Biocidal effect of lichen secondary metabolites against rock-dwelling microcolonial fungi, cyanobacteria and green algae

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Claudia Gazzano, Sergio E. Favero-Longo, Paola Iacomussi, Rosanna Piervittori

Abstract

The use of commercial biocides in outdoor environments is increasingly discouraged because of their ecotoxicity, new methods being thus invoked to control patinas of biological origin on the stone cultural heritage. The effects of secondary metabolites (usnic acid, norstictic acid, parietin) produced by saxicolous lichens, natural competitors of rock dwelling microorganisms, were investigated in vitro against microcolonial fungi (MCF: *Coniosporium apollinis, Coniosporium perforans, Coniosporium uncinatum, Phaeococcomyces*-like sp.), coccoid cyanobacteria (*Chroococcus minutus*) and green algae (*Scenedesmus ecornis*) which commonly occur on stonework. An acetone/water 10/90 vol/vol mixture was screened as suitable to solubilise the lichen metabolites and to not affect the bioassay results. Benzalkonium chloride 1% was used as positive control.

All the three metabolites (approx. 10^{-2} mM) inhibited the growth of the assayed MCF species, displaying the same effect of benzalkonium chloride. *Chroococcus* and *Scenedesmus* exhibited sensibility to the lichen metabolites when exposed to high incubation temperatures (35 °C), chemicals and temperature synergically yielding percentage decreases of intact cells with red chlorophyll epifluorescence. These findings suggest lichen secondary metabolites as allelopathic agents against rock dwelling microorganisms and as potential natural sources for their control on stone materials in restoration and conservation programmes. In this perspective, the detection of a negligible chromatic alteration ($\Delta E<0.5$) caused by LSM to the white Carrara marble is reported as the first step of the necessary extensive evaluation of the LSM-stone material interactions.

1. Introduction

Black patinas are a common biodeterioration phenomenon affecting the stone cultural heritage (Brimblecombe and Grossi, 2005; Caneva et al., 2008). Although the blackening of buildings and monuments can be also related to anthropogenic causes, as the deposition of gases and particles or sulphation mechanisms (Prieto et al., 2007), microorganisms as dematiaceous meristematic fungi, including microcolonial fungi (MCF), filamentous and coccoid cyanobacteria and, subordinately, green algae are also recognized as common agents of such threat to conservation (Gorbushina and Broughton, 2009; Macedo et al., 2009; Sterflinger, 2010). These organisms determine anaesthetic discoloration because of their dark pigments (Scheerer et al., 2009) and are often associated to physical and chemical deterioration processes because of their mechanical penetration and the release of acidic and chelating compounds (Macedo et al., 2009; Sterflinger, 2010; Favero-Longo et al., 2011).

The removal of these patinas is invoked in conservation programmes (Delgado Rodrigues and Valero, 2003), but biochemical and structural characteristics (e.g. EPS and/or mineral coatings, pigments) which make rock-inhabiting fungi, cyanobacteria and green algae adapted to the extreme microclimate conditions, including high temperatures, desiccation and osmotic stress (Sterflinger, 1998; Gorbushina and Broughton, 2009), also support a peculiar resistance to biocides (Gorbushina et al., 2003; Nugari et al., 2009). Anyway, the ecotoxicity of commercial biocides increasingly discourages their use in outdoor environments, where black patinas prevail (Scheerer et al., 2009).

On the other hand, natural products represent a huge potential source of compounds with biological activity, including phytotoxicity, which may be directly used as “pesticides” or represent a model to develop natural product-based pesticides (Duke et al., 2002). In this context, lichen secondary metabolites (LSM) have been suggested as potential natural herbicides because their chemical simplicity makes their synthesis potentially easy in the laboratory (Dayan and Romagni, 2001). LSM are a group of more than 800 compounds, in part exclusively synthesized by lichen-forming fungi, which include aliphatic, cycloaliphatic, aromatic and terpenic components (Huneck and Yoshimura, 1996; Elix and Stocker- Wörgötter, 2008). Many LSM are well known for determining allelopathic effects on bryophytes and vascular plants (Lawrey, 1984; Favero-Longo and Piervittori, 2010). Antibiotic, antiviral and anti-proliferative functions have been also recognized, suggesting their potential use for therapeutic applications (Oksanen, 2006; Boustie et al., 2011). The antimicrobial activity of LSM has been assessed against a wide set of bacteria and filamentous fungi, mainly of medical interest (Lawrey, 1984; Molnár and Farkas, 2010; Mitrović et al., 2011) or plant pathogens (Halama and Van Haluwin, 2004), but researches overlooked their effects against rock-dwelling organisms, thus preventing the evaluation of their potential use for the control of the biological colonization on stone materials.

This work aims to evaluate the effects of LSM on MCF, cyanobacteria and green algae which occur in black patinas and other biological patinas of different colours on the stone cultural heritage. The effects of usnic acid, norstictic acid and parietin on the fungal growth in axenic conditions and on the chlorophyll integrity in the photosynthetic organisms were assayed. These three LSM commonly occur in saxicolous lichens, in both silicicolous and calcicolous species (Smith et al., 2009). Usnic acid is a yellowish dibenzofuran, exclusive to lichen-forming fungi and widely distributed in the vegetative and reproductive structures of several species (e.g., species of genera *Lecanora, Ramalina*) (Ingólfsdóttir, 2002; Liao et al., 2010). It is well known for antibacterial and antifungal properties (and for antiprotozoal, antiviral, antiproliferative, antiinflammatory, analgesic, antipyretic, antitumor activities) which have been mostly studied for potential therapeutic applications (Cocchietto et al., 2002; Ingólfsdóttir, 2002), and is also known to play UV protection of thalli (McEvoy et al., 2006). Norstictic acid is a depsidone, exclusive to lichen-forming fungi (e.g., in species of genera *Buellia, Lecanora, Porpidia, Rhizocarpon*), which is mostly deposited as crystals in the apoplast and soluble in water in a small proportion, being likely involved in the metal uptake and homeostasis of lichens (Hauck et al., 2010). Parietin is an orange antraquinone, non exclusive to lichens, located as extracellular crystals in the top layer of the upper cortex of Teloschistaceae (e.g., in species of genera *Caloplaca, Xanthoria*), protecting the photobionts from excessive solar radiation (Gauslaa and McEvoy, 2005). The antimicrobial activity of both norstictic acid and parietin has been demonstrated against a small set of bacteria and fungi of medical interest (Tay et al., 2004; Manojlovic et al., 2005).

In order to assay the potential applicability of the investigated LSM to control biological patinas on the stone cultural heritage, their interaction with a historical and culturally significant stone material (the white Carrara marble) was analysed with regard to the chromatic alteration, having a high relevance in stone conservation (Prieto et al., 2007; Tretiach et al., 2007). The necessity to perform further tests on the effects of LSM on other physico-chemical properties of stone materials and to assess their biocide effects in situ, i.e. on microorganisms growing on rock substrates, is finally addressed.

2. Material and methods

2.1. Tested organisms and lichen secondary metabolites

Strains of the MCF *Coniosporium perforans* Sterflinger (CBS885.95), *Coniosporiumuncinatum* De Leo, Urzì, De Hoog (CBS100212) and *Coniosporium apollinis* Sterflinger (CBS109867) were purchased from Centralbureau von Schimmelcultures (CBS, The Netherlands) and maintained on Malt Agar Extracts (MEA) at 15 °C. Another MCF isolated from the travertine blocks of the Roman Theatre of Aosta, Italy (MCF-AO), whose ITS sequence (JF809604) matched most closely to GenBank accession sequences of the dematiaceous microcolonial fungus *Phaeococcomyces chersonesos* (AJ507323.4) (Gazzano, 2010), was also maintained in the same culture conditions (hereafter indicated as *Phaeococcomyces* sp.).

Strains of the green alga *Scenedesmus ecornis* (Ehrenberg) Chodat (SAG2332) and the cyanobacterium*Chroococcus minutus* (Kützing) Nägeli (SAG41.79) were purchased from Sammlung für Algenkulturen von Göttingen (SAG) and maintained on agarized Trebouxia Medium (TB; Ahmadjian, 1993) and BG11 (Castenholz, 1988), respectively, at 20 °C in the light.

Fungal, algal and cyanobacterial colonies were at first treated with mixtures having different acetone/H_2O vol/vol ratios (0/100, 10/90, 50/50, 100/0) to select a suitable solvent for the solubilisation of the lichen metabolites which are poorly soluble in water (Elix and Stocker-Wörgötter, 2008). Three lichen metabolites were thereafter assayed (Fig. 1): (+)-usnic acid, in some cases reported as the more active enantiomer (Cocchietto et al., 2002; Ingólfsdóttir, 2002), was purchased by Sigma–Aldrich (St. Luis, MO, USA); norstictic acid and parietin were extracted from lichen thalli. For all experiments purified water (ELGA Purelab, Elga, UK) was used.

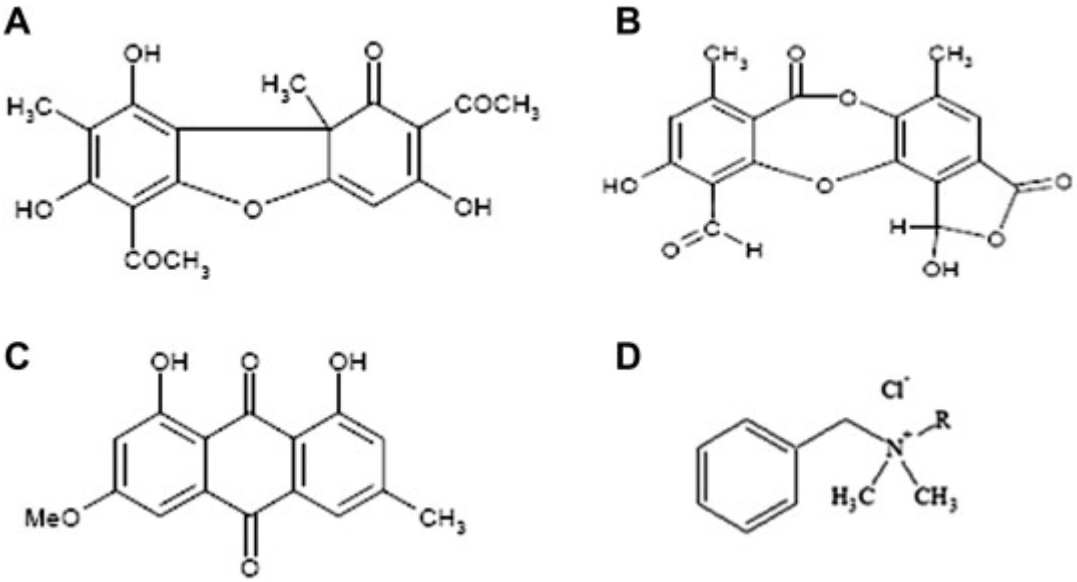

Fig. 1.

Chemical structures of usnic acid (A), norstictic acid (B), parietin (C) and belzalkonium chloride (D; R = –C_8H_{17}…$C_{18}H_{37}$).

2.2. Extraction and purification of lichen metabolites

Norstictic acid and parietin were extracted from *Pleurosticta acetabulum* (Neck.) Elix & Lumbsch and*Xanthoria parietina* (L.) Th. Fr., respectively. Thallus fragments of 20–40 mg were incubated for

1 h within 1 ml of acetone. The obtained solutions were filtered (0.45 µm; Albet, Barcelona, Spain) and the target metabolites were purified and collected using a HPLC system composed of Waters (Milford, MA, USA) components, including Waters 1525 binary HPLC Pump, Waters 2389 UV–Vis detector and Waters Fraction Collector-III. The column was a dC18 Atlantis ® reversed-phase column (150 × 4.6 mm, 5 µm). The mobile phase was 1 ml min^{-1} methanol (99.8%, Fluka, Buchs, Switzerland). According to Huneck and Yoshimura (1996), norstictic acid was detected on the basis of its UV absorption maxima at 212 nm, 239 nm, 270 nm and 317 nm; parietin was detected on the basis of UV–Vis absorption at 266 nm, 288 nm and 431 nm. The collected purified fractions were dried using a rotary vacuum evaporator (RE300, Stuart, UK).

2.3. Solubilisation of lichen metabolites

According to the preliminary assays on the acetone effects on the tested organisms, usnic acid and the obtained precipitates of norstictic acid and parietin were solubilised in an acetone/water 10/90 vol/vol mixture. The concentrations of the lichen metabolites were assessed using a DU530 Life Science UV–Vis Spectrophotometer (Beckman, Pasadena, CA, USA) on the basis of UV absorption at 270 ($\log_\varepsilon$ = 4.02) and 317 nm (3.70) for norstictic acid, at 266 nm (4.36) and 431 nm (4.20) for parietin, and at 290 nm (4.45) and 325 (3.85) for usnic acid. As acetone avoided the measure of UV absorption of water/acetone mixtures, sub-samples of these latter were dried with the evaporator and re-suspended in methanol for the analyses. The final concentrations of norstictic acid, parietin and usnic acid in the acetone/water 10/90 vol/vol mixture were 0.05 mM, 0.05 mM and 0.02 mM.

2.4. Evaluation of the effects of lichen metabolites on the tested organisms

Three mm diameter discs of mycelium were cut from the edge of young colonies of the MCF strains and inoculated on plastic Petri plates containing MEA (one inoculum per plate). Two mm diameter cyanobacterial and algal pellets were sampled from the edge of the stock colonies and inoculated on plastic Petri plates containing BG11 and TM, respectively (one inoculum per plate).

Fifty microliters of the metabolite solutions were poured on each inoculum of each organism, the liquid spreading on the culture medium up to a distance of approx. 0.7 mm from the inoculum. Water and the acetone/water 10/90 vol/vol mixture were used as negative controls. Benzalkonium chloride (1% in pure water: ~0.03 M; Fluka) was used as positive control. Five replicates were analysed for each study case.

Growth measurements on MCF were carried out 30 days after the incubation at 15 °C. Colony areas were measured by image analysis through colour-based pixel classification using WinCAM Pro 2007d software (Regent's Instruments, Nepean, Canada; technical details are reported in Supplementary Materials 1). The results were statistically analysed by means of ANOVA with post-hoc Tukey test using Systat 10.2.

The photosynthetic organisms were examined under a Nikon Eclipse 300 microscope, equipped with a B-2A filter block (450–490 nm excitation, 520 nm emission) and a Nikon Ds-5M digital camera, 15 days after the incubation. The algal and cyanobacterial viability was evaluated with reference to their chlorophyll epifluorescence (Tretiach et al., 2007). Representative photos of the colonies were acquired, the hue (colour model Hue-Saturation-Intensity) of the chlorophyll epifluorescence was measured through colour-based pixel classification using WinCAM Pro 2007d software (Regent's Instruments, Nepean, Canada) and the percentage of cells displaying red (Hue<15), orange-yellow (Hue between 16 and 50) and no epifluorescence (Hue>50), indicating no/low, medium and high sensitivity to the treatment, respectively, was calculated. The experiment

was performed by incubating colonies at 20 °C and thereafter repeated at 35 °C in order to mimic the high temperatures which often characterize the surfaces of stone materials during warm seasons (Camuffo, 1998).

2.5. Evaluation of the effects of lichen metabolites on the colour of rock surfaces

Polished surfaces of white Carrara marble coupons (approx. 20 cm^2) were poured with the metabolite solutions (25 µl cm^{-2}) and air dried at room temperature. Acetone/water 10/90 vol/vol and benzalkonium chloride 1% were used as controls.

The spectral reflection factor of the samples was evaluated with a portable spectro-colorimeter MiniScan XE by Hunterlab in 45/0 geometry (the sample is irradiated at 45° of incidence and the reflected radiation is evaluated at 0° of observation in order to avoid measurement of the specular radiation) with a circular measuring area of about 2.5 cm radius able to cover quite the whole surface of samples.

The CIE 1976 L*a*b* colorimetric coordinates with CIE D65 reference illuminant for Daylight and 2° observer were calculated and the colour difference ΔE evaluated on samples before and after the treatment with the LSM. Because of the homogeneity of the tested stone material, one sample was considered representative for the evaluation of each treatment. Every sample was measured in three different positions (rotated around the normal) in order to consider possible anisotropies due to polishing in the reflected spectrum.

3. Results and discussion

3.1. Microorganism sensibility to benzalkonium chloride and acetone

The growth of all the four MCF species was inhibited by benzalkonium chloride (Fig. 2). This widely used biocide also yielded a complete discolouration of both the cyanobacterial and algal colonies. Fifteen days after the treatment, the red epifluorescence typical of the intact cells and observed in the blanks (water-treated) turned to green, indicating a damage to chlorophyll molecules.

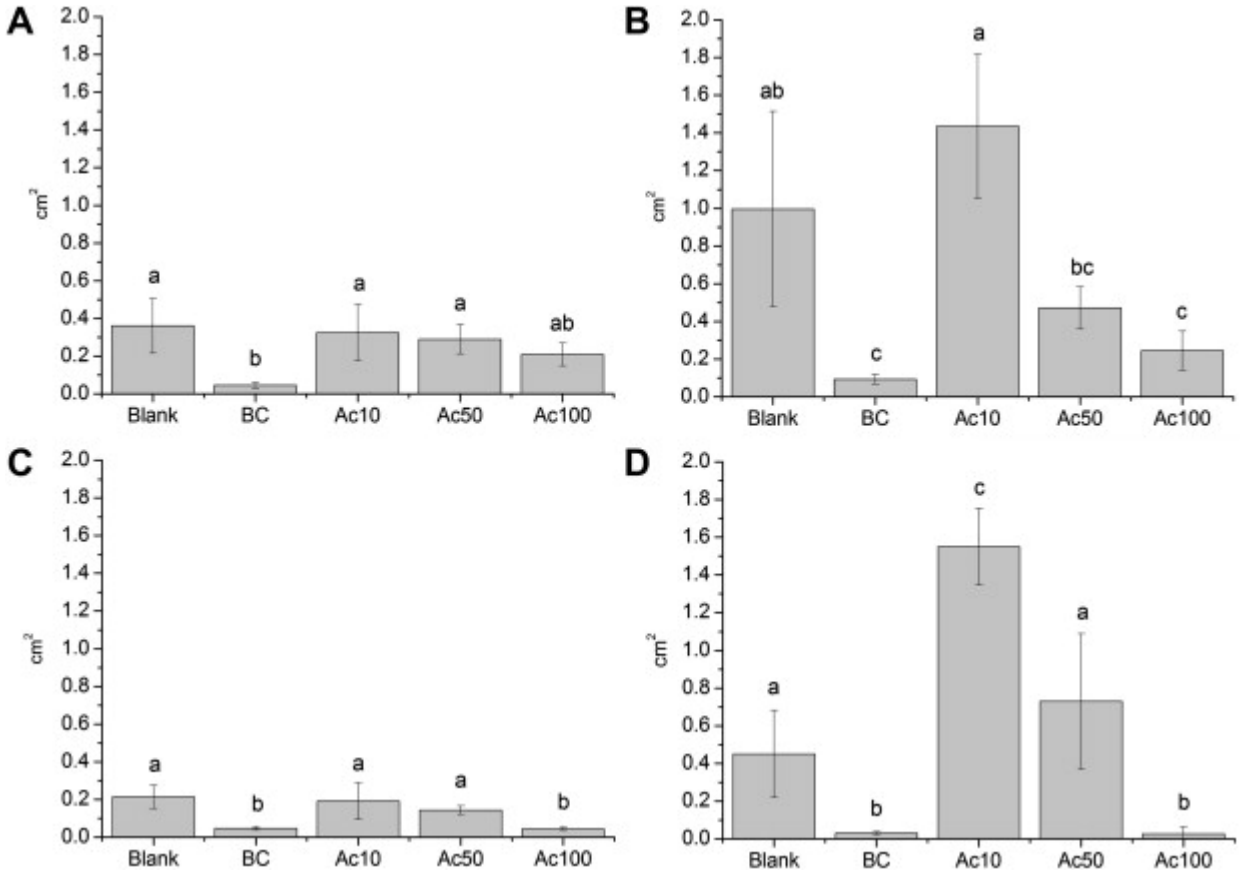

Fig. 2.

Effect of different acetone/H_2O vol/vol ratios on the areal growth of MCF. A, *Coniosporium apollinis*; B, *C. perforans*; C,*C. uncinatum*; D, *Phaeococcomyces* sp. Data are given as mean values ± SD. Blank = water; BC = benzalkonium chloride 0.03M; Ac10 = 10/90 acetone/H_20 vol/vol ratio; Ac50 = 50/50 acetone/H_20 vol/vol ratio; Ac100 = pure acetone. According to Tukey's test, columns which do not share at least one letter are statistically different

This result confirms the well-known efficacy of relatively low concentrations of benzalkonium chloride (1%) against both fungi and photoautotrophic patinas on stone materials (Caneva et al., 2008 with refs. therein). On the other hand, recent researches and field evidences increasingly discourage the widespread use of nitrogen containing biocides (as ammonium quaternary salts and, in particular, benzalkonium chloride), which should be avoided in order to not serve nutrients for micro- and macro-organisms and favour rapid recolonizations (Scheerer et al., 2009). Moreover, the wide use of sub-inhibitory concentrations of benzalkonium chloride has been suggested to induce a significant reduction of microbial sensitivity to biocides and to drive the evolution of polyextremotolerant fungi towards acquiring the missing virulence factors and further enhancing their stress tolerance (Gostinčar et al., 2011).

The evaluation of other chemicals to control rock dwelling organisms has also to consider the definition of a solvent which does not affect the reliability of the selected assays (Caneva et al., 2008). Acetone was previously suggested as one of the safest solvents to be used in fungal bioassays, its toxicity being mostly related to high concentrations (Eloff et al., 2007). In the case of the assayed MCF, each species showed a different sensitivity to the different acetone/H_2O vol/vol ratios (Fig. 2). All acetone mixtures did not significantly affect the growth of *C. apollinis* with respect to the blank, although a slight average decrease was detected in pure acetone. *C. perforans* was inhibited by both the 50/50 acetone/water mixture and the pure acetone, while it was slightly

stimulated by the 10/90 acetone/water mixture. *C. uncinatum* was inhibited by pure acetone as much as by benzalkonium chloride, while was not affected by the other acetone treatments. *Phaeococcomyces* sp. was significantly stimulated by the 10/90 acetone mixture, was not affected by the 50/50 mixture and was inhibited by pure acetone.

Different acetone concentrations, ranging from 2 to 50% were reported to inhibit the growth of fungi, each species showing a different sensibility (Burrell and Corke, 1980; Eloff et al., 2007). However, it is worth noting that the results of previous experiments dealt with bioassays in liquid media, while in the current research the acetone content is limited to the quantity of solution poured on the solid culture medium in order to mimic the application of chemicals on the colonized stone surfaces.

At both 20 °C and 35 °C, the 10/90 acetone/water ratio did not affect the *Scenedesmus* and *Chroococcus*viability estimated on the basis of the percentage occurrence of cells maintaining the red epifluorescence as indicator of intact chlorophyll (Fig. 3). Both inhibition and stimulation of algae and cyanobacteria by acetone were previously reported depending on the effects of solvent interactions with the cell membranes, inducing cytological damages or, oppositely, stimulating photosynthetic activity as a consequence of the increased permeability to CO_2 (Stratton and Corke, 1981; Stratton, 1987, 1989).

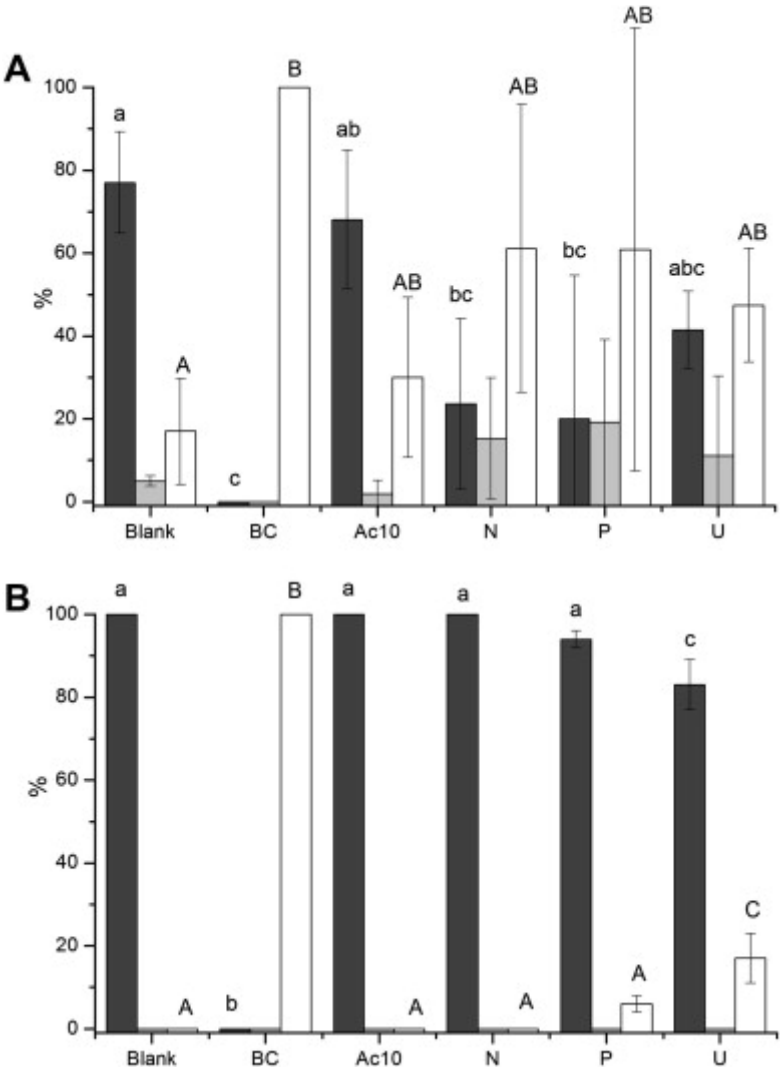

Fig. 3.

Effect of lichen secondary metabolites (dissolved in 10/90 acetone/H_20 vol/vol ratio) on the chlorophyll fluorescence of*Chroococcus minutus* (A) and *Scenedesmus ecornis* (B) incubated at 35 °C. %, percentage of cells showing red (black columns), orange/yellow (grey columns) and no chlorophyll epifluorescence (white columns). Data are given as mean values ± SD. Blank = water; BC = benzalkonium chloride 0.03M; Ac10 = 10/90 acetone/H_20 vol/vol ratio; N = norstictic acid; P = parietin; U = usnic acid. According to Tukey's test, columns which do not share at least one letter are statistically different

In the examined cases, the 10/90 acetone/water ratio did not negatively affect both MCF and photosynthetic organisms, appearing suitable to solubilise lichen metabolites and assay their effects on the selected species.

3.2. Microorganism sensibility to lichen secondary metabolites

The growth of all the MCF species was significantly inhibited by all the three assayed lichen metabolites, approx. 10^{-2} mM, which showed the same effect of benzalkonium chloride (Fig. 4). The average decrease in areal growth was higher for the three *Coniosporium* species (ranging from −85 to −95%) than in the case of *Phaeococcomyces* (−75%). The similar sensibility of the different species may be related with the high phylogenetic relatedness of the assayed species, which recently yielded their assignment to the same genus, i.e. *Knufia* (Tsuneda et al., 2011). Anyway, the homogeneous response of the tested fungi to the different substances is noteworthy in the complex framework of responses to different LSM exhibited by several fungal species previously assayed with usnic acid (Cocchietto et al., 2002; Ingólfsdóttir, 2002) and several other LSM. Similar or even higher concentrations of (+)-usnic acid were shown to not affect plant fungal pathogens, which were instead partially affected in their growth by similar concentrations (10^{-2} mM) of (−)-usnic acid (−10% growth) and evernic acid (from −40% to −60%) (Halama and Van Haluwin, 2004). On the other hand, (+)-usnic acid 0.002 mM and norstictic acid 0.1 mM were shown to inhibit the growth of*Candida* species, while no effect was detected against ten species of filamentous fungi (Tay et al., 2004). Parietin at concentrations ranging from 0.07 to 0.6 mM inhibited the growth of human fungal pathogens (Manojlovic et al., 2005).

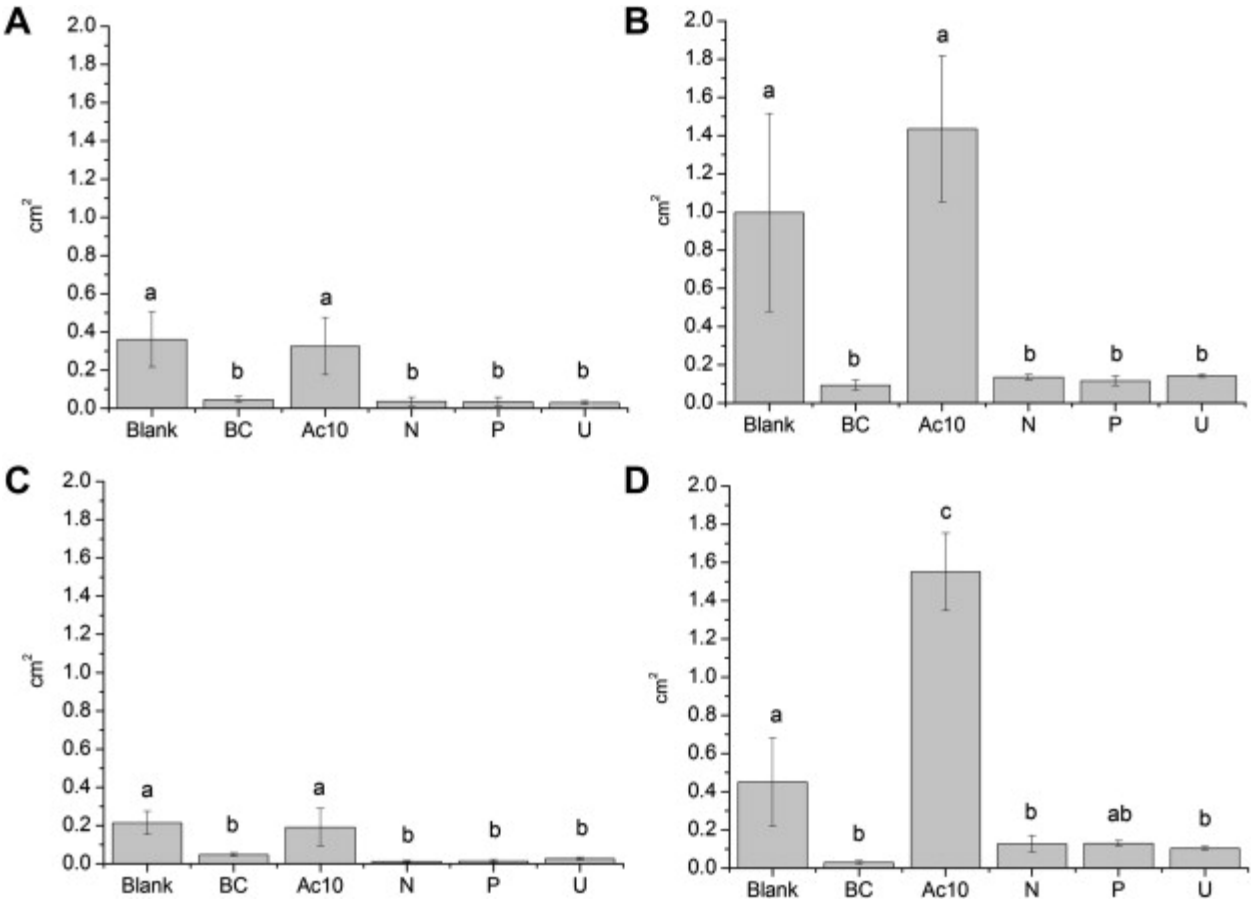

Fig. 4.

Effect of lichen secondary metabolites (dissolved in 10/90 acetone/H_20 vol/vol ratio) on the areal growth of MCF. A, *Coniosporium apollinis*; B, *C. perforans*; C, *C. uncinatum*; D, *Phaeococcomyces* sp. Data are given as mean values ± SD. Blank = water; BC = benzalkonium chloride 0.03M; Ac10 = 10/90 acetone/H_20 vol/vol ratio; N = norstictic acid; P = parietin; U = usnic acid. According to Tukey's test, columns which do not share at least one letter are statistically different

At both 20° and 35°, upon the treatment with benzalkonium chloride, the chlorophyll epifluorescence of the investigated cyanobacteria and green algae was not detected, indicating the death of the colonies. On the other hand, *Chroococcus* and *Scenedesmus* showed a different sensitivity to the lichen metabolites under the different temperature regimes of incubation. At 20 °C, both the green algae and the cyanobacteria maintained the red chlorophyll fluorescence upon all the treatments, including LSM and the 10/90 acetone/water control. At 35 °C, *Chroococcus* showed a high variability in the percentage occurrence of intact cells (red epifluorescence), suffering cells (orange-yellow epifluorescence) and dead cells (loss of epifluorescence) in the different replicates of each assayed case (Fig. 3A). The cyanobacterial colonies treated with norstictic acid and parietin displayed a significant decrease of intact cells (−70%) with respect to the water control, while a lower decrease was observed with usnic acid (−47%) and 10/90 acetone/water (−12%). Although no significant differences were detected between the treatments with LSM and the 10/90 acetone/water control, a trend in the reduction of intact cells is recognizable in the series: 10/90 acetone/water < usnic acid < norstictic acid, parietin < benzalkonium chloride. As temperatures of 35 °C or more often characterizes stone surfaces exposed to the sun radiation, where black patinas frequently occur (Caneva et al., 2008),

the results suggest a potential sensibility of cyanobacterial colonies to LSM in environmental conditions. At 35 °C, *Scenedesmus* showed a low, but significant decrease of intact cells after the treatment with usnic acid with respect to the water and 10/90 acetone/water controls, while was not affected by the other lichen secondary metabolites (Fig. 3B). High tolerance of usnic acid was previously shown for green algae (*Trebouxia* ssp.) being photobionts in the lichen symbiosis, while the photosynthetic activity of the free living *Chlamydomonas reinhardtii* was affected by usnic acid 0.14 mM (Takahagi et al., 2008).

It is worth noting that the tested MCF are known to stop their growth at high temperatures around 35 °C and to restart it when they return to the moderate temperatures (Sterflinger, 1998) at which we observed their sensibility to LSM. The sensibility of MCF to LSM is thus directly associated to a metabolically active condition, while the synergic effect of LSM and high temperatures is needed to affect cyanobacterial and green algal viability.

3.3. Chromatic alteration of white *Carrara* marble caused by lichen secondary metabolites

Historical and culturally significant white marbles are frequently spoiled by black patinas (Gorbushina and Broughton, 2009; Sterflinger, 2010), but may also be altered in colour by biocide treatments (Nugari et al., 1993). In the examined case, the application of all the three LSM caused negligible changes in colour to Carrara marble coupons (Table 1). The colour differences after the LSM treatments ($\Delta E<0.5$) were lower than that determined by benzalkonium chloride ($\Delta E = 0.9$) and in all samples under the just perceivable threshold (commonly defined $2\leq\Delta E\leq5$ depending on colour and visual adaptation). Moreover, the measured values generally fell in the lower range of chromatic variations reported for several biocidal treatments on different lithotypes (ΔE ranging from 0.24 to 18.45) (Tretiach et al., 2007 with refs. therein). The slight chromatic alteration was mainly correlated to a variation in lightness axis (L*) and can not be attributed to a different sampling area on the rock coupons (acceptance area of the measuring instrument was large enough to cover quite the whole sample). Variations only in lightness likely depended on the effects of acetone (similar ΔL values for acetone/water 10/90 and LSM treatments) on the gloss of the surface (Benavente et al., 2003) rather that on the dissolved LSM. Accordingly, LSM solutions at the assayed concentrations are not appreciably coloured, differing from markedly coloured lichen extracts in pure acetone which may yield remarkable chromatic variations on rock surfaces.

Table 1.
CIE 1976 lab colour coordinates of white Carrara marble before and after treatments with lichen secondary metabolites (45/0 geometry, D65 reference illuminant). BC = benzalkonium chloride 0.03M; Ac10 = 10/90 acetone/H_2O vol/vol ratio; N = norstictic acid; P = parietin; U = usnic acid.

	Untreated						After treatment						CIE ΔE color difference			
	L*		a*		b*		L*		a*		b*					
Sample	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	ΔL	Δa	Δb	ΔE
BC	89.98	0.13	0.11	0.03	0.66	0.02	89.07	0.53	0.13	0.02	0.84	0.15	0.91	−0.02	−0.19	0.93
Ac10	89.81	0.39	0.16	0.03	0.68	0.02	89.31	0.39	0.18	0.02	0.59	0.01	0.49	−0.02	0.09	0.50
N	89.76	0.15	0.17	0.01	0.86	0.01	89.30	0.13	0.16	0.02	0.84	0.01	0.45	0.01	0.02	0.45
P	87.72	0.27	0.28	0.01	0.83	0.02	87.38	0.16	0.30	0.02	0.75	0.05	0.34	−0.03	0.08	0.35
U	89.51	0.03	0.19	0.01	0.81	0.01	89.14	0.09	0.16	0.02	0.73	0.01	0.37	0.02	0.07	0.38

This information on the chromatic effect of the LSM application only represents a first step in the extensive evaluation of the LSM/substrate interaction where perceptive investigations (colour and gloss variations) should support the potential applicability of LSM on the stone cultural heritage (Caneva et al., 2008). The negligible chromatic alteration by LSM has to be confirmed upon

simulated aging of the treated surfaces, including exposition to wide temperature ranges and UV/Vis irradiation, and considering the influence of surface gloss (Benavente et al., 2003). Further physico-chemical rock parameters which may be affected by biocide application include mineral stability, water absorption capacity, permeability and surface tension (Tretiach et al., 2007 with refs. therein). In this context, it is worth noting that incubations of silicate (Turci et al., 2007) and carbonate (unpublished data) minerals with the assayed concentration of norstictic acid, the LSM mostly known for chelating properties, determined chemical modifications limited to the surface atomic layers, yielding an overall metal leaching negligibly higher than that detected in water incubations.

3.4. Conclusive remarks

The observed inhibitory effects suggest an allelopathic role of the examined LSM against MCF, cyanobacteria and free-living green algae which are the potential competitors of saxicolous lichens on the rock surfaces. Similar findings have long been argued with regard to higher plants and mosses which are potential competitors of terricolous lichens on soil (Lawrey, 1984; Favero-Longo and Piervittori, 2010). However, recent assessments on the mobilization of LSM in natural settings have suggested that, because of their low solubility in water (Elix and Stocker- Wörgötter, 2008), their allelopathic role should be less emphasized or even reconsidered (Stark et al., 2007). On the other hand, LSM occurrence has been detected within porous rocks colonized by lichens some millimetres below the surface (Bjelland and Thorseth, 2002), accounting for their mobility through the rock-surface micro-niche and not excluding a possible allelopathic role. In particular, the allelopathic effect may be directly related on the LSM concentration on the surface of the thalli, where they may inhibit the germination and growth of their potential competitors, as already suggested by Takahagi et al. (2008) with regard to free-living green algae. Accordingly, LSM thalline extracts were shown to inhibit the growth of a set of hypocrealean fungi isolated from non-lichen substrates, while they were better tolerated by other strains isolated from lichens (Lawrey et al., 1994).

In conclusion, the potential application of LSM for biocontrol may be possibly hypothesized not only in weed management (Duke et al., 2002), against plant pathogens (Halama and Van Haluwin, 2004), and bacterial biofilms on polymer surfaces of medical devices (Francolini et al., 2004), but also to inactivate rock dwelling microorganisms causing black patinas on the stone cultural heritage.

In this perspective, we report here that the investigated LSM, at the assayed concentration, do not determine a perceivable chromatic alteration on the white Carrara marble. An extensive screening of their potential impact on other physico-chemical properties of stone materials has now to be carried on in order to define the most suitable metabolite(s) to be tested in situ for assaying the inhibitory effects directly on the colonized rock surfaces.

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References

Ahmadjian, 1993 V. Ahmadjian **The Lichen Symbiosis** (second ed.)Wiley and Sons, New York (1993)

Benavente et al., 2003 D. Benavente, F. Martinez-Verdù, A. Bernabeu, V. Viqueria, R. Fort, M.A. Garcia del Cura, C. Illeuca, S. Ordonez **Influence of surface roughness on color changes in building stones** Colour Research and Application, 28 (2003), pp. 343–351

Bjelland and Thorseth, 2002 T. Bjelland, I.H. Thorseth **Comparative studies of the lichen-rock interface of four lichens in Vingen, western Norway** Chemical Geology, 192 (2002), pp. 81–98

Boustie et al., 2011 J. Boustie, S. Tomasi, M. Grube **Bioactive lichen metabolites: alpine habitats as an untapped source** Phytochemistry Reviews, 10 (2011), pp. 287–307

Brimblecombe and Grossi, 2005 P. Brimblecombe, C.M. Grossi **Aesthetic thresholds and blackening of stone buildings** Science of the Total Environment, 349 (2005), pp. 175–189

Burrell and Corke, 1980 R.E. Burrell, C.T. Corke **Interactions of the solvent acetone with the fungicides Benomyl and Captan in fungal assays** Bulletin of Environmental Contamination and Toxicology, 25 (1980), pp. 554–561

Camuffo, 1998 D. Camuffo **Microclimate for Cultural Heritage** Developments in Atmospheric Science, vol. 23Elsevier, Amsterdam (1998)

Caneva et al., 2008 G. Caneva, M.P. Nugari, O. Salvadori **Plant Biology for Cultural Heritage**The Getty Conservation Institute, Los Angeles (2008)

Castenholz, 1988 R.W. Castenholz **Culturing methods for cyanobacteria** Methods in Enzymology, 167 (1988), pp. 68–93

Cocchietto et al., 2002 M. Cocchietto, N. Skert, P.L. Nimis, G. Sava **A review on usnic acid, an interesting natural compound** Naturwissenschaften, 89 (2002), pp. 137–146

Dayan and Romagni, 2001 F.E. Dayan, J.G. Romagni **Lichens as a potential source of pesticides** Pesticide Outlook, 12 (2001), pp. 229–232

Delgado Rodrigues and Valero, 2003 J. Delgado Rodrigues, J. Valero **A brief note on the elimination of dark stains of biological origin** Studies in Conservation, 48 (2003), pp. 17–22

Duke et al., 2002 S.O. Duke, F.E. Dayan, A.M. Rimando, K.K. Schrader, G. Aliotta, A. Oliva, J.G. Romagni **Chemicals from nature and weed management** Weed Science, 50 (2002), pp. 138–151

Elix and Stocker- Wörgötter, 2008 J.A. Elix, E. Stocker- Wörgötter **Biochemistry and secondary metabolites** T.H. Nash III (Ed.), Lichen Biology (second ed.), Cambridge University Press, Cambridge (2008), pp. 104–133

Eloff et al., 2007 J.N. Eloff, P. Masoko, J. Picard **Resistance of animal fungal pathogens to solvents used in bioassays** South African Journal of Botany, 73 (2007), pp. 667–669

Favero-Longo and Piervittori, 2010 S.E. Favero-Longo, R. Piervittori **Lichen-plant interactions** Journal of Plant Interactions, 5 (2010), pp. 163–177

Favero-Longo et al., 2011 S.E. Favero-Longo, C. Gazzano, M. Girlanda, D. Castelli, M. Tretiach, C. Baiocchi, R. Piervittori **Physical and chemical deterioration of silicate and carbonate rocks by meristematic microcolonial fungi and endolithic lichens (Chaetothyriomycetidae)** Geomicrobiology Journal, 28 (2011), pp. 732–744

Francolini et al., 2004 I. Francolini, P. Norris, A. Piozzi, G. Donelli, P. Stoodley **Usnic acid, a natural antimicrobial agent able to inhibit bacterial biofilm formation on polymer surfaces** Antimicrobial Agents and Chemotherapy, 48 (2004), pp. 4360–4365

Gauslaa and McEvoy, 2005 Y. Gauslaa, M. McEvoy **Seasonal changes in solar radiation drive acclimatation of the sun-screening compound parietin in the lichen *Xanthoria parietina*** Basic and Applied Ecology, 6 (2005), pp. 75–82

Gazzano, 2010 Gazzano, C., 2010. Lichenized and non-lichenized fungi in the Roman Theatre of Aosta (NW-Italy): from biodiversity detection to biodeterioration mimicking and modelling. PhD thesis, University of Torino, Italy.

Gorbushina et al., 2003 A.A. Gorbushina, K. Whitehead, T. Dornieden, A. Niesse, A. Schulte, J.I. Hedges **Black fungal colonies as units of survival: hyphal mycosporines synthesized by rock-dwelling microcolonial fungi** Canadian Journal of Botany, 81 (2003), pp. 131–138

Gorbushina and Broughton, 2009 A.A. Gorbushina, W.J. Broughton **Microbiology of the atmosphere-rock interface: how biological interactions and physical stresses modulate a sophisticated microbial ecosystem** Annual Review of Microbiology, 63 (2009), pp. 431–450

Gostinčar et al., 2011 C. Gostinčar, M. Grube, N. Gunde-Cimerman **Evolution of fungal pathogens in domestic environments?** Fungal Biology, 115 (2011), pp. 1008–1018

Halama and Van Haluwin, 2004 P. Halama, C. Van Haluwin **Antifungal activity of lichen extracts and lichenic acids** BioControl, 49 (2004), pp. 95–107

Hauck et al., 2010 M. Hauck, S.-R. Jürgens, C. Leuschner **Norstictic acid: correlations between ist physico-chemical characteristics and ecological preferences of lichens producing this depsidone** Environmental and Experimental Botany, 68 (2010), pp. 309–313

Huneck and Yoshimura, 1996 S. Huneck, I. Yoshimura **Identification of Lichen Substances** Springer, Berlin (1996)

Ingólfsdóttir, 2002 K. Ingólfsdóttir **Usnic acid** Phytochemistry, 61 (2002), pp. 729–736

Lawrey, 1984 J.D. Lawrey **Biology of Lichenized Fungi** Praeger Publishers, New York (1984)

Lawrey et al., 1994 J.D. Lawrey, A.Y. Rossman, R. Lowen **Inhibition of selected hypocrealean fungi by lichen secondary metabolites** Mycologia, 86 (1994), pp. 502–506

Liao et al., 2010 C. Liao, M.D. Piercey-Normore, J.L. Sorensen, K. Gough ***In situ*** **imaging of usnic acid in selected *Cladonia* ssp. by vibrational spectroscopy** Analyst, 135 (2010), pp. 3242–3248

Macedo et al., 2009 M.F. Macedo, A.Z. Miller, A. Dionísio, C. Saiz-Jimenez **Biodiversity of cyanobacteria and green algae on monuments in the Mediterranean Basin: an overview** Microbiology, 155 (2009), pp. 3476–3490

Manojlovic et al., 2005 N.T. Manojlovic, M. Novakovic, V. Stevovic, S. Solujic **Antimicrobial metabolites from three Serbian *Caloplaca*** Pharmaceutical Biology, 43 (2005), pp. 718–722

McEvoy et al., 2006 M. McEvoy, L. Nybakken, K.A. Solhaug, Y. Gauslaa **UV triggers the synthesis of the widely distributed secondary lichen compound usnic acid** Mycological Progress, 5 (2006), pp. 221–229

Mitrović et al., 2011 T. Mitrović, S. Stamenković, V. Cvetković, S. Tošić, M. Stanković, I. Radojević, O. Stefanović, L. Čomić, D. Đačić, M. Ćurčić, S. Marković **Antioxidant, antimicrobial and antiproliferative activities of five lichen species** International Journal of Molecular Sciences, 12 (2011), pp. 5428–5448

Molnár and Farkas, 2010 K. Molnár, E. Farkas **Current results on biological activities of lichen secondary metabolites: a review. Zeitschrift fur Naturforschung** Section C - Journal of Biosciences, 65 (2010), pp. 157–173

Nugari et al., 1993 M.P. Nugari, S. D'Urbano, O. Salvadori **Test methods for comparative evaluation of biocide treatments** M.J. Thiel (Ed.), Conservation of Stone and Other Materials, vol. 1Spon, London (1993), pp. 295–302

Nugari et al., 2009 M.P. Nugari, A.M. Pietrini, G. Caneva, F. Imperi, P. Visca **Biodeterioration of mural paintings in a rocky habitat: the Crypt of the Original Sin (Matera, Italy)** International Biodeterioration and Biodegradation, 63 (2009), pp. 705–711

Oksanen, 2006 I. Oksanen **Ecological and biotechnological aspects of lichens** Applied Microbiology and Biotechnology, 73 (2006), pp. 723–734

Prieto et al., 2007 B. Prieto, N. Aira, B. Silva **Comparative study of dark patinas on granitic outcrops and buildings** Science of the Total Environment, 381 (2007), pp. 280–289

Scheerer et al., 2009 S. Scheerer, O. Ortega-Morales, C. Gaylarde **Microbial-deterioration of stone monuments – an updated overview** Advances in Applied Microbiology, 66 (2009), pp. 97–139Smith et al., 2009

C.W. Smith, A. Aptroot, B.J. Coppins, A. Fletcher, O.L. Gilbert, P.W. James, P.A. Wolseley **The Lichens of Great Britain and Ireland** The British Lichen Society, London (2009)

Stark et al., 2007 S. Stark, M.M. Kytöviita, A.B. Neumann **The phenolic compounds of *Cladonia* lichens are not antimicrobial in soils** Oecologia, 152 (2007), pp. 299–306

Sterflinger, 1998 K. Sterflinger **Temperature and NaCl-tolerance rock-inhabiting meristematic fungi** Antonie Van Leewenhoek, 74 (1998), pp. 271–281

Sterflinger, 2010 K. Sterflinger **Fungi: their role in deterioration of cultural heritage** Fungal Biology Reviews, 24 (2010), pp. 47–55

Stratton, 1987 G.W. Stratton **Toxic effects of organic solvents on the growth of blue-green algae** Bulletin of Environmental Contamination and Toxicology, 38 (1987), pp. 1012–1019

Stratton, 1989 G.W. Stratton **Effect of the solvent acetone on membrane integrity in the green alga *Chlorella pyrenoidosa*** Bulletin of Environmental Contamination and Toxicology, 42 (1989), pp. 754–760

Stratton and Corke, 1981 G.W. Stratton, C.T. Corke **Interactions between acetone and two pesticides towards several unicellular green algae** Bulletin of Environmental Contamination and Toxicology, 27 (1981), pp. 13–16

Takahagi et al., 2008 T. Takahagi, T. Endo, Y. Yamamoto, F. Sato **Lichen photobionts show tolerance against lichen acids produced by lichen mycobionts** Bioscience, Biotechnology, and Biochemistry, 72 (2008), pp. 3122–3127

Tay et al., 2004 T. Tay, A. Özdemir Türk, M. Yılmaz, H. Türk, M. Kıvanç **Evaluation of the antimicrobial activity of the acetone extract of the lichen *Ramalina farinacea* and its (+)-usnic acid, norstictic acid, and protocetraric acid constituents. Zeitschrift fur Naturforschung** Section C – Journal of Biosciences, 59 (2004), pp. 384–388

Tretiach et al., 2007 M. Tretiach, P. Crisafulli, N. Imai, H. Kashiwadani, K.H. Moon, H. Wada, O. Salvadori **Efficacy of a biocide tested on selected lichens and its effects on their substrata** International Biodeterioration and Biodegradation, 59 (2007), pp. 44–54

Tsuneda et al., 2011 A. Tsuneda, S. Hambleton, R.S. Currah **The anamorph genus *Knufia* and its phylogenetically allied species in *Coniosporium, Sarcinomyces*, and *Phaeococcomyces*** Botany, 89 (2011), pp. 523–536 and 887

Turci et al., 2007 F. Turci, S.E. Favero-Longo, M. Tomatis, G. Martra, D. Castelli, R. Piervittori, B. Fubini **A biomimetic approach to the chemical inactivation of chrysotile fibres by lichen metabolites** Chemistry – A European Journal, 13 (2007), pp. 4081–4093