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Innovative use of essential oil cold diffusion system for improving air quality on indoor cultural heritage spaces

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ABSTRACT

Bioaerosols play an important role in Cultural Heritage (CH) spaces air quality and biodeterioration risk and there is a growing interest to reduce them in actual conservation strategies. Essential oils (EOs) have been well-known in several fields for their antimicrobial properties and they have found use in a number of applications. In this work *Melaleuca alternifolia* (Tea tree) and *Thymus vulgaris* (Thyme) EOs are cold diffused in unventilated lab spaces for reducing air bacterial and fungi contamination. Moreover, Tea tree EO was also tested in an unventilated real CH space. The effectiveness of EOs in reducing air bacterial and fungi contamination in unventilated indoor spaces during 24 h was evaluated. Air quality was also analyzed by means of temperature, humidity and particulate matter variation. EOs' diffusion system CH artworks risks was studied by means of thermography, diffusion range and EOs compounds deposition on the artistic materials by GC–MS. Tea tree EOs vaporization showed the best results with 77,3% and 95,0% fungi and bacteria air contamination reduction, with no thermohydrometric alteration and absence of EOs components deposition on the artistic vault.

1. Introduction

Air quality refers to the degree to which the air is clean, clear and pollution free. Air pollution implies the introduction into the atmosphere of substances with a harmful effect, which, when they reach sufficiently high concentrations, have a strong impact on air quality and can adversely affect human health as well as Cultural Heritage assets (CH). Cities are home to a large number of Cultural Heritage that are subject to the continuous action of atmospheric agents and biological action (Hermosín 1995). These two factors facilitate the appearance of extrinsic damage and accelerate the degradation processes of the intrinsic matter of the CH, which in turn is facilitated by the action of environmental factors (humidity, light, temperature, etc.). One important CH indoor air quality complaints is the presence of high concentrations of fungi and bacteria, which increased risk of CH biodeterioration, if climatic conditions are favorable, and adverse human health effects (Ahmed et al., 2018).

Actually, due to the global pandemic situation, special relevance have taken the prevention of the presence of microorganisms in indoor air spaces. For reduction or elimination of microorganisms in indoor spaces, authorities recommend ventilation and/or the use of chemical products and sterilization systems. However, indoor CH spaces are frequently difficult to ventilate because they have few openable windows and artificial ventilation systems cannot be installed due to artistic coverings. Existing chemical products and sterilization systems can affect negatively artistic surfaces, and their use is not recommended on CH buildings (Valentín and Fazio, 2020). For this reason, there is a growing need to develop alternative control systems able to reduce air microorganisms on indoor CH spaces, especially in those with poor ventilation rate. These systems should ideally chance the following requirements: i) they should not be toxic for humans allowing their application in the presence of visitors; ii) should be innocuous for Artistic materials present on CH spaces; iii) and effective in reducing the microorganisms present in the air.

Based on these principles, this work focuses on the study of alternative control systems based on natural biocides able to meet the avobementioned requirements. A natural (or green) biocide is prepared in a manner that avoids waste formation, so that the lack of waste treatment

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and cleaning costs makes it economic. It should be prepared from the least toxic renewable raw materials, without solvents, consuming minimal energy and producing no by-products (Ashraf et al., 2014).

Essential oils (EOs) are natural products obtained from different plants. They can be extracted by water vapor distillation, dry distillation or mechanical distillation methods that do not involve temperature changes (Delmas 2016). The elaboration process therefore avoids the use of additives or diluents. EOs have been known for a long time, and they are used in several fields such as medicine and aromatherapy, as well as in the food, cosmetics and pharmaceutical industries (Campo Velasco and Vanega Mahecha 2012; Andrade-Ochoa et al., 2017; Kozics et al., 2019). These EOs have shown environmental compatibility due to their target-specific activities and their safety for humans and plants. These characteristics make essential oils to have a great potential for use as green biocides (Ashraf et al., 2014).

Essential oils usually contain different chemical substances (monoterpenes, sesquiterpenes, terpene alcohols and terpenoids) including odorants that vary depending on the plant species, the place of growth, environmental growth conditions and extraction parameters (Angulo-Milhem et al., 2021). Depending on their composition, the EOs can inhibit the metabolic functions of microorganisms, by solution or gaseous contact (Inouye et al., 2003). Some results suggest that vapor levels of 0.1–0.9 mg/L in air may suppress the growth of the bacterial pathogens of respiratory infection. In particular, Cinnamon bark, Lemongrass and Thyme oils are evidenced as the most effective for this purpose (Inouye et al., 2001). Some authors have also revealed a strong antimicrobial efficiency of Tea tree and Eucalyptus EOs vapors highlighting the use of EOs as natural disinfectants for the further development of air treatment technologies and indoor air quality improvement (Sailer et al., 1998; Pyankov et al., 2012; Usachev et al., 2013).

In the field of CH Conservation and restoration there is a growing interest in the search for non-toxic or low-risk alternatives for human, animal and environmental (España. Real Decreto-Ley 1054/2002, 2002). Therefore, in the last decade, EOs and other natural compounds like propolis, have been applied to contrast the biodeterioration of cultural heritage, representing a powerful resource in Green conservation strategies (Martín-Rey et al., 2011; Palla et al., 2020). A quick search on Scopus of the keywords related to this argument shows that there is a high number of publications related to “essential oils” alone (81,793 documents), and to “cultural heritage” or “artworks” terms alone (44,429 and 12,686 documents respectively), yet when a search using the terms “essential oils” and “cultural heritage” or “artworks” is carried out, very few documents were found (57 and 22 respectively). These studies are focused on the use of natural products obtained from plants with biocidal activity as an alternative and useful source in the control of biodeterioration, without negative environmental and human impacts.

These investigations show promising results for the use of Essential oils as Green biocides (antibacterial and antifungal) *in vitro* (on Petri dishes with microorganisms isolated from infected artworks) or applied on laboratory test tubes (simulating the biological attack on artistic material) in various artistic materials (stone materials and organic materials), (Gómez de Saravia et al., 2011; Borrego et al., 2012; Morales et al., 2013; Fidanza and Caneva 2019; Valentín 2019; Palla et al., 2020; Valentín and Fazio, 2020). Those studies indicated that due to the compatibility with artwork constitutive materials and the lack of negative effects on human health and environmental pollution, the use of EOs is a valid alternative to traditional biocides that might be considered (Borrego et al., 2012). However, as far as we know we do not have evidence that EOs have been applied in real artworks or in real CH indoor spaces (Rotolo et al., 2016).

In relation to the potential application of EOs on artworks, Palla et al. (2020) carried out studies on the use of EOs (*Origano vulgare* or *Thymus vulgaris*) dispensed in a glass container for the treatment of insect infestation by *Anobium punctatum* from wood artworks reproduction. Another published document shows the use of 0.1% *Artemisia absinthium* essential oil (dissolved in ethanol 70%) to remove fungi from paper material. In this study the Essential oil was applied in aerosol phase using high purity nitrogen bubbled into a solution of *Artemisia absinthium*/ethanol. The low concentration of Artemisia extract in aerosol spray did not affect the treated materials in terms of no pH changes and no colour change of the soluble pigments analyzed by spectrophotometry (Montero 2015; Valentín and Fazio 2020.).

In relation to the vaporized application of EOs in indoor spaces, there are multiple commercial products that indicate their benefits in improving air quality and its ability to sanitize the air. Moreover, certain scientific papers also address this issue. For instance, Gelmini et al. (2016) evaluated the efficacy of nebulized EOs (although the EO used was not indicated) combined with standard sanitization procedures in reducing the microbial contamination (yeasts and fungi) in residential health care house rooms (Gelmini et al., 2016). Other scientific studies have investigated the antiviral activity of EOs. Usachev et al. (2013) demonstrate that Tea tree and Eucalyptus EOs aerosol or vapor could inactivate Influenza A virus and *Escherichia coli* phage M13 with an efficiency of more than 95% after 5–15 min of exposure (Usachev et al., 2013).

Form another point of view, our team has been involved on the Santos Juanes Barroque church restoration process. This church is a BIC (Cultural Interest Heritage) and National Historical and Artistic Monument, located in the historical city of Valencia, Spain. The city of Valencia is the third most important city of Spain, where road traffic is continuous, with factories dedicated to industrial activity, an airport and a busy seaport. All this makes the amount of air pollution increase in the city affecting its rich CH buildings and their artistic assets. While carrying out previous studies of the air quality of the church by measuring the airborne microorganisms, particles, humidity and temperature, we realized that many parts of the church were in risk, especially inside the Camarín (Bosch-Roig et al., 2020). The Camarín is a space, without ventilation, with artistic oil mural painting in the dome and without access to the public.

In this scenario, we report herein for the first time, a study to evaluate the efficiency of nebulized selected EOs in reducing the microbiological air content present in CH confined areas. In particular, we report the results obtained in using nebulized selected EOs in the Camarín of Santos Juanes church of Valencia, Spain.

2. Materials and methods

2.1. Essential oils

Tea tree (*Melaleuca alternifolia*) and Thyme (*Thymus vulgaris*) 100% pure essential oils (EOs), without additives were acquired to PRANARÔM INTERNATIONAL. The EOs were selected for this experiment based on: i) their antimicrobial properties; ii) the literature evidence of their effectiveness applied to artistic samples; iii) their reported sanitation properties; and iv) its compatibility with the selected diffuser. Note that EOs are generally recognized as safe compounds (FEMA (flavor & extract manufacturers association) GRASS list).

2.2. Diffusor

The diffusion system selected was the Nobilé EOs diffuser (distributed by PRANARÔM INTERNATIONAL). This diffuser consists of a waterless diffusion device where pure essential oils are diffused as microparticles without heating them. Diffuser was used following manufacturer indications. Instructions suggest their use on indoor spaces with air volumes between 10 and 100 cubic meters. Pure EO was directly added on the diffuser crystal cone with a minimal dose of 10drops (approximately 0,5 mL) and maximal dose of 20 drops (approximately 1 mL) depending on the different experiments (described on section 2.4). Then, the diffusor was connected to the electricity and switched on. A complete diffusor session consisted of cycles of 2 min “ON” and 1 min

"OFF" for 2 h. Once the 2 h are up, the cycle ended automatically.

2.3. Gas mass chromatography tea tree essential oil chemical composition characterization

Tea tree essential oil composition were determined by gas chromatography–mass spectrometry (GC–MS). For the composition analysis of Tea tree essential oil, the oil was diluted in hexane at a concentration of 5 mg mL^{-1}. Then, 2 μL of the sample was directly introduced into the injector. The analysis was performed in a 6890/5975 inert GC–MS (Agilent Technologies, Santa Clara, CA, USA), equipped with a HP-5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm). Helium gas (ultrahigh purity grade, 99.999%) was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. The oven temperature was programmed initially 3 min at 60 °C. Then, from 60 to 100 °C at a ramp rate of 10 °C/min; oven temperature was then further increased to 140 °C at 5 °C/min and then increased until 240 °C at 20 °C/min; hold time 27 min. The injector and MS transfer line temperatures were set at 250 °C and 230 °C, respectively. The MS analysis parameters were the EI Ion source, electron energy 70 eV, solvent delay of 3 min and m/z40–550 amu. Tea tree essential oil components were identified according to their retention index and by matching mass spectra with the standard mass spectra from the NIST MS Search 2.0 library. The relative amounts of the individual components of tree essential oil were expressed as percentages of the peak area of total ion chromatograms.

2.4. In lab and real CH space EOs diffusion treatment experimentation

The EOs diffusion treatment was first tested in three unventilated air conditioning laboratory rooms with similar dimensions of that of the CH selected space. The best performed EO was then tested in-situ in the Camarín of Santos Juanes Church of Valencia. The 100% pure Tea tree (*Melaleuca alternifolia*) and Thyme (*Thymus vulgaris*) EOs, alone or blended were added to the diffusor according to the manufacturer's instructions and then it was switch on and leave active during the 2 h needed for a complete diffusor session. Three tests were conducted in three indoor unventilated (without windows and with only a door) and closed laboratory spaces the same day (September 2019): (i) Tea tree EO alone (20 drops, approximately 1 mL) was test on Lab 1 (89.9 m^3); (ii) Thyme tree alone (20 drops) was tested on Lab 2 (52.3 m^3); (iii) blend of Tea tree and Thyme (10 drops of each EO) was tested on Lab 3 (98.0 m^3). According to the in-lab results, the best performing EO was selected for the real CH indoor unventilated (without windows and with only a door) space tests (33.4 m^3). Two separate experiments were conducted on the Camarín space: first experiment (conducted on September 2020) 20 drops (approximately 1 mL) of the selected EO (maximum dose suggested by manufacturer) were added to the diffuser; while in the second experiment (conducted in November 2020, 72 days after the first one) 10 drops (approximately 0.5 mL, minimal dose suggested) were added.

2.5. Air quality measurements

Air quality measurements were conducted in order to determine the effect of the essential oils diffusion system. The measurements were conducted before any diffusion (t0), and at different times after the EOs diffusion: 2 h (t2), 3 h (t3), 4 h (t4) and 24 h (t24). Three types of air quality measurements were conducted: thermohydrometric, airborne particulate matter and microbiological air content. Thermohydrometric measurements were conducted with a Datalogger (Tzone TempU 03 multipurpose Temp and RH data logger for logistic temperature monitoring). Airborne particulate matter measurements were conducted by using a mobile laser particle counter (Airy Technology P311) which simultaneously samples 0. 3 μm, 0. 5 μm and 5 μm particles in the air for 1 min of absorption (flow rate of 2.83 L/min). Results were expressed in number of particles per minute. Values from the differential count (Δ PM: number of particles between the selected size and the next selected size) of the particles in the air (UNE 171330-2) were obtained. Microbiological air contamination measurements were conducted by using a Surface Air System (SAS SUPER 100/180) according to UNE 171330-2:2014 recommendations. Petri dishes with Sabouraud Chloramphenicol Agar (Scharlau) and with Plate Count Agar (Scharlau) were used for fungi and bacteria analysis respectively. An air flow of 100 L was sampled on each Petri dish. Petri dishes were incubated at 28 °C for 24 h to 5 days. Counts were adjusted according to manufacturer instructions in order to obtain quantitative results of microorganisms in each air cubic meter.

2.6. Cultural heritage EOs diffusion treatment risks assessment

The Camarín is a space with an oil mural painting and therefore CH risk assessment of EOs diffusion was evaluated. Three types of risk analysis were conducted: diffusion thermographic study, diffusion EOs range and EOs compounds deposition on the artistic vault. Thermographic study was conducted with a ThermoVision TM A20-V thermographic camera, from the FLIR SYSTEMTM brand in order to confirm the absence of heat emission when essential oils are diffused. A range study of the EOs diffused was carried out using absorbent papers placed over the diffusor at different heights (5 cm, 20 cm, 90 cm, 100 cm, 200 cm) for 10 min each. EOs residues have been visually checked on the absorbent papers. In order to evaluate the presence of EOs compounds on the Camarín artistic murals after the EOs diffusion treatment, seven microsamples from the artistic space, four before (1) and three after treatment (2), were taken with scalpel from the vault and analyzed GC-MS as described in section 2.3. Microsamples were taken from north, east and west orientations because Camarín south area is not decorated. Samples of wall paint fragments deposited on the floor were also taken. The samples were named according with the specific site where the scalpel was taken *north 1*, *north 2*, *east 1*, *east 2*, *west 1*, *west 2* and *floor wall 1*. In order to develop a calibration methodology for our study, Tea tree essential oil main peak area from GC-MS (at 11.89 min, which corresponds to the terpene compound 1-terpinen-4-ol) was plotted as a function of the concentration of Tea tree essential oil in hexane (from 0.01 to 5 mg/mL). A linear trend was observed (Fig. S1).

As it was mentioned above, for the EOs determination at the Camarín, seven samples from the Camarín artistic murals before and after EOs diffusion process were taken into account. Additionally, in order to determine the possible accumulation of EO on the samples surface, a positive control was also performed. For this purpose, one of the samples taken before EOs diffusion treatment, *floor wall1* sample, was divided in two parts: *floor wall1a* and *floor wall1b*. For the development of the analysis, three microsamples from the Camarín artistic murals after diffusion process (*north2*, *west2* and *east 2*) were put in contact with 1 mL of hexane. The positive control consisted of the application of 1 mL of a solution of 5 mg/mL of Tea tree essential oil in hexane on the top of the floor wall 1 b microsample which was additionally kept in contact with air for 2 h. Moreover, microsamples without EO's diffused (*north1*, *west1*, *east1* and *floor wall1a*) were also analyzed in order to obtain time zero data of the samples before diffusion process of the EO. Eight samples were analyzed by GC–MS as is described at section 2.3 and compared with the calibration analyzed. In order to quantify the EOs in each Camarín microsample, the main peak of each microsample at 11.89 min (1-Terpinen-4-ol), was integrated and compared with the integrated areas from the calibration method.

2.7. Statistical analysis

Triplicate data were obtained and presented in the results as means with standard deviation (SD). Significant differences were determined by analyses of Variance (ANOVA) at 95% significance level using Student T-Test. Differences between the analyzed EOs treatments were considered statistically significant at $p\text{-value} < 0.05$.

3. Results

3.1. In lab EOs diffusion treatment bioaerosol reduction

The initial results of biological particles content in indoor lab environments (t0) show an important variable presence of air microorganisms (Table 1). Lab 1 showed intermediate levels of fungi (267 ± 40 UFC m^{-3}) and bacteria (130 ± 10 UFC m^{-3}). Lab 2 showed the highest levels of fungi (677 ± 127 UFC m^{-3}) and bacteria (160 ± 36 UFC m^{-3}). While the lowest levels of both fungi (97 ± 35 UFC m^{-3}) and bacteria (20 ± 0 UFC m^{-3}) were found on Lab 3.

The bioaerosol reduction effect of Tea tree and Thyme EOs alone and their combination (1:1), through vapor phase against bacteria and fungi during 24 h was quantified (UFC m^{-3}) and air microbial reduction percentage over time was assessed and statistically compared with initial values (t0). Tea tree EO diffused in Lab 1 space resulted in an important and significant microbial reduction (66.3% and p-value 0.0208 for fungi and 61.5% and p-value 0.0184 for bacteria) just after the end of the diffusion cycle (t2). Microbial reduction was maintained or slowly reduced even more over the analyzed times (Fig. 1).

Thyme EO diffusion shows an important bacteria reduction over time (Fig. 1), with a maximal significant (p-value 0.0050) reduction of 79.4% (t4), that was slightly increased after 24 h but maintaining low levels (77 ± 38 UFC m^{-3}). Fungi shows an initial (t2 and t3) slow increase after the Thyme EO diffusion (−14.2% and −8.3% respectively), that was then significantly reduced (p-value 0.0206) at t4 and t24 (13.9% and 48.7%), but final levels were still high (347 ± 86 UFC m^{-3}).

The use of blend (1:1) EOs diffusion resulted in a significant (p-value = 0.0636) fungi reduction up to 51.5% at t3 (Fig. 1). Air fungi content then increased on t4 and t24 showing air fungi content at t24 higher than the initial one (t0). This blended mixture of EOs did not show significant bacteria reduction over the treatment time tested.

Among EOs vaporized in lab experiments, *Melaleuca alternifolia* Tea tree EO was the most effective in reducing both bacteria and fungi after the 24 h treatment time. The indoor laboratory spaces media temperature was about 19.01 ± 0.10 °C and the relative humidity was approximately 60.10 ± 0.20%.

3.2. Real CH space EOs diffusion bioaerosol reduction

Tea tree EOs was selected for real CH space diffusion experimentation. Two EOs doses were tested in two separate experiments inside the Camarín space. A first experiment (Camarín sept.) was conducted with 20 drops Tea tree EO cold vaporization in September 2020. The second experiment (Camarín nov.) was conducted with 10 drops Tea tree EO cold vaporization in November 2020. Initial microbial air content media value (t0) in the first experiment (Camarín sept.) shows intermediate (Table 1) microbial air content of bacteria (157 ± 116 UFC m^{-3}) and fungi (180 ± 53 UFC m^{-3}).

Table 1
Initial biological particles content in indoor tested environment: three laboratory spaces (Lab 1, 2 and 3) and one real CH space (Camarín sept. and Camarín nov.).

Indoor space	Microorganisms	UFC m^{-3}	Category	Recommendations
Lab 1	Fungi	267 ± 40	Intermediate	Over
	Bacteria	130 ± 10	Intermediate	Under
Lab 2	Fungi	677 ± 127	High	Over
	Bacteria	160 ± 36	Intermediate	Under
Lab 3	Fungi	97 ± 35	Low	Under
	Bacteria	20 ± 0	Low	Under
Camarín sept.	Fungi	180 ± 53	Intermediate	Under
	Bacteria	157 ± 116	Intermediate	Under
Camarín nov.	Fungi	410 ± 173	Intermediate	Over
	Bacteria	857 ± 145	High	Over

Category according to CEC 1993 Report No.12: Biological Particles in Indoor Environment (EUR 1488 EN) non-industrial indoor environments: Low< 25 UFC m^{-3} (fungi) and <50 UFC m^{-3} (bacteria); Intermediate<100 UFC m^{-3} (fungi and bacteria); high<2000 UFC m^{-3} (fungi and bacteria).
Recommendations according to *UNE 171330-2: 2014:* bacteria levels must be < 600 UFC m^{-3} and fungi levels must be < 200 UFC m^{-3}

The use of the highest (20 drops) Tea tree EO diffused dose (Camarín sept.) in real CH space resulted in an important microbial reduction. Fungi show an initial important reduction of 51.7% (t2) arriving to a statistically significant (p-value 0.0359) 90.6% reduction at the end of the treatment (t24). Initial bacteria air content was low (157 ± 116 UFC m^{-3}) and it was reduced to a 21.7% at t3, but later bacterial content augment at t4 (173 ± 107 UFC m^{-3}) and t24 (227 ± 133 UFC m^{-3}) showing a final content higher than the starting point (not significant differences have shown in bacterial reduction at any time). In no case was the existing recommendations exceeded (Fig. 2).

In the second experiment (Camarín nov.) a much higher initial (t0) media values microbial content was obtained both for fungi (410 ± 173 UFC m^{-3}) and for bacteria (853 ± 145 UFC m^{-3}). The use of the lower (10 drops) Tea tree EO nebulization (Camarín nov.) in the CH space resulted in an important microbial reduction over the treatment period. Fungi show a constant reduction over the time reaching the recommendations levels on t3 and with maximal and significant (p-value 0.0060) reduction of 77.3% after 24 h (t24). Bacteria air content media values, after the EO nebulization, decreased significantly (p-value 0.0008) up to 95% reduction on t3, maintaining significant (p-value 0.0007) low levels during the 24 h treatment (67 ± 12 UFC m^{-3}).

The indoor temperature media values of the Camarín in September 2020 were 26.76 ± 0.16 °C and relative humidity media values were 60.61 ± 1.04% with maximal values of 27.4 °C and 61.7% and minimal values of 26.5% and 55.1% respectively. In November 2020 temperature media values were 15.36 ± 0.11 °C and relative humidity media values were 65.19 ± 0.94% with maximal values of 15.8 °C and 66.6% and minimal values of 15.1 °C and 60.6% respectively.

3.3. EOs diffusion treatment PM air content evolution

EOs diffusion treatment introduce micro-drops into the studied spaces. In order to evaluate how this modifies the indoor spaces aerosols composition, air particles (particular matters: PM) content evolution was studied before EOs treatments (t0), and at different times after the EOs diffusion: 2 h (t2), 3 h (t3), 4 h (t4) and 24 h (t24). In all EOs diffusion treatments (in lab and in real spaces) the smallest particles (0.3 and 0.5 μm) media values show a significant increase (p-value 0.0001 in all cases) just after the EOs diffusion finished (t2), while 1 h after (t3) media values decreased over the time to values close to the initial ones (Figs. 3 and 4).

Highest analyzed PM (5.0 μm) showed a different behavior. In lab media values showed an initial decrease (t2) after the application of the essential oil (statistically significant only with thyme and blend p-value 0.0166 and 0.0470). While at t4 media values vary over the different spaces, showing a slow decrease (Lab 1 Tea tree and Lab 2 thyme), or slight augment (Lab 3 blend). After 24 h, in all cases particles were at levels slightly over the original ones (statistically significant only with Tea tree test p-value 0.0312) (Fig. 3). Similar statistically significant behavior showed the Camarín nov. with 10 drops Tea tree EO experiment (p-values 0.0001–0.0005), while the Camarín sept. experiment (Tea tree EO 20 drops) did not show statistically significant variations over time (Fig. 4).

3.4. Chemical composition of tea tree essential oils

The chemical composition of the Tea tree essential oils is shown at Table 2. The main tea tree EO compound was 1-Terpinen-4-ol (48%)

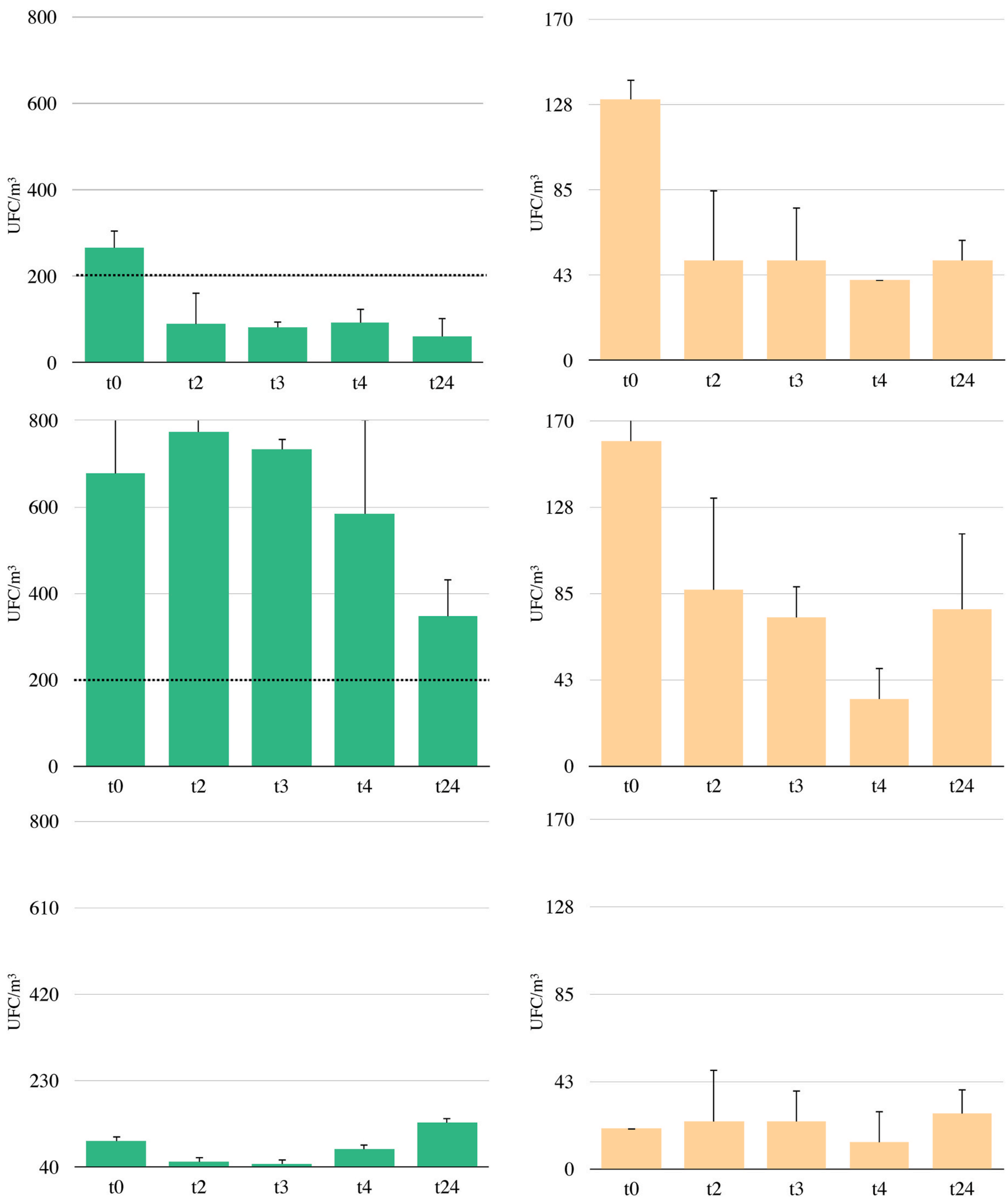

Fig. 1. In lab Bioaerosol reduction over time after EOs diffusion treatments. Media values and standard deviation errors of Fungi (in green at left side) and bacteria (in orange at right side). Top diagrams: Lab 1 Tea tree EO treatment; middle diagrams: Lab 2: Thyme EO treatment; bottom diagrams: Lab 3 blend EOs treatment. Recommendation (UNE 171330-2: 2009) levels are marked as discontinuous line.

(Table 2). Other Tea tree EO components included τ-terpinen (22%) and α-terpinen (10%). These data agree with the results obtained by Angullo-Milhem et al. that analyzed a Tea tree oil and found that it contained more than 60% of 4-terpineol and γ- and α-terpinene (Angulo-Milhem et al., 2021).

3.5. Cultural heritage EOs diffusion treatment risk assessment

Thermographic study allows us to corroborate that the use of the Novilé diffuser did not produce any heat (Supplementary Video 1) and therefore, the temperature of the rooms where the oil particles were released was not modified. In addition, from our studies it can be concluded that temperature changes did not affect the antimicrobial activity of the essential oil and increasing or decreasing the temperature in the Camarín only affected the number of microorganisms inside (Palla and Barresi 2017), not the effectiveness of the EO.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ibiod.2021.105251

The EOs diffusion range test reveal that microparticles of EOs were only evidenced on absorbent papers placed at a distance of 10 and 90 cm from the diffuser. This suggests that this diffusor system is viable for its use in CH spaced whose artistic surfaces are situated more than 1 m

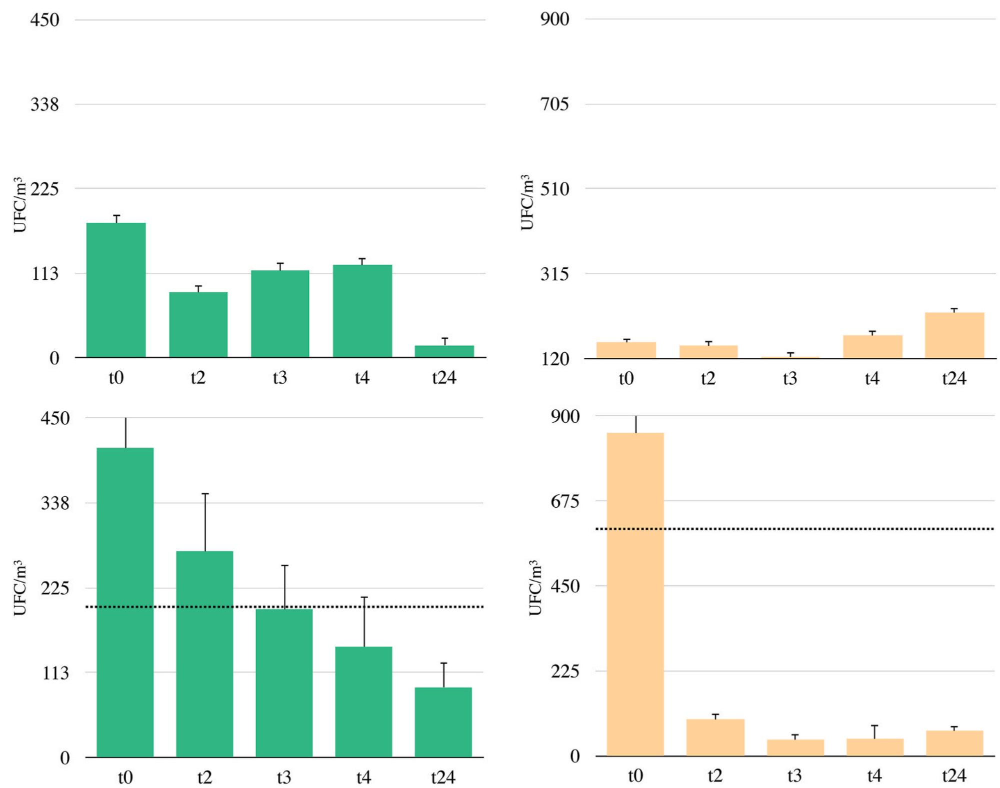

Fig. 2. Real CH space (Camarín) EOs diffusion bioaerosol reduction over time. Media values and standard deviation errors of Fungi (in green at left side) and bacteria (in orange at right side). Top diagrams: Camarín sept. Tea tree EO 20 drops treatment; Bottom diagrams show Camarín nov. Tea tree EO 10 drops treatment. Recommendation (UNE 171330-2: 2009) levels are marked as discontinuous line.

away from the diffuser.

In order to evaluate the presence of EOs compounds on the Camarín artistic murals after the EOs diffusion treatment, eight microsamples from the Camarín artistic murals before (1) and after (2) Tea tree EO diffusion treatment was evaluated. The main Tea tree EO compound 1-Terpinen-4-ol was quantified at GC-MS in the eight microsamples. The area peak of all samples tested was integrated and compared with the integrated areas from the calibration method. Area peaks are shown at Supplementary Table S1. In a first approach, four microsamples before EOs diffused (*north 1, east 1, west 1* and *floor wall1a*) were analyzed by GC-MS to obtain time zero data for the blank samples. All four microsamples showed 0% of presence of EOs as expected. Moreover, the possible accumulation of EO's on the samples surface was tested developing a positive control. For this propose, 5 mg/mL of Tea tree essential oil in hexane was applied on the top of the *floor wall 1b* microsample and exposed to the air for 2 h. Then, this positive control was analyzed by GC-MS and a peak corresponding to a 0.53 mg/mL of Tea tree essential oil concentration, 10% of the initial EOs concentration, was detected. The decrease of the EO presence is explained due to the volatile characteristics of the essential oils. Finally, the three microsamples from the Camarín artistic murals after diffusion process (*west 2, north 2* and *east 2*) were analyzed by GC-MS, none of them show any presence of EOs (Supplementary Table S1).

4. Discussion

The presence of high levels of air bioaerosols inside CH spaces is associated with higher risk of artistic surfaces biodeterioration. Moreover, high levels are associated to poor indoor air quality which can reduce visitors' comfort and augment health risks (Augustowska and Dutkiewicz 2006; Hoseinzadeh et al., 2013; Ahmed et al., 2018). Air monitoring in CH spaces has increased in the last years including temperature, humidity, light, PM and microbial content (Bosch-Roig et al., 2020). When high air bioaerosols are found, strategies have to be taken in order to reduce those levels. Those strategies are based on the natural ventilation of the spaces because the existing sanitation products are not recommended for spaces with artistic assets because of potential negative interactions (Valentín and Fazio 2020). EOs have shown a great potential as natural biocides in Green Conservation strategies due to its known antimicrobial and insecticidal activities (Palla et al., 2020). Manufacturers also promote their use to improve indoor air quality due to their purifying actions. However, to the best of our knowledge, no previous studies have analyzed their effects on CH spaces. The aim of the present study was to evaluate the feasibility of using cold diffusion of selected EOs on indoor unventilated CH spaces with artistic coverings.

In order to select the best performing EOs, three initial laboratory experiments were conducted. Three comparable spaces were chosen with similar dimensions and climatic conditions (by air-conditioning). Constant and stable climatic conditions were confirmed during treatments. Initial bioaerosol particles content in indoor tested spaces showed, in most cases, media values over the existing standard recommendations UNE 171330-2:2014 (AENOR. 2014). This norm indicates the methods of analysis and assessment criteria for indoor air quality. Following the norm, SAS equipment was used and evaluated: indoor fungi levels must be under 200 UFC m^{-3} and bacteria levels under 600 UFC m^{-3}. In particular, Lab 1 show initial (to) fungi and bacteria media values over those recommended levels and Lab 2 show only fungi media values over recommendations, while Lab 3 bioaerosol media values were according to recommendations.

Two EOs were chosen for this study: *Melaleuca alternifolia* (Tea tree) and *Thymus vulgaris* (Thyme). Tea tree and Thyme oils have been widely investigated for their acknowledged antimicrobial properties both by direct and by vapor-phase application (Bakkali et al., 2008; López et al., 2007), and the research shown their positive application for controlling artistic assets biodeterioration organisms (Rakotonirainy and Lavédrine, 2005; Gómez de Saravia et al., 2011; Veneranda et al., 2018; Fidanza

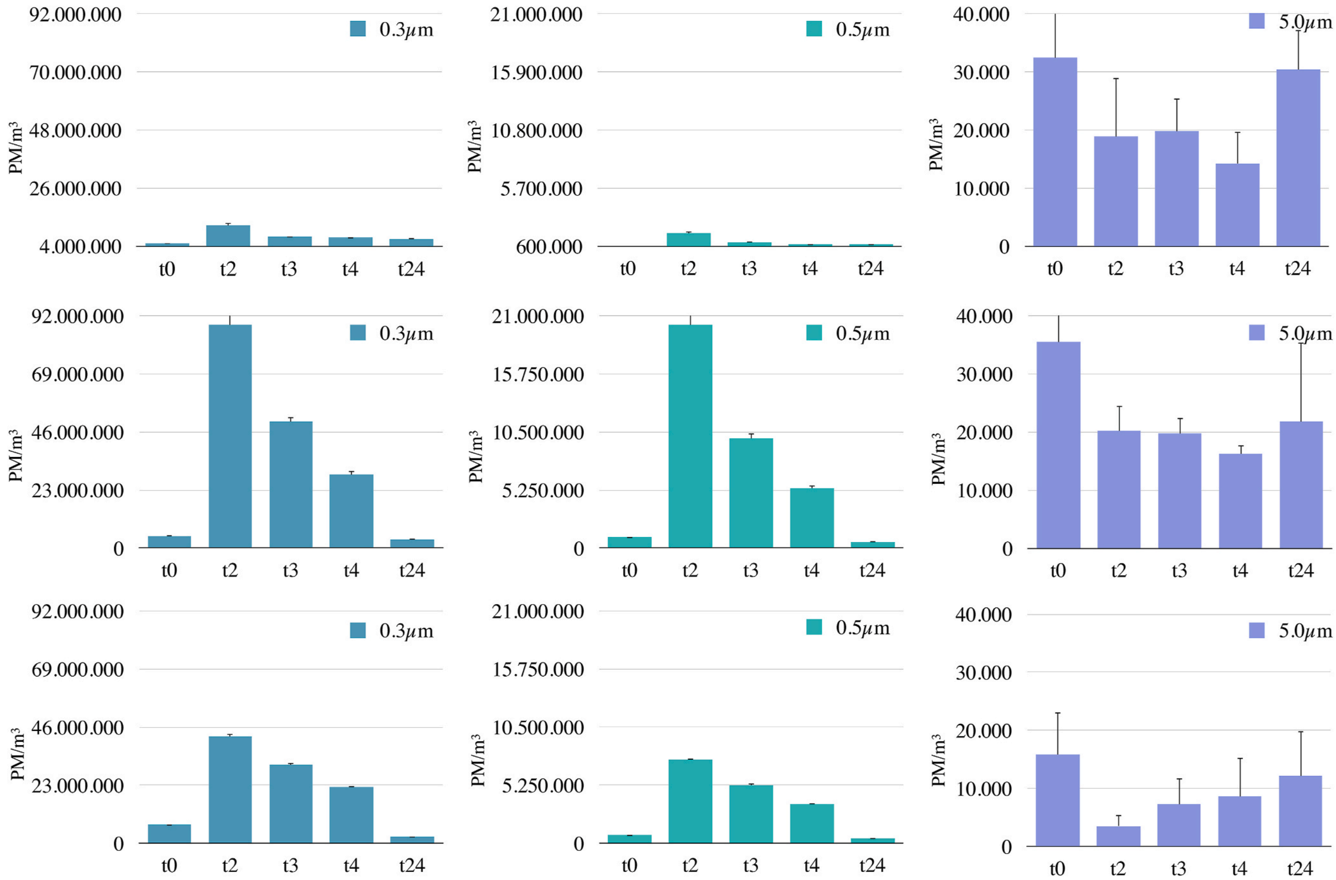

Fig. 3. In lab EOs diffusion treatments air particulate matter (PM) variation over time. Media values and standard deviation errors of 0.3 μm (left diagrams); 0.5 μm (middle diagrams); and 5.0 μm (right diagrams). Top diagrams: Lab 1 Tea tree EO treatment; middle diagrams: Lab 2: Thyme EO treatment; bottom diagrams: Lab 3 blend EOs treatment.

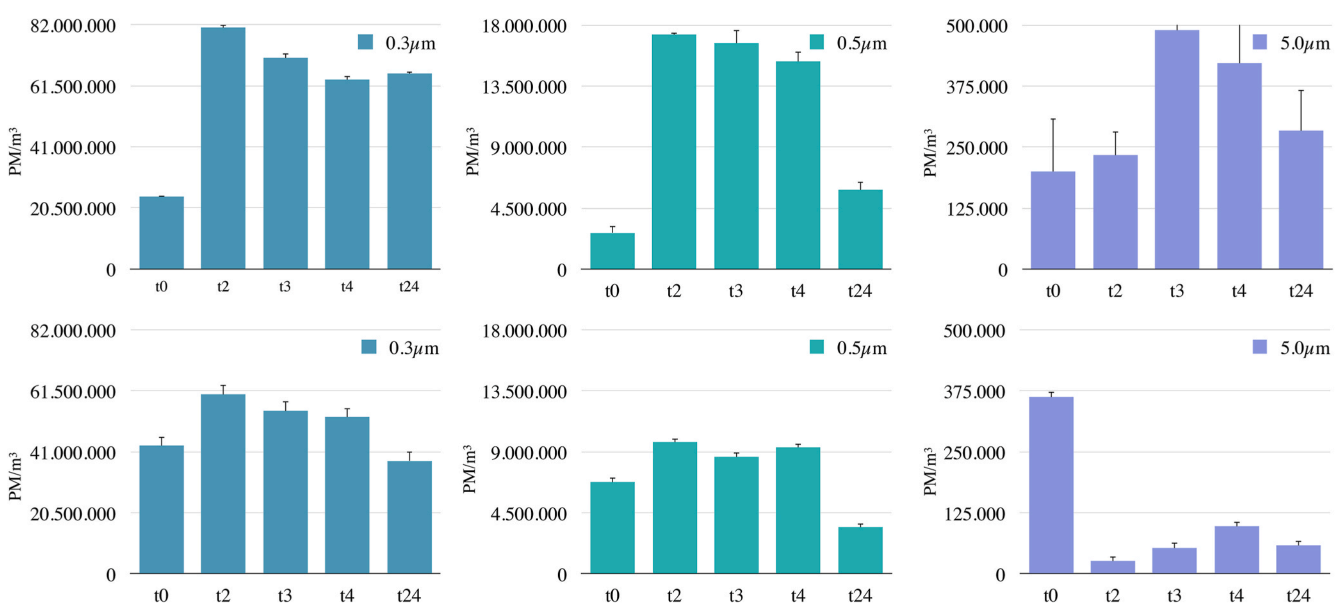

Fig. 4. Real CH space (Camarín) EOs diffusion treatments air particulate matter (PM) variation over time. Media values and standard deviation errors of 0.3 μm (left diagrams); 0.5 μm (middle diagrams); and 5.0 μm (right diagrams). Top diagrams: Camarín sept. Tea tree EO 20 drops treatment; Bottom diagrams show Camarín nov. Tea tree EO 10 drops treatment.

and Caneva, 2019; Palla et al., 2020). Lab experiments were conducted with the selected EOs alone and in combination, as combined treatments with EOs have been reported to affect their antimicrobial activities with synergic, neutral or antagonistic effects (Ultee et al., 2000; López et al., 2007).

Tea tree EO vaporization treatment in Lab 1 was able to significantly

Table 2
Composition of the tree essential oil (*Melaleuca alternifolia*).

Peak number	Component	CAS number	Rca (%)	m/z	Molecular weight g/mol (theory)	Molecular Structure (theory)	Retention time (min)
1	Cyclofenchene	7785-70-8	3	136	136	C10H16	5.770
2	α−Terpinen	99-86-5	10	136	136	C10H16	7.446
3	Benzene, 2-ethyl-1,3-dimethyl-	535-77-3	3	134	134	C10H14	7.661
4	τ−Terpinen	99-85-4	22	136	136	C10H16	8.397
5	p-Mentha-1,4(8)-diene	586-62-9	4	136	136	C10H16	9.088
6	1-Terpinen-4-ol	20126-76-5	48	154	154	C10H18O	11.987
7	1-Terpinen-8-ol	98-55-5	4	154	154	C10H18O	12.360
8	(+)-Aromadendrene	489-39-4	2	204	204	C15H24	19.222
9	(+)-Ledene	21747-46-6	2	204	204	C15H24	20.445
10	Cadina-1(10), 4-diene	523-47-7	2	204	204	C15H24	28.887

[a] Relative composition of the compounds identified in the essential oil.

reduce fungi and bacteria content below recommendations after 2 h treatment and maintain them over 24 h, with a reduction content of 66.3% for fungi and 61.5% for bacteria. Thyme EO vaporization treatment in Lab 2 took longer time (24 h) to significantly reduce fungi content (48.7%) and media values over the analyzed times were over recommendations. This result could be related to the important high initial levels found on this space (677 ± 127 UFC m^{-3}). Instead, Thyme EO diffusion showed an important significant bacteria reduction over treatment time up to 79.4% (t4). Combined Tea tree and Thyme blend EOs diffusion treatment was able to significantly reduce fungi values (51.5%) after 3 h but later fungi content increased over the initial values. In contrast, the blended mixture did not show significant bacteria reduction over the treatment time tested. These results show that Tea tree and Thyme EOs blend did not increase the air microbial reduction compared with the EOs alone. This agrees with other authors' results that show how EOs vapor phase mixture can produce synergism or antagonism antimicrobial effects (Goñi et al., 2009). These effects are reported to be dependent to the particular microorganisms (Fu et al., 2007) agreeing with our results that show that blend treatment was able to reduce fungal airborne but not bacterial airborne.

These in lab tests allow us to select *Melaleuca alternifolia* Tea tree EO for further real CH space tests due to its effectiveness on air bacteria and fungi reduction and its maintained effect over 24 h. Two Tea tree EOs dose were tested in order to minimize EO sensory (organoleptic) effect on the real CH visited space and in order to assess the minimal dose needed. Thermohydrometric conditions on the CH real space was evaluated during the 24 h treatment time because it was not air-conditioning and therefore impossible to maintain constant. Media values showed 11.4 °C higher temperatures media values on the first experiment, conducted in September 2020, and 5.58% lower relative humidity media values than those conducted in the second experiment, November 2020. Initial bioaerosol media values (fungi and bacteria) and PM media values also varied significantly between experiments. Initial media values of fungi, bacteria and PM air content were higher in November than in September, with 230 UFC m^{-3} and 696 UFC m^{-3} subtraction values for fungi and bacteria respectively. In particular, Camarín nov. showed initial fungi and bacteria media values over those recommended levels, while Camarín sept bioaerosol media values were according to recommendations.

Even though the highest levels of microorganisms shown in November, lower dose (10 drops) of Tea tree EO showed a high and gradual air microbial reduction statistically significant during the treatment time, with reductions up to 77.3% and 95.0% for fungi and bacteria, respectively. Previous literature reported that EOs airborne pathogenic bacteria (like *Streptococcus pyogenes, Streptococcus pneumonia, Hemophilus influenzae*) inhibition is related to the EOs concentrations (Yang et al., 2012). These authors showed a decreased bacterial colony growth higher with higher EOs concentrated treatments. However, our results showed an opposite trend as we found a higher microbial reduction when the lower EO diffusion dose was applied. This could be due to a different EOs behavior on diverse microorganisms' strains linked to different susceptibility related to membrane properties (López et al., 2005); or because of diverse environmental conditions between experiments that could affect EOs effectivity, stability and performance as it has been observed with light exposure (Veneranda et al., 2018) and with high temperatures in diverse oil and gas biocides (Williams and Schultz., 2015). Our results are in line with other authors that show that various Tea-tree oils are able to reduce bacteria (*E. coli*) and yeast (*S. cerevisiae*) even at low concentrations (even 30 μL) after few minutes (even 5 min) incubation due to instantaneous and irreversible damage of the cells (Schmolz et al., 1999). Further investigations should be done in order to explore this dose-response behavior.

The active principles of the essential oil of the Tea tree are terpinene derivatives that have antibacterial and fungicidal properties (Angulo--Milhem et al., 2021). As it is described at Table 2, the chemical composition of the Tea tree essential oils analyzed by GC-Ms shows that the main Tea tree EO compound is 1-Terpienen-4-ol; 48% of the relative total composition of the Tea tree oil sample if compared with the other nine main Tea tree EO compound. Moreover, other terpenes were also identified: τ-Terpinen (22%); α-Terpinen (10%); 1-Terpienen-8-ol (4%) or p-Mentha-1.4(8)-diene (4%), among others. As stated above, these data are in concordance with other paper already published (Angulo--Milhem et al., 2021). Tea tree EO main compound 1-Terpienen-4-ol is a terpene alcohol with broad-spectrum antimicrobial activity against different species like *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Cryptococcus neoformans*, *Malassezia* spp., *Rhodotorula* spp., *Trichosporon* spp., *Aspergillus* spp., *Penicillium* spp., and *dermatophytes* (Cox et al., 2000; Hammer et al., 2004; Carson et al., 2006; Morcia et al., 2012; Nogueira Brilhante et al., 2016). Different authors have investigated its antimicrobial mechanisms of action demonstrating that they act by inhibiting respiration and increasing cell membrane permeability. Terpinen-4-oil have a lipophilic characteristic that interact directly with cell membrane structure and associated enzymes. It has been reported that terpenes are able to cross the microbial cell wall and insert between the lipid bilayer membrane changing its permeability and fluidity, leading to membrane destabilization, cell wall degradation, lysis and cell death. (Cox et al., 2000; Bakkali et al., 2008; Marcos-Arias et al., 2011; HĿc-Wydro and Szydɩo, 2016; Nogueira Brilhante et al., 2016; Behbahani et al., 2019). Císarová et al., 2020 even demonstrate an antisporulant action of vapor EOs using gas diffusion method (Císarová et al., 2020).

The general decreased levels of air microorganisms content showed in this work agree with previous studies that confirm vapor EOs can act as antimicrobial agents interfering with any stage of their life cycle (Avila-Sosa et al., 2012; Stupar et al., 2014). Our results also show a variable microorganisms reduction effectiveness of diffusion EOs depending on the microorganism's type. This agrees with other authors results that state that EOs antimicrobial effectiveness depended on the microorganism and even on strains of same microorganisms' specie (Pietrzak et al., 2017; Císarová et al., 2020). This variable effect has been investigated by some authors indicating that it is due to a different rate of terpene penetration through microorganisms' cell wall (Cox et al., 2000). And this different penetration rate determined by the diverse lipid composition of microorganism's membranes (Połeć et al., 2019).

PM air content evolution study during EOs nebulizing treatments indicated that the cold diffusion system produced EOs micro-drops sizes between 0.3 μm and 0.5 μm; and that they were maintained on the air over the 24 h treatment. This agrees with results by Angulo-Milhem et al. (2021) that found that Tea tree EOs diffusion in confined environments can persist on the air between 5 h and 60 days depending on the diffusion mechanism used (Angulo-Milhem et al., 2021).

Our CH risk assessment results allow us to confirm that the EOs diffusion system method proposed did not alter the thermohydrometric conditions of the artistic space, neither reach directly the artistic surfaces present on its vault. Even though maximum diffuser range has been determined as 1 m and artistic vault is 2.77–4.74 m high, the passive movement of the EOs micro-particles within the space is unknown and therefore there was some possibility that they could reach the vault. However, GC-MS studies showed no presence of EOs components on the artistic murals of the Camarín after EOs treatment. The absence of EOs deposition on the vault is very important because diverse works have shown that EOs direct application to some artistic materials can produce chromatic alterations. For instance, Pietrzak et al., (2017) reported that direct application of Thyme to fungal and bacteria altered historical books was able to disinfect them, yet lightly altering the paper structural and mechanical properties and yellowing it. Our results are in line with Palla et al. (2020) that show that wooden artworks exposed to Oregano and Thyme volatile compounds, in an ad-hoc-assembled chamber for insect infestation treatment, show compatibility with artwork material.

5. Conclusions

Our results confirm the known efficacy of EOs diffusion on reducing bioaerosols. The bioaerosols reduction potential of a Tea tree and Thyme EOs alone or blend cold diffusion system is preliminarily tested in lab spaces. Tea tree show better performance against fungi and bacteria and is assayed on indoor unventilated CH real space. Our results confirm the Tea tree EOs cold diffusion system feasibility on effectively reducing high microbial air content on indoor unventilated CH spaces, without damaging artistic surfaces. This research is a first step towards improving air quality in indoor CH spaces with natural biocides and eco-sustainable approaches as a valid alternative to traditional biocides. Further studies are required focused on: (i) the development of appropriate diffusion systems for larger artistic spaces; (ii) analyze the risk absence also on other type of artistic materials; and (iii) evaluate EOs vapor organoleptic impact in visitors.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2021.105251.

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