected, the not treated areas with a visible biological growth (NT) showed the highest values for the ATP assessments, while the NT areas with low or no visible biological growth (NT- and NT–, respectively) showed lower values of ATP (Fig. 2), with the most recent piece of marble (NT –; Fig. S1) showing the lowest values.

The extra NT positive reference named NT + (2.7.2; Fig. S1) showed the closest ATP values to those of the NT at both sites (12,750 RLU/cm^2 at SE and 11,147 RLU/cm^2 at NW).

Except E0.5 (emulsifier), almost all the treatments showed lower values of ATP presenting significant differences (p value <0.05) with respect to the NT values at both the NW and SE sides. The E2 treatment, with a higher concentration of the emulsifier, showed an inhibition effect 5 days after the treatment at both sites, while 4 months later the effect was still observed at NW.

The OE, OTE, OT, BIOT and TE were the treatments showing a significant effect on the vitality (Fig. 2a,b), detectable 5 days as well as 4 months from the treatment application, with a long-term effect more pronounced at the SE site. In the case of BIOT, a slight decreasing of the ATP values was observed after 4 months for both NW and SE areas (Fig. 2c,d); for all the other treatments a slight increasing of the vital activity was registered after 4 months, higher at NW; however, >85 % of vitality reduction with respect to NT was maintained (Fig. 2d). Among EOs, OE is the treatment with the highest efficiency in both the NW and SE areas, followed by OTE.

Table 3
Bacterial and fungal viable titer of the treated and untreated marble surfaces.

Sample	Bacteria (CFU/g ± sd)		Fungi (CFU/g ± sd)	
	NW	SE	NW	SE
NT	$4.4 \times 10^6 \pm 3.1 \times 10^5$	$2.2 \times 10^6 \pm 3.8 \times 10^5$	$1.0 \times 10^4 \pm 4.1 \times 10^3$	$5.5 \times 10^3 \pm 4.7 \times 10^3$
E0.5	$4.1 \times 10^6 \pm 2.7 \times 10^6$	$5.6 \times 10^5 \pm 1.7 \times 10^5$	$4.8 \times 10^3 \pm 2.4 \times 10^3$	$6.5 \times 10^3 \pm 3.0 \times 10^3$
OE	$6.5 \times 10^6 \pm 3.5 \times 10^5$	$5.7 \times 10^3 \pm 2.3 \times 10^3$	Nd	$1.5 \times 10^3 \pm 3.0 \times 10^3$
TE	$4.3 \times 10^6 \pm 3.8 \times 10^5$	$5.8 \times 10^3 \pm 2.5 \times 10^3$	$8.0 \times 10^2 \pm 5 \times 10^2$	$1.0 \times 10^3 \pm 1.1 \times 10^3$
OT	$1.6 \times 10^5 \pm 2.7 \times 10^4$	$3.4 \times 10^4 \pm 2.2 \times 10^3$	$5.0 \times 10^2 \pm 1 \times 10^3$	$5.0 \times 10^2 \pm 1.0 \times 10^3$
OTE	$1.2 \times 10^5 \pm 1.6 \times 10^4$	$2.5 \times 10^4 \pm 2.9 \times 10^3$	Nd	$1.5 \times 10^3 \pm 1.9 \times 10^3$
BIOT	$7.8 \times 10^3 \pm 5.1 \times 10^2$	$8.0 \times 10^2 \pm 1.0 \times 10^2$	Nd	Nd
E2	$8.1 \times 10^4 \pm 1.8 \times 10^4$	$3.6 \times 10^5 \pm 8.2 \times 10^4$	$1.3 \times 10^3 \pm 5 \times 10^2$	$3.3 \times 10^3 \pm 2.2 \times 10^3$

sd = standard deviation; Nd = not detectable, no colonies grew plating 0.1 ml of marble suspension 0.01 g/ml.

3.4.3. Microscopy

The microscopic observations made *in situ* before and 5 days after the treatment showed changes in the initial colour and colonization appearance of the treated areas in the case of BIOT, OE and OTE, at both the SE and NW sites. In Fig. S4 some representative subareas for all the treatments are reported. In the case of E0.5 the biological colonization seems to increase, while in the case of the other treatments the morphological aspect of the investigated surface seems to remain quite stable.

3.4.4. Colorimetry

All the selected areas were tested to individuate possible colour changes after the treatments. The measurements revealed some changes for all the treated areas, with a higher colour variation generally observed in the SE areas.

The addition of EOs solutions (OE, TE, OT, and OTE) led to total colour differences (ΔE*, Fig. 3a). The solutions producing the highest colour variation were BIOT, TE, OE and OTE, especially at the SE site. The lowest total colour variation (ΔE*) has been observed in the E2-treated area at the NW site, NT at both sites and E0.5-treated area at the SE site (Fig. 3a).

Generally, for all treatments the global colour variation is mainly due to the increase of the lightness (ΔL*), indicating a slight whitening effect, Δa* (red colour) and Δb* (yellow colour) (Fig. 3b,c,d).

4. Discussion

This work reports the description and the results of a trial *in situ* with the use of EOs to control the growth of the microbial community inhabiting the external white marble of Santa Maria del Fiore Cathedral. The trial was

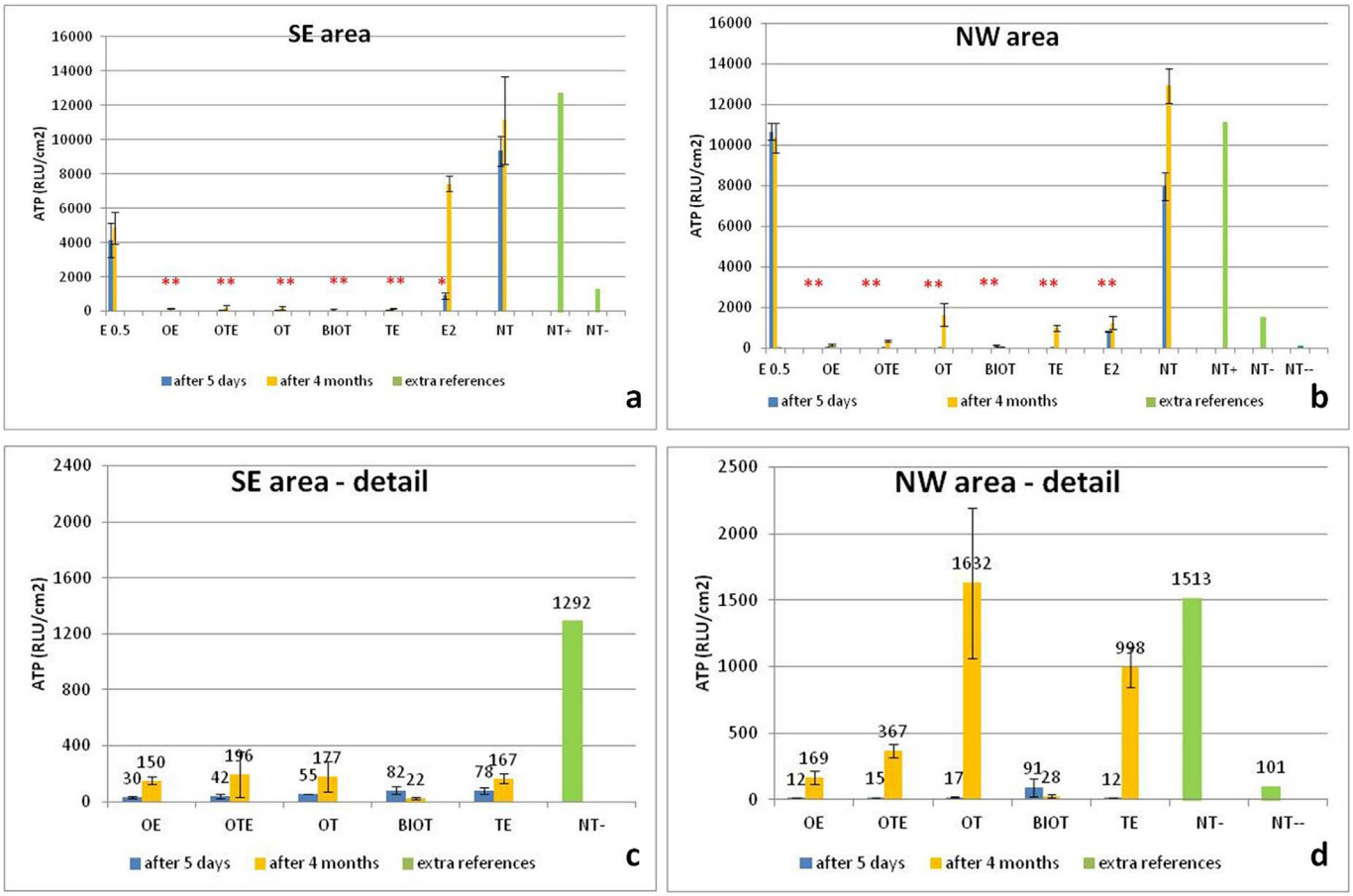

Fig. 2. The ATP values registered for the investigated SE and NW areas at two different time lapses (5 days and 4 months) from the treatments, as well as the values registered for some extra references. The asterisk indicates the values statistically significant with respect to the NT; the statistical analysis does not include the extra references NT+, NT- and NT– (see Section 2.7.2 for details).

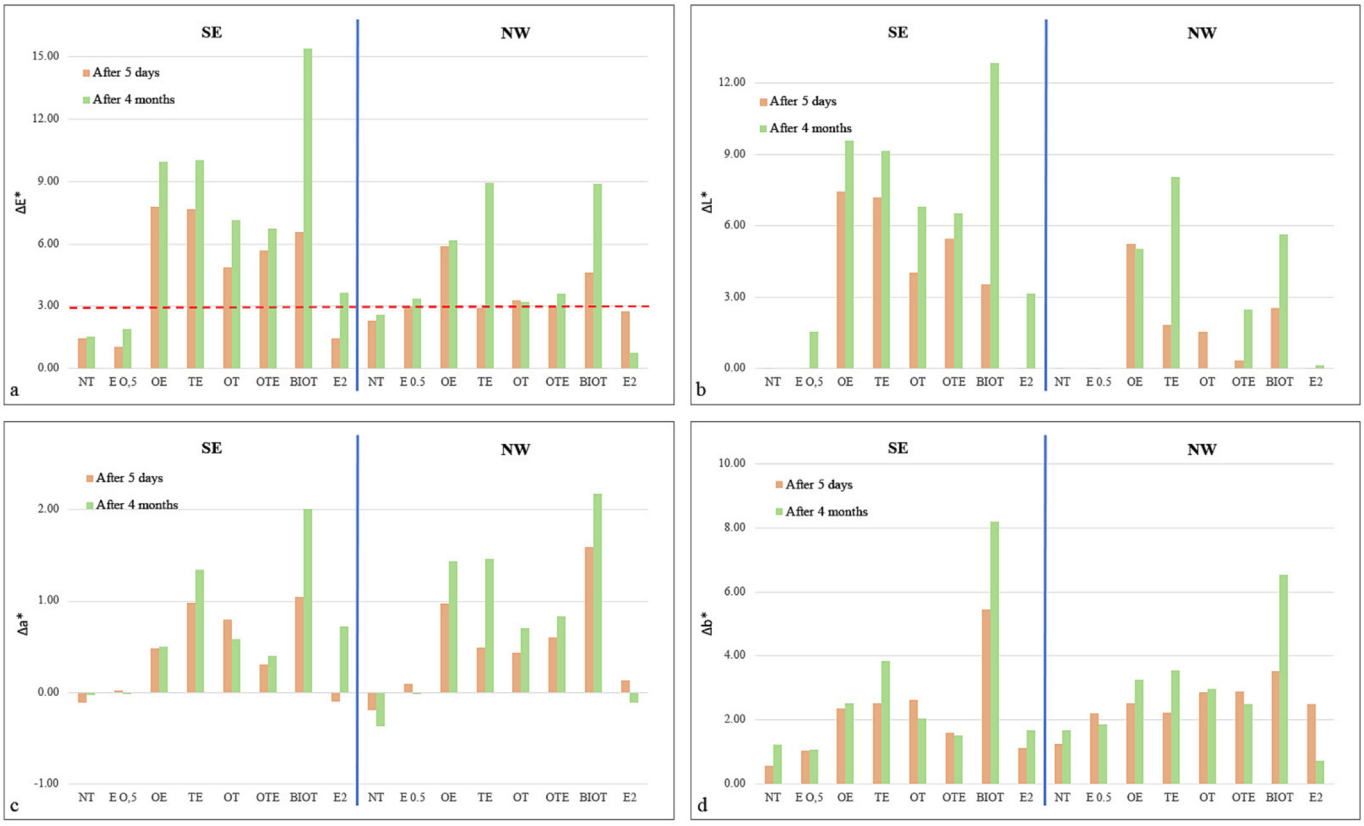

Fig. 3. Colorimetric values of ΔE* (a), DL* (b), Da* (c) and Db* (d) values for the SE and NW areas after 5 days and 4 months of treatments. The red dotted line represents the colour change perception limit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

carried out in two study sites, having different exposition, where the most spread biodeterioration phenomenon is the darkening of marble. We previously investigated this phenomenon as well as the composition of the marble microbial community by means of microscopic, cultivation and metataxonomic techniques (Santo et al., 2020; Santo et al., 2021; Checcucci et al., 2022).

We conducted the present work first performing preliminary tests on the SMFC cultivable microbiota and on marble specimens to verify the effectiveness and the absence of side-effects of the EOs treatments on marble, then applying the treatments on selected areas of the two study sites. In all cases we compared the action of EOs with that of Biotin T, a broad-spectrum commercial biocide commonly used in cultural heritage. While in the literature sensitivity tests are usually reported against single strains isolated from cultural heritage, we tested the sensitivity of the whole cultivable community of bacteria and fungi inhabiting the white marble in the two SMFC study sites. We used oregano and thyme EOs, since they are among the most used against stone microorganisms, especially oregano (Fidanza and Caneva, 2019). Oregano and thyme EOs revealed to be very effective in the sensitivity test against bacterial and fungal microbiota cultivated from marble: no colonies grew at a concentration (0.025 %) 80-fold lower than that of the working solutions used for marble treatment.

We then determined the chemical composition of these two EOs and tested their effects on uncolonized Carrara marble specimens. The GC–MS analysis showed no qualitative but quantitative differences in terpene diversity of the two EOs. *T. vulgaris* EO was marked by the presence of high amounts of p-cymene (39.4 %) and thymol (34.3.0 %), while carvacrol accounted for 60.5 % of *O. vulgare* EO. Indeed, it is well known from the literature that terpene profiles show high variability between different plant species and these compounds have been largely used as biochemical markers in chemotaxonomic studies of forest and aromatic plants (Hanover, 1992; Langenheim, 1994; Barbero and Maffei, 2016; Nikolić et al., 2021).

For the EOs working solutions, we used Tween 20, a product commonly used on stone, as emulsifier to facilitate the dispersion and penetration of EOs into the stone porosity. We chose 0.5 % as the minimum concentration giving a visible emulsifying effect in an EO solution, since no information was available about the possible inhibition effect on stone microbiota by Tween 20. Then we applied the EOs, Biotin T and control solutions on marble specimens at the concentration selected for *in situ* trial (2 %, according to the minimum EOs concentration applied on stone in other trails, *e.g.*, Devreux et al., 2015). The chosen treatments did not lead to an oily appearance of the surface nor caused significant changes in the porosity of marble specimens in laboratory conditions, as revealed by water absorption measurements after one month from the treatment, or colour modification up to two years of outdoor exposure of the samples. These results are among the very few contributes to the investigation of the effects of EOs on stone (*i.e.*, Bartolini and Pietrini, 2016; Spada et al., 2021b). The need to explore the possible interactions between EOs and building materials is a point raised by different authors as quite a priority in research on EOs before considering their usage at larger scales (Fidanza and Caneva, 2019; Boccalon et al., 2021; Pinna, 2022; Romani et al., 2022); measures of surfaces colorimetry and capillary water absorption capability are among the interference tests strongly recommended in research on stone restoration using EOs (Fidanza and Caneva, 2019). Moreover, we evaluated colour changes on marble specimens exposed to the outdoor atmospheric agents up to two years under natural aging conditions.

We then transferred the treatment *in situ* to the larger marble surfaces of Florence Cathedral. We applied treatment solutions by brush as described in other papers (Devreux et al., 2015; Bartolini and Pietrini, 2016), since our goal was to test the effectiveness of the treatment in controlling microbial activity. Testing different methods of EOs application and combination with other cleaning phases of the restoration procedures (as done by Spada et al., 2021b) is among the perspectives of our work.

To evaluate the effectiveness of the EOs treatments, we used multidisciplinary tests both *in situ* and *ex situ*. The vitality of microorganisms colonizing the marble treated surfaces was evaluated quantitatively by determining the viable titer of bacteria and fungi and the total ATP content and comparing the obtained values with those of reference not-treated surfaces. Moreover, we performed microscopic observations and colorimetric measurements of the marble surfaces before and after the treatment to evaluate the changes in the appearance of the colonized surfaces.

The viable titer of bacteria was of the order of magnitude of 10^6 CFU/g at both NW and SE, while the viable titer of fungi was of the order of magnitude of 10^3–10^4 CFU/g (NT values, Table 3). Both bacteria and fungi showed a viable titer double at NW respect to SE, according to the prevailing epilithic growth style of the NW community that we have previously discussed as related to the wetter marble surface habitat at NW (Santo et al., 2021).

Significant reductions of the bacterial viable titer respect to that of NT were found for the OT, OTE, BIOT and E2 treatments at NW and for all treatments at SE, where the most effective were oregano (OE) and thyme (TE) (Fig. S3). The wider and greater effectiveness of the treatments at SE could be imputed to the higher development, compactness, and stability of the epilithic biofilm at NW that can hinder the diffusion of EOs (protecting more the bacterial community).

With few exceptions, the effectiveness of the OE and TE treatments against bacteria and fungi was quite similar and was not improved by using them in combination (OT and OTE). On the other hand, we found high amounts of monoterpenes with well-known antimicrobial activity (*e.g.* Cristani et al., 2007) in both EOs (p-cymene and thymol in the thyme EO and carvacrol in the oregano EO). Moreover, when in combination, the use of the emulsifier (OTE) did not seem to increase effectiveness, except against fungi at NW.

It is worth noting that, while the emulsifier (E0.5) at the concentration used for the EOs solutions did not influence the effect of the treatments, the increase of its concentration in the E2 treatment showed a reduction of the bacterial viability at both NW and SE as well as of the fungal viability at NW (Fig. S3 and Table 3).

Concerning fungi, the inhibition effect of the EOs treatments resulted to be greater at NW, with the best performance of the oregano treatment both in single and in combination with thyme and comparable to that of BIOT. Since the growth conditions we used favoured filamentous fungi, the better performance of the EOs on fungi at NW could be partially due to a greater presence of fungi in the vegetative form sensitive to biocides in the wetter biofilm; on the contrary, the prolonged irradiation of the SE-facing marble could favour a greater presence of fungi in a dry form, such as spores, more resistant to the environmental stress as well as to biocides. This hypothesis can also be suggested by the endolithic growth of black fungi we observed inside the SE marble and interpreted as a protection against the irradiation stress (Santo et al., 2021). On the other hand, for both bacteria and fungi, we cannot exclude that the differences in the sensitivity to the EOs could depend on the different relative abundance found for some components of the NW and SE microbial communities (Checcucci et al., 2022).

The differences in the sensitivity results obtained on cultivable community in the laboratory (3.2) and *in situ* conditions are not surprising since the response of *in situ* microorganisms is influenced by their living in a complex biofilm on stone and other environmental factors.

While the viable titer refers to the viability of specific components of the microbial community (bacteria and fungi), the ATP content refers to the activity of more components of the whole community, including for example the cyanobacteria. The treatments BIOT, OE, OTE, OT and TE showed an ATP content significantly lower than NT for both the NW and SE areas and at both times of the measurements (5 days and 4 months after the treatments). The ATP values were quite stable in time up to four months, especially at SE, indicating mid-term maintenance of vitality inhibition due to the treatments.

The EOs action mechanism on microbial cells is mainly due to its lipophilic character and includes one or more impacts, simultaneously or not, on the cells walls, on the cytoplasmic membranes and proteins, as well as on the ATP synthesis process (Nazzaro et al., 2017; Nazzaro et al., 2019). The Gram-negative bacteria are more resistant to the EOs stress with respect to the Gram-positive bacteria due to their different cell wall organization (Nazzaro et al., 2019). The slightly higher values of ATP observed after the treatments at the NW site with respect to the SE site could be related not only to a higher amount of biological growth but also to its microbial composition with organisms more resistant to EOs action, such as the Gram-negative cyanobacteria. Cyanobacteria resulted indeed abundant on SMFC marble, with a relative abundance up to about 53 % at the NW site (Checcucci et al., 2022).

In accordance with the bacterial and fungal viable titer results, Tween 20 at 2 % (E2) gave an ATP content significantly lower than that of the NT up to 4 months at NW and after 5 days at SE, while at the lower concentration used to emulsify the EOs solutions (E0.5) did not. To our knowledge, this is the first report where the possible antimicrobial activity of Tween 20 has been tested on stone. This result should be considered since Tween 20 is a product commonly used for stone treatment. Besides those of E2, the results of the ATP determination generally agree with the results of the viable titer determination, especially those of the BIOT, OT, OTE (bacteria and fungi at both NW and SE), OE, TE (bacteria at SE and fungi at NW). As found with the viable titer, the effectiveness of the OE and TE was not improved by using them in combination nor, when in combination, the use of the emulsifier (OTE) seemed to increase the efficacy. This further confirms the reliability of the ATP test for monitoring the vitality of lithobiontic communities. Moreover, the sensitivity of this test was supported by the correspondence of the ATP values in similarly colonized areas (*i.e.*, NT, NT +) as well as by the differences in not-treated areas with different levels of colonization (*i.e.* NT, NT-, NT–).

In accordance with the ATP results, the microscopic observations performed *in situ* before and 5 days after the treatment revealed that the BIOT, OE and OTE treatments were those producing visible changes in the initial colonization appearance of the treated areas at both the SE and NW sites. However, as no mechanical treatment was applied to remove debris from the treated surfaces, a change in the whole appearance of the biofilm because of the biocide treatment would not be necessarily expected.

Colour spectrophotometry is used to evaluate the interference of treatments with the substrate as well as, by some authors, the effectiveness of the applied biocides on microbial colonization of stone through the chromatic changes of the biological patinas (Devreux et al., 2015; Bartolini and Pietrini, 2016; Sanmartín et al., 2020). From the latter point of view, in our work, the solutions producing the highest global colour variation (ΔE^*) of the treated marble surfaces were BIOT, OE, TE, and OTE, especially at the SE site, showing correspondence with the reduction of microbial activity (ATP test). Generally, this variation was mainly due to the increase of the lightness (ΔL^*), indicating a whitening effect, of Δb^* tending to the yellow, and to a slighter increase of Δa^* tending to the red. The lightening of the surface was obtained after the application of EOs solutions in other *in situ* trials on marble (Devreux et al., 2015; Bartolini and Pietrini, 2016) and, in general, it is associated to marble less colonized areas (Silva et al., 2022). This could indicate a decrease of the initial colonization leading to a progressive cleaning of the SMFC marble (NT and E0.5 showed no or a slight increase of ΔL^*). Concerning the increase of Δb^*, treatments with Biotin T and other commercial biocides resulted to induce a yellowing of the stone surface (Sanmartín et al., 2020; Bartolini and Pietrini, 2016) and this effect was confirmed in our work as well. Even if less pronounced than for BIOT, a similar colour change behavior was observed for TE and OE. However, the correspondence between colour change and the antimicrobial activity is not so obvious (*e.g.* E2 gave very little colour change and showed some antimicrobial activity) and, according to other authors, the use of colour measurements to evaluate the colonization of stone surfaces must be done carefully (Silva et al., 2022). Beyond the possible significance of microbial inhibition, the yellowing of the stone surface, if not confined to the biological patina, can be an undesirable side-effect that should be considered also for commercial biocides. Since we did not find chromatic interference of EOs treatments with uncolonized marble surfaces, this aspect must be further investigated on colonized marble.

5. Conclusions

This work represents one of the few studies available in the literature on the effects of the treatment of marble with EOS regarding both possible interference with marble appearance or properties and the response of the resident microbial community through laboratory as well as *in situ* tests.

In laboratory conditions, the oregano and thyme EOs resulted to be very effective against the whole cultivable community of bacteria and fungi at very low concentration (0.025 %). When applied at the 2 % concentration, the oregano (OE) and thyme (TE) EOs solutions did not lead to oily appearance of the surface neither interfere with the water absorption capacity nor with colour of uncolonized marble. Based on these results, the EOs solutions were then applied on marble surfaces of the Cathedral of Santa Maria del Fiore, one of world's architectural masterpieces, and the antimicrobial effectiveness was evaluated by multiple tests. We found a good correspondence between parameters for evaluation of viability (bacterial and fungi viable titer) and activity (ATP determination) and some correspondence among these and microscopy and colorimetry. These results suggest the quantification of the viability/activity of the microbial community as a more appropriate tool to evaluate the antimicrobial effectiveness of biocide treatments.

Considering the whole data, OE and TE treatments were effective against microbial community, in more cases comparably to BIOT, and their effectiveness was not improved by using them in combination. The differences found, particularly by viable titer, in the two study sites or in the two components (bacteria and fungi) of the microbiota can be partly imputed to the different structure and colonization pattern of the microbial community due to the peculiar climatic conditions of the differently exposed study areas.

Even if the BIOT resulted the most effective treatment, the added value of our trial consists in using a sustainable tool for contrasting biodeterioration, that is the rationale of this work.

The results obtained encourage us to develop the treatment with EOs by applying them in combination with other restoration cleaning procedures also to better investigate and avoid possible side-effects, like the yellowing of marble.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162913.

CRediT authorship contribution statement

Alba Patrizia Santo: Conceptualization, Methodology, Investigation, Writing – review & editing, Funding acquisition. **Beatrice Agostini:** Conceptualization, Funding acquisition. **Oana Adriana Cuzman:** Methodology, Investigation, Formal analysis. **Marco Michelozzi:** Investigation, Formal analysis. **Teresa Salvatici:** Methodology, Investigation, Formal analysis. **Brunella Perito:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Opera di Santa Maria del Fiore funds. The authors wish to thank Archt. Francesca Mannucci for her help during *in situ* trial and Dr. Maria Landolfi for technical help *in situ* and in laboratory.

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