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A novel approach for *in-situ* assessment of the efficacy of biocides on building of historical interest by bioluminescence

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***Abstract* – Growing interest is now addressed to using essential oils (EOs) as antimicrobial substances, in medical, industrial, food, and many other sectors. Oregano and thyme EOs are among the most effective oils and, for this reason, they have also been investigated in the field of cultural heritage. This work reports some preliminary results of a new approach for monitoring *in-situ* the biocide action on biological patinas by a fast analysis based on bioluminescence. In this research, we evaluated the efficacy of a commercial biocide based on EOs, in comparison with common commercial biocides containing hazardous formulations for human health and environment.**

Keywords: essential oil; biocide; *Origanum* spp. oil; bioluminescence; biological patina cleaning; historical buildings.

I. INTRODUCTION

Biodeterioration is any undesirable change in the properties of a material caused by the vital activities of organisms, as defined by Hueck in 1965 for the first time [1]. The magnitude and the nature of this change can vary greatly, from breakage and loss of cohesion of the substratum (mechanical damage), to chemical alteration due to excretion of metabolites (chemical damage), to formation of patinas and crusts (aesthetic damage) [2].
Cultural heritage materials are affected in different ways by detrimental organisms, the so-called biodeteriogens. They colonise materials for: i) using the substratum as nutritional source; ii) exploiting the material as support for their growth. The latter is the case of stone materials, which typically serve as growing supports, ruling out the case of absorption of mineral salts [3].
Control of biodeterioration phenomena can be achieved by means of direct or indirect methods. The latters act upon environmental and/or ecological factors, and/or upon substrates. Direct methods include mechanical, physical, chemical and biochemical treatments [4]. Choosing chemicals and biochemical approaches means, most of the time, adopting biocides. In fact, significant effort has been put in order to control and hinder biodeterioration phenomena over the years, often exploiting biocides, which have successfully been used for long time. Unfortunately, some traditional biocides are potentially dangerous. Their extensive or inappropriate use can adversely implicate human and environmental health. Extensive research is ongoing and long overdue, aiming to find alternative and eco-friendly substances or methods to reduce biodeterioration phenomena. With the aim to improve the releasing time of the antifoulant, it may be loaded in nanocontainers as silica nanospheres, reducing the frequency of the treatments [5]. Another eco-friendly approach is the employing of organic waste for the production of natural biocides active against bacteria [6].
A biocide for application on stone cultural heritage should: i) be environmentally sustainable, ii) not interfere with stone material, iii) be safe for human health, iv) be easy to apply, v) have long-lasting effect, vi) be cost-efficient.
In recent years, growing interest is addressed to the use of essential oils (EOs) as biocidal substances, in medical, industrial, food and many other sectors. In cultural heritage field, several research groups used EOs for their strong antimicrobial activity [7]–[12]. Oregano and thyme are among the most effective EOs and, for this reason, have been sporadically investigated in the field of cultural heritage [13]–[16].
The most used methods for studying the growth and/or the cleaning of biological patinas require sampling collection and analysis with bench instruments: i) fluorescence optical microscopy, using either fluorochromes or autofluorescence [17], [18]; ii) digital microscopy [19]; iii) cell culturing techniques [9], [20], [21]; iv) ATP extraction and determination in laboratory [19], [22]. This work reports some preliminary results of

a new approach for monitoring *in-situ* the biocidal action over time, through a portable device based on bioluminescence, that permits to evaluate the amount of biological patinas by measuring their metabolic activity. Bioluminescence is the production and emission of light by a living organism. The phenomenon occurs widely in marine organisms, as well as in some bacteria, fungi, and terrestrial insects such as fireflies. Bioluminescence is due to a chemical reaction that involves some light-emitting molecules, enzymes and the energy-carrying molecule adenosine triphosphate (ATP), present in cells. If the light emission is sufficiently intense, it may be revealed by a bioluminometer [23].

The main aim of the present study is to compare the efficacy of three commercial chemicals for inhibiting biological growth, potentially dangerous for environmental and human health, with that of a new commercial blend based on an EOs formulation.

The specific objectives of this work are:

1. monitoring over time the activity of four commercial biocidal mixtures applied on three different stone typologies of historical manufacts, aiming to test the biocidal efficacy.
2. investigating the reliability of the *in-situ* analysis with a portable bioluminometer as a novel assessment approach.

II. MATERIALS AND METHODS

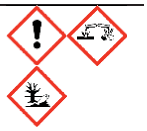
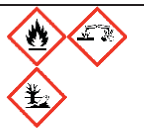
A. Specimens

The biocidal activity was tested on three kinds of substratum presenting biological patinas: fired clay brick (brick), marble, and carbonate sandstone (Pietraforte). The brick selected for the present test was a typical production of Bologna (Italy) (Fig. 1a), dating back to XIV and XV centuries, part of the sloping windowsill of a church exposed to west. It had intense red colour, compact matrix, and visibly attached biological patinas, due to the hygroscopicity of the material, which favours biological growth. The marble stone, employed as decorative element around a window, showed a decent state of conservation, with the presence of superficial biological growth (Fig. 1b). The Pietraforte (carbonate sandstone) specimen was utilised as church step, and it was affected by water stagnation and rising damp phenomena. It presented erosion that created cavities in which the biological patina grew (Fig. 1c). In conclusion, for all the analysed samples, the continuous exposure to pollutants, atmospheric particles, wind, rainwater and air moisture, favoured the growth of biological patinas.

B. Biocides

Four kinds of commercial biocide mixtures (M1, M2, M3, M4) were used on each substratum. Each biocide was applied by brush twice consecutively, according to the technical data sheet, pure or diluted, using the concentration recommended for the maximum efficacy. The bioluminescence was recorded 5 days after the application. The active components of the mixtures, together with their CLP (Classification, Labelling and Packaging) hazard pictograms, are detailed in Table 1.

Table 1. Chemical composition and CLP hazard pictograms of the commercial biocide mixtures.

Mixture	Components	CLP Pictograms
M1	quaternary ammonium salts (2.5 v/v%) in aqueous solution	
M2	n-ottil-isothiazolinone (OIT) (< 5 v/v%) and quaternary ammonium salts in aqueous solution	
M3	iodiopropinilbutilcarbamate (IPBC) (< 5 v/v%) and n-ottil-isothiazolinone (OIT) (<5 v/v%) in ethanol-acetone solution	
M4 (EOs)	*Origanum* spp. essential oil (monoterpenes, monoterpenoids, phenols) (0.5-3.0 v/v%) in aqueous solution	

The CLP pictograms, were introduced by the REGULATION (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 [24]. They provide information about the damage that a particular substance or mixture can cause to human health or to the environment.

M1 mixture is a biocide with a neutral/slightly basic pH based of quaternary ammonium salts with disinfectant and detergent actions. As reported in the technical data sheet, the product has a broad-spectrum efficacy against bacteria, fungi, algae, lichens. It has a rapid action and does not stain the substrates. For these characteristics, it is widely used. Quaternary ammonium compounds damage the cytoplasmic membrane and the enzymatic system, bringing to cellular death [25].

M2 formulation is a mixture of n-ottil-isothiazolinone and quaternary ammonium salts in aqueous solution, and, as reported in the technical data sheet, has an effect on lichens, bacteria, fungi and algae. It has a weakly acidic pH (5.5). Isothiazolinones utilise a two-steps

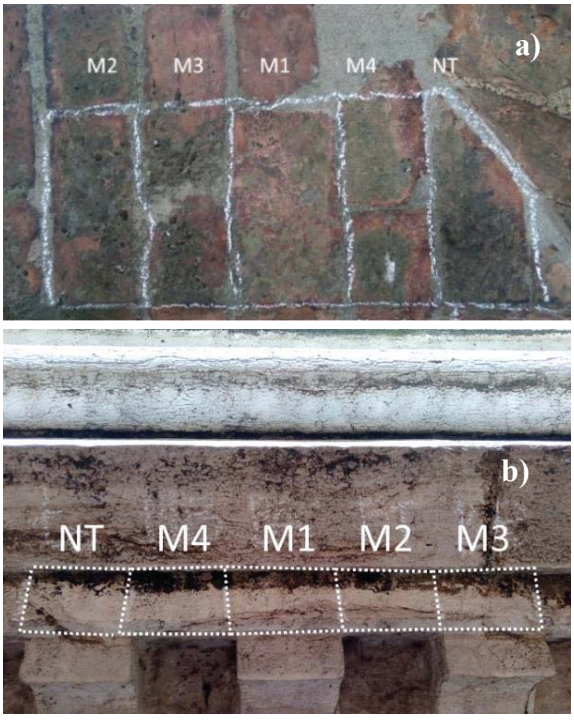
mechanism involving rapid inhibition of growth and metabolism, followed by irreversible cell damage, resulting in the disruption of metabolic pathways involving dehydrogenase enzymes, and inhibition of energy production (ATP synthesis) [26].
M3 formulation consists of carbamates and isothiazolinones as active compounds, in organic solvent. As reported in the technical data sheet, M3 mixture have an effect on actinomycetes and lichens as well as bacteria, fungi and algae. This formulation has, however, some side effects: some microorganisms, like algae and lichens, can release organic pigments with colours ranging from yellow, to orange, red or brown after death. These pigments (melanins and carotenoids), are partially soluble in organic solvents and migrate on the surface of the material staining the substrates. The inhibition process involves the formation of some cellular enzyme-inhibitor complexes, with subsequent carbamylation of the serine hydroxyl, resulting in the inhibition of the enzymes [27].
M4 is a blend of essential oils (*Origanum* spp.). with weakly acidic pH (4.5 - 4.9) and, as reported in the technical data sheet, acts on bryophytes, mosses, lichens and algae. Operator safety requires only the use of safety glasses or wrap-around goggles, whereas respiratory protections and glows are optional for odour control. Applied to a certain dose, essential oils interfere with cell membranes, affecting their structure and function, damaging transport systems, enzymes, ion channels or receptors [28].

C. Instrumentations

The Bioluminometer System SURE Plus Hygenia was used for analysing microbial activity on the surface, using sterile swabs to collect samples. The technical characteristics of the System SURE II are: detection threshold in 1 femtomole of ATP; led time 15-20 sec; automatic calibration; pre-dosed disposable swab. Bioluminescence values are expressed in RLU (Relative Light Units), which are proportional to the ATP concentration. The analysis was performed using three swabs per area, obtaining the average value and the respective standard deviation. The analysis was performed before and after biocide application. The method provides information about the presence of ATP molecules, related to the vitality of the cells.
USB digital microscope was employed to investigate colorimetric changes of biological patinas. The digital microscope has resolution of 2 megapixels (1600 x 1200 pixels), CMOS sensor type; magnification in the range 10-150X; 10-150 mm of focus Range and LEDs light source.

III. RESULTS

The four mixtures, named M1, M2, M3 and M4, were applied on four delimited areas, identified by the same name tags. Bioluminescence was recorded for each sample in 10x20 cm (brick), 10x7 cm (marble) and 10x25 cm (Pietraforte) areas, by scraping the sterile swab on the surface (Fig. 1). The not treated section (NT) delimited in the pictures, is a macroscopic reference. The NT values (RLU) reported in Table 2, were collected in the corresponding area before the treatment with M1, M2, M3 and M4 mixtures. The bioluminescence values of the not treated specimens were in the range of 6960-3620 RLU.
The biolumiescence values of each specimen before and after the biocide application are reported in Table 2.
The residual bioluminescence values after 5 days are reported as percentage of the initial intensity in Fig. 2. The biocide efficacies follow the orders: (i) M3 ≈ M4 ≈ M1 > M2 for the brick specimen, (ii) M3 > M1 > M4 > M2 for the marble specimen, (iii) M3 ≈ M4 ≈ M1 > M2 for the Pietraforte specimen. The M3 appears to be the optimal biocide for all the specimens, decreasing the initial bioluminescence up to a residual 7 % on brick, 15 % on marble and 13 % on Pietraforte. The M2 mixture appears to be the less effective chemical composition for all the specimens. The M4 mixture, containing *Origanum* spp. oil, shows a biocide action similar to that of M1 and M3 on brick (residual patina 7,7 %) and on Pietraforte (residual patina 14 %), whereas, in the present test, it seems to have less efficacy on marble (residual patina 44 %) (Fig. 2). These results suggest that all biocides are affective; however, M4 composition is more environmentally sustainable and safer for human health than M1 and M3.



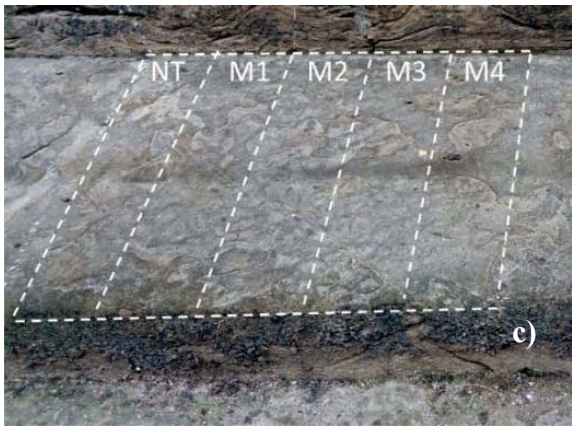


Fig. 1. Delimited areas on brick (a), marble (b), and Pietraforte (c) before the treatment.

Table 2: Bioluminescence of the specimens: brick, marble and Pietraforte; non treated (NT) and treated, after 5 days.

Brick	NT		Treated	
Biocide	(RLU)	st.dev.	(RLU)	st.dev.
M1	6626,3	26,5	610,0	67,8
M2	6877,7	92,2	1664	306,7
M3	6803,3	61,8	492,3	46,2
M4	6737,0	97,3	519,3	30,7

Marble	NT		Treated	
Biocide	(RLU)	st. dev.	(RLU)	st. dev.
M1	3648,0	105,8	1203,7	150,2
M2	3749,7	393,9	2450,7	164,6
M3	3960,3	246,4	577,3	153,1
M4	3620,7	137,6	1597,3	196,1

Pietraforte	NT		Treated	
Biocide	(RLU)	st. dev.	(RLU)	st. dev.
M1	6804,7	442,4	715,3	179,1
M2	6147,7	579,6	1395,7	575,0
M3	4169,3	557,2	415,0	284,1
M4	4521,3	791,8	628,3	131,1

The comparison between NT area and areas treated with different biocides was performed, also, by observation with digital microscopy at a magnification of 150 X. The images of the different areas, treated with the four biocides, are reported in Fig. 3. The biological patinas treated with M1, M3 and M4 biocides, showed a brown colour indicating an initial biocide action on all the samples. On the contrary, the patina treated with M2 biocide showed only a weak change of the colour with large portions of the original green colour. That is in agreement with the higher residual bioluminescence of samples treated by M2.

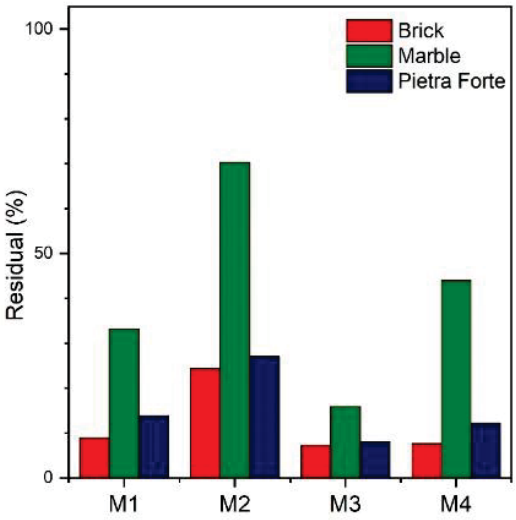


Fig. 2. Residual bioluminescence of brick, marble and Pietraforte specimens (%) after 5 days.

IV. CONCLUSION

The utilisation of a portable bioluminescence device can represent a valid approach for monitoring *in-situ* the efficacy over time of applied biocides. The analysis is fast and cost-efficient, and it permits to verify the presence of residual biological activity on the specimen also if macroscopically invisible.

For the texted materials (brick, marble and Pietraforte) the EOs mixture was efficacious. In fact, after the treatment, the bioluminometer signal evidenced low residual biological patinas: 7,7 % on brick; 13,9 % on Pietraforte, whereas it was less efficacious on marble (44 %).

The comparison of EO-based mixture with traditional chemical biocide mixtures, suggests that the essential oil inhibits the microbial activity of the biological patina with an efficacy comparable to that of common biocides. Furthermore, with the intention of developing a proper and more long-lasting biocidal treatment, a characterisation of the biological patinas, with microbial identification, will be carried out in the near future. The identification of the different microorganisms, which have different mechanisms of resistance to biocides, will lead to the optimisation of the treatments

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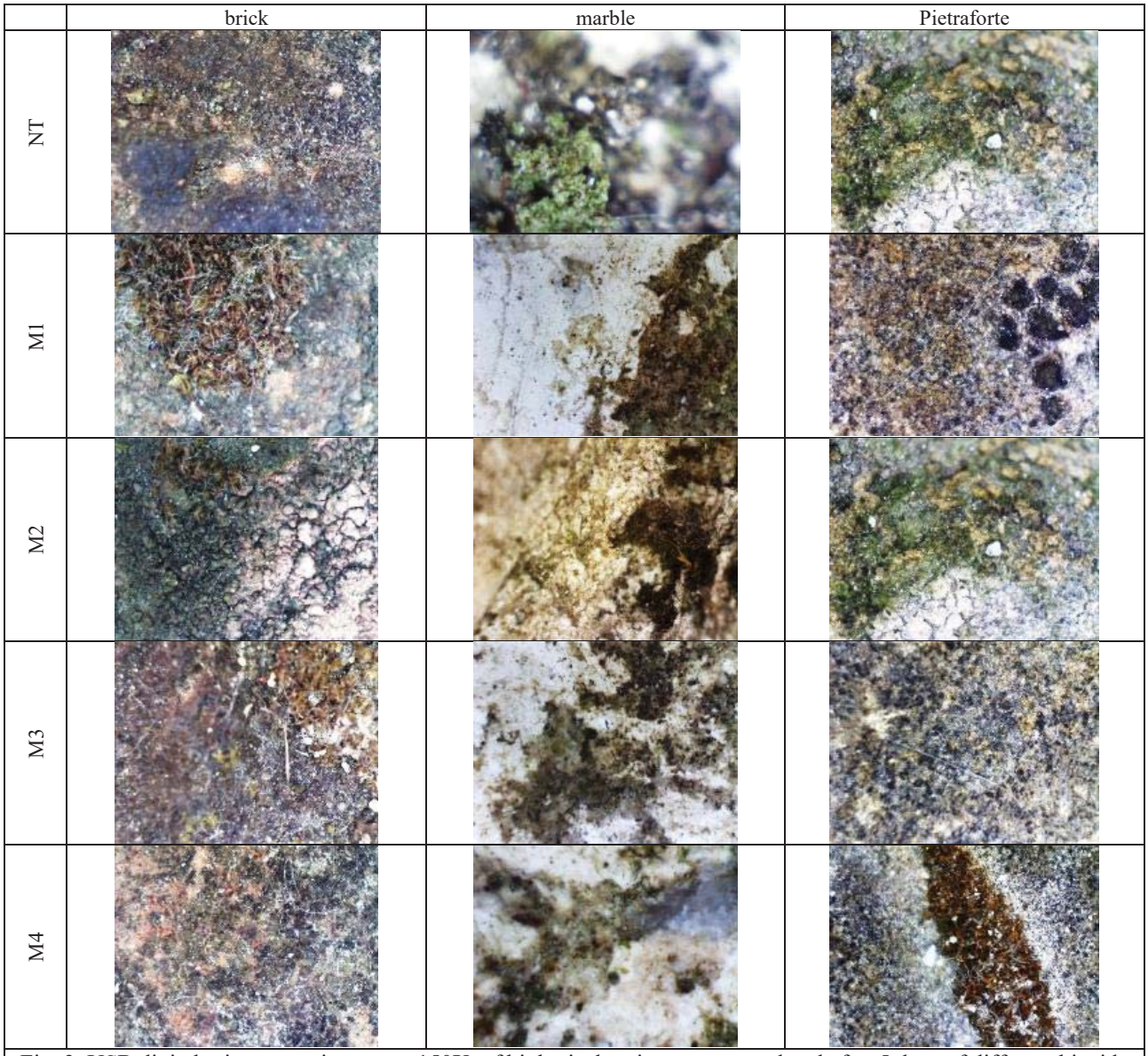

Fig. 3. USB digital microscope images at 150X, of biological patina, non treated and after 5 days of different biocide treatments for the three specimens.

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