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BIOFILMS ON EXPOSED MONUMENTAL STONES: MECHANISM OF FORMATION AND DEVELOPMENT OF NEW CONTROL METHODS

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Esame finale anno 2009

In memory of my professor,
Ion I. Bără

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ABREVIATIONS

A	Algophase
ABAs	AntiBiofouling Agents
AHL	Acylated Homoserine lactone
ARISA	Automated rRNA Intergenic Spacer Analysis
au	absorbance units
BA	Barrier filter emission
bp	base pairs
CBE	*Ceramium botryocarpum* Extract
CCD	Charge Coupled Device
CI	Cinnamaldehyde
CLSM	Confocal Laser Scanning Microscopy
CS	Capsaicin
DAPI	4',6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to DNA, used in fluorescence microscopy
ddNTP	dideoxyribonucleotide triphosphate
DM	Dichroic Mirrror
DNA	Deoxyribonucleic Acid
EM	Epiflourescence Microscopy
EPS	Extracellular Polymeric Substances
ESI-MS	Electrospray Ionization-Mass Spectrometry
EtBr	Ethidium Bromide, a fluorescent stain for nucleic acids
EtOH	Ethanol
FT-IR	Fourier Transform Infrared Spectroscopy
ITS	Internal Transcribed Spacer, used to know the genetic diversity among different strains of microorganisms by sequencing the ITS gene
LSM	Laser Scanning Microscopy
MeOH	Methanol
OD	Optical Density
OM	Optical Microscopy
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pAPS	Poly-Alkyl Pyridinium Salts
QQ	Quorum Quenching
QS	Quorum Sensing
rfu	relative fluorescent units
RH	Relative Humidity
rRNA	ribosomal Ribonucleic Acid
S	Silres BS OH 100
SEM	Scanning Electron Microscopy
SEM-EDX	Energy-Dispersive X-ray spectroscopy analysis
SYTO	a group of fluorescent nucleic acid stains
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
UV	Ultraviolet
ZA	Zosteric Acid
W pure	Silres BS 290
W	7% Silres BS 290 in isopropyl alcohol

ABSTRACT

The biodeterioration processes are wide spread in the natural environments and therefore all the monumental assets are susceptible to this kind of risk. Within the stone monumental artefacts artistic fountains can be considered the most vulnerable because of their particular exposure conditions which are extremely favorable to formation of phototrophic biofilms, giving rise to biodegradation processes related with physical-chemical and visual aspect alterations. Microbial diversity of five fountains (two from Spain and three from Italy) was investigated. It was observed an ample similarity between the biodiversity of monumental stones reported in literature and that one found in studied fountains. The achievement of microbial ecological data for this artifacts are very useful to identify the most diffuse microorganisms dwelling on these monumental assets, to investigate their possible risk for stone conservation and to understand and predict, with some approximation, their behavior when maintenance intervention of a fountain is required.

Mechanical procedures and toxic chemical products are usually employed to remove such phototrophic patinas. Alternative methods based on natural antifouling substances are recently experimented in the marine sector, due to their very low environmental impact and for the bio settlement prevention on partially immersed structures of ships. In the present work groups of antibiofouling agents (ABAs) were selected from literature for their ability to interfere, at molecular level, with the microbial communication system "quorum sensing", inhibiting the initial phase of biofilm formation. The efficacy of some natural antibiofoulants agents (ABAs) with terrestrial (Capsaicine - CS, Cinnamaldehyde - CI) and marine origin (Zosteric Acid - ZA, poly-Alkyl Pyridinium Salts – pAPS and *Ceramium botryocarpum* extract - CBE), incorporated into two commercial coatings (Silres BS OH 100 - S and Wacker Silres BS 290 - W) commonly used in stone conservation procedures were evaluated. The mixing of ABAs with a coating was considered necessary because their water solubility and considering the aqueous conditions of their application. The formation of phototrophic biofilms in laboratory conditions (on Carrara marble specimens and Sierra Elvira stone) and on two monumental fountains (Tacca's Fountain 2 - Florence, Italy and Fountain from Patio de la Lindaraja - Alhambra Palace, Granada, Spain) has been investigated in the presence or absence of these natural antifouling agents. Recent investigation methods for the biofilm development and efficacy evaluation of selected ABAs were

used as useful tools in understanding the morphology and behavior of biofilms and treatments, such as various microscopical techniques (OM, EM, CLSM, SEM), molecular biology (ARISA fingerprint method, molecular identification of the isolated strains), image analysis, FTIR and SEM-EDX.

The natural antibiofouling agents, at tested concentrations, demonstrated a certain inhibitory effect. The silane-siloxane based silicone coating (W) mixing with ABAs was more suitable with respect to ethyl silicate coating (S) and proved efficacy against biofilm formation only when incompletely cured. The laboratory results indicated a positive action in inhibiting the patina formation, especially for poly-alkyl pyridinium salts, zosteric acid and cinnamaldehyde, while on site tests revealed a good effect for zosteric acid.

However this partial curing most probably favors its removal in water environment with the consequence of eliminate the ABAs products, and hence more suitable supporting product must be investigated for this kind of application.

I. RESEARCH OBJECTIVES

Special attention to monumental fountains is given only when some others alteration types appear and their state of conservation must be evaluated (Not R., 1995; Cardilli L., 1979; Pietrini A.M., 1991; Peraza Zurita Y., 2002a). Usually, detailed data related with the microbial composition are not considered necessary for further restoration processes, biological patina/biofilms being controlled in the same way, by mechanical removal or biocide treatments. Taking into account that the colonization mechanisms, in exposed environment, is an endless process that restarts after each removal, the study of microbiological components can contribute to understanding which are the most adapted genera/species dwelling on these kind of artefacts, which are the most dangerous as biodeteriogens, and consequently to develop new methods for control this unwanted biological growth. The most suitable approach should be to block its initial formation. In order to achieve this aim, groups of antibiofouling agents (ABAs) were selected from literature for their ability to interfere, at molecular level, with the microbial communication system "quorum sensing", inhibiting the initial phase of biofilm formation. Such kind of innovative products were for the first time tested in the monumental heritage field.

The main objectives of this research activity are:

- to study the main microbial components (algae, cyanobacteria and fungi) of the phototrophic biofilms developed on monumental fountains by traditional and molecular methods and to compare the biodiversity of different fountains biotopes;
- to study the biological patina structure and the mechanism of its development on sound stone material;
- to characterize the selected natural antibiofouling agents and to develop and verify their efficiency and behavior in laboratory and on site, as innovative natural antibiofouling agents for inhibition of patina formation.

II. INTRODUCTION

II.1. Biofilms formation and evolution

Biofilms occur on all solid surfaces in aquatic habitats. This complex biocenosis consist principally of water (70 to 95% of the fresh weight), extracellular polymeric substances (Flemming H.C., 1993; Wahl M., 1998) and microorganisms such as phototrophs (algae, cyanobacteria, diatoms) and heterotrophs (bacteria, fungi, protozoa, nematods), which are embedded in this hydrated matrix (Norton T.A., 1998). The proportion of each group is varying seasonally and it is influenced by different habitats (Underwood A. J., 1984; Anderson M. J., 1995; Roeselers G., 2007). The biofilm contains cells debris, airborne particles, bacteria and spores together with amounts of inorganic material adsorbed from the substratum (Warscheid Th., 2008). The biopolymers act also in sticking the cells to the substratum, and their adhesive properties contribute to the formation and cohesion of biofilms (Albertano P., 2003).

The extracellular polymeric substances (glyocalyx, sheath or envelope) protect the microorganisms composing a biofilm by balancing changes in: humidity, temperature, osmotic pressure and pH. The ability of bacteria and cyanobacteria to release EPS is related to their nutritional status. In cyanobacteria, particularly, limitation in light or nutrients can increase production of polysaccharides (Albertano P., 2003). Based on its ion-exchange capacity, the biofilms even resist to the penetration of biocides, detergents or antibiotics, hindering the possible control of microbial biodeterioration processes in the long-term (Warscheid Th., 2008).

It is found that the surfaces exposed to river water are covered with bacteria and microcolonies after only 3 days (Lawrence J. R., 2003). The photosynthetic microorganisms develop easily on the stone surfaces and, once established, allow the growth of more complex microbial consortia formed by heterotrophic microroganisms which can exercise stronger deteriorating activity (Tiano P., 1993; Tomaselli L., 2000a; Crispim C. A., 2003; Zurita Y. P., 2005). A study related with the early stages of biofilm succession in a lentic freshwater environment reports the abundance of the green algae in the first 1-4 days, followed by the one of diatoms (5-7 days) and cyanobacteria (10-15 days) (Sekar R., 2004). Some authors give an important role to diatoms in the first steps of colonization (Wetherbee R., 1998; Peraza Zurita Y., 2002a).

Organisms or cells may be attracted by a substratum in a variety of ways, especially if they are motile, whereas not motile microorganism (many cyanobacteria, and diatoms) presumably are deposited on a substratum by gravity or water movement. This initial contact may result in a transitory chemical attraction that is difficult to characterize. Cell control attachment and release are depending on the habitability of the microhabitat, non-motile microorganism, such as diatoms, adjust their position on substrata by a complex mode of cell motility called "gliding" (Edgar L. A., 1984). The adhesive molecules of diatoms, have been fully characterized and seems that the polysaccharides are the candidates for the binding domain of the initial adhesion (Wetherbee R., 1998). Many authors consider very relevant the role of EPS in early development stages of a biofilm also for blue-green algae (Scott C., 1996). It seems that the capsular EPS has an important role during the initial stages of biofilm formation, since it facilitate the attachment of cells to the substrate (Decho A. W., 2000, Barranguet C., 2005).

Many bacterial species, in a population density-dependent, coordinate their responses to environmental changes using complex cell-to-cell communication mechanism, by secretion and uptake of small diffusible molecules. These molecules, used as signals, are oligopeptides in Gram-positive bacteria, AHL derivatives (Acyl Homoserine Lactone) in Gram-negative bacteria (including cyanobacteria) and γ-butyrolactones in *Streptomyces* species. Recent studies in medical field revealed that fungi, like bacteria, use quorum regulation to affect population-level behaviors such as biofilm formation and pathogenesis, being most extensively studied in *Candida albicans* (Hogan D. A., 2006).

Since biofilms typically contain high concentration of cells, cell-to-cell signals are believed to play an important role in its development . This phenomenon , known as Quorum Sensing (QS), influence the bacterial population coordinating important biological functions including motility, swarming, aggregation, plasmid conjugal transfer, virulence, sporulations, antibiotics biosynthesis, symbiosis, biofilm maintenance and differentiation (Gray K. M., 1997; Jiang X., 2006). In addition, recent findings have shown the ability to produce AHL autoinducers in bacteria isolated from prehistoric caves with wall paintings and in biofilms dominated by cyanobacteria (Laiz L., 1999).

The organization of fouling consists of three main levels: molecular fouling, micro-fouling and a macro-fouling (Barrios A. C., 2004). It is noted that the formation

of an organic molecular layer should be realized before the attachment of the microorganisms on a solid substrate (Yebra D. M., 2003). After that a reversible stage of adhesion of the primary colonisers starts, followed by their fixing and propagation. Finally a EPS matrix is formed and a three-dimensional structure is auto-organised (Fig. 1). The mature biofilm has a complex heterogeneity which confers stability and resistance to the biocoenosis (Nikolaev Y. A., 2007).

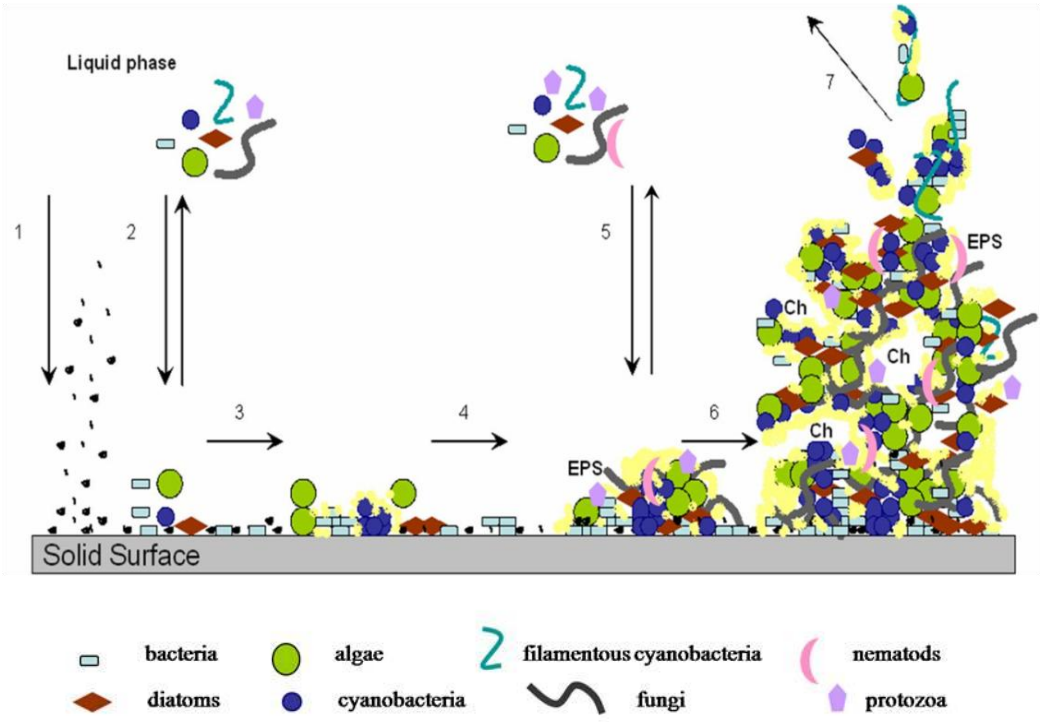


Fig. 1. Biofilm development : (1) - the establishment of the organic molecules (black spots); (2) – the reversible adhesion of micro-organisms – primary colonizers (bacteria, green algae, coccoid cyanobacteria and diatoms); (3) – the transition to the irreversible adhesion, multiplication, the start of EPS (extracellular polymeric substances) production; (4) – the start of a three-dimensional development of the biofilm structure; (5) - adhesion of secondary colonizers (filamentous cyanobacteria, fungi) and continuous development of the biofilm; (6) – the mature biofilm with a expressed structure forms a specific micro-ecosystem; (7) - the homeostasis phase keeps in equilibrium the biofilm with a continuous growth of the structure and detachment of small parts which become free-living; some macro-organisms (i.e. invertebrates) can adhere.

The concept of biofilm architecture was introduced by Lawrence et al. in 1991 to define the unique structures formed by pure culture bacterial biofilms grown in continuous flow slide culture. The specific architecture of biofilm is a consequence of the interaction between the community members, mediated by the quorum sensing

communication and influenced by the physical-chemical and genotypic factors. Stevenson R. J. (1986) grouped periphyton community based on microbial components immigration and growth rate: the pioneers have a high initial abundance but decrease with time, the relative abundance of the late colonizers increases with time, and the intermediates have a relatively more stable abundance than the other groups. Biofilm evolution is also dependent by stochastic and mechanical processes, deterministic phenomena and temporal changes (Wimpenny J., 2000).

II.2. Biofilms interaction with the substratum, biodeterioration processes

The biological colonization of stone artifacts exposed under uncontrolled out-door conditions is always influenced by the favorable microclimate and the high biodiversity of the opportunistic airflora (Caneva G., 2007). Phototrophic biofilms can develop on monumental stone surface influencing their state of conservation both by aesthetical and physical-chemical point of view. The phototrophic microorganisms like phototrophic bacteria, algae, cyanobacteria and diatoms are the first colonizers of the stone surfaces followed, in favorable environmental conditions, by heterotrophic ones as associate consumers. This colored biocoenosis is stuck together by gel-like extracellular polysaccharide matrix (EPS) which confers resistance to dried periods and cleaning treatments.

The development of photosynthetic microorganisms depends on the combination of environmental location and climatic conditions, in addition to the chemical-physical properties of stones. The whole properties that contribute to biological colonization has been defined as “bioreceptivity” by Guillitte in 1995, who further defined different types, such as primary, secondary and tertiary bioreceptivity. The first pioneering microorganisms of a stone surface include ubiquitous photosynthetic microorganisms such as cyanobacteria, green algae, diatoms and lichens (Darlington A., 1981; Warscheid Th., 2000). A synergistic biodeteriorative effect on stone surfaces can be started by the concomitant growth of phototrophic and heterotrophic populations. In fact, bacteria and fungi metabolize the organic matter produced by phototrophs and release organic acidic compounds able to solubilise the minerals components of the substratum (Warscheid Th., 2000). The formation of biofilms intensifies microbial attack by weakening the mineral matrix through repeated

wetting and drying cycles and subsequent expansion and contraction (Warscheid Th., 1996).

The biofilm formation is related with the primary bioreceptivity. Some studies revealed that the primary receptivity is mainly controlled by chemical composition and physical characteristics of the substrates such as mineral composition, structure-texture, permeability, porosity, roughness, pH (Krumbein W. E., 1988; Albertano P., 2000; Miller A., 2006; Tiano P., 1995a). Naturally, also the intrinsic properties of the microorganisms appear to have an important role on the development of photosynthetic biofilms. The anionic nature of exopolymers can strongly adsorb cations and dissolve organic molecules from the materials, and can stabilize dust particles. The biofilm properties such as ion-exchangers and metabolic end-products like acids (and derived salts) can led to an increasing of water content of porous materials (Sand W., 1997). It was observed that calcium ions may be easily subtracted from rocks and precipitated on polysaccharide sheaths of cyanobacteria in the form of calcium carbonate (Albertano P., 2003, Crispim C. A., 2005). The metabolic activities of the organisms that are forming a biofilm are related with the production of extracellular polymeric substances, liberation of chelating compounds and organic/inorganic acids, the presence of coloured pigments and mechanic pressure exerted by shrinking/swelling phenomena. Therefore, the stony structure can be interested to pitting, ion transfer, leaching processes and dwindling (Tiano P., 1998). Through their growth, biofilms can change the chemical and mineralogical composition of the original rock. They can change the stability, permeability and colour of the stone as well the density (Kovacic L., 2000).

The monumental fountains, due to the constant contact with water, are particularly affected by the microbial colonization giving rise to biodegradation processes combined with visual aspect alterations. The different microenvironmental conditions with some areas constantly in contact with water or others only sporadically wet and some perpetually dry, offer ideal growth substrata for many microorganisms and organisms. This various habitats determine a different distribution of organisms on the fountain according to their specific requirements (Pietrini A.M., 2005). The most important factors determining biological growth are (Walsh J. H., 1968): primary energetic input (light), secondary energetic input (nutritive factors) and climate. In urban and industrial areas must be also considered the environmental pollution.

The biofilm directly participate in stones decay processes, causing both aesthetic and physical-chemical biodamages (Tomaselli L., 2000a), inducing alterations due to the fluid retention, to the aggressive action of both metabolic products and to atmospheric pollutants entrapped in the EPS, which can increase the chemical corrosion process. Besides the discoloration and staining processes (Fig. 2) produced by biogenic pigments (e.g. the green chlorophyll, brownish melanin, red carotenoids), the microflora leads to the change of materials characteristics with regard to their mechanical properties, superficial absorbency/hydrophobicity, diffusivity and thermal-hydric behavior (Warscheid Th., 2008). Within the complex biofilm not all inhabiting microorganisms that can affect the stone surface need to be in direct contact with the substratum (Gaylarde C. C., 1999).

Fig. 2. Biodeterioration aspects present on the monumental fountains: discoloration (a, b, c, d); staining (b, arrow); encrustation (c, d).

A particular aspect of biodeterioration of stone monuments is linked to endolithic organisms colonizing the interior of rocks (Salvadori O., 2000). The presence of endolithic community, which can penetrate up to depths of several millimetres (Saiz-

Jimenez C., 1999), with the diffusion of their excreted products into the intergranular matrix enhance by weathering reactions and decrease mechanical properties, such as cohesion between grains (Alakomi H. L., 2004.). The endolithic microhabitat gives protection from intense solar radiation and desiccation, and also provides mineral nutrients (Walker J. J., 2005).

II.3. Biomolecules and biofilm control

The microorganisms growing on the artistic fountains can be suppressed by indirect (e.g. water chlorination) or direct methods such as mechanical action (e.g. abrasion) and/or biocides treatments. The application of toxic substances can be hazardous to the environment and to public health other than for the stone itself. The biocide application can be harmful for conservators and the environment (Price C. A., 1996) and little is known about the consequences of repeated applications (Fortune I. S., 2008). The EC regulations (BPD 98/8/EC n 20 June 2004) have had as consequence the elimination from the market of the most active (and toxic) compounds applied to this aim and new approaches are made in several sectors in order to overcome this problematic.

In nature some organisms may be heavily fouled on much of their surfaces, while others can be totally fouling-free. This has generated interest in identifying the secondary metabolites that might repel or inhibit fouling organisms. Recently the discovery of biomolecules involved in cell-to-cell signaling has opened new possibilities for the study of microbial communities. Autoinduction has been defined as a form of communication, in which cells monitor the population density via small autoinducer signal molecules and regulate the expression of various genes in a QS manner (Davies D.G., 1998). Formation of autoinducer molecules (AHLs) has been reported to be widely distributed among bacteria and cyanobacteria. The presence of QS molecules for all cultures isolated from the catacombs of Rome (N-acyl homoserine lactone type) have been evidenced using both chemical and function-based assays. It still remain uncertain if the presence of these signal molecules is due to the cyanobacteria themselves or to contaminating microorganisms, or both are responsible for this production (Albertano P., 2003).

The possibility to interfere with this cell density-dependent communication mechanism constitutes a novel and promising strategy to control biofilm formation. The

inhibition of QS is a new concept, known as a Quorum Quenching (QQ) (Romero M., 2008). An example of QQ is the production of acyl homoserine lactone (AHL) antagonists, for example halogenated furanones are produced by the red alga *Delisea pulchra* to inhibit its surface colonization by AHL-based 'quorum sensing' microorganisms (Givskov M., 1996). Other marine organisms, such as sponges (*Reniera sarai, Aaptos suberitoides., Acanthella cavernosa, Crella incrustans*), corals (*Sinularia polydactyla, Leptogorgia virgulata*), seeweeds (*Ceramium botryocarpum, Sargassum muticum, Polysiphonia lanosa*) and marine fungus (*Ampelomyces* sp.) are reported for their antifoulant properties, attributed to the presence of various antifouling compounds (aaptamine and analogs, 3-alkylpyridinium derivatives, diterpenoid hydrocarbons – pukalide and exopukalide, respectively, 3-chloro-2,5-dihydroxybenzyl-alcohol) (Reddy M. V. R., 1993; Sepčić K., 1997; Herlt A., 2004; Hellio C., 2001; Kwong T. F. N., 2006). Most recently, various higher plants (*Zostera marina, Pisum sativum, Cinnamonum sp., Capsicum anuum*) and freshwater alga (*Chlamidomonas reinhardtii*) were also shown to secrete AHL signal mimic substances and to interfere with the QS mechanism (Zimmerman R. C., 1995; Teplitski M., 2000; Teplitski M., 2004; Xu Q., 2005; Niu C., 2006). The production of diverse AHL mimic compounds by various organisms seems reasonable if the biological aim of these eukaryots is to disrupt AHL-mediated QS. Therefore, the possibility of interfering with QS using mimic signals constitutes a novelty and a promising strategy to control biofilm development.

Another set of biomolecules that seem to offer promising possibilities for the control of biofilms in terrestrial environment are the siderophores. These are low molecular weight, high affinity chelators of ferric iron, synthesized and secreted by many microorganisms in response to iron deprivation. The presence of siderophores was reported for non-pathogenic bacteria (*Staphylococcus carnosus, S. Xylosus*) under low-iron conditions in growth media. The siderophore preparations inhibit the growth of Gram-negative microorganisms from catacombs (Albertano P., 2003).

Alternative new approach based on natural antifouling substances, are recently experimented in the marine sector for the prevention of bio settlement on ships submerged structures. Natural sources or synthetic analogues must be found to ensure supplying at a reasonable cost (Yebra D. M., 2004). These natural antifouling agents are active substances found in marine animals, plants microorganisms, and in terrestrial plants, which prevent the settlement of microorganisms and the patina formation on the

surface of their structures, and they are believed to function as natural chemical defence against fouling. These chemical agents are in fact mimic bacterial signals that interfere with the cell-to-cell communication network (QS), which plays an important role in the biofilm formation. Therefore, these substances may be expected to be new environmentally friendly antifouling agents (de Nys R., 2002; Omae I., 2003). In the last period many studies related with the new environmental-friendly antifouling technologies were reported especially in the marine sector, but also in the medicine, food or water industries. All this studies are based on the antibiofouling strategies in the natural environment (Evans L.V., 1993; Yebra D. M., 2004; Fusetani N., 2004).

Since 'quorum sensing' plays such a substantial role in biofilm formation, one strategy considered for preventing biofilm formation is to coat or embed surfaces with compounds capable of interfering with related signaling mechanisms. Recent studies in the marine biofouling sector consider the fluoropolymers and silicones the best materials that can be used as fouling-release coatings (Yebra D. M., 2004).

In order to be applied in the cultural heritage field these new substances must be, other than active as settlement inhibitors, not dangerous for the integrity of the stone surface, i.e. not change the colour and without reaction with the stone components.

III. MATERIALS AND METHODS

III.1. Investigated fountains

In this research five monumental fountains (Plate 1) were selected for studying the phototrophic biofilms composition. Two of them (Tacca's Fountain 2 - Florence and Fountain from Patio de la Lindaraja - Granada) were chosen for the in situ experiments in order to evaluate the efficacy of the innovative antibiofouling treatments in real cases. A literature survey related with biodeterioration of fountains studied by various authors is presented in Table 1.

The five investigated fountains are:

Tacca's Fountains (1 and 2) - Florence, Italy. These two bronze baroque fountains with marine monsters were set up in 1620 by Pietro Tacca, one of the foremost fountain designer in Florence. They are located in a typical urban area in Santissima Annunziata Square. With an air of gothic exuberance, two fantastic creatures support each other back to back, while the basins of the fountain swell out like organic forms. In the centre of the two shells Tacca carved a water-filled run-off, which, being solid, is like an allegory of water. The pedestals and the border of the basins are made by Carrara marble.

Second Fountain from Villa la Pietra - Florence, Italy. The original villa was built in the fifteenth century by the Macinghi. In 1490 substantial renovations transformed the villa in the present state. During the period in which Florence was the capital of the Kingdom of Italy, the villa housed the Prussian Embassy. In 1904 Sir Arthur Acton, owner of the villa, started to build the garden located on the back of the building. This garden proposes the classical plan, based on three sloping terraces and on the topiary art shaped in a romantic manner, with various sculptures and two fountains. The so called Second Fountain is made by concrete.

Fountain from Patio de la Sultana, Generalife - Granada, Spain. This fountain is located in the Sultana Court at Generalife, Granada (Spain). The arcaded structure dates back to 1584. The area was originally the site of the now disappeared Palace Bath. Water from the irrigation canal, which at one time probably filled it while flowing to the adjacent courtyard, can still be seen pouring through a gap in the side wall. In the centre is a U-shaped pool of water, in the middle of which in the 19th century was placed a smaller pool, with a stone fountain, made on Macael marble and Sierra Elvira stone.

Fountain from Patio de la Lindaraja, Alhambra - Granada, Spain. It is located in the Patio de la Lindaraja, an open air garden with arcaded galleries on the ground floor. The effect is cloister-like, which is further enhanced by the design of the garden with its fountain in the middle. The base, ridge and pilaster of the fountain, in baroque style are made of stone from the Elvira Mountains. From around 1626 to 1995 the fountain had an adorned marble basin in Nasrid style, with epigraph inscriptions, which is currently kept in the Museum of the Alhambra.

Table 1. The investigated fountains reported by various authors

Fountain	References
A. Trevi Fountain, Rome, Italy	**A.** Pietrini (1991); Nugari et al. (1997)
B. Pretoria Fountain, Palermo, Italy	**B.** Not et al. (1995)
C. Fountain of Tritone , Rome, Italy	**C.** Barcellona Vero et al. (1979)
D. Four Rivers Fountain, Rome, Italy	**D.** Ricci et al. (1994)
E. Fountain of Bibatauin, Granada, Spain	**E.** Peraza Zurita et al. (2005)
F. Lions Fountain, Alhambra Palace, Granada, Spain	**F.** Sarro et al. (2005)
G. Fountain from Patio de la Lindaraja, Alhambra Palace, Granada, Spain	**G.** Bolivar et al. (1997)
H. El Bano de Comares and La sala de los Abencerrajes, Alhambra Palace, Granada, Spain	**H.** Bolivar et al. (1998)
I. Patio del Cuarto Dorado and Patio de los Arrayanes, Alhambra Palace, Granada, Spain	**I.** Sanchez-Castillo et al. (1997)
J. Tacca's Fountains	**J.** Tomaselli, et al. (2000b)
K. Alcazares and Alhambra's Fountains	**K.** Peraza Zurita (2002b, 2004)

III.2. Biofilms development

II.2.1. *Natural biofilm observation*

Many components of the biofilm contain different pigments, like chlorophylls, bacteriochlorophylls, carotenoids and phycobilins. The green algae contain chlorophyll a, b and carotenoids, the diatoms contain chlorophyll a, c, carotenoids and xanthophylls, and the cyanobacteria contains chlorophyll a and phycobilins (phycocyanins and phycoerytrins) (Neu T. R., 2003). Due to the specific autofluorescence of the pigments present into the different phototrophs they could be distinguished very easily by Epifluorescence Microscopy (EM) or Confocal Laser Scanning Microscopy (CLSM).

CLSM allows elimination of out-of-focus haze, horizontal and vertical optical sectioning, determination of 3D relationship of cells, and 3D computer reconstruction from optical thin section. Laser microscopy can be used to detect signals resulting from both reflection and autofluorescence in biofilm. Reflection provides information on the presence of colloidal and mineral materials or cell inclusions. Autofluorescence may be used to monitor specific population such as autotrophic algae and cyanobacteria. Most frequently, specific fluorescent molecules may be used directly to stain microorganisms (e.g. DAPI, SYTO series) or they may be conjugated to probes to identify specific biofilm targets such as the lectins used for examine the extracellular polymeric substances (Neu T. R., 1997; Lawrence J. R., 1998; Lawrence J. R., 2003, Wierzchos J., 2004). This technique was successfully applied for studying the aerophytic biofilms in cultural heritage (Hernández-Mariné M., 2002; Roldán M., 2004).

Epifluorescence microscopy, developed in the first half of this century and wide used with the beginning of 1990 for measuring the biomass of plankton (Sieracki M. E., 1990), was chosen to evaluate the covered area of the phototrophic patina, while the confocal laser microscopy allows the spatial semi-quantification of the micro-communities of the fully hydrated biofilms (Rodriguez S. J., 2007).

Aliquots of the biofilm samples collected from the investigated fountains (Table 3) were carefully observed by optical microscopy (Nikon Eclipse E600) in order to distinguish the main microbial components that are constituting phototrophic patinas and to observe their structure. Data related with the three-dimensional biofilm structure was collected using Zeiss LSM 510 META confocal laser scanning microscope. The autofluorescence of phototrophs and substratum was recorded in three channels using 488 nm, 543 nm and 633 nm excitation beams, with emission at 475-525 nm, 560-615 nm and 650 nm, respectively.

Cross-section of small solid parts of samples collected from Sultana Fountain were observed by epifluorescence microscopy (Nikon Eclipse E600) using UV-2A (excitation 330-380 nm, DM 400 nm, BA 420 nm) and TRITC (excitation 450-490 nm, DM 565 nm, BA 610 nm) filter cubes. A polystyrene resin (Mecaprex MA2, Presi, France) was used for samples embedding. The images were recorded using a Nikon DXM1200F digital CCD camera.

II.2.2. *Laboratory experiments*

Carrara marble specimens completely (1x1x0.5 cm and 5x5x1 cm) or partially (10x5x1 cm) immersed in well water were used for these experiments. The biological inoculum (≈ $17x10^5$ cells/ml) was composed mainly by *Chlorella sp., Cosmarium sp., Navicula sp., Nitzchia sp., Leptolyngbya sp., Aphanocapsa sp., Gloeocapsa sp., Oscillatoria sp.,* protozoa (Fig. 3). The same mixed biofilm inoculum was used for the experiments carried out under indoor and outdoor conditions (Table 2). The outdoor experiment was started after one month after the water inoculation, and therefore the principal microbial components in the two simulation fountains was more stable and presented some differences: diatoms, filamentous algae and coccoid cyanobacteria were dominant in the simulation fountain with sprinkling water, while the palmeloid type algae and coccoid cyanobacteria were preponderant in the simulation fountain with stagnant water (Fig. 4).

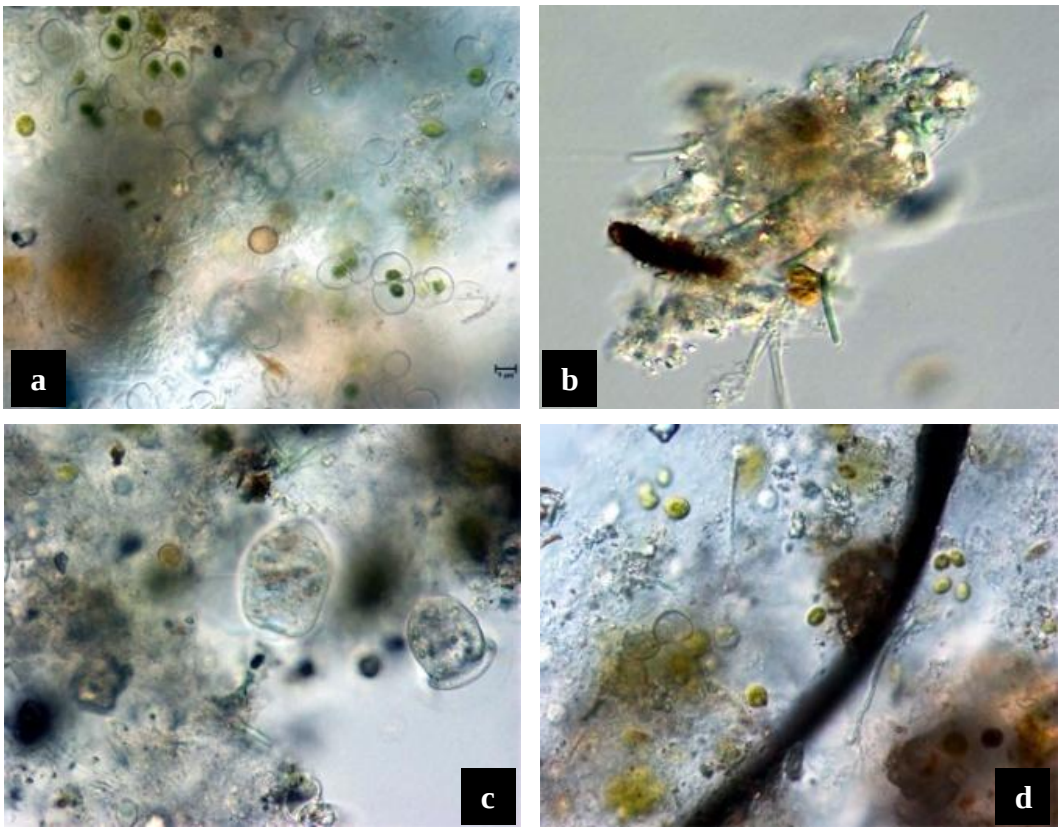


Fig. 3. Biological start inoculum for the indoor and outdoor experiments with conjugatoficeae and palemloid algal types, diatoms (a); filamentous cyanobacteria (b); protozoa (c) and coccoid cyanobacteria and fungal hypha(d).

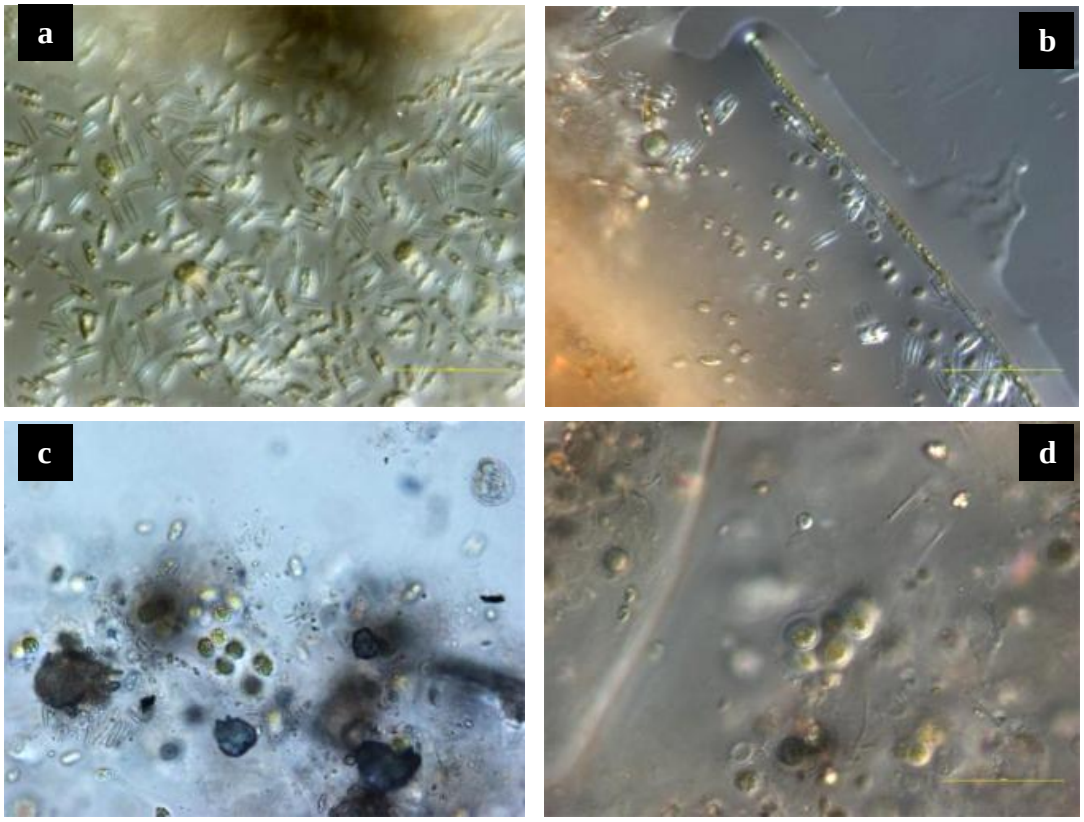


Fig. 4. Main microbial components after 1 month from the inoculum and before the immersion of stone specimens in the two simulation fountains: with sprinkling water (a,b) and with stagnant water (c,d).

Table 2. The experimental conditions for indoor and outdoor experiments

Experimental conditions	Indoor experiment	Outdoor experiment	
	stagnant water	sprinkling water	stagnant water
Materials	- plastic container (33x27x13 cm) filled with well water - Carrara marble (1x1x0.5 cm)	- plastic container (150 cm Ø, 50 cm height) filled with well water, refreshing of water for 5 min every hour - Carrara marble (10x5x1 cm and 1x1x0.5 cm)	- plastic container (150 cm Ø, 50 cm height) filled with well water - Carrara marble (5x5x1 cm)
Exposure	low artificial lighting with continuous white fluorescent light at photosynthetic photon flux density of 10 µmol photon $m^{-2}.s^{-1}$ in conditioned room at 27 ºC	east orientation, day/night cycle, sheltered from direct sun after midday, temperature ranging between 9ºC and 37ºC during the whole test	

The phototrophic biofilm development in the first stages was investigated on Carrara marble (1x1x0.5 cm) using the Leica TCS SP5, Leica TCS SP2, confocal laser scanning microscopes (CLSM) in fluorescence and reflection modes. Focusing the instrument at different depths it can be obtained optical slices without any mechanical

interference with the patina structure and the colours chosen for each channel are all false colours.

As regard the indoor experiments, the images were recorded on collected samples with both microscopes. For the Leica TCS SP5 the following conditions were used: the reflective signal of the substratum was captured in the blue channel with the excitation at 488 nm and the emission at 490 to 500 nm; the auto fluorescence of photosynthetic organisms was observed in the green and red channel, using the excitation at 561 and 564 nm and emission between 565-615 nm and 665-790 nm respectively; extracellular polymeric substances (EPS) were labelled with the lectin Concanavalin-A conjugated with Alexa Fluor 488 (Invitrogen, Molecular Probes) and observed in the green channel (excitation 488 nm and emission at 493 to 517 nm). With the microscoope Leica TCS SP2 the following conditions were used: excitation at 488 nm and emission at 480 to 499 nm in the reflectance mode; excitation at 351-364 nm, 488 nm, 543 nm and emission at 400-508 nm, 500-561 nm, 555-700 nm respectively for capturing the natural fluorescence of the phototrophs.

The results of outdoor experiments were obtained by capturing images of phototrophic microorganisms autofluorescence, present in the collected samples, using Leica TCS SP5 (excitation beams at 488 nm, 543 nm, 458 nm and emission at 500-531 nm, 680-784 nm and 459-469 nm, respectively) in three channels (for phycoeritryn, chlorophyll *a* and substratum). The images from stacks were captured at 0.6 µm intervals, in RGB scale, filed in TIFF format.

The biofilm observation of microbiological development on 10x5x1 cm and 5x5x1 cm Carrara marble specimens was assessed through visual observations.

III.3. Study of biofilm composition

III.3.1. *Isolation and identification of microorganisms by traditional and molecular methods*

A total of 35 samples were collected by scraping small areas of stone surface from different locations (Plate 2 and 3) of the five artistic fountains, using sterile scalpels and sterile vials. Table 3 contains the description of the collected samples. The samples coming from fountains of Florence, were immediately transported to the

laboratory for microbiological analysis, while the ones coming from Spain arrived as soon as possible, conserved in 2% formalin, to our laboratory by air express courier. Aliquots of the samples were processed for microscopical analysis, others were used for the inoculation in agarized cultural media for the growth and isolation of phototrophs and fungi, and others aliquots were used for the microbial community analysis by ARISA fingerprint method.

Table 3. Characteristics of the collected samples from five investigated monumental fountains

No.	Fountain	Encodes, typology and sampling position
1.	**Tacca's Fountain 1, Florence, Italy**	**1T** – light green biofilm continuous wet because of the flowing water, cylindrical marble pedestal, east orientation **2T** – dark green biofilm sporadically wet due to the splashing of water, right corner of the parallelepiped pedestal, east orientation
2.	**Tacca's Fountain 2, Florence, Italy**	**O1**– brown biofilm below the water level, internal part of the basin, south orientation **O2** – green-brown biofilm below the water level, internal part of the basin, south orientation **O3** – brown biofilm above the water level, internal part of the basin, south orientation **N1** – brown biofilm below the water level, sporadically wet due to the water splashing, internal part of the basin, north orientation **N2** – green-brown biofilm above the water level, sporadically wet due to the water splashing, internal part of the basin, north orientation **N3** – green-brown biofilm above the water level, sporadically wet due to the water splashing, internal part of the basin, north orientation
3.	**Second Fountain of Villa la Pietra, Florence, Italy**	**VP1** – grey powder above the water level, dry internal part of the basin, north-east orientation **VP2** – black crust, dry internal part of the basin, north-east orientation **VP3** – brown-green biofilm below the water level, internal part of the basin, north-east orientation **VP4** – brown-green biofilm, interface water/air, intermittent wetted area, internal part of the basin, north-east orientation **VP5** and **VP6** – light green and turbid deposition, bottom of basin
4.	**Fountain from Patio de la Sultana Generalife, Granada, Spain**	**S1a** – green biofilm under the water level, internal part of the central fountain, east orientation **S1b** – green biofilm, low part of the pedestal trunk of the central fountain with constant high wetness, south orientation **S1c** – green biofilm, the same level with the water one, corner of the pedestal basement, north orientation **S1d** – green biofilm, low part of the pedestal trunk of the central fountain with constant high wetness, east orientation **S1e** – green biofilm, low part of the pedestal trunk of the central fountain with constant high wetness, north orientation **S1f** – green biofilm, low part of the pedestal trunk of the central fountain with constant high wetness, west orientation **S2** – green spots on grey patina, sporadically wet by splashed water, continuous shaded 'surtidore', west orientation

No.	Fountain	Encodes, typology and sampling position
4.		**S5N** – grey patina, rarely wet, continuous unshaded 'surtidore', north orientation **S5W** – brownish patina, rarely wet, continuous unshaded 'surtidore', west orientation **S6N** – dark-green patina, rarely wet, continuous unshaded 'surtidore', north orientation **S6S** – grey patina, rarely wet, continuous unshaded 'surtidore', south orientation **S9W** – red brownish patina, continuously wet, continuous shaded 'surtidore', west orientation **S9N** – dark-green patina, continuously wet, continuous shaded 'surtidore', north orientation **S9S** – green patina, continuously wet, continuous shaded 'surtidore', south orientation **S9E** – green patina, continuously wet, continuous shaded 'surtidore', east orientation
5.	**Fountain from Patio de la Lindaraja Alhambra, Granada, Spain**	**1L** – brown powdering patina, external part of the basin, north-west orientation **2L** – green patina, sporadically wet, internal part of zig-zag ornaments of superior marble basin, south-east orientation **3L** – green patina, sporadically wet, internal part of superior marble basin, south orientation **4L** – green patina, internal part of the central fountain, west orientation **5L** – green biofilm, sporadically wet, internal horizontal part of pilaster ornaments, west orientation **6L** – green patina, continuously wet, internal part of foundation, west orientation

III.3.1.1. Traditional methods

Aliquots of the collected samples (Tacca's Fountain 1, Second Fountain from Villa la Pietra and Fountain from Patio de la Sultana) were suspended in standard saline solution and sown in Petri dishes containing the respective solid medium for the isolation of: (i) *cyanobacteria* (BG-11), (ii) *nitrogen fixing cyanobacteria* (BG-11_0), (iii) *algae* (modified BG-11 (5 ml/l $NaNO_3$ diluted 1:1 with sterile water)) and (iv) *fungi* (PDA Difco). BG-11 type media were described in Rippka R. et al (1979).

The Petri dishes containing cultural media for phototrophs were incubated under continuous low white fluorescent light at photosynthetic photon flux density of 10 μmol photon m^{-2} s^{-1} at 27 ºC, while the ones containing PDA for fungi were incubated in darkness, at 26 ºC. The developed cyanobacteria, algae and diatoms colonies were transferred into tubes containing the same specified liquid cultural media, while the fungal strains were isolated on the PDA agar medium. All the isolated strains were maintained on slants with their specific cultural medium in appropriate conditions.

The morphological characterization and morphotypes identification were carried out with optical microscopy (Nikon Eclipse E600) according to Geitler L. (1932), Komarek J. and Anagnostidis K. (1988, 1998, 2005), Barnett H. L. (1998) and Bourrelly P. (1966) for all collected samples. Representative isolated cyanobacterial strains were also characterized by phylogenetic analysis of the 16S rRNA gene.

III.3.1.2. DNA extraction

DNA of isolated cyanobacterial strains and of collected samples from two fountains (Second Fountain of Villa la Pietra, Florence, Italy and Sultana Fountain from Generalife, Granada, Spain) were extracted. 30 ml of liquid cultures (BG-11, or $BG\text{-}11_0$ media) of the isolated cyanobacterial strains were incubated with agitation in a Gallenkamp Orbital Incubator (CO_2 4%, 27 ºC, light/dark cycles 14/10 hours). The biomass was harvested by centrifugation and the pellet was washed with standard saline solution (0.1% w/v) for two times or even more in case of the mucilaginous cultures, and then the pellet was divided in aliquots and frozen at - 20 ºC. For DNA extraction, pellets were thawed and treated with PowerPlant™ DNA Isolation Kit, according to the manufacturer's protocol (Mo Bio Laboratories Inc.).

Total community DNA of samples collected from the fountains was extracted using PowerSoil™ DNA Isolation Kit, according to the manufacturer's protocol (Mo Bio Laboratories Inc.).

All the DNA extracts were laden into 0.5% agarose gel, previously stained with ethidium bromide (10 mg/ml). The quality of DNA extracts was examined under UV light after 15 minutes at 150V of DNA molecules migration.

III.3.1.3. PCR amplification and sequencing

To amplify the rRNA 16S gene plus the ITS region, the encoding gene fragments of cyanobacteria, template DNA, universal primer 16S27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and cyanospecific primer 23S30R (5′-CTTCGCCTCTGTGTGCCTAGGT-3′) were used. (Edwards U., 1989; Taton A., 2003). The genomic DNA (1 µl) was amplified using Hot Start Master (Larova GmbH, Germany). This PCR was carried out with an activation and initial denaturation step of 2 min at 94 ºC, followed by 30 cycles of denaturation for 45 sec at 94 ºC, annealing of 1

min at 55 ºC, and elongation of 2 min at 72 ºC, followed by a final extension step of 7 min at 72 ºC. All amplification reactions were performed in a TGradient Thermal Cycler (Biometra, Goettingen, Germany). The amplicons were visually quantified by comparison with DNA Molecular Weight Marker VI (0.15 - 2.1 kpb) (Roche, Mannheim, Germany) on agarose gel electrophoresis. The amplified DNA fragments were purified using ExoSAP-IT Clean-Up kit following the manufacturer's instructions (USB Corporation, Affimetrix, Inc, USA) and then sent to BMR Genomics, Padua, Italy (www.bmr-genomics.it) for sequencing.

The gene sequences for rRNA 16S were obtained using the three primers 16S979F, 16S544R and 16S1092R (Hrouzek P., 2005). Single reads were aligned in a unique 16S rRNA gene sequence using the software suite PHRED, PHRAP CONSED developed by the University of Washington (Gordon D., 2004) and consensus sequences imported in ARB (Ludwig W., 2004), which was used for the subsequent analytical steps. Most similar sequences included in the analysis were retrieved from the most recent SILVA database. The aligner on the SILVA website was also used to produce the sequence alignment for the ARB software. The alignment was later visually investigated and corrected under ARB. Phylogenetic relationships of the sequences were calculated using Neighbor-joining algorithm (Saitou N., 1987) using sequences over 1300 base pairs.

Sequencing and phylogenetic analysis were used for molecular identification of 35 cyanobacterial strains isolated from the monumental fountains. DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. It has been performed using the chain termination method developed by Frederick Sanger (1977). This method consist in annealing and extension of a short complementary primer to a single stranded template DNA by DNA polymerase. The reaction is split into 4 tubes (called A - adenine, C - cytozine, G – guanine or T - thymine) each containing a low concentration of the indicated dideoxy nucleotide, in addition to the normal deoxynucleotides. Dideoxynucleotides, once incorporated, block further chain extension and so each tube accumulates a mixture of chains of lengths determined by the template sequence. The reactions are performed in a single tube containing all four ddNTP's, each labeled with a different color dye. Since the four dyes fluoresce at different wavelengths, a laser then reads the gel to determine the identity of each band according to the wavelengths at which it fluoresces (Howe C. J., 1995). The results are then depicted in the form of a chromatogram, which is a diagram

of colored peaks that correspond to the nucleotide in that location in the sequence, specific for each organism (Fig. 5) Therefore, these data are used for molecular phylogenetic analysis expressed in a phylogenetic tree, comparing the obtained sequences with the ones from public and specific databases.

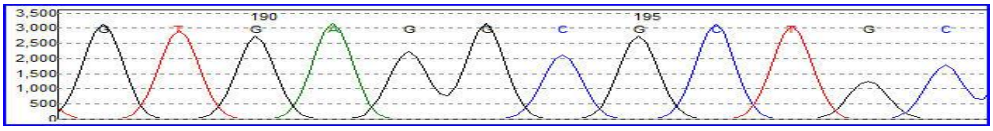


Fig. 5. Chromatogram of a DNA sequence (…GTGAGGCGCTGC…)

III.3.2. *Microbial community analysis by ARISA fingerprint method*

III.3.2.1. DNA extraction

Total community DNA of the samples collected from the fountains was extracted using PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc.), according to the manufacturer's protocol. The quality of DNA extracts was observed as described in III.3.1.2.

III.3.2.2. PCR amplification and ARISA

PCR amplification of rRNA 16S gene for ARISA was performed using two specific primers sets, for eubacterial and cyanobacterial domain, respectively (Table 4). The reaction mixture (50µl each) for the PCR amplification contained: 5µl 10x DyNAzyme EXT buffer (with 15 mM $MgCl_2$), 5µl dNTPmix (2mM), 1µl of each primer (10pmol/µl), 2.5µl BSA (Bovine Serum Albumin, BioLabs, 10 mg/ml), 33.5µl distillated water. Before adding 1µl of *Taq* DNA polymerase (Amersham-Pharmacia) and 1µl of genomic DNA, aliquots of the mix were placed in PCR tubes and sterilized under UV light (312 nm) for 8 min. Reactions (50µl final volume) were initially denaturated for 5 min at 94ºC, followed by 35 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min, and then a final extension step at 72ºC for 10 min was performed. The PCR products (5 µl each) mixed with bromophenol blue were visualized after 45 min of electrophoresis on 1.2% agarose gel stained with ethidium bromide (10 mg/ml). DNA Molecular Weight Marker VI (0.15 - 2.1 kpb) (Roche, Mannheim, Germany) loaded in the same gel (3 µl and 6 µl) was used for the approximate quantization of amplified DNA.

Table 4. Primer sequences and target sites

Primer[a]	Sequence (5'→ 3')	Target site	Group	Reference
16S1515F	AGT CGT AAC AAG GTA GCC GTA CC	1492-1515	cyanobacteria	Cardinale et al., 2004 (modified)
23S30R-6FAM	CTT CGC CTC TGT GTG CCT AGG T	30-52	cyanobacteria	Lepère et al., 2000
ITSF	GTC GTA ACA AGG TAG CCG TA	1494-1513	eubacteria	Cardinale et al., 2004
ITSReub-HEX	GCC AAG GCA TCC ACC	23-37	eubacteria	Cardinale et al., 2004

[a] R (reverse) and F (forward) designations refer to the primer orientation in relation to the rRNA
[b] *E.coli* numbering of 16S rRNA or 23S rRNA nucleotides

The amplified DNA (2 ng/ µl each) was then sent to BMR Genomics for fragment separation in a capillary chromatographer. A fluorescent dye-labeled size standard GeneScan™ ROX500 (35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, 500 base pair fragments) was added and the analyze was carried out with a GeneScan 3100 ABI Automated Capillary DNA Sequencer (Applied Biosystems).

ARISA (Automated rRNA Intergenic Spacer Analysis) is a fingerprint molecular method for analyzing the community heterogeneity. This approach consist in extraction of total DNA from an environmental sample and then isolate homologous genes (usually rRNA genes) from the DNA mixture. Fig. 6 illustrates the ARISA principle. Each peak of an electropherogram represent an operational taxonomic unit, and therefore valuable information about the biodiversity of specific groups (e.g. using specific primers for eubacteria and cyanobacteria) present in the analyzed sample is obtained.

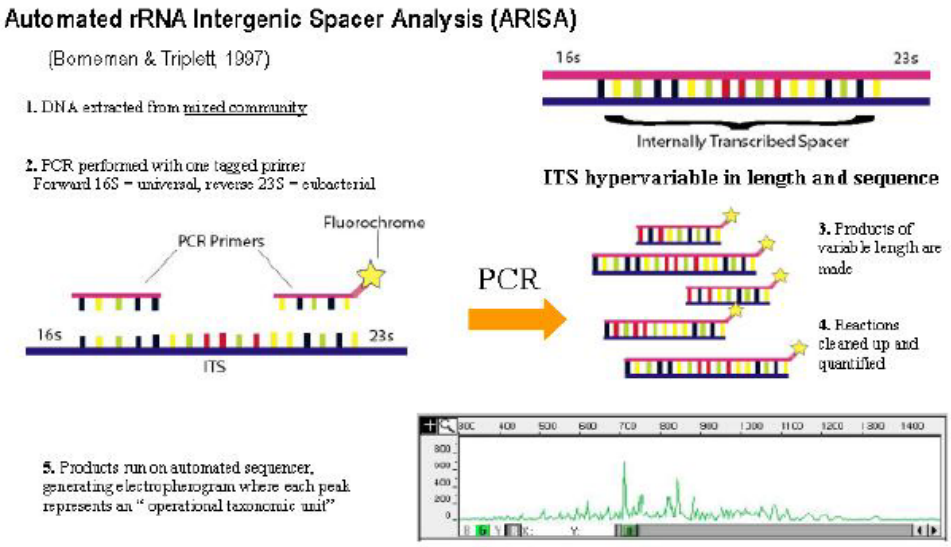


Fig. 6. The scheme of ARISA molecular fingerprint method for microbial community analysis

The computational analysis were performed with Peak Scanner ™ (Applied Biosystems 1.0, 2006) and BioNumerics 2.5, (Applied Maths, Belgium) software. Only peaks higher than 50 relative fluorescence units and fragments between 100-650 base pairs were considered.

Principal component analysis (PCA) was performed on ARISA profiles obtained with BioNumerics software, in order to evaluate the similarities of microbial communities for fountains samples. The peaks intensities for each band were normalized in accordance with the total fluorescence of each sample ARISA profile. PCA was carried out using Unscrambler® Camo Software AS with the default parameters.

III.4. Stone material and color measurements

Tests were carried out with two different types of stones (Fig. 7) commonly used for creating artistic monuments in Italy and south of Spain:

- Carrara marble type P, a white metamorphic stone with a mean grain size <100 μm with an unimodal distribution. The water porosity was 0.57% (hydrostatic balance method- ISO 6783:1982). Specimens dimension: 1x1x0.5 cm or 5x5x1 cm.
- Sierra Elvira stone classified as pelsparite (Folk R. L., 1959; Folk R. L., 1962) or grainstone (Dunham R. J., 1962), a grey sedimentary stone with a pellet structure (pellet dimension 150-250 μm), presenting diffuse phenomena of dolomitization. The water porosity was 0.31%. Specimens dimension: 1x1x0.5 cm.

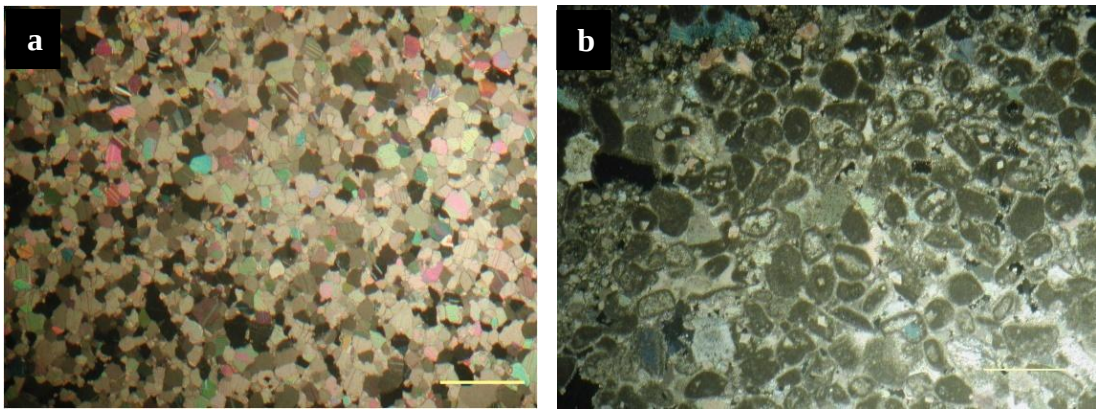


Fig. 7. Thin sections (crossed Nicols) for Carrara marble type P (a) and Sierra Elvira stone (b); scale bar 500μm**.**

To quantify the color change of the stones, before and after the applied treatments containing ABAs, the color parameters were calculated using CIELAB 1976 formula (Ohno Y., 2000). Reflectance values of the stone were measured with a Minolta Chroma Meters CR-200, a compact tristimulus color analyzer which measures the reflected colors of surfaces. The readings were taken three times and an average value was considered. Three main parameters (L*, a* and b*) were recorded, where L* is the lightness/darkness coordinate; a* the red/green coordinate, with + a* indicating red and - a* indicating green; b* the yellow/blue coordinate, with + b* indicating yellow and - b* indicating blue. ΔE is the total color difference calculated from CIELAB 1976 color difference formula: $\Delta E = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$.

III.5. Antibiofouling agents, coatings preparations and their characterization

Five natural antibiofouling agents (ABAs) were selected as potential inhibitors of biofilm formation – three of them are present in marine environment (pAPS in a sponge, ZA in a marine plant and CBE in a red alga) and the others have a terrestrial origin (CI in cinnamon and CS in chilli pepper) (see Plate 4). Algophase (A) was used as a positive control even if this product has a biocide action and not an antibiofouling one.

(i) Poly-alkyl-pyridinium salts (pAPS) [1] (*3-alkylpyridinium* active compound) isolated from a marine sponge *Reniera sarai* (Sepčić K., 1997). In fact, the surface of this sponge is smooth and clean, veiled in a greasy secretion containing large amounts of pAPS that probably act as a protective coating against fouling (Turk T pers. obs.). Poly-APS are a mixture of two main polymers with molecular weights of 5520 and 18900 Da, corresponding to polymers composed of 29 and 99 3-octylpyridinium units, respectively (Fig. 8). Purified pAPS are soluble only in water. In aqueous solutions pAPS behave similarly to detergents that are classified as quaternary ammonium compounds. According to their critical micelle concentration and charge, pAPS resemble well-known cationic detergents like cetylpyridinium chloride and cetyltrimethylammonium bromide (Malovrh P., 1999). Therefore, these detergent-like properties of pAPS are capable of exercising a number of biological activities. The

[1] kindly provided by Sepčić K (Univ. of Ljubliana, Biotechnical Faculty, Slovenia)

surfactant activity towards artificial and natural membranes and acetylcholynesterase inhibitory activity might be involved in antifouling molecular mechanisms that prevent settlement of bacteria and metazoan onto the sponge surface (Sepčić K., 2006).

The aqueous extracts from this sponge possesses strong anticholinesterase and helmolytic activity as well as moderate antimicrobial and cytotoxic activities (Sepčić K., 1997). pAPS were tested for their potential anti-microfouling activity, for example their ability to prevent the formation of biofilm on submerged surfaces under laboratory conditions (Garaventa F., 2003). pAPS and some other natural 3-alkylpyridines were found to be very effective in preventing of microbial film formation (Sepčić K., 2006) and was also tested to prevent diatoms, algae and fungi settlement (Faimali M., 2003; Eleršek T., 2008). It was used as a water solution in the concentrations reported in Table 6.

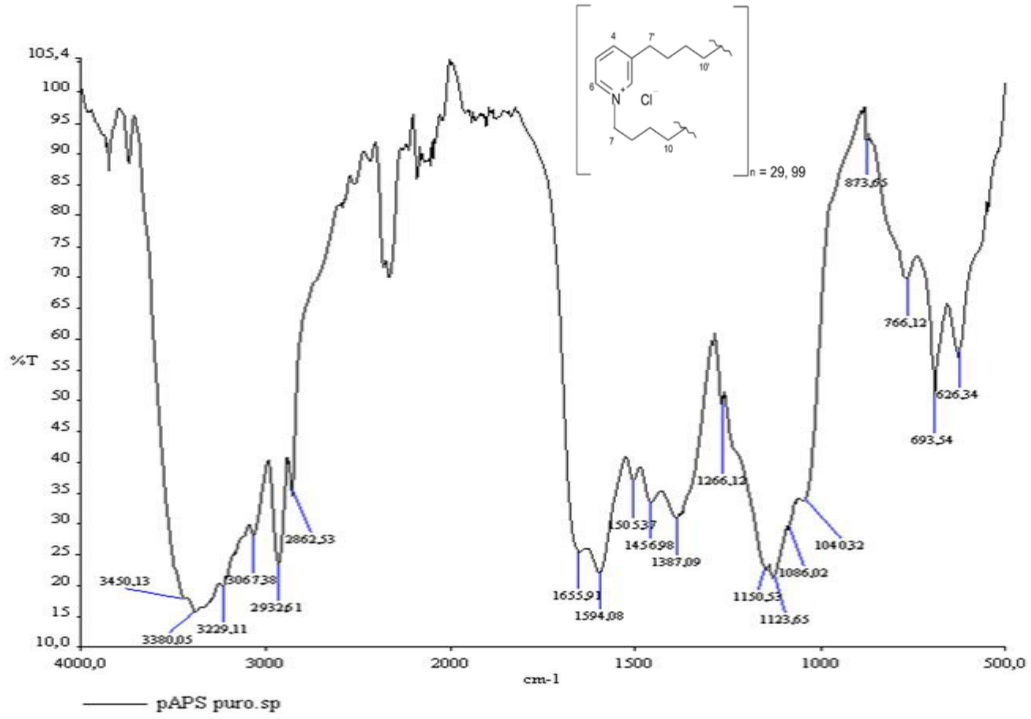


Fig. 8. The FT-IR spectrum and the chemical formula of poly-alkylpyridinium salt

(ii) Zosteric acid (ZA) (*p-sulphoxy-cinnamic acid*) is a natural product present in the eelgrass *Zostera marina.* The eelgrass produces and continuously releases the water-soluble antifouling compound ZA. This antifoulant does not kill microorganisms but inhibits their adhesion trough its binding to special sites on cell-surfaces. The precise mode of action in producing the antifouling response remains undetermined; however, it should be noted that the extracellular polysaccharides

produced by many microorganisms are highly sulphated, and these sulphate esters play an important role in polymerisation (i.e. glue/gel formation) and therefore ZA could be operating at the atomic level by blocking sulphate-binding surface sensors, or by inhibiting the polymerization of extracellular glue (Zimmerman R., Patent 1995). Other authors have been reported also the preventive efficiency of ZA in microbial attachments and the antifouling effect has been attributed to the sulphate ester group (Todd J. S., 1993; Stanley M. S., 2002; Newby B. Z., 2006). ZA is very water soluble and generally, easily washes off the surfaces or leaches out of coatings (Elder S. T., 2007). ZA has shown to prevent biofouling from some marine and freshwater bacteria, algae and algal spores, barnacles, tubeworms and fungal spores adhesion (Todd J. S., 1993; Callow M. E., 1998; Stanley M. S., 2002; Newby B. Z., 2006).

Zosteric acid was synthesised in our laboratory according to previously reported procedures with little modifications (Alexandratos S. D., 1999). The modifications were related with the incomplete purification of zosteric acid, performed only with diethyl ether and methanol. The synthesised chemical compound was characterised by ESI-MS[2] (Fig. 9) and FT-IR (Fig. 10) and the resulting spectra were in agreement with the presence of zosteric acid. It was used as a water or methanol solution, in the concentrations reported in Table 6.

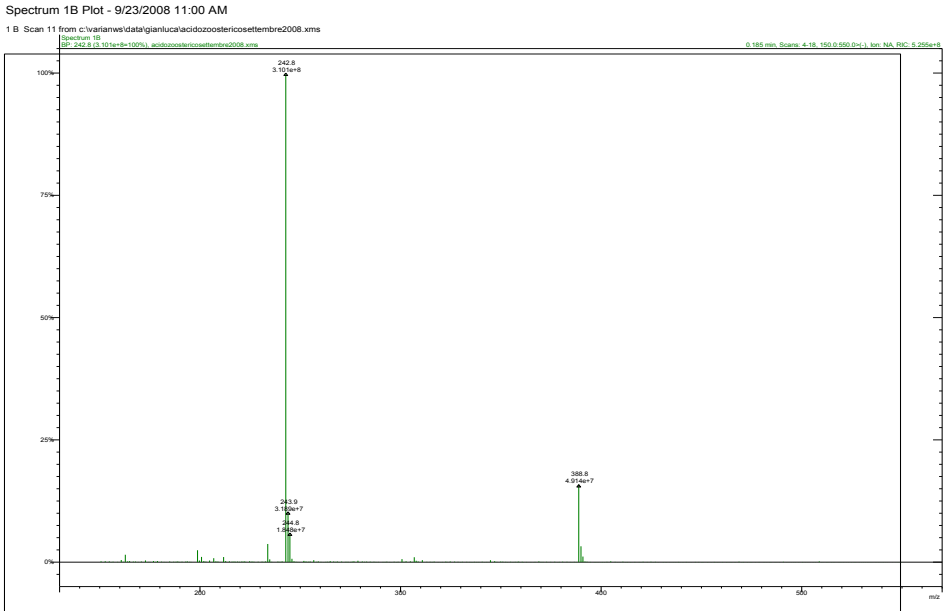


Fig. 9. ESI-MS spectrum of synthesized zosteric acid

[2] GC-MS analysis was made by Prof. M. Bambagiotti of the Department of Pharmacology, University of Florence, Italy

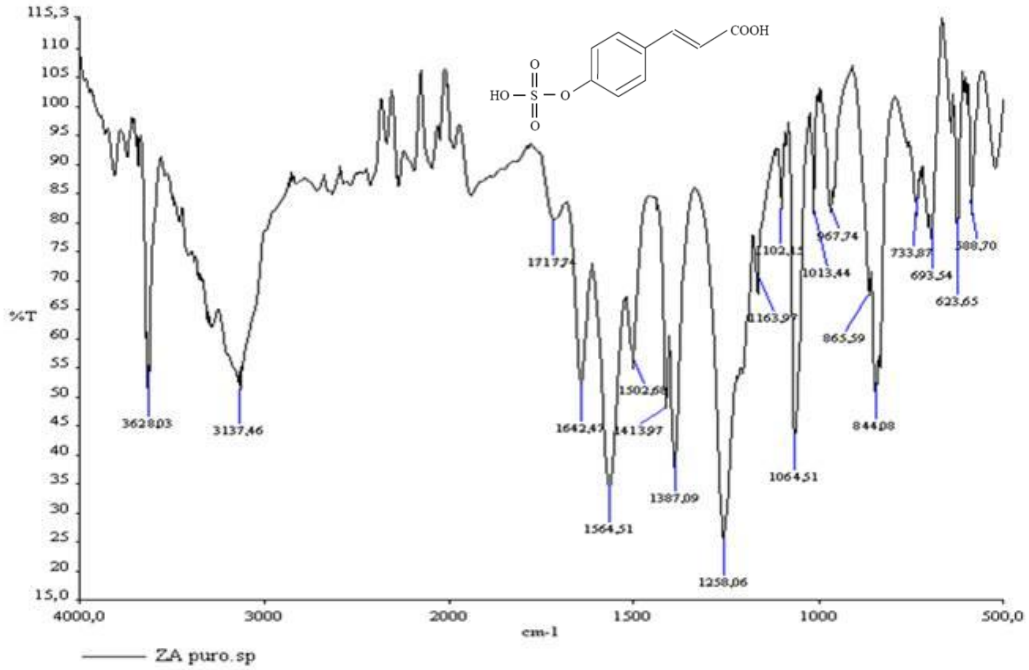


Fig. 10. FT-IR spectrum and chemical formula of zosteric acid

(iii) Extract of ***Ceramium botryocarpum*** **(CBE)**[3] is a dichloromethane extract of a marine rhodophyta (*Ceramium lanciferum* var. *monstruosum* – this name is currently regarded as a synonym for C. *botryocarpum*). This extract and two other ones (ethanol and methanol) of this alga were successfully tested against marine bacteria, diatoms and microalgae (*Ulva* sp.) which are involved in the microfouling process (Bazes A., 2006). The effectiveness of this antifoulant was attributed to the releasing of allelopathically active compounds interfering with settlement and growth competitors. Allelopathy describes any direct or indirect effect of a living organism on another one through biochemical compounds released in the environment, including therefore all biochemicals interactions, both stimulatory and inhibitory (Gross E. M., 2003). The active compound is still unknown. CBE is soluble in water and presents a very high hygroscopicity. The FT-IR spectrum (Fig. 11) has similarities with one of pAPS, and therefore can be suggested its belonging to salts group, with characteristic signals at 1637 and near 3000 cm^{-1}. Very recent studies report the presence of natural digeneaside in salt form in the extracts of red alga *Ceramium botryocarpum* (Claude A., 2009). It was used as a water or methanol solution in the concentrations reported in Table 6.

[3] kindly supplied by A.Bazes (Univ. of South-Bretagne, LBCM, France)

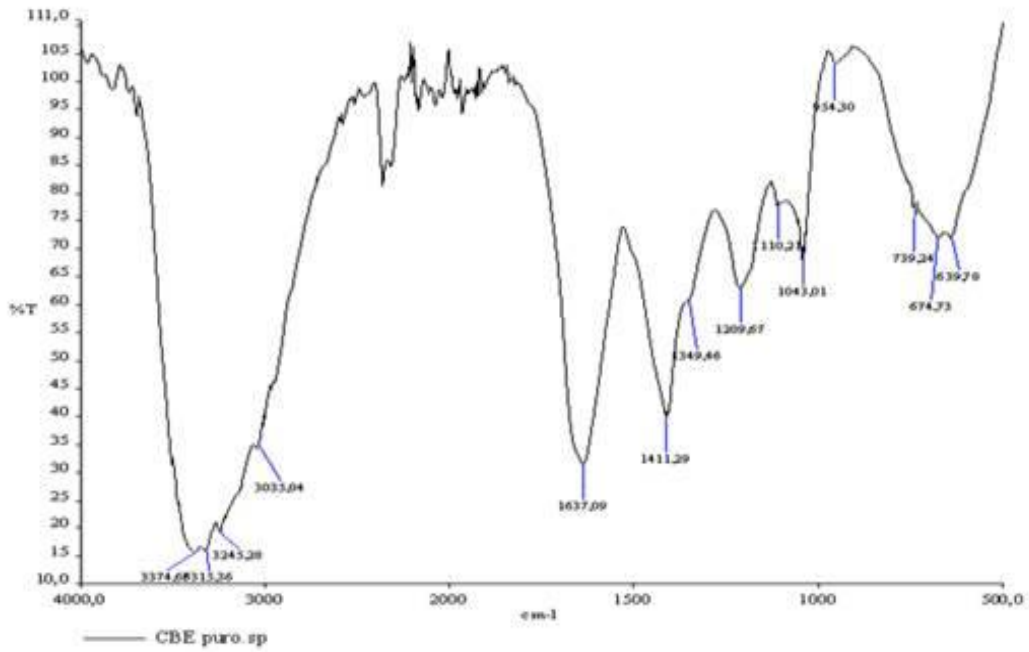


Fig. 11. FT-IR spectrum of *Ceramium botryocarpum* extract

(iv) Capsaicin (CS) (*8-methyl-N-vanillil-6-nonenamide*) is the natural extract from chilli pepper responsible for the 'hotness' of the pepper, being concentrate mostly in the placental tissue of the pepper. Two very pungent synthetic capsanoid compounds are capsaicin and dihydrocapsaicin. It is an incredibly powerful and stable alkaloid seemingly unaffected by heat or cold (Watts J. L., US Patent, 1995). It has limited water solubility (≈60 mg/L). The best co-solvent for obtaining higher concentration of capsaicin in water was ethanol (Turgut C., 2004). It is registered by the US Environmental Protection Agency (EPA) in 1999, as a bird, animal and insect repellent and it was classified as a biochemical pesticide. Capsaicin has previously been assayed for antimicrobial activity (Cichewicz R. H., 1996; Molina-Torres J., 1998) and the efficacy against freshwater bacteria is also reported (Xu Q., 2005). Studies related with the toxicology and metabolism of capsaicin (Surh Y. J., 1995) indicated that capsaicin was toxic only when it was oxidized into an epoxy intermediate. The capsaicin employed in this study was purchased from Aldrich Chemical Co., and its composition was 65% capsaicin and 35% dihydrocapsaicin. Chemical formula and FT-IR spectrum are reported in Fig. 12. It was used as a water or ethanol solution in the concentrations reported in Table 6.

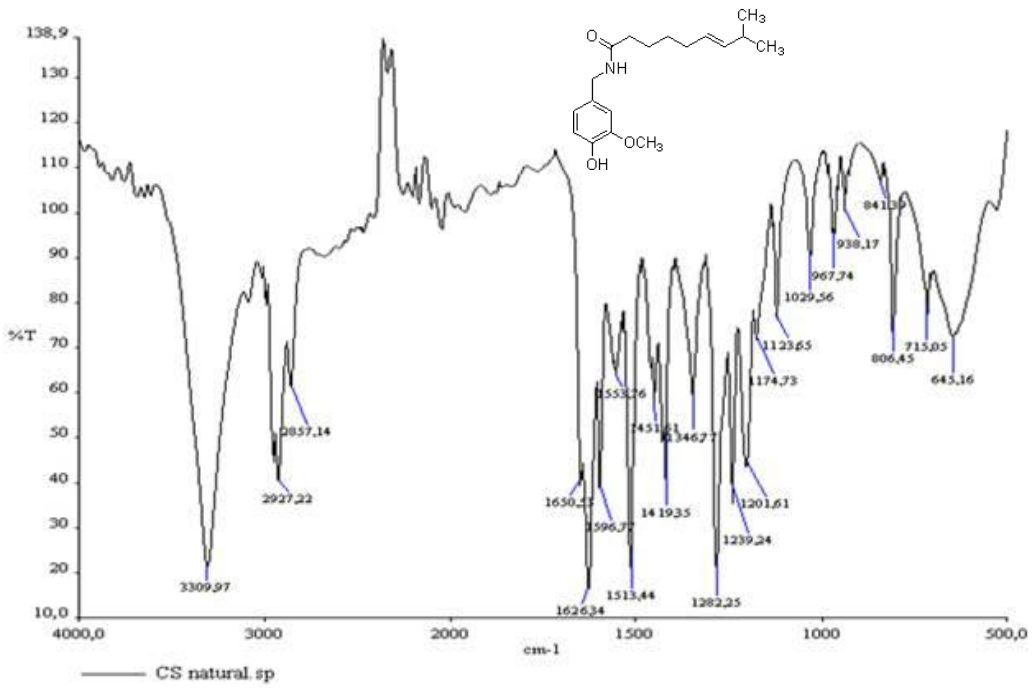


Fig. 12. FT-IR spectrum and chemical formula of capsaicin

(v) Cinnamaldehyde (CI) (*trans-3-phenyl-2-propenal*) is isolated by steam distillation from cinnamon bark. It was especially investigated in the food sector for its antimicrobial activity for both gram-positive and gram-negative bacteria (Bowles B. L., 1995; Helander I. M., 1998). It was found to interfere with quorum sensing of prokaryotes (Niu C., 2006), inhibiting the bacterial and fungal growth (Lee H. C., 2005). Also previous studies have shown that leaf essential oils from *C. osmophloeum* have excellent anti-termite, anti-mildew, anti-mite and anti-mosquito activities (Chang S. T., 2001; Chang S. T., 2002; Cheng S. S., 2004). The mechanism of antimicrobial activity of cinnamaldehyde consist in the interaction with the cell membrane causing disruption sufficient to disperse the proton motive force by leakage of small ions without leakage of larger cell components, such as ATP (Gill A. O., 2004). For this research, the pure cinnamaldehyde was purchased from Aldrich Chemical Co., and it was used as a methanol solution in the concentrations reported in Table 6. FT-IR spectra and chemical structure is presented in Fig. 13.

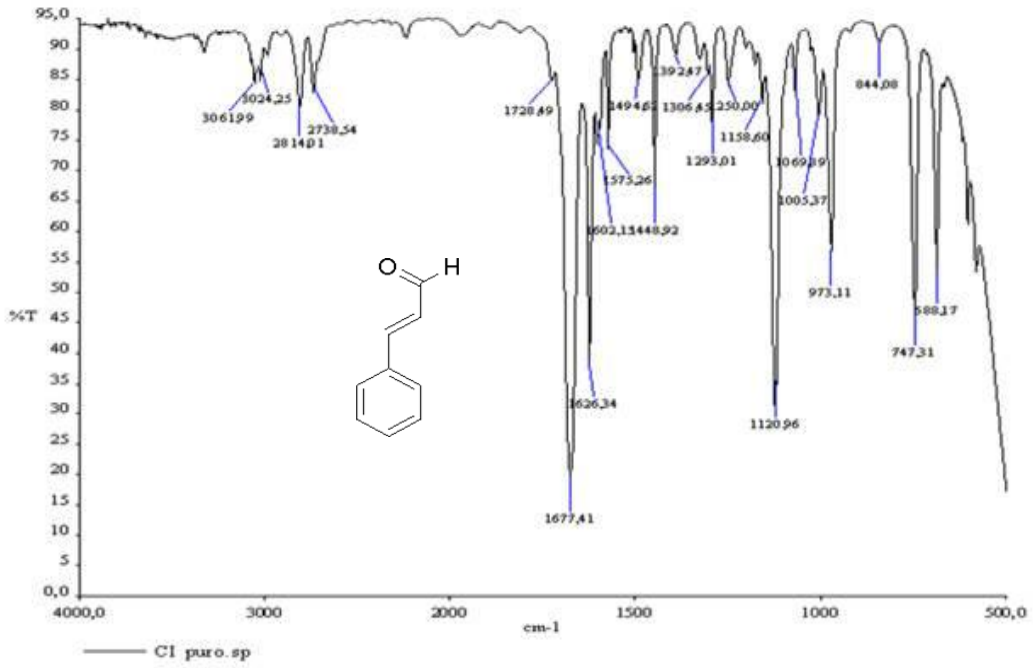


Fig. 13. FT-IR spectra and chemical formula of cinnamaldehyde

Concentrate Algophase (A)[4] (*N-butyl-1,2 benzisothiazolin-3-one*) is a biocide with a large spectrum of action (bacteria, cyanobacteria, algae, fungi). This product was developed by PHASE. Chia et al. has been validated (US Patent 0197366/2005) for its antimicrobial efficiency of *N-butyl-1,2 benzisothiazolin-3-one* against bacterial and fungal strains. Literature reports the use of Algophase® (Tiano P., 1995b; Urzi C., 2007) which had a different active principle (*2,3,5,6-tetrachloro-4 (methyl sulphonyl) pyridine*). It was used as supplied, according to the manufacturer protocol, in the concentrations reported in Table 6. Chemical formula and FT-IR spectrum of A is depicted in Fig. 14.

The mixing of ABAs with coatings was considered necessary due to the fact that most of them have high water solubility and to the aqueous conditions of their application. Possible coatings containing ZA and CS (poly[3-hydroxyalkanoates] and silicones such as Sylgard 184) were currently investigated as environmentally friendly coatings to protect surfaces from biofouling (Geiger T., 2004; Hany R., 2004; Barrios C. A., 2005; Xu Q., 2005; Newby B. Z., 2006). Fluorinated and other coatings silicone-based products were tested by Mussalli Y. G. (1989) over a 2-year period, but significant engineering development is still required.

[4] kindly provided from Phase - Proind Srl. Italy

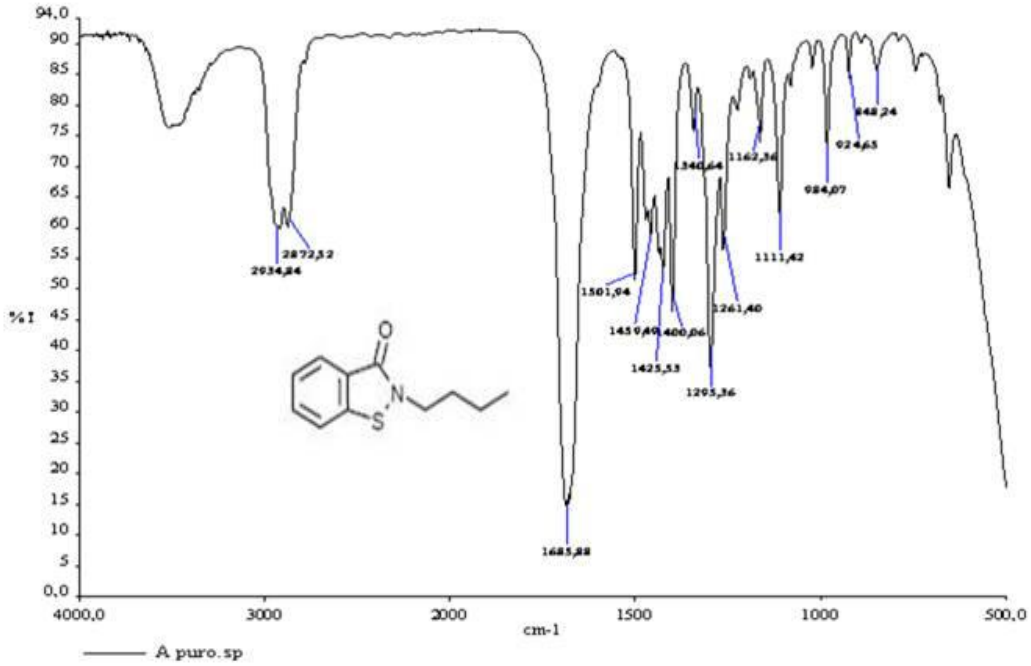


Fig. 14. The FT-IR spectrum and chemical formula of algophase

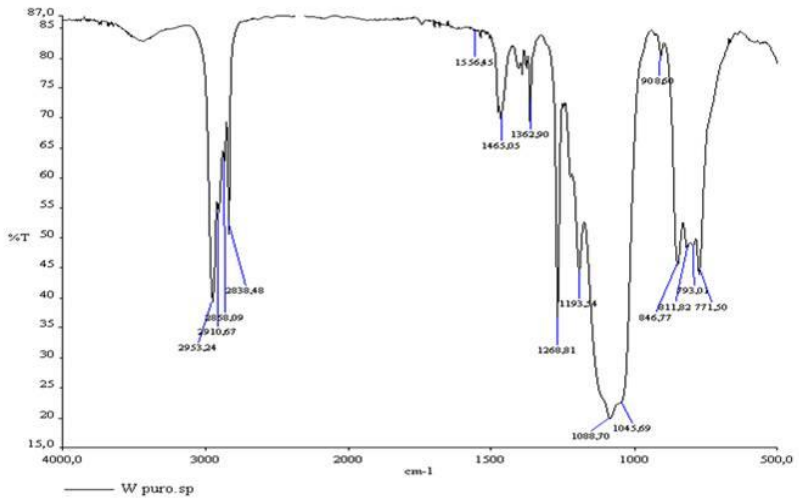
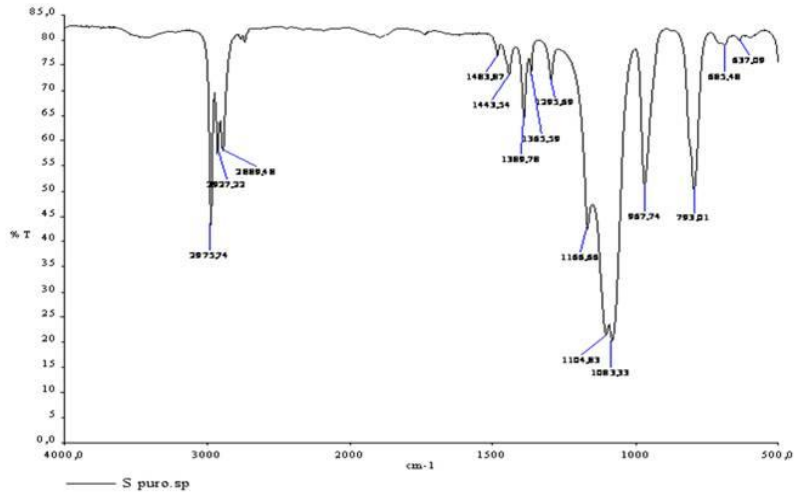
Two common silicone-based coatings (Silres® BS OH 100 and Silres® BS 290) employed in stone conservation (Esbert R. M., 1991; Carò F., 2002; Behamondez M. P., 2007; Tsakalof A., 2007) were chosen in this research as incorporating agents for the selected ABAs. Silicones were among the first synthetic polymers to be used for preservation historic and artistic monuments. The main characteristic of silicones are derived from their organometallic nature and the bonding between silicon and oxygen. The advantage of silicone-based treatments is due to the fact that these polymers render the impregnated material water-repellent but not water-proof (Amoroso G. G., 1983). The silicones were purchased from Wacker Chemie A.G. In Table 5, the chemical formula and the Fourier transform infrared spectra (FT-IR) of tested coatings are reported. The ABAs efficiency were also tested without any coating.

(i) Silres® BS OH 100 (S) is an ethyl-silicate, solventless product, ready-to-use as light consolidant for construction materials. The product contains a catalyst that promotes the reaction between the ethyl silicate and water coming from atmospheric humidity or the moisture present in the capillary pores. A glass-like silica gel binder is formed. Under standard conditions (20°C/50% RH), final hardness is reached after two weeks, when most of the ethyl silicate has been converted to silica gel.

(ii) Silres BS® 290 (pure W) is solventless concentrate silicone, based on a silane/siloxane mixture used as a water-repellent for mineral substrates. It is dilutable

with organic solvents and has a good penetration power and an outstanding beading effect. The hydrophobic silicone resin network is formed within a few hours, even on non-alkaline substrates. After application, it reacts with the atmospheric moisture or with water present in the pores of substrate, generating the active ingredient while alcohol is formed. This coating greatly lowers the water absorbency of the substrate. Concentration tested was 7% in 2-Propanol (W).

Table 5. Structural formula and FTIR spectra of the ABAs and coatings

Coatings	FT-IR spectra
Silres BS 290 (pure W) [-Si(CH_3)(OCH_3)-O-] – [-Si(R_1)(R_2)-O-], solventless R_1, R_2 = alkyl groups	
Silres BS OH 100 (S) Si (OCH_2-CH_3)$_4$	

Supporting agents with different curing periods (5, 15 or 30 days) were tested in order not to completely block the release of ABAs active compounds. The application of treatments on the stone specimens was done brushing four applications at 5 minutes intervals (≈ 20 mg/cm^2). W containing or not ABAs were poured (4ml) in Petri dishes (90mm Ø) and were used for treatment characterization at different curing

times (5 days, 1 month). All treated samples and Petri dishes were let to cure in plastic boxes at room temperature and 50% RH.

The ABAs concentration, type of solvent and coating are reported as operative conditions in Table 6.

Table 6. The operative condition of the experiments

ABAs	Coating	ABAs solution	ABAs concentration	Curing	Experimental application
pAPS	W	water	0.001 % w/v	5 days	indoor/outdoor (immersion method)
			0.003 % w/v	30 days	indoor (diffusion method II)
				5 days	outdoor (immersion method)
			0.005% w/v	30 days	indoor (diffusion method II)
			0.006 % w/v	30 days	outdoor (immersion method)
				15 days	in situ
			0.01 % w/v	30 days	indoor (diffusion method II)
	without coating	water	0.003 % w/v	-	indoor (diffusion method I)
			0.005% w/v	-	indoor (serial dilution method, diffusion method I)
			0.01 % w/v	-	indoor (diffusion method I)
CBE	S	methanol	0.06 % w/v	5 days	indoor/outdoor (immersion method)
			0.2 % w/v	5 days	outdoor (immersion method)
	W	water	0.06 % w/v	5 days	indoor/outdoor (immersion method)
			0.2 % w/v	30 days	indoor (diffusion method II)
				5 days	outdoor (immersion method)
			0.5 % w/v	30 days	indoor (diffusion method II)
			1 % w/v	30 days	indoor (diffusion method II)
	without coating	water	0.2 % w/v	-	indoor (diffusion method I)
			0.5 % w/v	-	indoor (serial dilution method, diffusion method I)
			1 % w/v	-	indoor (diffusion method I)
ZA	S	methanol	0.06 % w/v	5 days	indoor/outdoor (immersion method)
			0.2 % w/v	5 days	outdoor (immersion method)
	W	water	0.06 % w/v	5 days	indoor/outdoor (immersion method)
			0.2 % w/v	30 days	indoor (diffusion method II)
				5 days	outdoor (immersion method)
			0.5 % w/v	30 days	indoor (diffusion method II)
				30 days	outdoor (immersion method)
				15 days	in situ
			1 % w/v	30 days	indoor (diffusion method II)
	without coating	water	0.2 % w/v	-	indoor (diffusion method I)
			0.5 % w/v	-	indoor (serial dilution method, diffusion method I)
			1 % w/v	-	indoor (diffusion method I)

ABAs	Coating	ABAs solution	ABAs concentration	Curing	Experimental application
CS	S	ethanol	0.006 % w/v 0.02 % w/v	5 days 5 days	indoor/outdoor (immersion method) outdoor (immersion method)
	W	water	0.006 % w/v 0.02 % w/v 0.08% w/v 0.1 % w/v 0.16 % w/v	5 days 5 days 30 days 30 days 15 days 30 days	indoor/outdoor (immersion method) outdoor (immersion method) indoor (diffusion method II) outdoor (immersion method) in situ indoor (diffusion method II)
	without coating	ethanol	0.02 % w/v 0.16 % w/v 0.08% w/v	- - -	indoor (diffusion method I) indoor (diffusion method I) indoor (serial dilution method, diffusion method I)
CI	S	methanol	0.4 % v/v 1.5 % v/v	5 days 5 days	indoor/outdoor (immersion method) outdoor (immersion method)
	W	methanol	0.4 % v/v 0.5 % v/v 1.5 % v/v 3 % v/v	5 days 30 days 30 days 5, 30 days 30 days	indoor/outdoor (immersion method) indoor (diffusion method II) indoor (diffusion method II) outdoor (immersion method) indoor (diffusion method II)
	without coating	methanol	0.5 % v/v 1.5 % v/v 3 % v/v	- - -	indoor (diffusion method I) indoor (serial dilution method, diffusion method I) indoor (diffusion method I)
A	S	as supplied	5 % v/v	5 days	indoor/outdoor (immersion method)
	W	as supplied	3 % v/v 5 % v/v 15 % v/v	30 days 30 days 5 days 15 days 30 days	indoor (diffusion method II) indoor/ outdoor (diffusion method II) outdoor (immersion method) in situ indoor (diffusion method II)
	without coating	methanol	3 % v/v 5 % v/v 15 % v/v	- - -	indoor (diffusion method I) indoor (serial dilution method, diffusion method I) indoor (diffusion method I)

All FT-IR spectra have been recorded using a Perkin Elmer System 2000 FT-IR spectrophotometer. FT-IR is an analytical technique that provides information about the chemical bonding or molecular structure of materials, both organic or inorganic. It was used to characterize the ABAs and treatments.

An ESEM Quanta-200 FEI environmental scanning electronic microscope equipped with EDX microanalysis system was used for elemental and morphological investigations. The microanalysis were carried out at 1 Torr, while the quantitative analysis were performed in 0.1 Torr vapour pressure. SEM-EDX allows to determine the chemical composition of a specimen. The **s**canning electron microscope is a type of

electron microscope that collect images of the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals containing information about the sample's surface topography, composition and other properties such as electrical conductivity. We used it for observing the morphology and distribution of silane-siloxane based silicone mixed with some natural antifouling agents.

III.6. Laboratory experiments

III.6.1. *Growing conditions*

A logarithmic phase of a mixed natural biofilms growing in BG11 and composed by samples collected from the Second Fountain – Villa la Pietra (Florence, Italy) and Fountain from Patio de la Sultana - Generalife (Granada, Spain) was used as inoculum for the laboratory experiments, both under indoor and outdoor conditions. The main microorganisms present in the inoculum were: green algae (*Conjugatophyceae*, *Chlorella* sp., *Cosmarium sp.*, *Palmela* sp.), cyanobacteria (*Leptolyngbya* sp., *Aphanocapsa* sp., *Gloeocapsa* sp.), diatoms (*Navicula* sp., *Nitzchia* sp.) and some protozoa.

The growing conditions for all experiments are reported in Table 7. The stone samples treated with lower concentrations of ABAs were kept under laboratory controlled conditions. The stone samples treated with higher concentrations were exposed outdoor to the local environmental conditions. In both cases the samples were completely immersed into well water sown with biofilm inoculum.

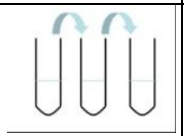
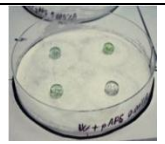
III.6.2. *Indoor experiments*

III.6.2.1. Immersion method

Marble specimens (50x50x10 mm) and glass microscope slides (7,5x2,5x0,1 mm) treated with the ABAs mixed with two coatings (S and W) were cured for 5 days and therefore they were completely immersed in well water sown with the biological inoculum and kept under controlled conditions (Table 7).

The concentrations of ABAs that have been used are reported in Table 6.

Table 7. Experimental conditions for ABAs efficiency assessment.

Conditions	**Indoor experiments**				**Outdoor experiments**			
Method	**Immersion method**	**Serial dilution method**	**Diffusion method I**	**Diffusion method II**	**Immersion method**			
Materials	 plastic container (33x27x13 cm) filled with water well	 sterile tubes and Petri dishes (90 Ø)	 Sterile Petri dishes (90 Ø)	 Sterile Petri dishes (90 Ø)	 plastic container (150 cm Ø, 50 cm height) filled with water well, stagnant water	 plastic container (150 cm Ø, 50 cm height) filled with water well, refreshing of water for 5 min every hour	 plastic container (68x48x20 cm) filled with water well	 plastic container (150 cm Ø, 50 cm height) filled with water well, refreshing of water for 5 min every hour
Substratum	• microscope glass slides • Carrara marble ($5x5x1cm^3$)	• Water well	• Agarized specific cultural media	• Carrara marble powder mixed with W containing ABAs	• Carrara marble ($1x1x0.5\ cm^3$ and $5x5x1\ cm^3$) and Sierra Elvira stone ($1x1x0.5\ cm^3$)	• Carrara marble ($10x5x1\ cm^3$)	• Carrara marble ($5x5x1\ cm^3$)	• Carrara marble ($5x5x1\ cm^3$)
ABAs and coatings	• ABAs incorporated into S and W coating, cured for 5 days	• ABAs without coating agent	• ABAs without coating agent	• ABAs incorporated into W coating, cured for 30 days	• ABAs incorporated into S and W coating, cured for 5 days		• ABAs incorporated into W coating, cured for 30 days	
Exposure	low artificial lighting with continuous white fluorescent light at photosynthetic photon flux density of 10 µmol photon $m^{-2}.s^{-1}$ in conditioned room at 27 ºC				east orientation, day/night cycle, temperatures between 9ºC and 37ºC (the average for T_{min} and T_{max} during the two months of experiment being 17.4ºC and 29.9 ºC, respectively)			
Inoculum	Mix of biofilm (≈ $17x10^5$ cells/ml)	Mix of biofilm (≈$98x10^4$cells/ ml)	Cyanobacteria(≈$57x10^5$ cells/ml) Algae (≈$38x10^5$ cells/ml) Fungi (≈$15x10^5$ cells/ml)		Mix of biofilm (≈ $16x10^5$ cells/ml)		Mix of biofilm (≈ $47x10^5$ cell/ml)	

Epifluorescence microscopic technique was used for recording the autofluorescent signal of the phototrophs (Nikon Eclipse E600, objectives (4x, 10x), TRITC filter with excitation at 450-490 nm and emission in red region, > 610 nm). The evaluation of the biofilm growth, on the treated glass slides after 10 days of incubation, was done observing them under epifluorescence microscopy. Each treated glass slide was divided into 25 areas totting up a 290.32 mm^2. The fluorescent image of each area was elaborated and the number of fluorescent spots (corresponding to at least one microorganisms) counted.

As regard the Carrara marble specimens, the fluorescent images, photographed with the same digital CCD camera (Nikon DXM1200F) were elaborated using the ImageJ® program, and a two-dimensional semi-quantitative evaluation of phototrophic community colonization on the stone specimens was performed. A total of 30 images were collected for the 5x5x1 cm stone samples using the 4x objective. All the images were captured in RGB scale and saved in JPG format and therefore elaborated in order to estimate the area covered by phototrophs.

ImageJ® is e free Java-based image processing program developed at National Institute of Health. It can display, edit, analyze, process, save and print many image formats including the ones recorded by EM or CLSM. It supports "stacks", a series of images that share a single window, can calculate area and pixel value statistics of user-defined selections. ImageJ® was designed with an open architecture that provides extensibility via Java plugins. Custom acquisition, analysis and processing plugins can be developed using ImageJ's built in editor and Java compiler. User-written plugins make it possible to solve almost any image processing or analysis problem.

III.6.2.2. Serial dilution method

Two sets of 9 tubes each were prepared for each antibiofouling agent, containing triplicates of three different concentrations of aqueous dilutions. One set was used for spectrophotometrical analysis and the other one for dry biomass quantification. The initial concentration for each ABAs was: 0.5% w/v for ZA and CBE, 0.005% w/v pAPS, 0.08% v/v CS, 1.5% v/v CI, 5% v/v A and 15% v/v MeOH as a control, because it was used as a solvent for CS and CI. These concentrations were each diluted ten and hundred times, respectively (e.g. the following concentrations of zosteric acid were used: 0.5% (dil 0), 0.05% (dil 1), 0.005% (dil 2)). 100µl of biological inoculum (a

mixed biofilm derived from samples collected in real fountains) was added in every tube, reaching a concentration of ≈98x10^4cells/ml (Neubauer count chamber).

The biological growth was evaluated by "in vivo" measurements of chlorophyll *a* and optical density (680 nm, 750 nm) (Fig. 15), at 6 time intervals (0, 5, 10, 20, 40, 60 days), using a Beckman DU 640 spectrophotometer. The estimation of pigment content was corrected by subtraction of residual absorbance at 750nm (Lee T., 1995). Media and standard deviation of three measurements for each dilution was considered. Small aliquots (0.75ml) from each tube were diluted with 0.75 ml H_2O, sonicated in the cuvette for 10 seconds in continuous pulse at 10 watts, using a MISONIX Sonicator, in order to disperse clumps and chains (Petersen F. C., 2004), and therefore immediately measured. Some ABAs (capsaicin, *C. botryocarpum* extract, cinnamaldehyde, algophase) induced an opaqueness or a bit yellowing of the water at the start concentrations, in this case the spectrophotometric measurements cannot be considered, being out of range.

A qualitative analysis of the dominant components present in each dilution and type of ABAs was done, sowing 200µl on the specific agarized cultural media (BG_{11}, BG_{11} modified and diluted 1:1, PDA) for cyanobacteria, algae and fungi, respectively.

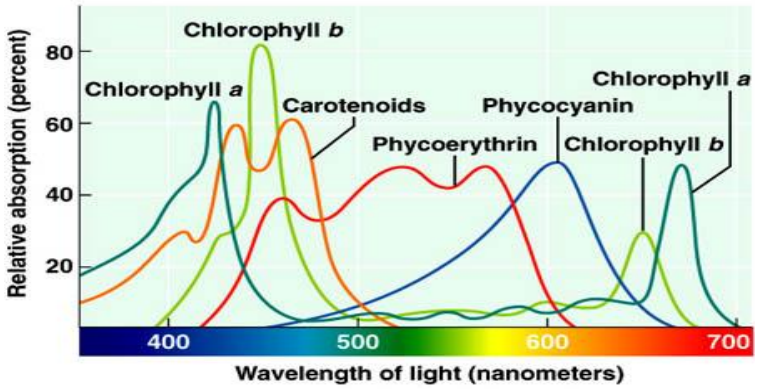


Fig. 15. The absorbance of various pigments of phototrophic microorganisms in visible light

Dry weight was determined filtering a known volume (9 ml) of aqueous ABAs solutions containing the biofilm, by means of nitrocellulose acetate filters (0.22 µm). For the dry weight determination, filtered cells were kept at 105°C for 2 hours.

The growing conditions are reported in Table 7.

III.6.2.3. Diffusion method I

For each ABA a set of 3 Petri dishes were used for testing the efficiency against 3 types of microorganisms (a cyanobacteria; a green alga; and a fungus (Fig. 16)) using specific cultural media for each of them (BG_{11}; BG_{11} modified and diluted 1:1; and PDA respectively). Petri dishes with the appropriate cultural media were inoculated with specific isolated strain of above mentioned 3 microorganisms, using 0.25ml inoculum with a concentration of $\approx 57x10^5$ cells/ml for cyanobacteria, $\approx 38x10^5$ cells/ml for algae and $\approx 15x10^5$ cells/ml for fungi. Sterile small cylinders (≈4 mm diameter) were sunk into the inoculated cultural medium and filled with 0.1 ml ABA, W at different concentrations, as can be seen the Table 8. Then, the Petri dishes with phototrophs were incubated in continuous low light at 28°C, while the one with fungi at 25°C, in darkness. The valuation was done by visual inspection at different time intervals (7, 24 and 75 days).

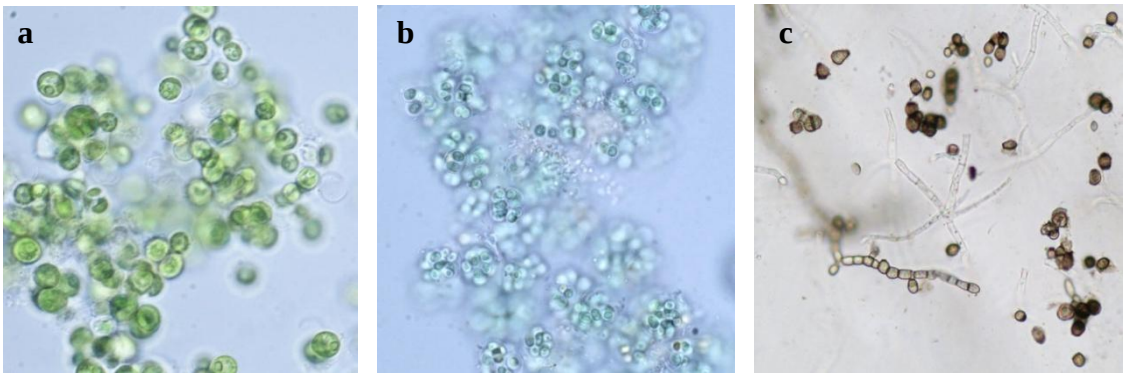
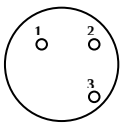
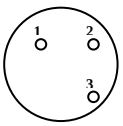
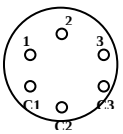
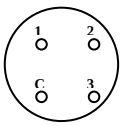
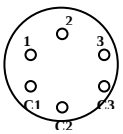
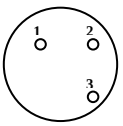
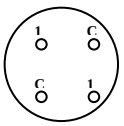


Fig. 16. The algal (a), cyanobacterial (b) and fungal (c) strains used as inoculum for the experiments of Diffusion methods

Table 8. Location and ABAs concentrations of cylinders used in the experiment 'Diffusion method I'. Cylinders marked with numbers represent various ABAs concentrations, and the ones with C are the controls in which were added: pure 2-Propanol for A and W; MeOH (30%, 15%, 5% v/v) for CS and CI dishes. The empty space, without sunken cylinder, was considered the control for the others ABAs which were dissolved in water (ZA, pAPS and CBE)

						
ZA **1%** **0.5%** **0.2%**	**pAPS** **0.01%** **0.005%** **0.003%**	**CS** **0.156%** **0.078%** **0.02%**	**A** **15%** **5%** **3%**	**CI** **3%** **1.5%** **0.5%**	**CBE** **1%** **0.5%** **0.2%**	**W** **7%**

III.6.2.4. Diffusion method II

This method was thought as a complementary method to the Diffusion method I. In this case the microorganisms diffuse on the substratum containing ABAs, while on the Diffusion method I, are the ABAs diffusing on the surface containing microorganisms. The ABAs products mixed with W and W alone (2 ml) were amalgamated with Carrara marble powder (1.5g with grains size <0.63mm) into Petri dishes (90 mm) and let to cure for 1 month in plastic boxes at room temperature and 50% RH. Three different concentrations were used for each ABAs, as the one reported in Table 8. In every Petri dish were placed 4 drops (200µl each) of 4 different inoculum type (the same strains as in the Diffusion method I: an alga, a cyanobacteria, a fungus plus a mix of all three). The drops were let to dry for few hours under sterile conditions. Sterile 90 mm filter paper discs (3 for each Petri dish) were placed on the loose-fitting plastic cover and humidified with 3 ml of BG_{11} and then, the Petri dishes were sealed with parafilm, settled upside-down and incubated in continuous low light, 28°C. The visual inspection, after 1 and 15 days, was carried out using a portable Celestron Handled USB Digital Microscope. The results from the mixed inoculum were not considered relevant because of the fungal dominant influence.

III.6.3. *Outdoor experiments – immersion method*

Carrara marble (50x50x10 mm, 10x10x5 mm) and Sierra Elvira stone specimens (10x10x5 mm for both types of stones) treated with the ABAs mixed with two coatings (S, W cured for 5 days) were completely immersed in well water sown with the biological inoculum and kept under natural outdoor climatic conditions in the plastic basin simulating a fountain with stagnant water (Table 7). Other Carrara marble specimens (100x100x10 mm) treated with W containing ABAs and cured for 30 days have been partially immersed in the simulation fountain with sprinkling water. The concentrations of ABAs that have been used are reported in Table 6.

The control of phototrophic growth was done by visual examination and EM as described for the indoor experiments (III.6.2.1.), 5 images being collected for each 1x1x0.5 cm stone samples and 30 images for 50x50x10 mm marble specimens, using the 10x objective.

The three-dimensional growth of phototrophic biofilm was quantified on the image stacks captured by confocal laser scanning microscopy (Leica TCS SP5, excitation beams at 488 nm and 543 nm and emission at 500-531 nm and 680-784 nm) in two channels (for phycoeritryn and chlorophyll *a*) using the same small Carrara marble specimens (1x1x0.5 cm). One stack images acquisition was performed in central point of each stone specimens. The images from stacks were captured at 0.6 µm intervals, in RGB scale, filed in TIFF format, 40x magnification lens (captured area of 144400 μm^2). The same program for image analysis was used for calculating the covered area and an ImageJ® special plug-in for voxels counting was applied for evaluating the biovolume of each stack.

The fluorescence spectra of stone material and W coating was recorded using lambda scan function of CLSM, using λ_{exc} of 458nm. Due to a strong autofluorescence of Carrara marble, Sierra Elvira stones and Silres BS 290 in the green region, it was noticed an interference with the autofluorescent signals of some phototrophs (cyanobacteria), limiting also the possibility of using fluorescent labels. The recorded fluorescence intensity of lambda scans are reported in Fig. 17.

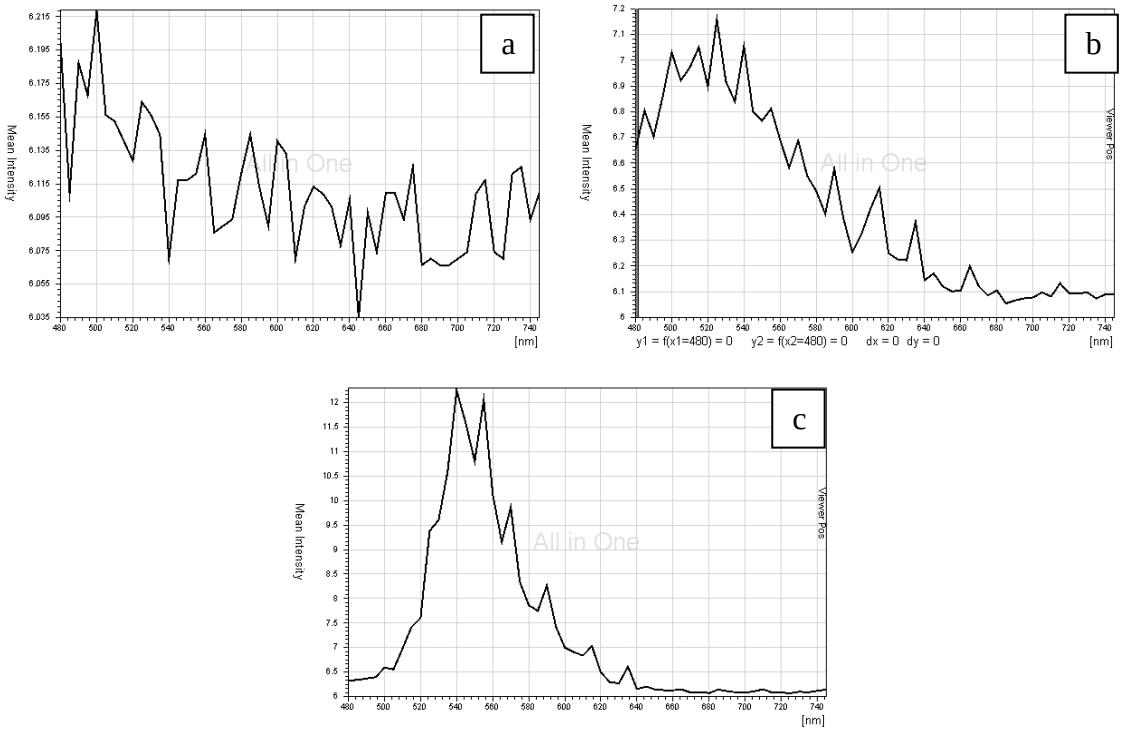


Fig. 17. Two-dimensional plots with the spectra of Carrara marble (a), Sierra Elvira stone (b) and Silres BS 290 coating (c)

III.7. In situ experiments

III.7.1. *Cleaning procedures, application of treatments and optical evaluation*

The monumental Fountain from Patio de la Lindaraja, placed in the Alhambra Palace in Granada, Spain and Tacca's Fountain 2 from Florence, Italy (Fig. 18) were chosen as representative case studies for the in situ experiments. Both experiments were carried out for six months, from October 2008 to April 2009.

Fig. 18. The monumental fountains chosen for in situ experiments: (a) Fountain from Patio de la Lindaraja and (b) Tacca's Fountain 2

The internal part of lower basin of both fountains was picked out for the experiments and the selected areas were cleaned before the ABAs application in order to remove the biological patina. The cleaning procedure on Patio de la Lindaraja Fountain consisted of mechanical removal by abrasion, performed by Alhambra staff. The structure of the lower basin of Lindaraja Fountain, made of Sierra Elvira stone, still maintain, after cleaning, its surface covered by a calcareous concretions layer.

Tacca's Fountain 2, made of pietra serena and Carrara marble was cleaned with the help of OPD staff from Florence, conforming to the following operations (Fig. 19, 20):

- repeated applications of H_2O_2 by brushing, for elimination of superficial calcareous layer together with the biological patina;

- mechanical removal of the thick calcareous layer by using a micro sand blasting device;
- new applications of H_2O_2 by brushing, for removal the remaining biological patina;
- poultices of ions-exchange resins and Japanese paper, and rubbed then with a bristle brush;
- new application of H_2O_2, and the rinse with water next day;
- every intervention was followed by rising with deionized water;
- application of acetone by brushing in the last step, for humidity leftover elimination.

Fig. 19. The cleaning procedures performed on Tacca's Fountain, Florence, Italy

Fig. 20. Selected area of Tacca's Fountain 2, before (a) and after (b) cleaning

Taking into account the laboratory results, 3 ABAs (ZA, pAPS and CS), a biocide (A) and W coating were used for in situ application tests in the concentrations reported in Table 6.

Six areas (30x20 cm^2 each, separated by a 2 cm of neutral zone) were delimitated on the internal surface and the chosen treatments were brushed in 4 applications (≈ 20 mg/cm^2) (Fig. 21). The treatments were let to cure in natural conditions for 15 days before the reopening of the water flux.

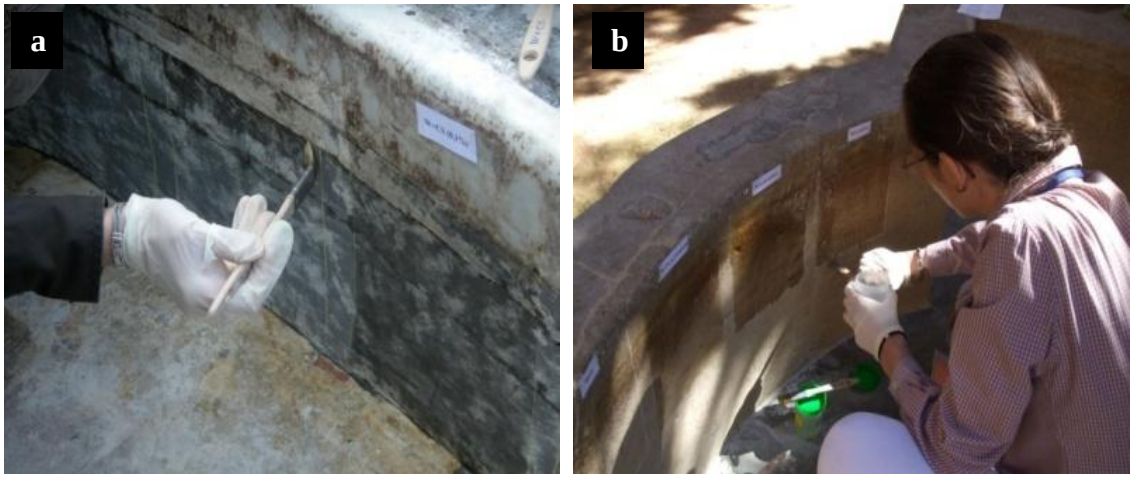


Fig. 21. Application of selected treatments containing ABAs on the internal part of the basin of Tacca's Fountain 2 (a) and Fountain of Patio de la Lindaraja (b)

The evaluation was carried out by visual inspection, using a portable Celestron Handled USB Digital Microscope and by molecular analysis with ARISA fingerprint method. Samples of biological patina present on the selected zones were collected before starting the experiments and after 4 and/or 6 month after the application of treatments for morphotypes identification by optical microscopy (Nikon Eclipse E600) the molecular characterization of biodiversity, by ARISA fingerprint. The schemes for both fountains with the selected areas, sampling zones and microscopical images capturing sites are depicted in Fig. 22.

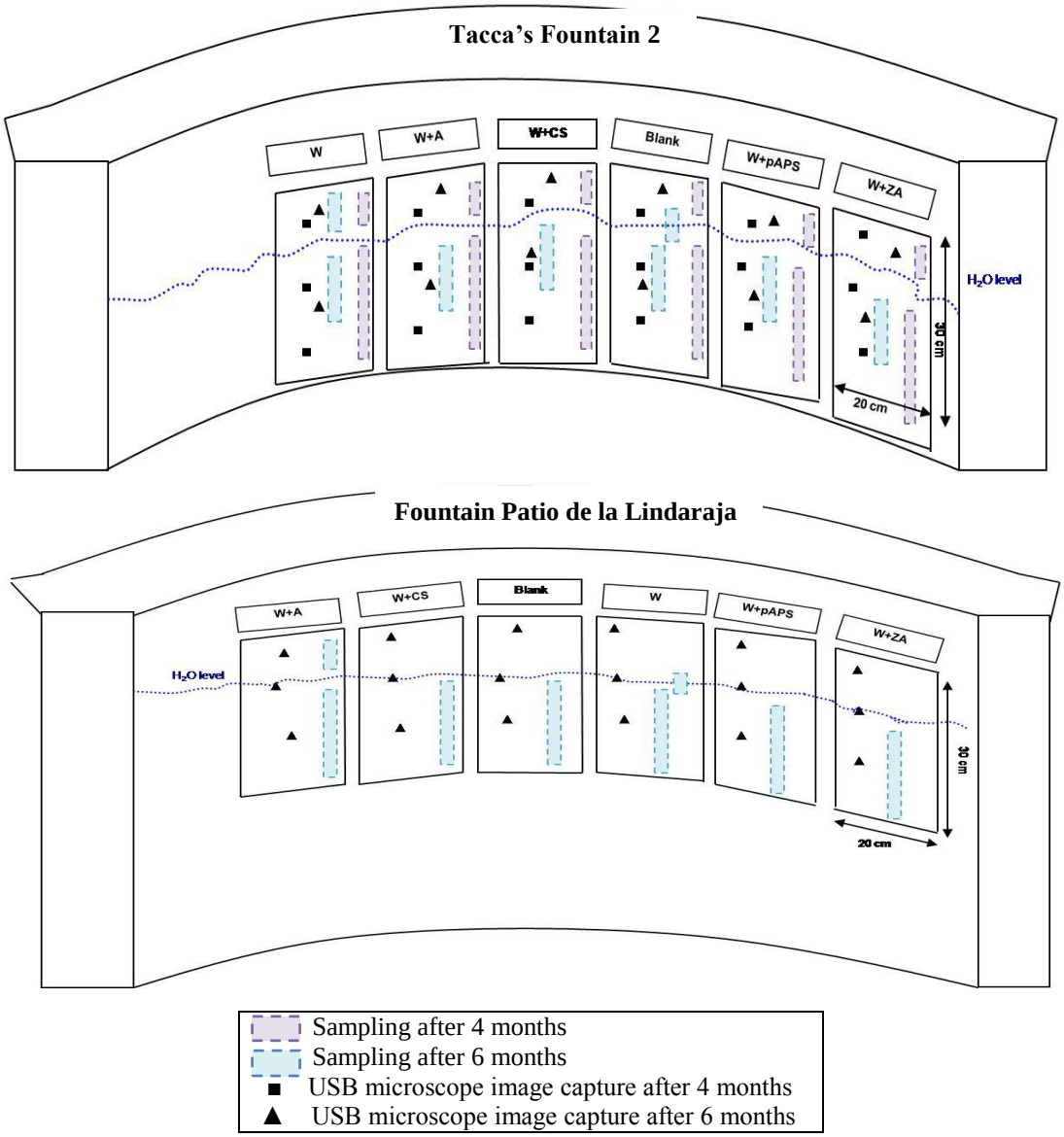


Fig. 22. Selected area, sampling zones and location of microscopical images capturing in the Tacca's Fountain and Fountain from Patio de la Lindaraja

III.7.2. *Molecular evaluation of treatments efficacy*

Total community DNA of the samples collected from the fountains was extracted using PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories Inc.), according to the manufacturer's protocol. The quality of DNA extracts was observed as described in III.3.1.2. PCR conditions that have been used are reported in III.3.2.2. The amplified DNA (2 ng/ µl each) was then sent to BMR Genomics for fragment separation in a capillary chromatographer. A fluorescent dye-labeled size standard GeneScan™ ROX1000BV (50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600,

650, 700, 750, 800, 850, 900, 950, 1000 base pair fragments) was added and the analyze was carried out with a GeneScan 3100 ABI Automated Capillary DNA Sequencer (Applied Biosystems). The computational analysis were performed with Peak Scanner ™ (Applied Biosystems 1.0, 2006). Only peaks higher than 100 relative fluorescence units and fragments between 100-1000 base pairs were considered. The evaluation of efficiency was performed by comparing the total number of peaks present in each sample. The cyanobacterial primer (23S20R) may also amplify the DNA of chloroplasts, providing information about all phototrophs (including algae and diatoms) (van Gremberghe I., 2008; SILVA sequences database). The ARISA electroferograms consist in peaks, each peak represents a different taxonomical unit from the sample. Each peak height and area are related to the amount of DNA in the peak and a correspondence between these values and the taxon abundance in the sample can be deduced.

IV. RESULTS AND DISCUSSIONS

IV.1. Biofilms on monumental stones

IV.1.1. *Biofilm development*

Observing the patina composition on marble specimens with the confocal microscope we can confirm that the first stone colonisers are the green algae, cyanobacteria and diatoms. These phototrophic pioneers are observed in the young biofilm just after 6 days of incubation in contaminated water (Fig. 23) and it can be seen that the cyanobacteria prefer to colonize deeper irregularities of the stone substrate. The presence of the EPS and heterotrophs can be observed just after 22 days of immersion in the contaminated water, under indoor laboratory conditions (Fig. 24).

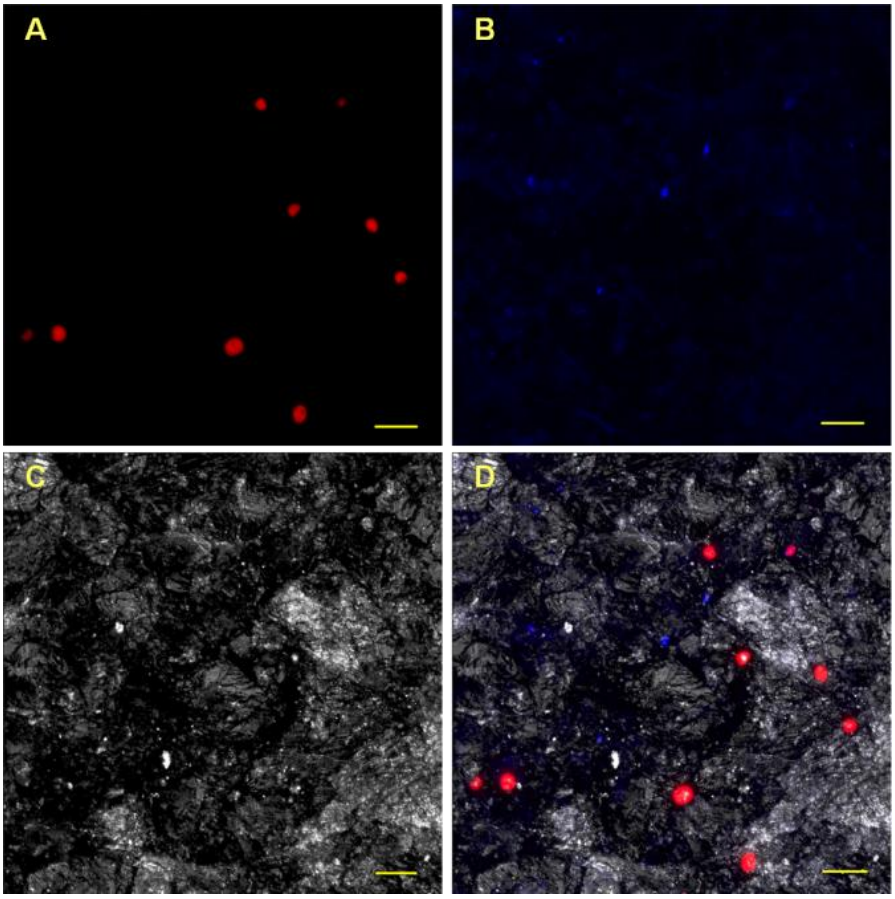


Fig. 23. Maximum intensity projection images of 6 days old biofilm developed on the marble sample examinate by CLSM. Images showing single channel signals of (A) autofluorescence of individual algae in the red channel, (B) filamentous bacteria and microorganisms in the blue channel, (C) reflection of the substratum in the blue channel, and (D) the resulting overlay of the four channel (in the green channel was not recorded any signal). Scale bar 20 μm.

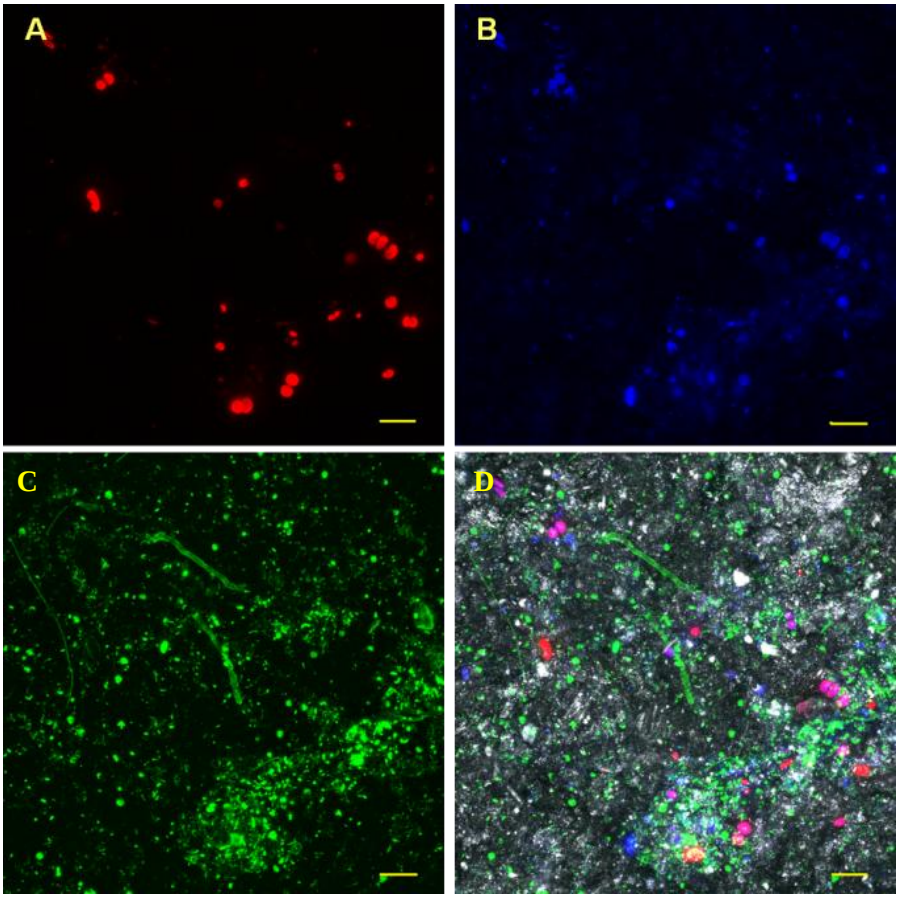


Fig. 24. Maximum intensity projection images of 22 days old biofilm developed on the marble sample examinated by CLSM. The single channel signals show (A) the algae and diatoms in division captured in the red channel, (B) the algae and the cyanobacteria autofluorescence in the green channel, (C) the polysaccharidic matrix and fungi hyphae stained with concanavaline A cojugated with Alexa Fluor 488 recorded in the blue channel, and (D) is the resulting overlaying of all four channels (including the channel used for capturing the substratum signal, in the reflectance mode). Scale bar 20 μm.

The formation of a visible biofilm on a stone surface in natural environment is a quite longer process, even months, and its proliferation is strongly dependent by the environmental conditions (especially light, temperature and water content) and on the concentration of the microorganisms that can adhere on. Therefore, the external factors can enhance or hamper the development of phototrophic biofilms. For example, under outdoor conditions, the biofilm grew exponentially in the first few days on Carrara marble specimens kept in the simulation basin with sprinkling water while the growth on the stone specimens maintained in stagnant water was not so aggressive (Fig. 25). The thickness of the formed biofilm was also considerable different in the two situations: 14μm for the biofilm formed in stagnant water, with respect to 25μm for that one developed in the simulation fountain with continuous supplying of fresh water. This

behaviour is certainly due to the continuous renew of nutrients and oxygen. The presence of capsulate EPS (Fig. 25 bA and bC) focused its important role in the initial stages of biofilm formation (Decho A. W., 2000; Barranguet C., 2005). The filamentous algae (*Mougeotia* sp.), filamentous cyanobacteria and (*Leptolyngbya* sp.) round shape cyanobacteria (*Chroococcus* sp.) were preponderant in the biofilms developed in the fountain with water renewal, while the biofilm developed in the other simulation fountain was composed mainly by cyanobacteria and algal forms with sheaths (e.g. *Palmela* sp., *Chroococcus turgidus*).

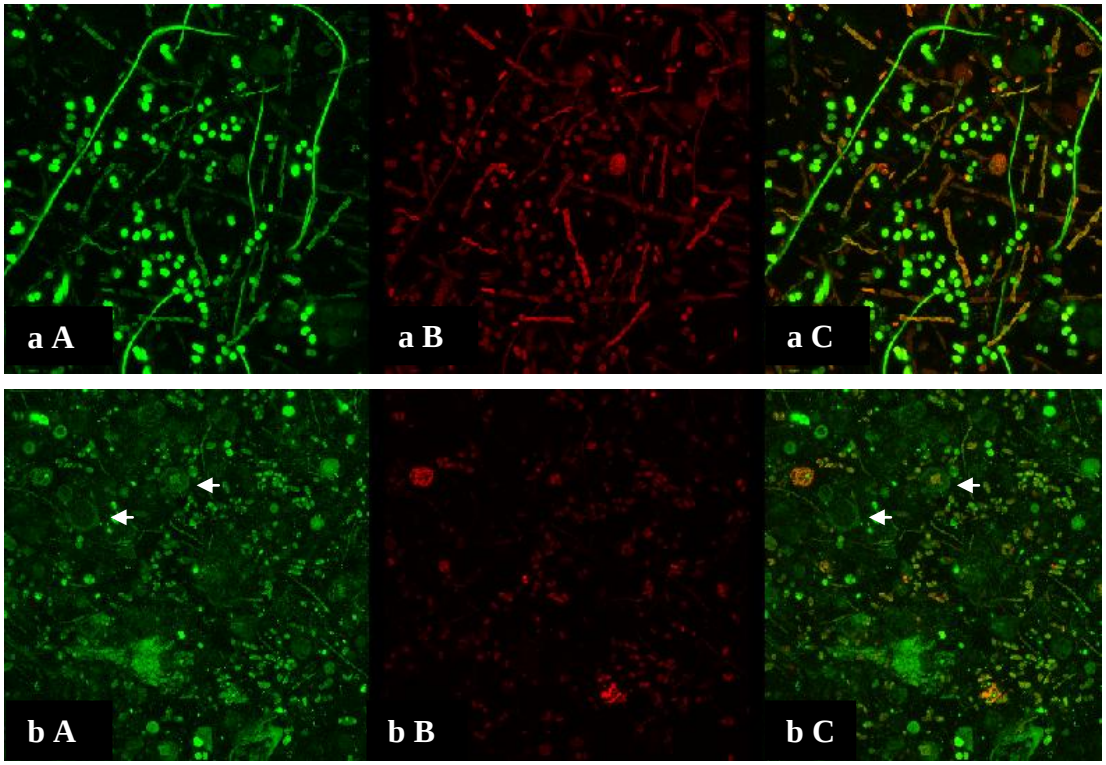


Fig. 25. Confocal microscope images of phototrophs recorded in green (A) and red channel (B) and the overlay of both channels (C) for biofilms developed on Carrara marble specimens under outdoor conditions in the simulation fountain with constant refreshing of water (a) and in that one with stagnant water (b). Key to colour and symbols: green – cyanobacteria and unwanted fluorescence of the marble, red – algae, orange – phototrophs containing different pigments which have been emitted fluorescence in both channels, arrows – capsulate cyanobacteria. 70 optical section (z step = 0.40 μm) with 27.53 μm total thickness of the biofilm were recorded for set (a) and 38 optical section (z step = 0.40 μm) with 14.76 μm total thickness of the biofilm were recorded for each set (b)

The formation of biofilm prevailed at the water/air interface on Carrara marble specimens (10x5x1 cm) immersed only for a half in the simulation fountain with sprinkling water. Biofilm spread especially where the stone was permanently in contact with the water than in the areas sporadically wet, e.g. above the water level.

In Fig. 26 it can be seen the one month old phototrophic biofilm that have been formed with respect to the water availability.

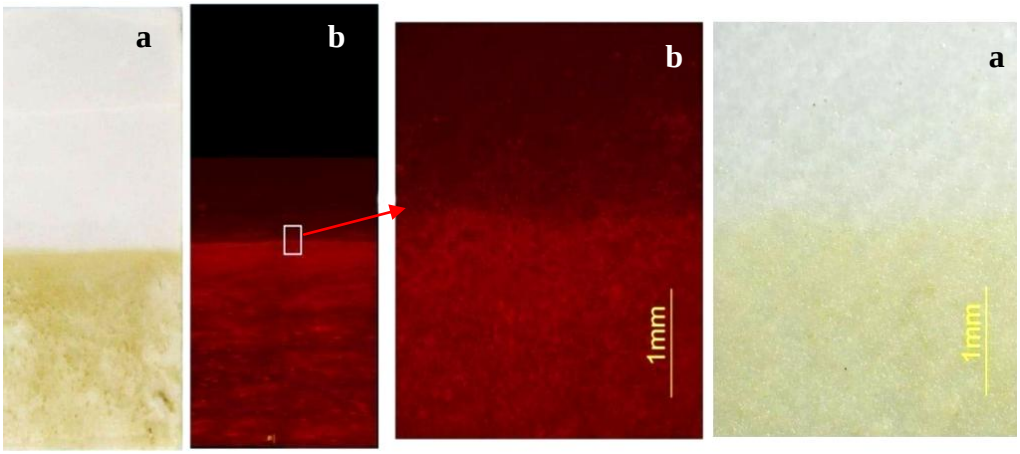


Fig. 26. Images in visible (a) and red light (b) of a 30 days old biofilm developed on Carrara marble specimens (10x5x1 cm) partially immersed in water with a detail at the air/water interface

The back side of stone specimens, sheltered by direct sunlight shown much green patina than the front side, for all samples, partially or completely immersed into well water for 5 months (Fig. 27). This behaviour suggest the preference of biofilm for the lower light intensities and constant high humidity. A difference in the microbial composition developed on Carrara marble specimens, kept in the two simulation fountains was also observed. Diatoms prevailed in the simulation fountain with sprinkling water, especially under the water level, while the cyanobacteria were dominant above the water level and on the samples completely immersed in the simulation fountain with stagnant water.

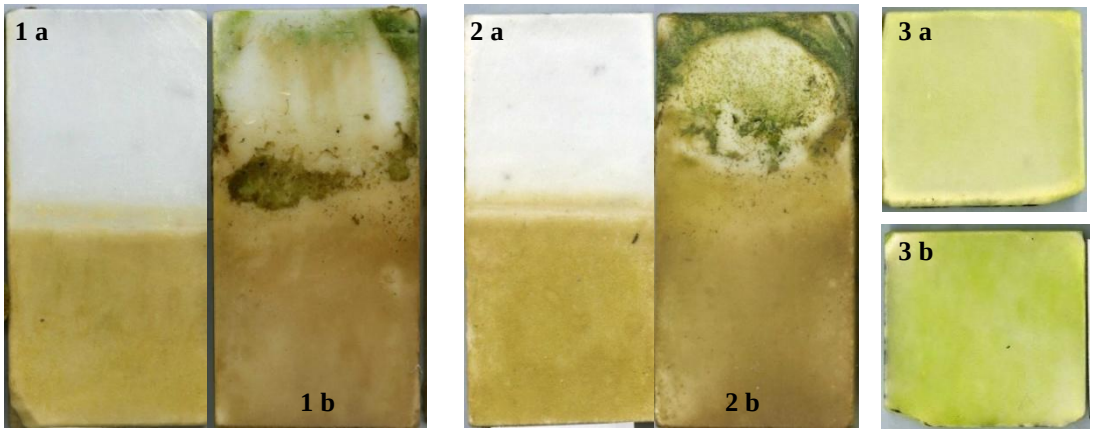


Fig. 27. Biofilm development on both sides of Carrara marble specimens, 10x5x1 cm (1 and 2) partially immersed and 5x5x1 cm (3) completely immersed into well water, for 5 months: (a) front side; (b) back side.

Epifluorescence observations of the zones in which the biofilm was detached, revealed the presence of coccoid phototrophs inside of the micro-irregularities of the stone (Fig.28), confirming the biodeteriogenic risks induced by endolithic and chasmolithic microorganisms for the monumental stones and artistic fountains.

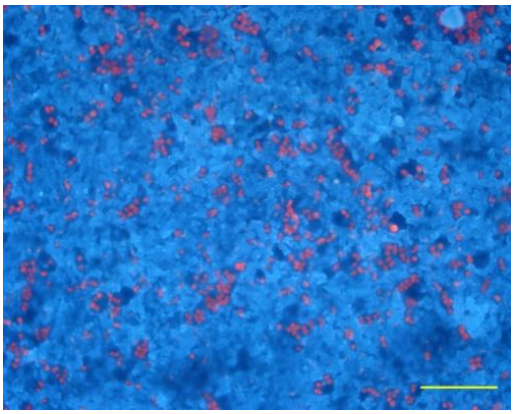


Fig. 28. The phototrophic presence within the micro-irregularities of Carrara marble stone under UV light. Scale bar 100 µm.

Some cross-section of collected samples (S2, S1c) from Fountain from Patio de la Sultana (Alhambra Palace, Granada) revealed the presence of calcium carbonate layers alternating with the microorganisms ones. In Fig. 29 is clearly visible a stratification which can be due to the alternation of dry/wet conditions which favored the precipitation. This process could also be enhanced by the presence of the microorganisms.

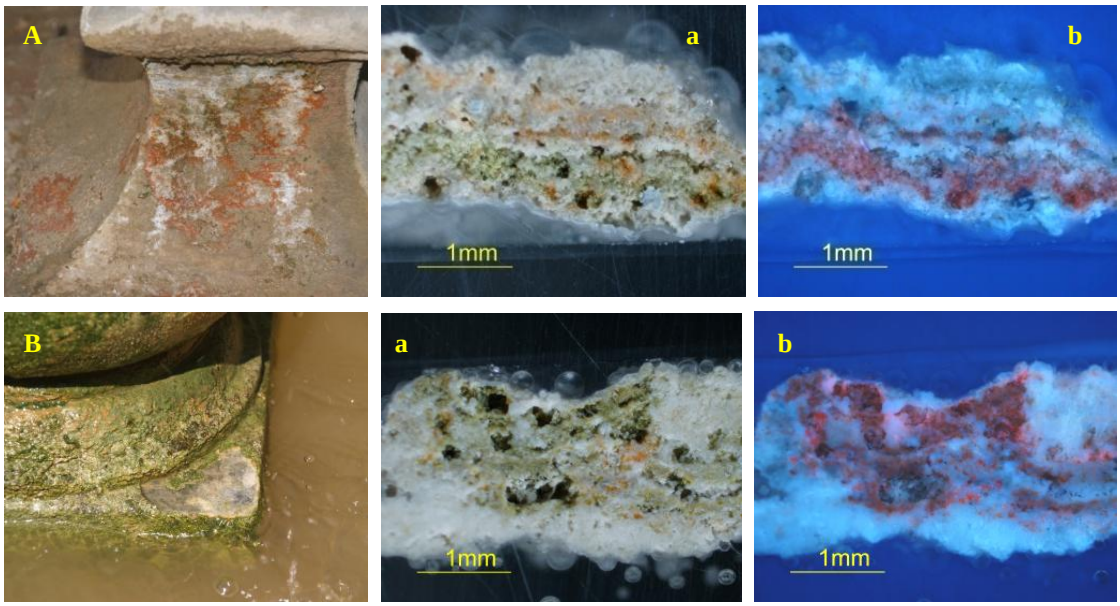


Fig. 29. Biofilm cross-section of the samples S2 (A) and S1c (B) collected from Sultana Fountain (Granada, Spain) observed in visible (a) and UV (b). The thick microorganisms layer (green in visible light and red UV light, respectively) is the last one developed.

In fact, the dominance of various cyanobacterial types of *Phormidium* sp. , reported for its ability of favouring calcium carbonate precipitation in layers (Brehm U., 2004), was noticed in the sample S1c by optical microscopy (Fig. 30). By CLSM was observed that the microbial phototrophic community consists especially of this oscillatoriacean cyanobacteria, considered epilithic and partially endolithic (Fig. 30 d).

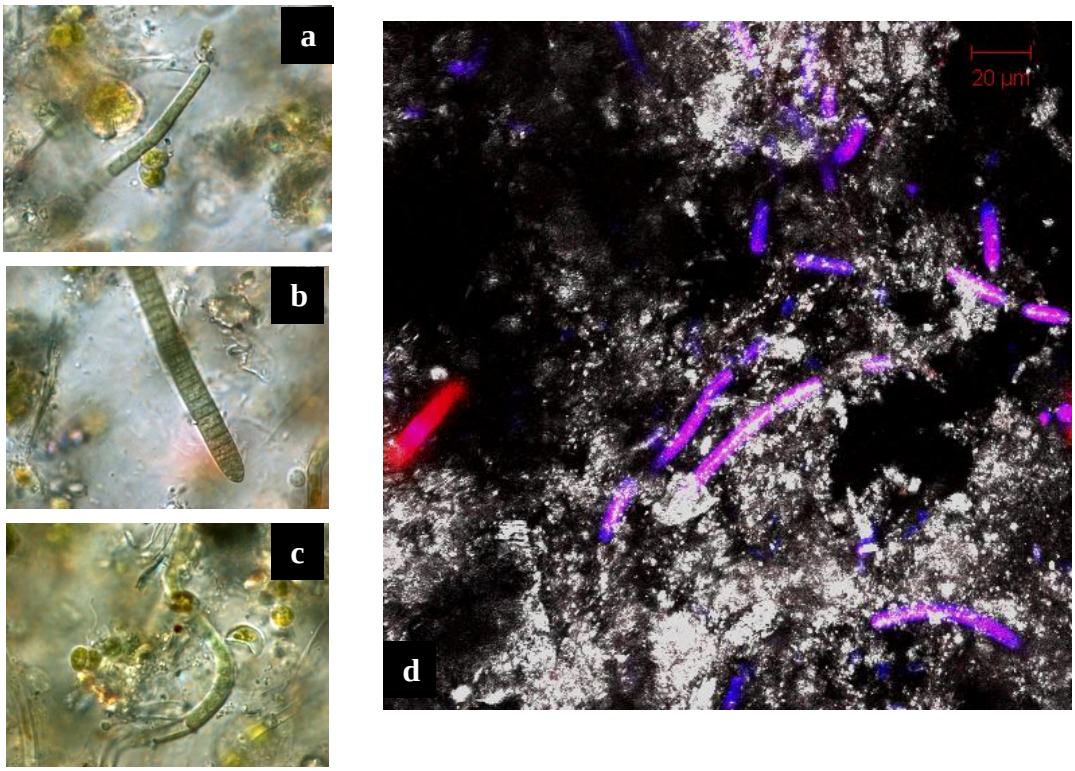


Fig. 30. The phototrophic diversity of sample S1c with different species of filamentous cyanobacteria *Phormidium* sp. and algae observed under optical microscope (a,b,c) and under CLSM - maximum projection of 31 stack images, step size 0.70 µm, with 20.95 µm thickness of the biofilm (d).

The phototrophic biofilms form colored patinas and incrustations. Some cyanobacteria are capable of directly precipitate of calcium carbonate onto/into their sheaths but many other species, even if without this capability, can induce calcium carbonate precipitation into the biofilm by providing nucleation sites for its crystallization (Ortega-Calvo J. J., 1993). This behavior is especially attributed to the presence of extracellular polymeric substances (EPS) and to biofilms ability of locally grain-trapping together with various metabolic processes such as photosynthetic uptake of CO_2 and/or HCO_3^-, ammonification, denitrification, sulphate reduction. Also (Riding R., 2000) biomineralization processes (concretion, accretion) were reported for cultural assets both in marine and freshwater environments (Bolivar F. C., 1997; Tiano P., 2006; McNamara C. J., 2009).

IV.1.2. *Biodiversity of microorganisms on monumental fountains*

The main groups occurred on the investigated monumental fountains belong to Cyanophyta, Chlorophyta and Bacillariophyta (Plate 5). Table 9 contains the list of the microorganisms observed on the fountains investigated in this research, including the isolated strains of cyanobacteria (Plate 6, 7), algae, diatoms (Plate 8) and fungi (Plate 9). Cyanobacteria represent the main group, both with coccoid (*Chroococcus* sp.,*Gloeocapsa* sp., *Nostoc* sp.) and filamentous forms (*Calothrix* sp., *Leptolyngbya* sp., *Phormidium* sp., *Pseudophormidium* sp.). Diatoms were dominant in the Fountain from Patio de la Lindaraja which is a regularly maintained fountain and its surfaces is periodically mechanically cleaned. The presence of a great diversity of diatoms on this sound stone material corroborate their role as primary inhabitants. The algae are very well represented in all fountains by *Chlorella* sp. and *Cosmarium* sp. In both fountains from Spain *Apatococcus* sp. is the most common genera, not observed in the Italian fountains. The heterotrophic fraction formed by fungi was composed especially by black molds (*Alternaria* sp., *Aspergillus* sp., *Papulospora* sp., *Phoma* sp., *Torula* sp., *Ulocladium* sp.). The most common genera for all the biofilms collected from the monumental fountains were *Acremonium* spp. and *Phoma* sp.

Table 9. Occurrence of Cyanobacteria, Chlorophyta, Bacillariophyta and Fungi in the investigated fountains: (1) Tacca's Foountain 1; (2) Tacca's Fountain 2; (3) Second Fountain from Villa la Pietra; (4) Fountain from Patio de la Sultana; (5) Fountain from Patio de la Lindaraja

Genus	**Fountain**				
	1	**2**	**3**	**4**	**5**
CYANOBACTERIA					
Aphanocapsa sp.	+			+	
A. novacekii			+		
A. grevillei			+		
Aphanothece sp.					+
A. stagnina			+		
Borzia trilocularis			+		
Calothrix spp.	+	+		+	
Calothrix lacustris			+		
Chroochoccus sp.	+	+	+	+	
C. turgidus	+		+		
Cyanosarcina sp.			+	+	
Dermocarpa sp.				+	
Gloeobacter violaceus			+		
Gloeocapsa sp.	+	+	+	+	
G. sanguinea			+		
Leptolyngbya sp.	+	+	+	+	
Lyngbya sp.				+	
Nostoc spp.			+	+	

Genus	1	2	3	4	5
Pseudanabaena sp.				+	
Pseudophormidium sp.	+	+	+	+	
Phormidium sp.	+		+	+	
Schizotrhix lacustris			+		
Synechocystis sp.	+				
Staniera sp.				+	
CHLOROPHYTA					
Apatococcus sp.				+	+
Apatococcus lobatus				+	
Chlorella sp.	+	+	+		
Chlorella saccharophila				+	
Cosmarium spp.	+	+		+	
Dilabifilum prinzii				+	
Monoraphidium contortum			+		
Scenedesmus spp.	+		+		
Palmella sp.	+	+			
BACILLARIOPHYTA					
Achnanthes sp.	+	+		+	
Achnanthes affinis	+				
Aulacoseira varians					+
Cymbella sp.				+	
Diatoma sp.				+	
Epithemia zebrina			+		
Navicula spp.	+	+	+	+	+
Nitzschia sp.	+			+	
Pinnularia sp.					+
Synedra sp.					+
FUNGI					
Acremonium spp	+		+	+	
Alternaria sp.			+	+	
Arthrinium sp.			+		
Aspergillus spp.			+	+	
Aureobasidium sp.			+		
Bispora sp.			+		
Epicoccum sp.			+	+	
Fusarium sp.			+	+	
Papulospora sp.			+	+	
Penicillium spp.	+			+	
Pestalotia sp.			+		
Phialophora sp.	+				
Phithomyces sp.	+		+		
Phoma sp.	+		+	+	
Thielaviopsis sp.	+				
Torula sp	+		+		
Trichoderma sp.				+	
Ulocladium sp.	+				

From ecologically point of view, algae such as *Apatococcus* sp., *Chlorella* sp., *Cosmarium* sp., palmeloid types ones and diatoms as well, such as *Achnanthes* sp., *Melosira* sp., *Navicula* sp., *Nitzschia* sp., have been preferentially detected in the samples collected from immersed zones or continuously and abundantly wetted areas. Cyanobacteria are also represented in this types of habitats by genera such as

Aphanothece sp., *Borzia* sp., *Cyanosarcina* sp., *Pseudoanabaena* sp. Some microorganisms such as *Epithemia zebrina*, *Gloeocapsa sanguinea*, *Staniera* sp. have been observed only on samples collected from dried substrates.

The phylogenetic tree (Fig. 31) illustrates the relation among 30 cyanobacterial isolated strains with the reference ones from the public database. The isolated strains formed clusters with high homology. Strains such as *Nostoc* spp., *Calothrix* spp. and *Pseudoanabaena* sp. form very homogeneous clusters. *Nostoc* spp. were isolated from Fountain from Patio de la Sultana and Second Fountain from Villa la Pietra, from different habitats and was observed a very homogenous phylogeny and morphology. The strain VP4-08 isolated from Second Fountain, Villa la Pietra, characterized as *Calotrhrix* sp. was phylogenetic identified with *Rivularia* sp., but a clear separation between these two species is still problematic. Many oportunistic strains such as *Leptolyngbya* spp. have been easily isolated, but in fact they are less representatives for natural community. The strain VP3-01 morphological characterized as *Chroococcus* sp. corresponds with maximum sequence similarity with *Gloeobacter violaceus*. In fact, the color of this strain is blue-violet. The main taxonomic groups represented in this analysis are Nostocales (I) with heterocytous species (*Nostoc* spp., *Calothrix* spp.), Oscillatorialles (II) with filamentous non-heterocytous species (*Phormidium* sp., *Leptolyngbya* spp., *Pseudoanabaena* sp.) and Chroococcales (III) with coccoid cyanobacterial species (*Gloeocapsa* spp., *Chroococcus* spp., *Dermocarpa* sp., *Gloeobacter violaceus*).

Many papers dealing with photosynthetic microorganisms dwelling on stone monuments report mainly the occurrence of cyanobacteria and algae as colonizers of stone cultural assets (Tomaselli L., 2000). Ecologically, cyanobacteria, algae and diatoms may belong to two main groups: one is composed of species that can stand extreme conditions such as high light intensity and drought periods, while the second group contains species living under dim light and constant humidity conditions (Ortega-Calvo J. J., 1993). Both situations are found in the artistic fountains, and therefore many microorganisms that are reported for the stone artefacts (*Aphanocapsa* sp., *Calothrix* sp., *Chroococcus* sp., *Gloeocapsa* sp., *Nostoc* sp., *Oscillatoria* sp., *Phormidium* sp., *Synechosystis* sp., *Synechococcus* sp., *Chlorella* sp., *Scenedesmus* sp., *Achnanthes* sp., *Navicula* sp., *Nitzschia* sp., *Pinnularia* sp.) (Crispim C. A., 2003; Crispim C. A., 2004; Flores M., 1997; Lamenti G., 1997; Ortega-Calvo J. J., 1993; Tomaselli L., 2000; Uher B., 2005) can dwell on the monumental fountain, as ubiquitous members. The

phototrophs reported in the literature for monumental fountains are listed in Table 10. These data can be confronted with those reported in Table 9 that contains the list of the microorganisms isolated from fountains investigated in this research.

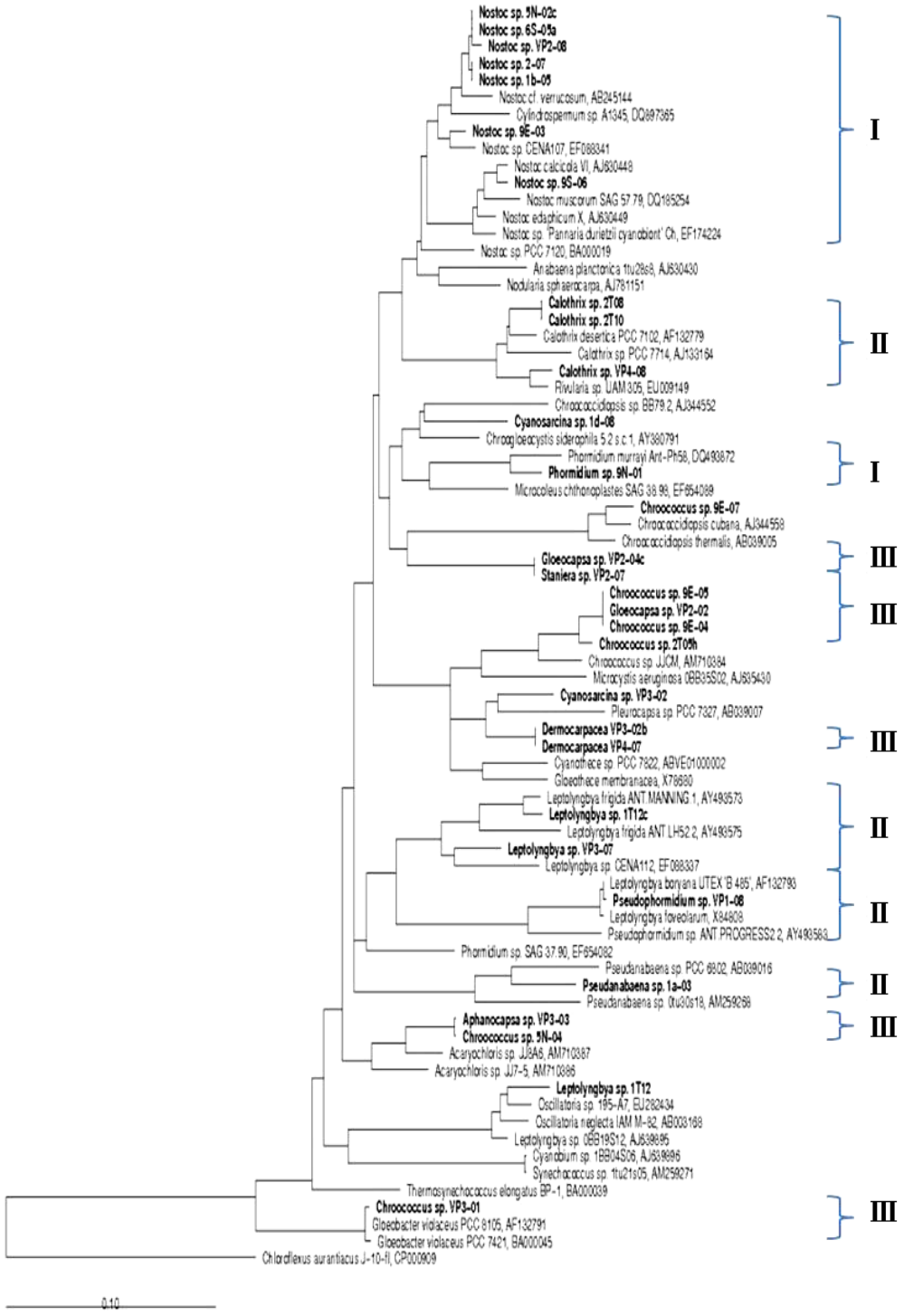


Fig. 31. Phylogenetic tree from 16S rRNA sequences from 30 cyanobacterial strains isolated from three monumental fountains (in bold). Roman numbers indicate the taxonomic groups. Scale bar represents 0.1 substitution per nucleotide position.

Table 10. Most common taxa of Cyanobacteria, Chlorophyta and Bacillariophyta occurring in the fountains reported in literature by the various authors, synthesized in Table 1

Genus	Frequency (%)	Species (no)	Species	Reference
CYANOBACTERIA				
Aphanothece sp.	22	2	*A. saxicola*	120, 123
Chamaesiphon sp.	33	4	*C. incrustans, C. polonicum, C. polymorphus*	15, 103, 120
Chlorogloea sp.	44	3	*C. microcystoides, C. purpurea*	15, 103, 123, 120
Chroococcidiopsis sp.	44	1		15, 16, 103, 142
Chroochoccus sp.	67	4	*C. minutes, C. turgidus, C. varius*	94, 95, 102, 103, 105, 109
Gloeocapsa sp.	33	2	*G.compacta*	94, 103, 120
Hyella sp.	11	1	*Hyella fontana*	15
Myxosarcina sp.	33	2	*M. chroococcoides*	15, 94, 120
Pleurocapsa sp.	56	3	*P. minor, P. fluviatilis*	15, 94, 103, 120, 123
Nostoc sp.	11	1		120
Lyngbya sp.	33	3	*L. martensiana, L.tenue*	94, 95, 105, 120
Oscillatoria sp.	33	3	*O. agardhii, O. lacustris*	94, 95, 105, 109
Phormidium sp.	89	9	*P. ambiguum, P. autumnale, P. cali dum, P. favo sum, P. foveolarum, P. retzii, P. subfuscum, P. uncinatum*	16, 95, 101, 102, 103, 105, 109, 120, 123
Schizothrix sp.	22	3	*S. gomontii, S. tenuis*	15, 16
Symploca sp.	44	2	*S. elegans, S. muralis*	16, 103, 102, 120
Calothrix sp.	44	3	*C. elenkinii, C. fusca*	94, 102
Plectonema sp.	11	2	*Plectonema battersii*	95
CHLOROPHYTA				
Apatococcus sp.	44	2	*Apatococcus lobatus*	15, 16, 101, 102, 120, 142
Chlorella sp.	76	1		8, 15, 94, 95, 102, 105, 123, 142
Chlorococcum sp.	55	1		16, 94, 103, 123, 142
Chlorosarcina sp.	22	1		101, 102
Chlorosarcinopsis sp.	44	2	*Chlorosarcinopsis minor*	101, 103, 120, 123
Cosmarium sp.	22	4	*C. depressum, C. granatum, C. reniforme*	103, 109
Palmela sp.	22	1	*Palmela miniata*	15, 120
Planophila sp.	22	1		102
Pleurastrum sp.	22	1		103, 120
Poloidion sp.	22	1	*Poloidion didymos*	16
Scenedesmus sp.	22	1	*S. ecornis, S. obliquus, S. smithii*	95, 120
Stichococcus sp.	22	4		95, 103, 105
Ulotrix sp.	44	1		94, 102, 103, 120
Spirogyra sp.	22	1		94, 120
BACILLARIOPHYTA				
Achnanthes sp.	44	2	*A. lanceolata*	103, 109, 120
Amphora sp.	33	4	*A. ovalis, A. perpusilla, A. veneta*	95, 101, 102, 109, 123
Cymbella sp.	33	5	*C. affinis, C. cystula, C. helvetica, C. hustedtii, C. ventricosa*	101, 102, 109, 120

Genus	Frequency (%)	Species (no)	Species	Reference
Diatoma sp.	11	1	*Diatoma vulgare*	120
Epithemia sp.	22	1	*Epithemia sorex*	101, 102, 109
Gomphonema sp.	22	2	*G. intricatum, G. olivaceum*	109, 120
Navicula sp.	76	7	*N. cari, N. criptocephala, N. gracilis, N. menisculus, N. rhyncocephala, N. seminulum* var. *frag.*	15, 16, 101, 102, 103, 109, 120, 123
Melosira sp.	22	1	*Melosira varians*	102, 120
Nitzschia sp.	44	3	*N. linearis, N. palea*	95, 102, 105, 109
Pinnularia sp.	11	1		95, 105
Synedra sp.	33	2	*Synedra ulna*	95, 101, 102, 109

IV.1.3. *Microbial community study by ARISA fingerprint method*

The complex ARISA profiles of cyanobacteria and eubacteria of the microbial communities from two monumental fountains (Second Fountain from Villa la Pietra and Fountain from Patio de la Sultana) were compared using the PCA (Fig. 32).

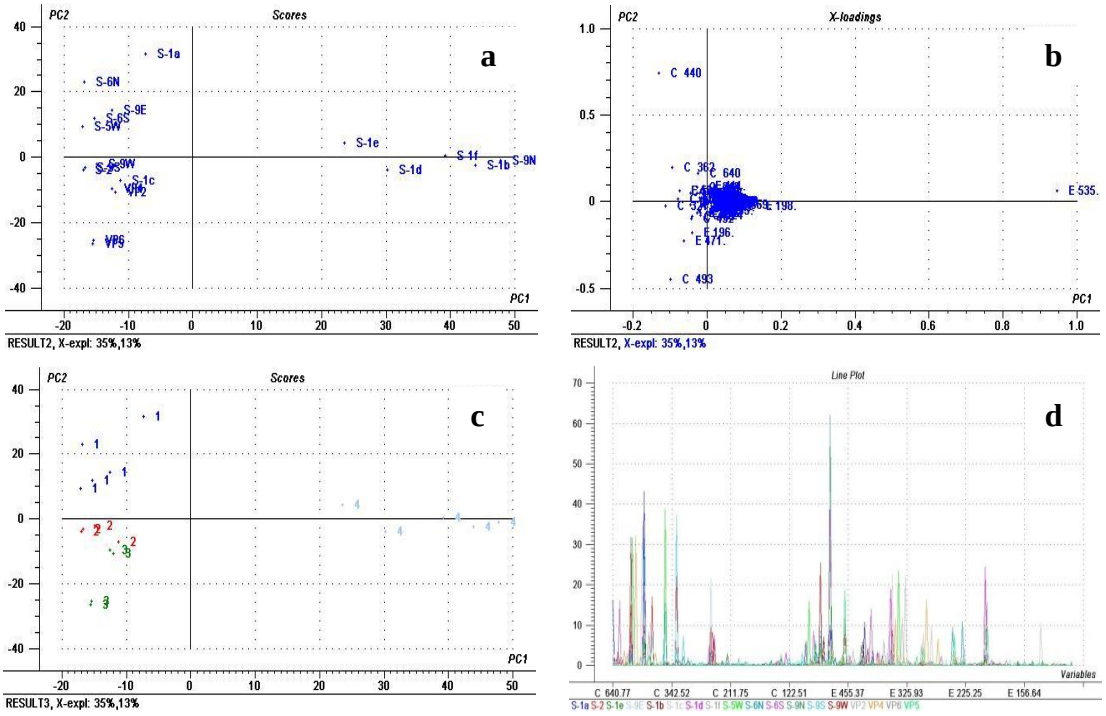


Fig. 32. Principal component analysis (PCA) generated from ARISA profiles for monumental fountains microbial communities. (a) Score plot (PC1-PC2) of the samples collected from fountains; (b) x-loading plot of the representative base pairs length of the cyanobacterial (C) and eubacterial (E) ARISA profiles; (c) clusters of the microbial community identified in the artistic fountains habitat; (d) distribution of all eubacterial and cyanobacterial amplicons present in the investigated samples.

The results indicated four clusters of microbial diversity. The samples collected from Second Fountain formed a separated group, having also similarities with the microbial communities from Sultana fountains. The microbial community which is growing under the water level is completely different from the rest of the others sites, being characterized by the dominance of two cyanobacterial operational taxonomic and showing the peculiar presence of ARISA fragments with 440 and 493 base pairs respectively. The microbial communities of the pedestal (samples S1b, S1d, S1e and S1f) collected from N, S, E and W orientations are very similar and form a separate group. The microbial community of sample S9N, located in shadow and with a north orientation is very similar to this cluster maybe because of the humidity retention. This group is distinguished by the presence of an eubacterial operational taxonomic unit with an ARISA fragment 535 base pair long. The presence of these peculiar components made the data related with the others samples very little perceivable, due to compression in very similar groups. Therefore, elimination from the plot of these specific samples (S-1b, S-1d, S-1e, S-1f, S-9N) were performed.

After removing these samples from the analysis, the PCR of the ARISA profiles individuated other 2 groups of samples containing specific cyanobacteria and eubacteria respectively (Fig. 33).

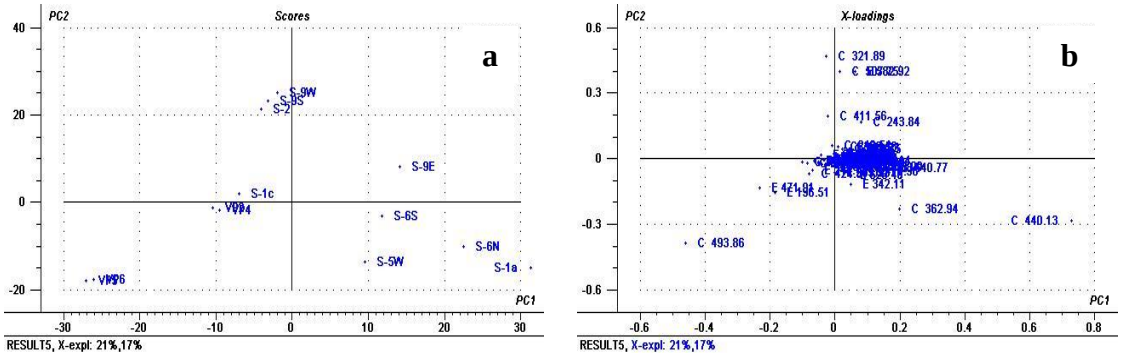


Fig. 33. Second PCA generated from ARISA profiles for monumental fountains microbial communities, after the remouving of samples containing specific eubacterial operational taxonomic unit. (a) Score plot (PC1-PC2) of the samples collected from fountains; (b) x-loading plot of the representative base pairs length of the cyanobacterial (C) and eubacterial (E) ARISA profiles.

The first group include three samples collected from shadow (S-9W, S-9S and S-2) which are differentiated by three operational taxonomic units of cyanobacteria. The second group include the samples collected at the air/water interface (S-1c, VP4), plus the sample collected from the dried zone above the water level. This group is

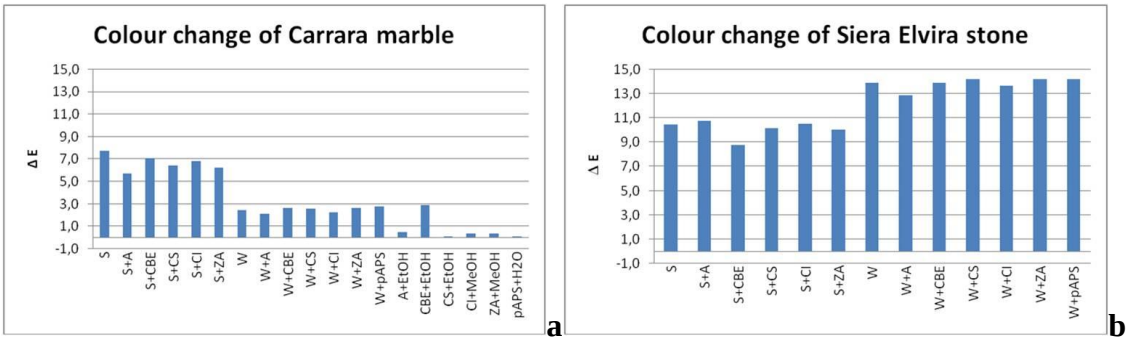
characterized by the presence of two eubacterial operational taxonomic units (471 and 196 baise pairs). The x-loading plot indicate a high similarity as regard the eubacterial and cyanobacterial operational taxonomic units, that are present in the analysed samples.

IV.2. Antifouling agents as an alternative method for prevention the biofouling on artistic fountains

IV.2.1. *Characterization of antibiofouling agents and coatings*

IV.2.1.1. Color tests

The difference of the colour before and after treatments for both types of stones was detected calculating the ΔE values. The two silicone coatings used as carriers (S and W) induced some changes in the color of the stones while the ABAs do not have an effective influence in color change (apart CBE). The S treatment induces an appreciable color change in Carrara marble while the W treatment slightly influenced the chromatic aspect of the stone (Fig. 34a). The high values of ΔE in treated Sierra Elvira stone (Fig. 34b) are due mainly to the shift of L* value (evident darkening) and also to a slight shifting of the blue and green hues.



a **b**

Fig.34 The colour modification induced by the treatments on Carrara marble (a) and Sierra Elvira stone stone (b), evidenced by ΔE.

IV.2.1.2. FT-IR and SEM-EDX analysis

Coating behavior have been investigated only for Silres BS 290, containing or not ABAs, because proved a better efficiency against microbial attachment, under the first indoor laboratory experiment (see IV.2.3.1.1).

The FT-IR spectra of pure W, the solution of W 7% in 2-Propanol (W), and those of 1 month cured product are presented in Fig. 35**.** The W characteristic peaks are similar with the ones of pure W. It can be observed that the polymerization of the resin start quite immediately after the solvent removal, the specific peaks being well distinguished after 30 days of curing (1129, 1023 and 777 cm^{-1}) while the intensity of the 849 peak in W pure is lower.

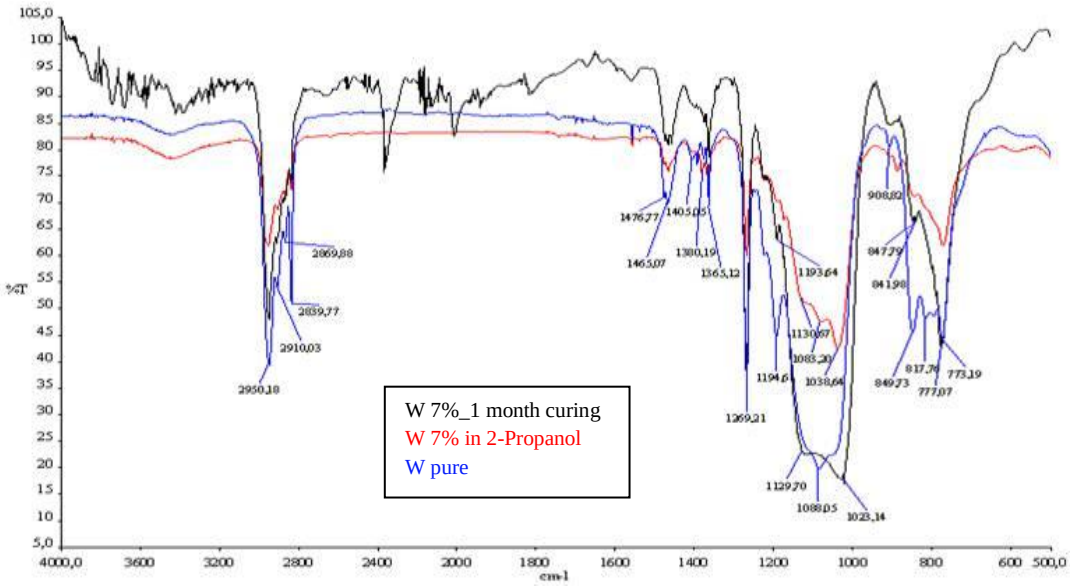


Fig. 35. The FT-IR spectra of pure Wacker 290 and diluted 7% with 2-Propanol (uncured and cured for 1 month).

Taking into consideration the polymerization status for the silane siloxane based silicone (W) containing the antibiofouling agents, after 30 days of curing, the FT-IR spectra indicated the presence of same characteristic peaks as for W without any ABA (1129, 1023 and 777 cm^{-1}), which confirm that the silicon network was formed.

In Fig. 36 are shown the SEM microphotographies of the W coating, mixed or not with A and pAPS, at two different curing times. W coating, cured for 30 days, forms on the stone surface a continuous film, while that one cured only for 5 days has a broke up aspect, with small holes, probably due to the solvent removal in the course of analyzing SEM chamber. The latter morphological aspect was also observed for mixed

W with A and pAPS, cured for 5 days as well. The 30 days cured pAPS incorporated in W coating present an interrupted surface with respect to the W alone. This can be due to the presence of water, used as a solvent for pAPS dissolution, the applied treatment containing ≈30% H_2O.

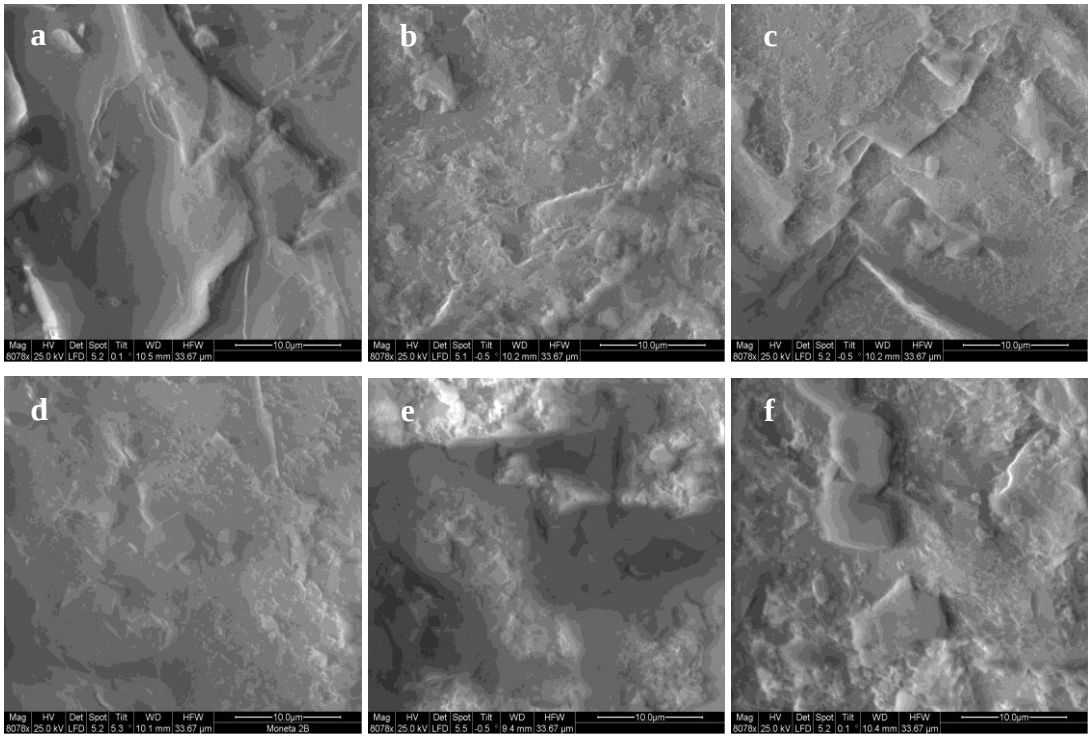


Fig. 36. SEM micrographs with the surface morphology of the treatments applied on Carrara marble specimens: (a) stone surface without any treatment; (b) with W cured for 5 days; (c), with W cured for 30 days; (d), with W+A cured for 5 days; (e), with W+pAPS cured for 5 days; (f), with W+pAPS cured for 30 days.

The elemental SEM-EDX analysis revealed differences in what the presence of silicon and other elements on the treated stone surfaces are concerned (Table 11). All treatments were analyzed in various areas: the uniform coated ones and those less coated (with holes); and in zones where the product did not present a film aspect. The coatings cured for 5 days (W, W+pAPS, W+A) evidenced a very discontinuous distribution. The coatings cured for 30 days showed a uniform distribution, with less silicon in the W+pAPS, probably due to the interaction of W with the H_2O used as solvent for pAPS before application.

Table 11. Elemental SEM-EDX analysis on the surface of the untreated Carrara marble and treated one with various coatings cured for different periods (5 and 30 days).

Element (%)	Carrara Marble	W (5 days curing)		W (30 days curing)		W+pAPS (5 days curing)		W+pAPS (30 days curing)		W+A (5 days curing)	
		coated	less coated	coated	less coated	coated	less coated	coated	less coated	coated	less coated
C	30.29	35.12	31.22	31.44	29.22	28.67	29.02	29.48	28.42	30.54	31.12
O	50.21	47.34	51.57	49.74	51.34	48.92	50.52	48.33	48.54	49.96	50.1
Mg	10.66	7.59	8.57	8.58	9.92	7.19	10.59	7.58	7.20	9.18	9.39
Si	0.07	1.45	1.09	1.61	1.57	0.87	0.27	0.76	0.55	1.46	0.69
Ca	8.76	8.50	7.58	8.63	7.96	14.36	9.61	13.85	15.31	8.86	8.68

Because the ABAs containing treatments were especially planned for underwater application in the monumental fountains, the W coating behavior in aqueous environment was previously investigated on Petri dishes. Under optical microscopy, crystal-like formations that took shape on the surface of all coatings (with or without ABAs) were observed with an increase in their volume in time (Fig. 38). The FT-IR (Fig. 37) and SEM-EDX (Table 12) analysis revealed the presence, on these new formations, of carbonates ($CaCO_3$) that concentrate in time because of the water evaporation. It seems that the W coating function as nucleation site for the carbonates crystallization, being the concentration of Ca in the crystals higher than on the surface.

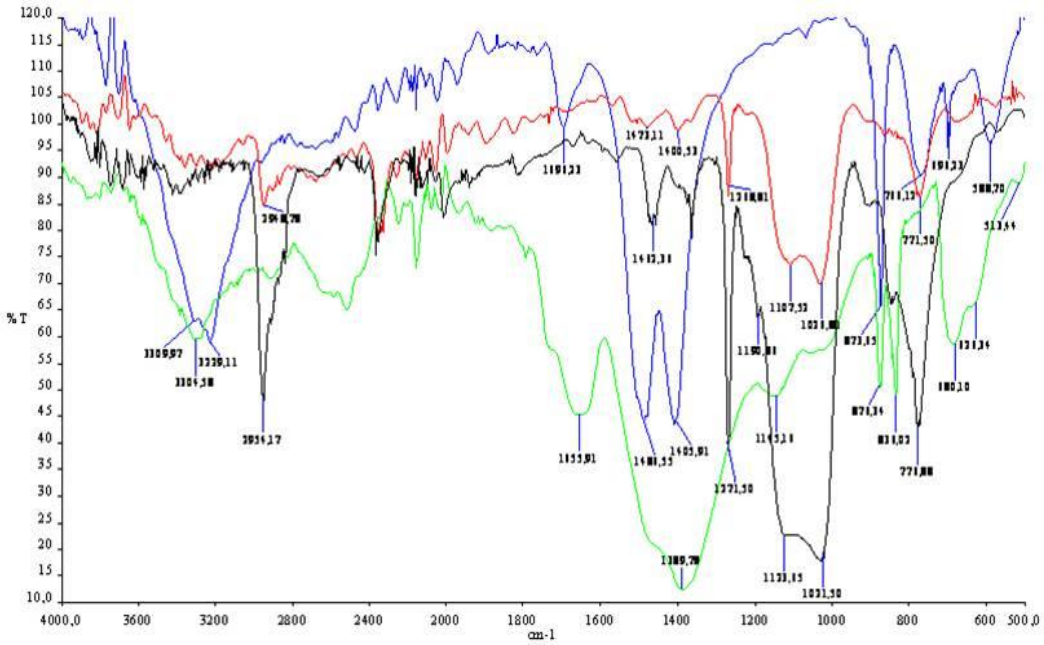


Fig. 38. The FT-IR spectra with the W coating behavior in aqueous environment.

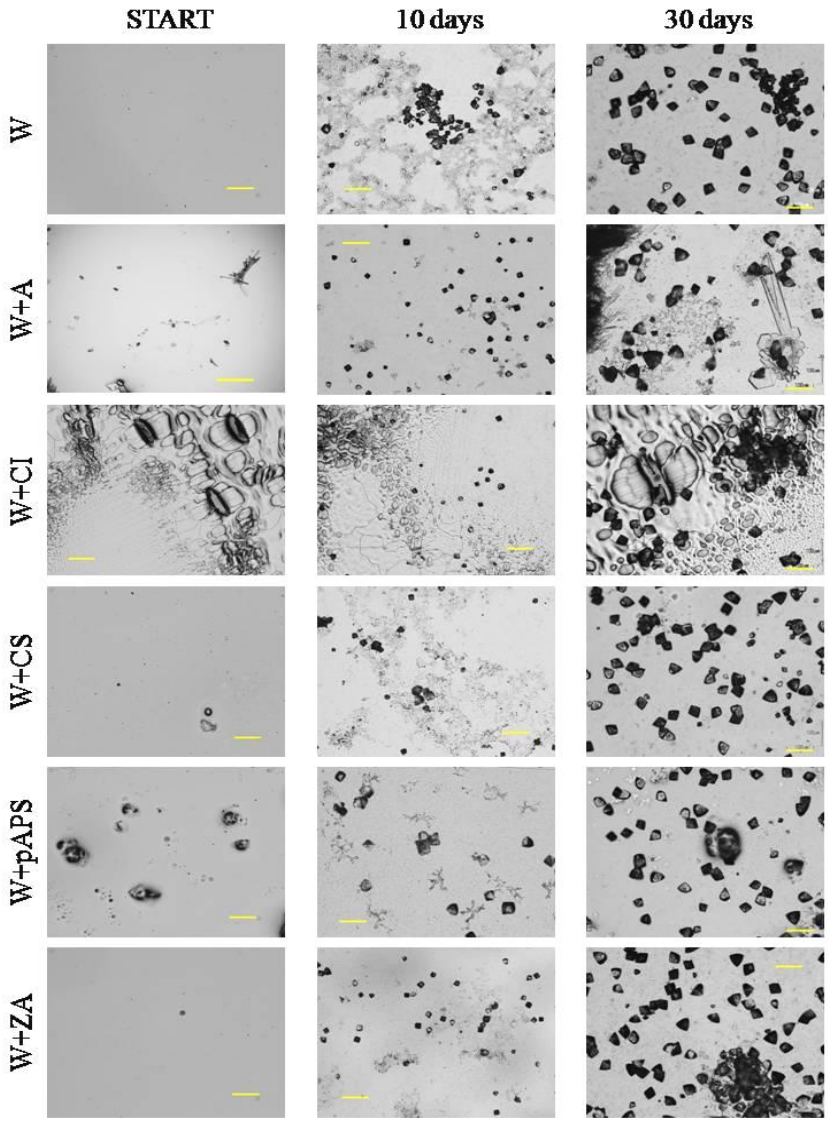
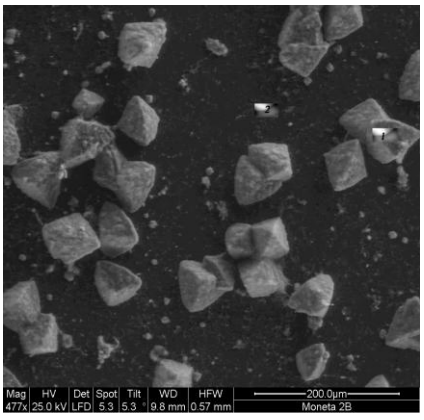


Fig.37 The morphological aspect of the treatments before and after 10 and 30 days under the water immersion. Scale bar 100µm.

Table 12. The elemental composition in weight percent, measured by EDX and the micrograph of W coating surface (1) and new cristals formed on it (2) after 2 months of water immersion.

Element	W coating	
	Cristals like (1)	Surface (2)
C	58.37	55.39
Na	2.68	8.40
Si	3.78	35.54
Ca	35.17	0.68



It seems that the W coating have the same behavior on the treated stone, and the formation of carbonates is depending on the water quality and on the dry/wet cycles that can occur. This phenomenon can take place on the monumental fountains, especially on the areas at the interface between air and water and is depending on the water mineral content in the latter.

In Fig. 39 are the SEM micrographs of Carrara marble specimens coated with W and cured for 5 and 30 days, before and after water immersion. New formations were observed on the surface after 12 days of immersion, with irregular shapes and dimensions. In Table 13 can be observed that Ca ions increase (weight percentage) after the water immersion, and the new formation that took shape contained a higher concentration with respect to the surface.

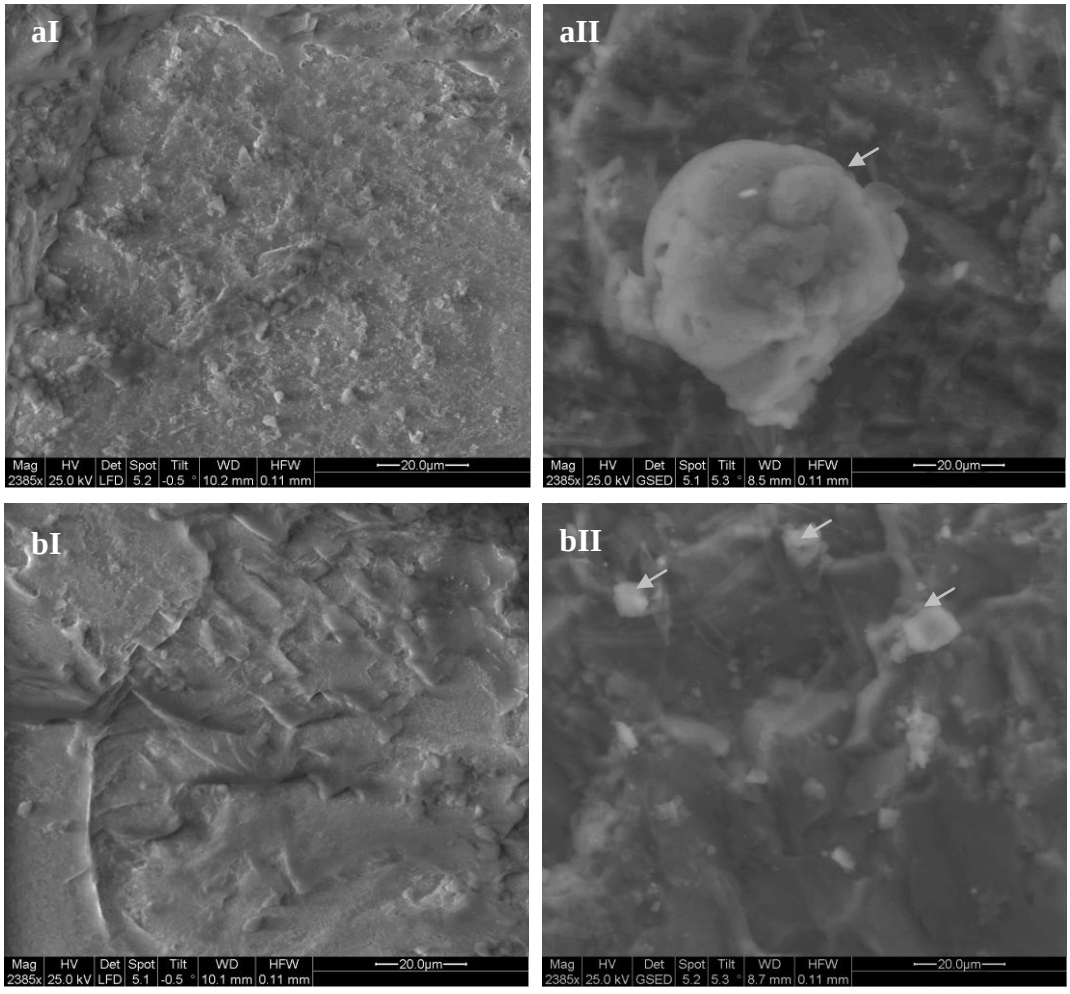


Fig. 39. The SEM observation of the W coating cured for 5 (a) and 30 days (b) before (I) and after (II) water immersion. The arrows indicate the new carbonates formations.

Table 13. The elemental composition in weight percent, of the Carrara marble and coated Carrara marble with Silres BS 290 (W), before and after water immersion.

Elements (%)	Carrara Marble	W 5 days			W 30 days		
		before	after 12 days of immersion		before	after 12 days of immersion	
		Surface	Surface	New formation	Surface	Surface	New formation
C	60.84	66.70	61.81	61.78	62.55	55.86	58.83
Na	0.00	0.00	0.71	0.61	0.00	0.65	0.49
Mg	21.42	14.41	16.25	8.13	17.07	16.38	12.62
Si	0.14	2.75	3.02	2.23	3.20	3.63	2.77
Ca	17.60	16.13	18.22	27.25	17.18	23.48	25.29

The presence of ABAs in the mixture it is hardly evidenced by FT-IR analysis for almost all inhibitors tested (pAPS, CBE, ZA, CS). This is due to the very low concentration used and/or because the characteristic regions for ABAs spectra overlaid with that one of W which has a strong signals between 1300-800 cm^{-1}. In Table 14 are listed the concentrations of W and ABAs used in the solutions that were applied on stone specimens and the concentration of ABAs referred to 100% of W, after the complete solvent removal, therefore after the complete curing.

Table 14. The concentration of ABAs and W before and after the solvent evaporation

ABAs	solution		after solvents removal	
	ABAs (% v/v)	W coating (% v/v)	ABAs (%)	W coating (%)
pAPS	0.01	7	0.14	100
ZA	0.5		7.14	
CBE	0.5		7.14	
CI	1.5		21.42	
CS	0.16		2.28	
A	5		71.42	

Considering the CI and the biocide A it seems that both of them reacted with the coating (Fig. 40, 41). In fact, the double olefinic bound of CI reacted, as indicated by the presence of peaks at 3080-3090 cm^{-1} in the W containing CI. It is possible that longer chains have been formed by the reaction of the OH group from silicate with the O from olefinic group, because the peaks from 1627 and 1449 cm^{-1}

disappeared. The peaks corresponding to the aromatic ring had a banding change (Fig. 40). Considering the product Algophase (A) mixed with W, it can be observed that the signals at 1428, 1438 and 1500 cm^{-1} disappeared and some new peaks at 1317, 1331, 1352 and 1364 cm^{-1} appeared. A possible change corresponding to C=O bond can be suggested by the variance of signal 1682 cm^{-1} present only in the pure Algophase (Fig 41).

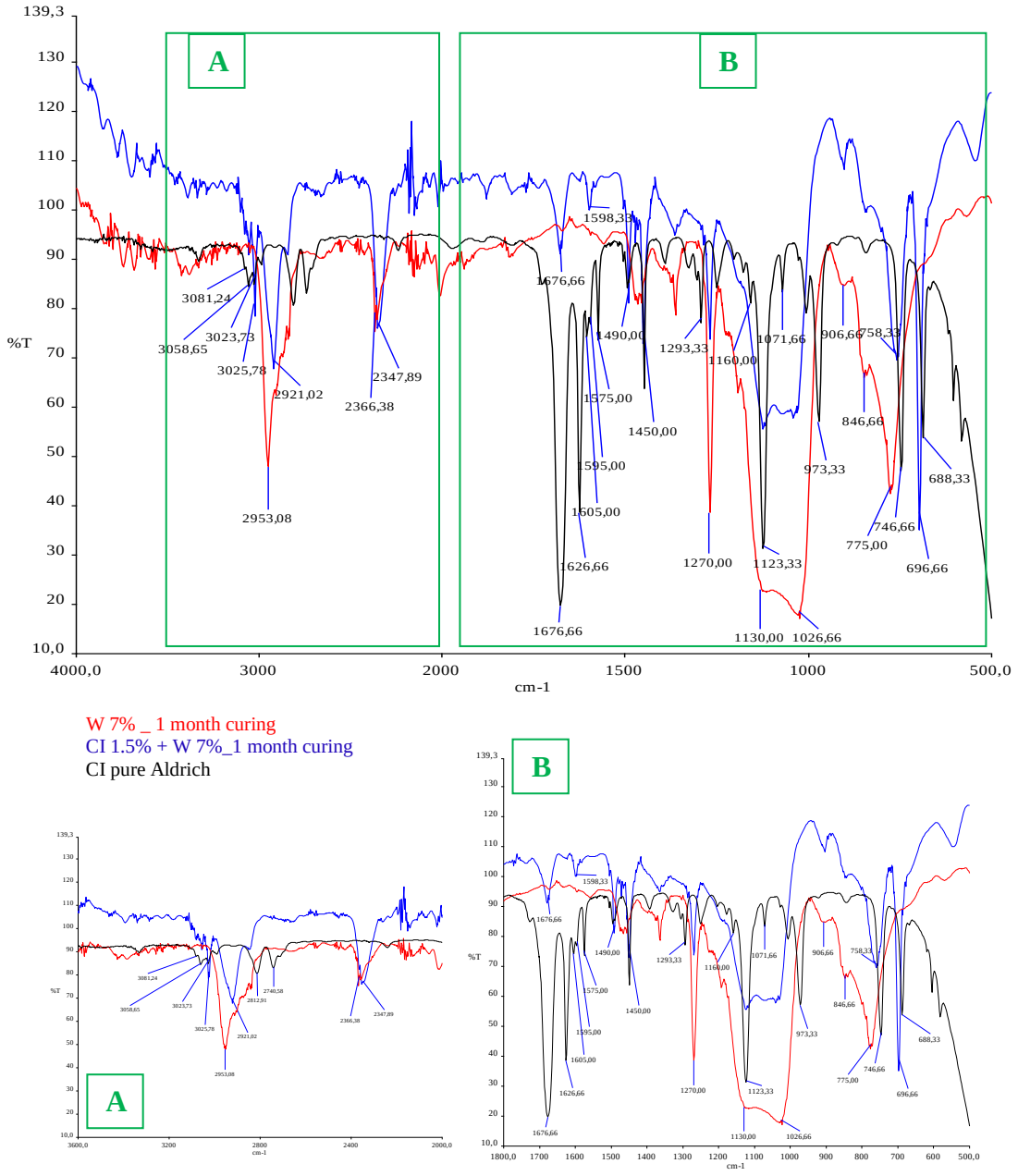


Fig. 40. The FT-IR spectra of Cinnamaldehyde incorporated into Silres BS 290 coating after 1 month of curing, with details of zones 3600-2000 (A) and 1800-500 (B).

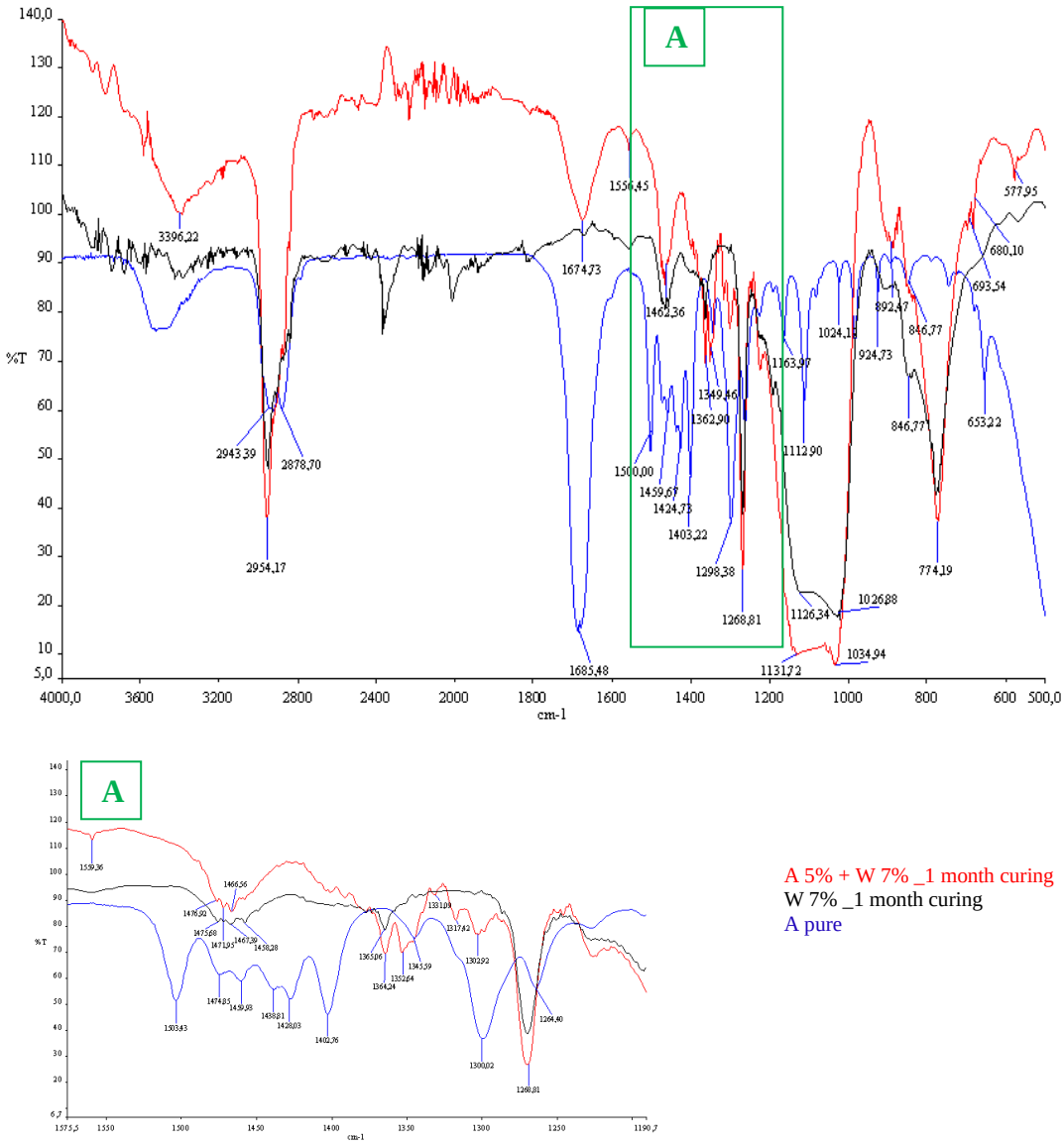


Fig. 41. The FT-IR spectra of Algophase incorporated into Silres BS 290 coating after 1 month of curing, with a detail (A) of the differences between the pure Algophase and biocide mixed with the coating

IV.2.2. *Efficacy of antibiofouling agents against biofilm development*

IV.2.2.1. Indoor laboratory experiments

IV.2.2.1.1. Immersion method

The results obtained on microscope glass slides indicated a good antibiofouling activity of all products tested (Tables 15,16). These preliminary results

have suggested that the ABAs can be used as effective regulators of the biological settlement process, being receptor-active molecules for the primary colonizers and, as consequence, able to inhibit their attachment on the substrate. The most effective seems to be the CI incorporated into S and ZA and Poly APS into W.

Table 15. The average estimation of the autofluorescent cells on the treated glass slides with the AA incorporated in Silres BS OH 100 (S)

S	S+ZA	S+CS	S+CI	S+CBE	S+A
223.7 cells/cm^2	124..1 cells/cm^2	107.0 cells/cm^2	83.7 cells/cm^2	136.5 cells/cm^2	105.8 cells/cm^2

Table 16. The average estimation of the autofluorescent cells on the glass slides treated with the AA incorporated in Silres BS 290, 7% in isopropyl alcohol (W)

W	W+ZA	W+CS	W+CI	W+CBE	W+pAPS	W+A
324.8 cells/cm^2	70.8 cells/cm^2	120.1 cells/cm^2	114.7 cells/cm^2	133.2 cells/cm^2	78.1 cells/cm^2	141.4 cells/cm^2

Considering the Carrara marble specimens, treated with ABAs incorporated into the two coatings and cured for 5 days, no significant difference between treated specimens and reference was observed after the first month of immersion, especially for S coating containing ABAs. The Carrara marble specimens treated with ABAs incorporated into W carrier exhibited less growth with respect to the ones treated with S carrier. After the second month, the phototrophic development on treated samples was higher than in the reference ones for both types of coatings containing ABAs (Fig. 42). This results are probably due to the very low concentration of ABAs used in this first experiment.

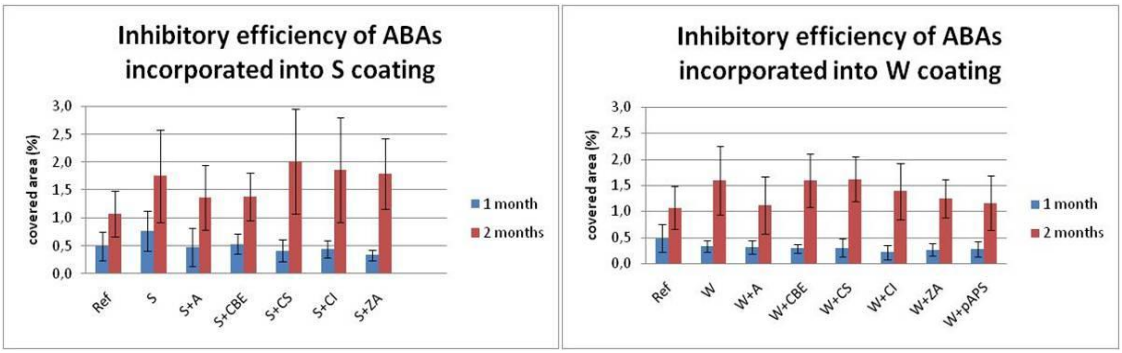


Fig.42. Inhibitory efficiency of ABAs incorporated into S and W coatings applied on Carrara marble specimens under indoor laboratory conditions

IV.2.2.1.2. Serial dilution method

This method gave an information about the ABAs influence against the microorganisms that are present in phototrophic biofilms that have been collected from monumental fountains. By comparing the optical density (OD) results obtained at 750 nm (Fig. 43) with the ones obtained by dry weight quantification (Fig. 44) can be clearly seen that most of the ABAs possess an inhibitory effect at higher tested concentrations (dilution 0 and 1). The only exception was for the ZA which proved a positive effect at the lowest concentration. This behavior can be attributed to the reaction ability of the active sulphate esteric group, which is free of intermolecular hydrogen bonds at lower concentrations. The most efficient ABAs testes seems to be pAPS and CI at all tested concentrations. The solvent MeOH did not showed any significant inhibitory effect even at the most higher concentration tested (15%) which means that the positive effect of CI and CS can be assigned to their actions. The control A was efficient at all tested concentrations.

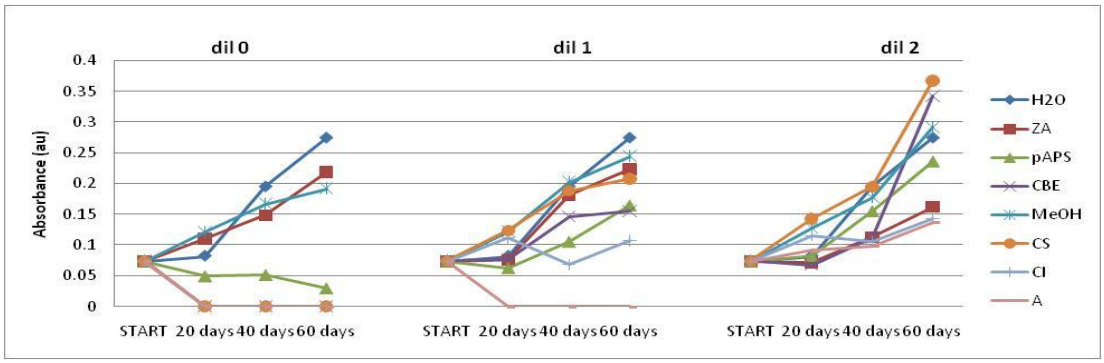


Fig. 43. Quantification of microbiological development by in vivo measurements of optical density (750nm) in different aqueous solutions containing ABAs

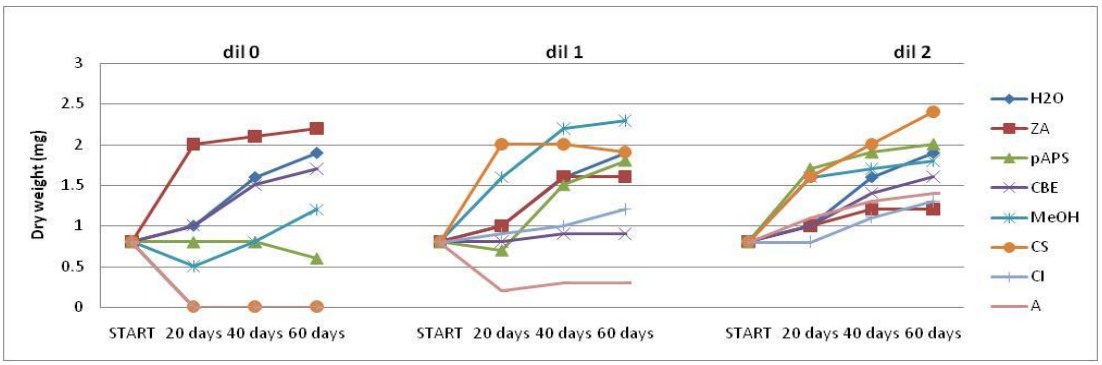


Fig. 44. ABAs efficacy evaluation by dry weight biomass

The efficacy evaluation, by in vivo measurements of chlorophyll *a* content, revealed a inhibitory action for all ABAs that have been tested (Fig. 45) against phototrophs (algae, diatoms and cyanobacteria). ZA exhibited a contrary behavior with respect to the others inhibitors, as described above, being less efficient at the highest concentration. CI and A strongly inhibited the phototrophic growth even at the lower concentrations that have been used. The phototrophic development slightly varied for all the ABAs in the 20 days. Clearly differences between phototrophic community behavior at the several dilutions examined have been noticed after 40 days. CS, CBE and MeOH were the less efficient at the lowest concentration.

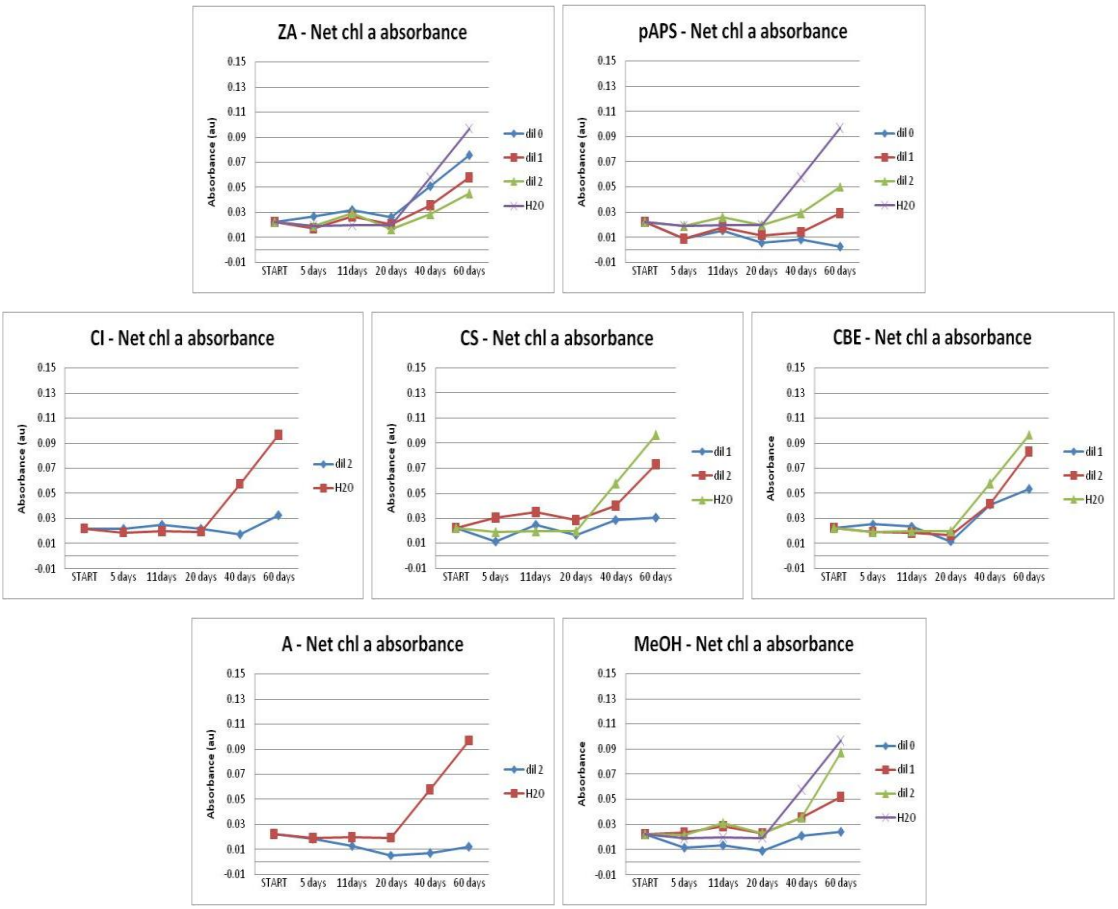


Fig.45. In vivo fluorescence of chlorophyll *a* detected for phototrophic development in aqueous solutions containing ABAs. The ABAs efficacy against phototrops after two months was: pAPS > MeOH > ZA > H2O (dil 0); CS > pAPS > MeOH > CBE > ZA > H_2O (dil 1); A > CI > ZA > pAPS > CBE > MeOH > H_2O (dil 2). Data is not shown in case of CI, CS, CBE, A (dil 0) and CI, A (dil 1) because the registered values were out of range due to the induced opaqueness and/or yellowish colour of the water solution containing ABAs.

The qualitative analysis of the microbial community present in the aqueous solutions containing the inhibitor agents and assessed by sowing an aliquots in Petri dishes, as described at materials and methods (see III.6.2.2), indicated a variability in the number of various groups such as the phototrophic (diatoms; algae – *Chlorella* sp., *Scendesmus* sp.; cyanobacteria – two filamentous and two coccoid types, phototrophic bacteria) and heterotrophic ones (fungi, bacteria, protozoan cysts) (Table 17). CI (dil 0 and dil 1) and CS (dil 0) completely repressed the phototrophic development. Microbial diversity are reduced with the others ABAs at the highest concentrations and especially in the first 20 days. It seems that ZA, pAPS and CBE cause an inhibitory effect of some microbial groups by reducing the diversity, while CI, CS and A have a toxic behavior, the Petri dishes remaining empty or only bacteria were developed. Cyanobacteria was the group less found and diatoms were that one more occurred.

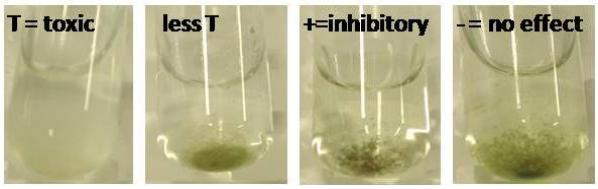
Table 17. The qualitative evaluation of microbial diversity in the aqueous solutions containing natural inhibitors: Key to symbols: "*" - heterotrophs (bacteria, protozoan cysts); "F" – fungi; "+" – phototrophs (algae, diatoms, cyanobacteria, phototrophic bacteria); "-" – no growth. Each symbol corresponds to a different taxon.

	20 days			**2 months**		
	dil 0	**dil 1**	**dil 2**	**dil 0**	**dil 1**	**dil 2**
H2O	** ++++++	-	-	** +++++++	-	-
ZA	** +++	** +++F	* +++F	** +++++	* +++++	* +++++
pAPS	* ++	** ++++	** ++++F	* +++	** ++++F	** ++++++
CS	*	** +++	* ++++	*	** +++	** +++++++
CI	-	*	*	-	*	** +
CBE	** +	** +++F	*** +++F	** +++	** +++++	** +++++++
A	-	-	*	-	-	*
MeOH	**	** ++++	* +++++	*	** +++++F	** ++++++F

The visual appraisal of presence/absence of the green color in all the tubes used in this experiment sustain the toxic behavior of CI, CS, MeOH and A. ABAs efficiency related with the inhibitory or toxic behavior is presented in Table 18.

Table 18. The behavior of selected active substances against the phototrophic components of biofilm. Key to symbols: see photos below.

	dil 0 (start)	dil 1	dil 2
ZA (0.5%)	-	-	+
pAPS (0.005%)	+	+	+
CBE (0.5%)	+	+	-
CI (1.5%)	T	T	T
CS (0.08%)	T	less T	-
A (5%)	T	T	T
MeOH (15%)	T	less T	-
H2O	-		



IV.2.2.1.3. Diffusion method I and II

The visual evaluation of ABAs efficacy ("+" = efficient;"-" = no efficient; "+/-" = uncertain) was done for both experiments in relation to the reference (Fig. 46, 47). The results of the diffusion methods at the end of experiments revealed that most of the ABAs are efficient against cyanobacteria rather than algae, and only CI showed a inhibitor effect against fungi as well, at tested concentrations (Table 18). The behavior of W and CBE in both experiments presented same contradictions as regard the efficacy against fungi. For W, these behavior can be attributed to the different curing time, while for CBE can be due to the mixing with the W coating. The Petri dishes with A and W did not present any growth of algae and cyanobacteria, as on the control. It seems that isopropyl alcohol has an efficacy action against microorganisms. A and W with the Diffusion method II, presented an inhibitory effect, without any solvent, being isopropyl alcohol completely evaporated during the curing period. For this result we can consider valid the positive influence of A and W against cyanobacteria and algae obtained in the Diffusion method I, even if in that case the data interfered with the isopropyl alcohol.

Table 18. The efficiency of the ABAs, A and W against three microbial strains (an alga, a cyanobacteria and a fungus) isolated from the bioflms developed in monumental fountains, evaluated by diffusion methods (I and II). Key to symbols: see photos below in Fig. 46 and Fig. 47.

		ZA		pAPS		CBE		CI		CS		A		W	
CONCENTRATION USED	C1	1%		0.01%		1%		3%		0.16%		15%		7%	
	C2	0.5%		0.005%		0.5%		1.5%		0.08%		5%			
	C3	0.2%		0.003%		0.2%		0.5%		0.02%		3%			
DIFFUSION METHOD		I	II	I	II	I	II	I	II	I	II	I	II	I	II
ALGA	C1	+/-	+/-	+/-	+/-	+/-	+/-	+	+	+/-	+	+	+	+	+
	C2	+/-	+/-	+/-	+/-	+	+/-	+	+	+/-	+	+	+		
	C2	+/-	+	+/-	+	+	+/-	+	+	-	+/-	+	+		
CYANOBACTERIUM	C1	+	+	+	+/-	+/-	+/-	+	+	+	+	+	+	+	+/-
	C2	+	+/-	+	+/-	+/-	+/-	+	+	+	+	+	+		
	C3	+	+/-	+	+	+	+/-	+	+	+	+	+	+		
FUNGUS	C1	+/-	-	-	-	+	-	+	+	-	-	+	+	+	-
	C2	+/-	-	-	-	+	-	+	+	-	-	+	+		
	C3	+/-	-	-	-	+	-	+/-	-	-	-	+	+		

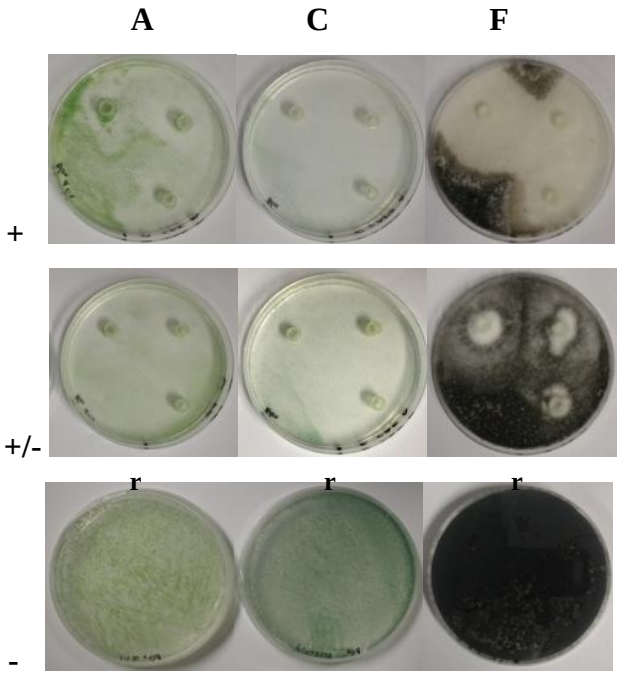


Fig. 46. The evaluation of ABAs efficacy with the Diffusion Method I by visual aspect of the microorganisms strains development in the presence or absence of ABAs, after 25 days for fungi and 75 days for algae and cyanobacteria. Key to symbols: + = efficient; - = no efficient; +/- = uncertain; r = reference; A = alga; C = cyanobacterium; F = fungus.

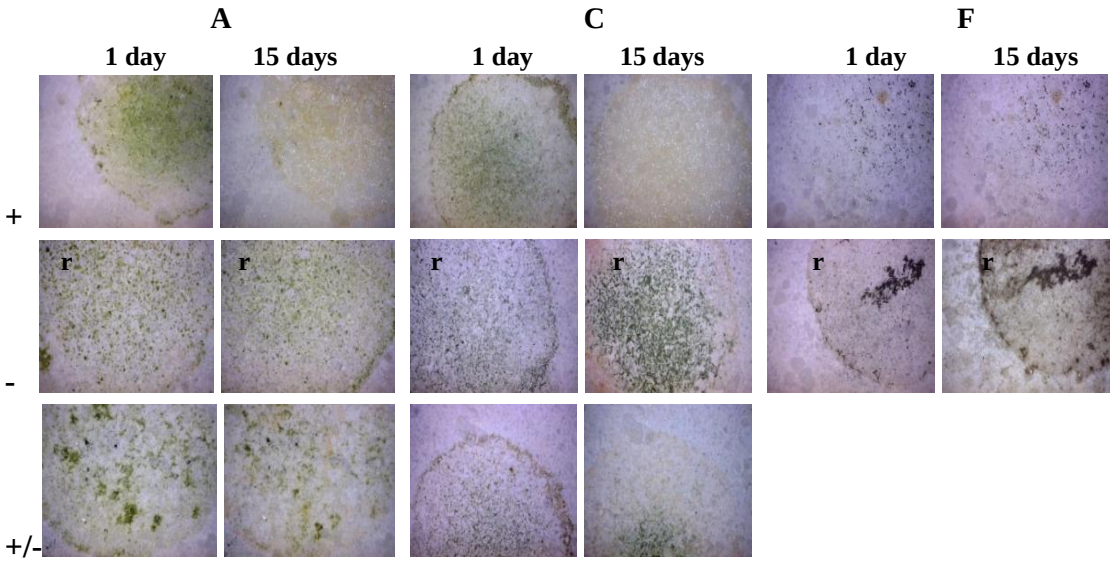


Fig. 47. The evaluation of ABAs efficacy with the "Diffusion Method II" by visual aspect of the microorganisms strains development in the presence or absence of ABAs, after 1 and 15 days. Key to symbols: "+" = efficient; "-" = no efficient; "+/-" = uncertain; "r" = reference; "A" = alga; "C" = cyanobacterium; "F" = fungus.

CS showed a certain efficacy against fungi only in the first 4 days and only at the concentrations of 0.16% and 0.08%, respectively. In the Petri dish containing the pAPS small drops of exudates were observed on the surface of the fungus, and therefore pAPS may induce a specific reaction of the fungal strain. CI and W showed and inhibitory effect while no fungal development was present in the Petri dish with A (Fig. 48).

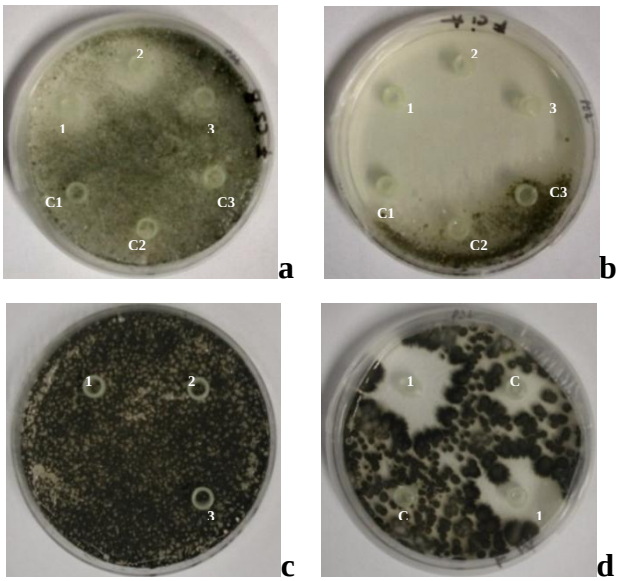


Fig. 48. The efficacy of CS (a), CI (b), pAPS (c) and W (d) against the fungal strain tested with the "Diffusion method I". Key to symbols: 1-3 represent different concentrations of ABAs; C 1-3 represent different concentrations of solvent.

IV.2.2.2. Outdoor experiments – immersion method

The same concentrations of ABAs incorporated into W and S coatings tested with the immersion method under indoor conditions were also tested under natural exposure, using the simulation fountains with sprinkling and stagnant water. The results obtained clearly evidenced that the W coating is more suitable for the ABAs incorporation than the S one and the concentrations that have to use must be increased (Fig. 49).

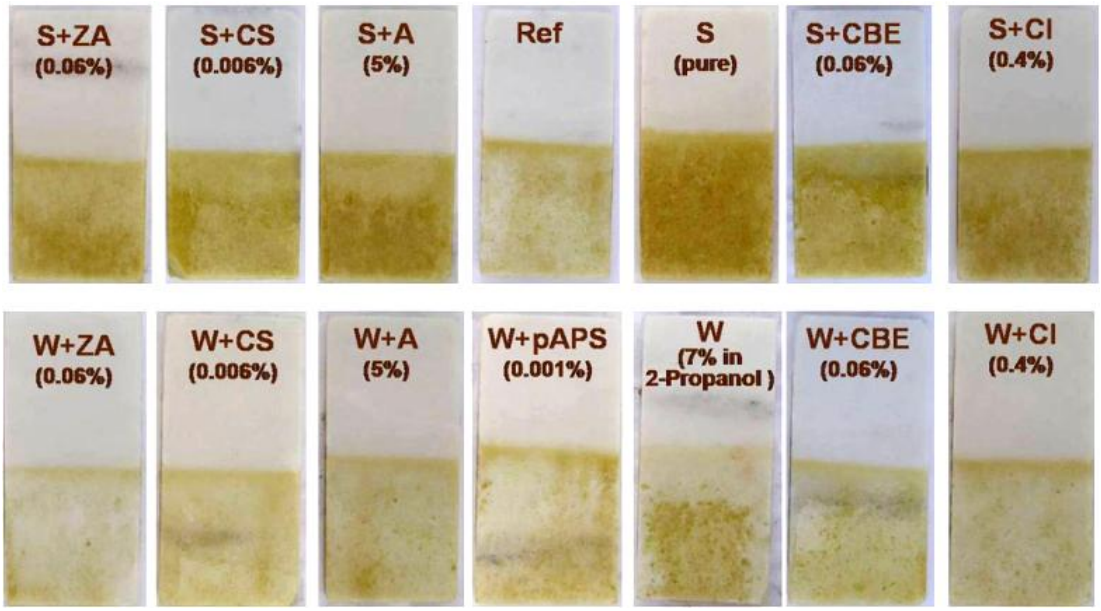


Fig. 49. The visual aspect of the Carrara marble specimens after 1 month of partial immersion in water

Taking into consideration of the results obtained using higher concentration of ABAs, the phototrophic development on Carrara marble specimens was slightly higher than for the Sierra Elvira stone. Some of the incorporated ABAs (CI, CS and pAPS) into both types of coatings cured for 5 days (S, W) seem to have an inhibitory effect on biofilm development in the first 15 days (Fig. 50, 51).

After the first and the second month, some treated stone specimens with S coating showed a surprising diminution of the patina (S-A, S-CBE, S) with respect to the reference and with the correspondent ABAs in W coating (Fig 50). It seems that the S coatings swelled forming a silica gel and, as consequence, the microbiological patina and/or the silica gel film is randomly detached.

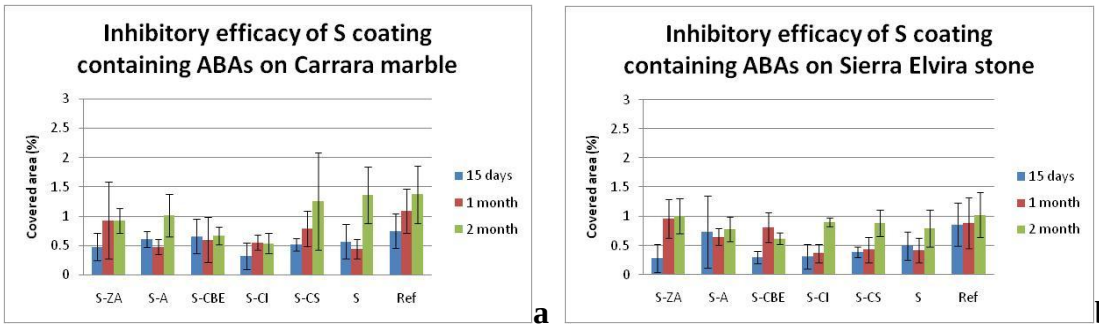


Fig. 50. The efficiency of ABAs incorporated into S coating applied on Carrara marble (a) and Sierra Elvira stone (b) specimens, under outdoor conditions.

The W carrier with some of incorporated inhibitors (W-pAPS, W-CI and W-CS) demonstrated a good inhibitory effect on phototrophic growing. It seems that W alone has a little inhibitory effect for the development in the first 15 days, however after 2 months the growth of the phototrophic biofilm on this coating was similar to that one developed on the untreated specimens (Fig. 51).

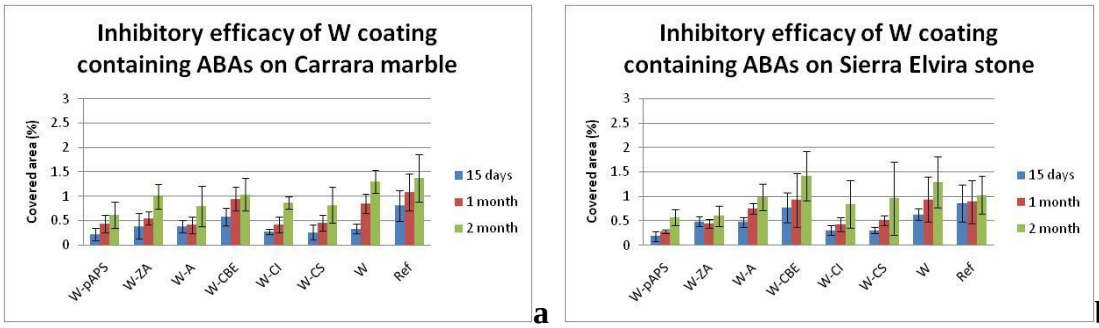


Fig. 51. Efficiency of ABAs incorporated into W coating applied on Carrara marble (a) and Sierra Elvira stone (b) specimens under outdoor conditions.

The phototrophic microorganisms start to colonize the surfaces on horizontal plane rather than on vertical one as shown by CLSM observations. This behavior was observed for all the specimens analyzed. In Fig. 52, one xz section, taken vertically along the phototrophic biofilm developed on Carrara marble, treated with CI incorporated into W, is presented.

Fig. 52. CLSM xz section taken vertically trough the biofilm showing the horizontal distribution of the phototrophs on Carrara marble specimen treated with CI incorporated into W coating, after 15 days of immersion. Scale bar 10 μm.

The results of the biovolume estimation revealed a lesser growth for all the treatments containing ABAs after 15 days of immersion with respect to the reference and the specimen treated only with W coating. CI and ZA incorporated into W remained efficient against biofilm development even after 2 months of immersion (Fig. 53). The pAPS incorporated into W revealed a higher biovolume with respect to the others ABAs and the reference. This result is in contrast with that one obtained by epifluorescence analysis, when pAPS mixed with W shown one of the lowest covered area by the phototrophs. This could be due either to the fact that the CLSM analysis was carried out only into a single point (144400 μm^2), while the epifluorescence analysis was performed on many regions of the same sample, or/and to a different behavior of the biofilm development after the initial colonization.

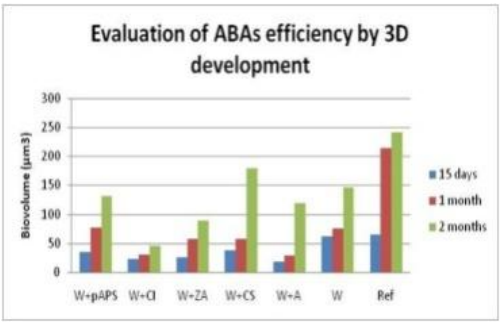


Fig. 53. The efficacy of antibiofouling treatment on Carrara marble assessed by occupied biovolume under outdoor conditions

The presence of some inhibitors on the surface may induce an heterogenous development of the biofilm, especially on z axis, while on the surfaces without any treatments, the biofilm will grow in a more homogenous manner, developing on all three space direction (x, y and z) (Fig. 54).

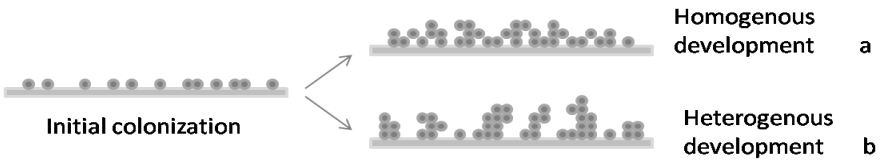


Fig. 54. Biofilm development in the absence (a) or presence of inhibitors (b)

As regard the treatments cured for 1 months, the results after 15 days of water immersion revealed an ample phototrophic growth on all coated stone specimens as the one observed on the reference (untreated specimen). After 30 days biological

development could be seen by naked eye (Fig. 55). These results led to the assumption that the complete curing of the incorporating agent Silres BS 290 blocks the ABAs efficacy.

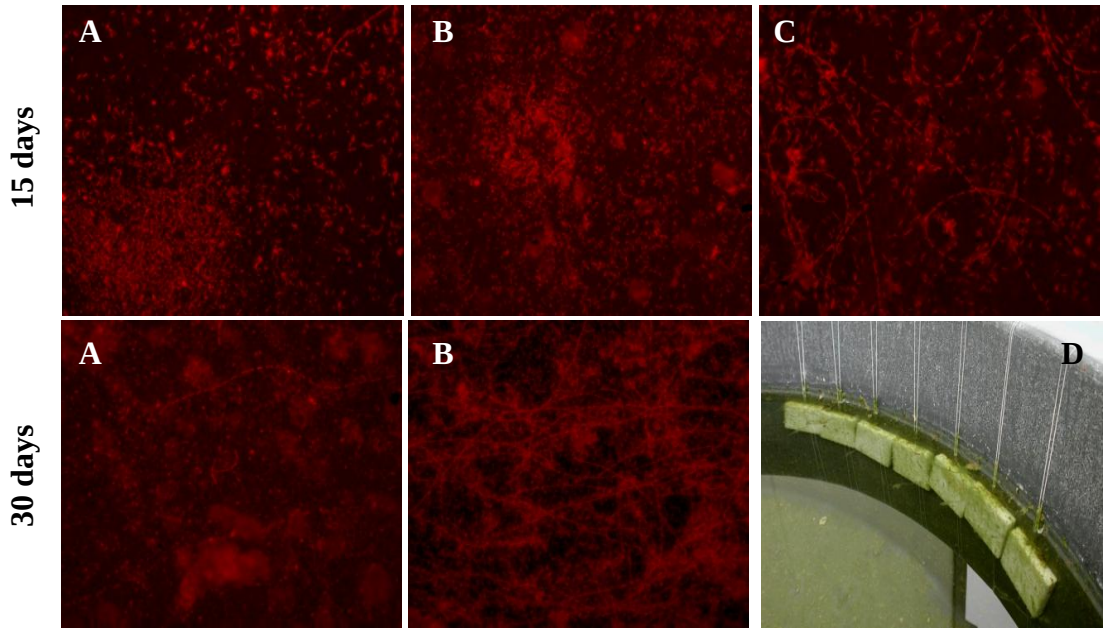


Fig. 55. The autofluorescent images of the biofilm growing on Carrara marble specimens treated or not with ABAs incorporated in W and cured for 30 days, after 15 and 30 days of immersion in well water: (A) untreated stone, (B) Silres BS 290; (C) pAPS incorporated into Silres BS 290; (D) the aspect of the samples in the simulation basin after 30 days of immersion

IV.2.2.3. In situ experiments

IV.2.2.3.1. Optical observations

Immediately after the application of the treatments on the lower basins of both fountains the stone surface darken. The treated areas turned lighter after 10 weeks on Fountain from Patio de la Lindaraja, while after 4 months in both fountains, the limits between treated and untreated zones start to blur and therefore, after 6 months any difference could not be observed by naked-eye. Unfortunately not-foreseen interferences occurred after the application of ABAs on the two fountains even if for short periods, such as the water suppression due to some technical reasons (on Tacca Fountain) and a light cleaning intervention (on Lindaraja fountain). The fouling process, more aggressive on the Fountain from Patio de la Lindaraja (Fig. 56) than the one from Tacca's Fountain 2 (Fig. 57), was due to the more favourable climate.

Fig. 56. Fountain from Patio de la Lindaraja after 1 month (a), 2 months (b) and 6 months (c) from reopening the water flux

The macroscopically evaluation of Tacca's Fountain (Fig. 58) was quite difficult to perform due to uneven colour of the substratum. After 4 months the zones treated with W+CS, W+A and W+pAPS seemed to be less covered by biological patina and after 6 months an evident difference between visual aspect of treated and untreated areas was not observed.

Fig. 57. Tacca's Fountain 2 after 1 day (a) and 6 months (b) from reopening of the water flux

The images captured by the USB portable microscope (Fig. 59) revealed the presence of microorganisms on the stone surfaces. The biological growth has a spotted aspect (light or dark brown, and yellowish) and its development occurred both above and under the water level and also at the interface between air and water. A continuous deposit containing microorganisms was observed especially on the lowest part of the

wall, probably due to the accumulation of detached microorganisms and dirt (e. g. soil particles, hair) on the ground level of the basin.

Fig. 58. Macroscopical aspect of the examined area for "in situ" experiment on Tacca's Fountain: **a.** before treatment; **b.** after the treatments (W, W+A, W+CS, Blank (untreated), W+pAPS, W+ZA - from left to right); **c.** after 4 months under water; **d.** after 6 months under water.

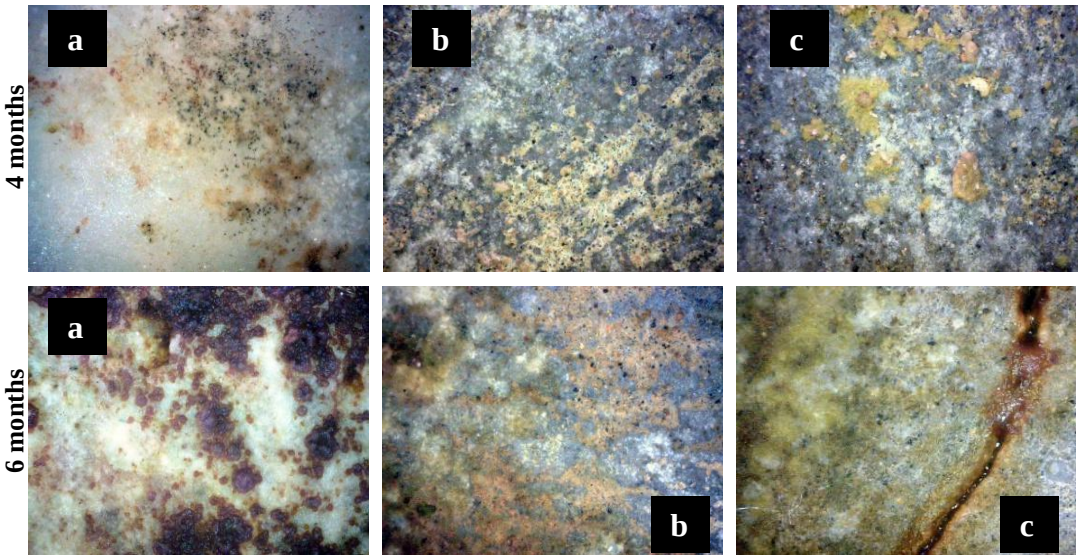


Fig. 59. The microscopical aspect (20x) of biological growth on selected areas observed by USB portable microscope after 4 and 6 months of running water on Tacca's Fountain 2: (**a**) above the water level, (**b**) at the air/water interface and (**c**) below the water level.

The microscopical observations (Fig. 60) of the collected samples revealed a high biodiversity before the cleaning procedures of the fountains, with phototrophs

(algae, cyanobacteria) and heterotrophs (nematodes, protozoa, fungi). Diatoms were dominant, as the most abundant microbial group after 4 and 6 months, both above and below the water level, on all treated and untreated areas. This behavior suggest a high settlement ability in new or re-colonization processes. The presence of filamentous cyanobacteria and algae was noticed on the untreated surfaces, below the water level, especially after 6 months and less after 4 months. Beside diatoms, round shape phototrophs were observed on treated areas after half year from starting the experiment.

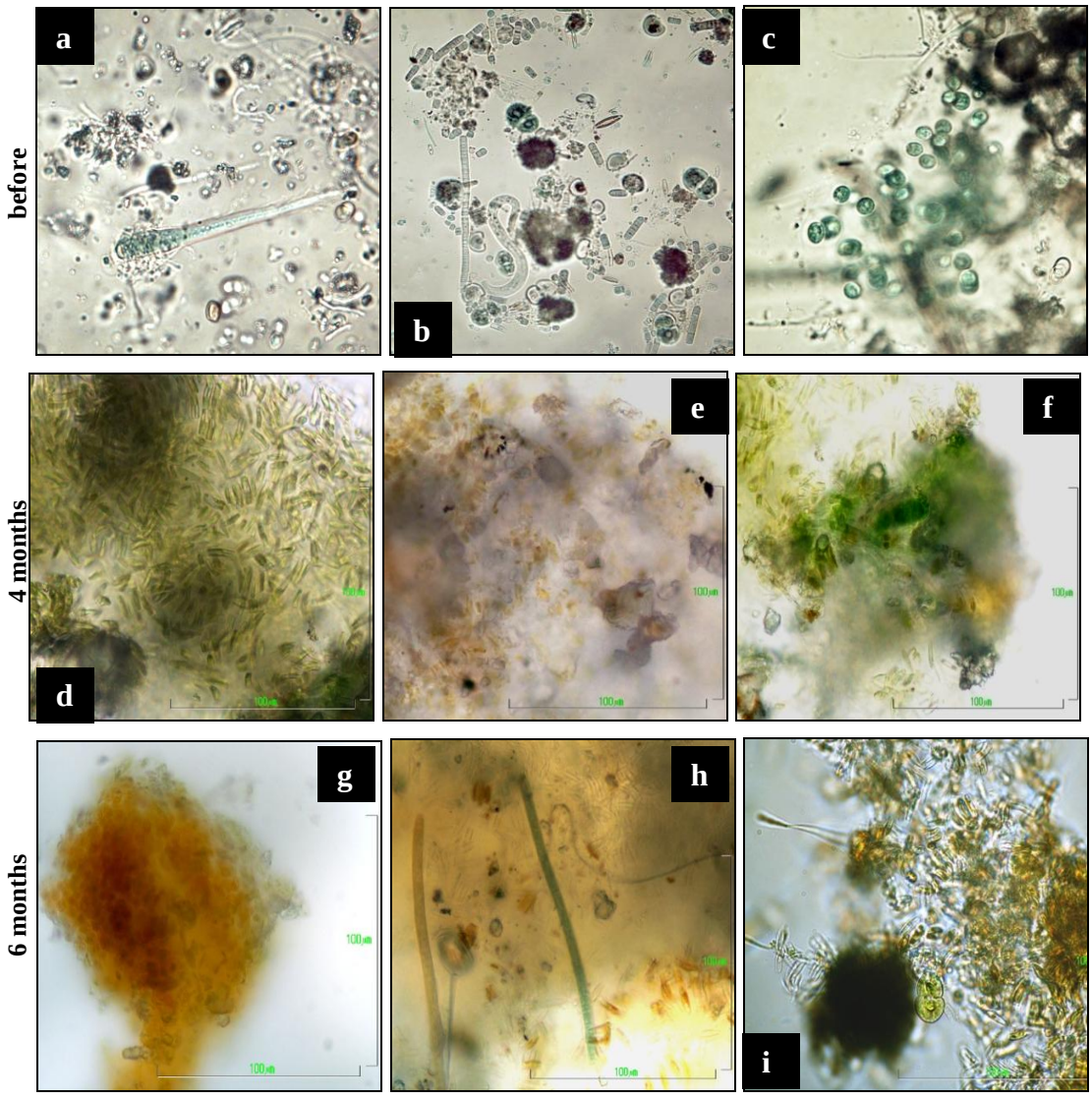


Fig. 60. The microbiological components of the biofilm developed on Tacca's Fountain 2, before the treatment and after 4 and 6 months from the reopening of water flux: (**a**) *Calothrix* sp., *Leprolyngbya* sp.; (**b**) *Cosmarium* sp., *Phormidium* sp., *Gloeocapsa* sp., diatoms, nematode; (**c**) *Palmella* sp.; (**d, e**) diatoms; (f) diatoms and *Calothrix* sp.; (**g**) coccoid phototrophs; (**h**) *Phormidium* spp., *Leptolyngbya* sp., diatoms; (**i**) diatoms, *Leptolyngbya* sp., *Cosmarium* sp.

Considering the fountain from Patio de la Lindaraja, the visual observations (Fig. 61) of the selected areas showed scarce phototrophic growing with respect to the reference one and other non treated areas of the internal fountain basin after 10 weeks. After 6 month from the beginning of experiment, the entire surface of the lower basin that have been immersed into water, was uniform and completely covered by a green biofilm composed mainly by filamentous phototrophic microorganism, as the onsite microscopical observation (Fig. 62) revealed. A visible biological growth was not observed by naked-eye above the water level.

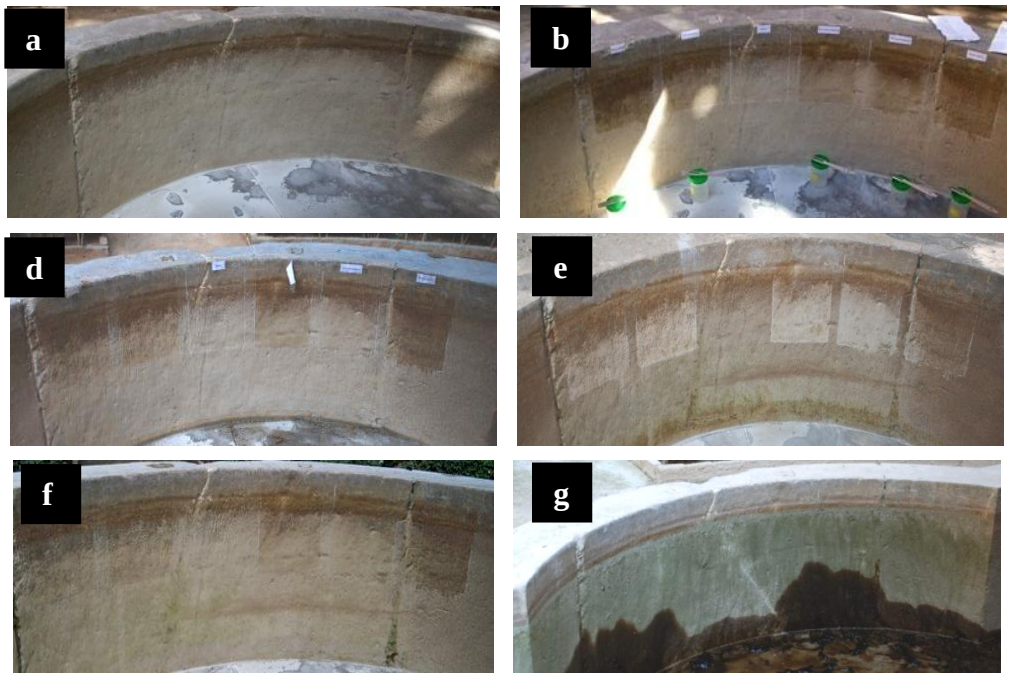


Fig. 61. Macroscopical aspect of the examined area for "in situ" experiment: **a.** before treatment; **b.** after the treatments (W+A, W+CS, Blank (untreated), W, W+pAPS, W+ZA - from left to right); **c.** after 15 days from treatments; **d.** after 10 weeks under water; **e.** after 4 months under water; **f.** after 6 months under water.

The presence of the various types of diatoms (*Navicula* spp., *Pinnularia* sp., *Synedra* sp., *Aulacoseira* sp.) was noticed on the samples collected after 6 months by optical microscopy (Fig. 63). The dominant species was the bacillariophyte *Aulacoseira* sp., floating or freely motile genus, common found in eutrophic waters, which forms filamentous colonies with cells linked cell-to-cell in chains. Taking into account the ecological characteristics of this genus and the dark green color of the water fountain, it can be supposed that its presence on the wall surface is due to filaments attachment on the irregularities of the stone, when the fountain basin have been emptied. The same

types of diatoms and the green alga (*Apatococcus* sp.) were found in the samples that have been collected before the cleaning procedures.

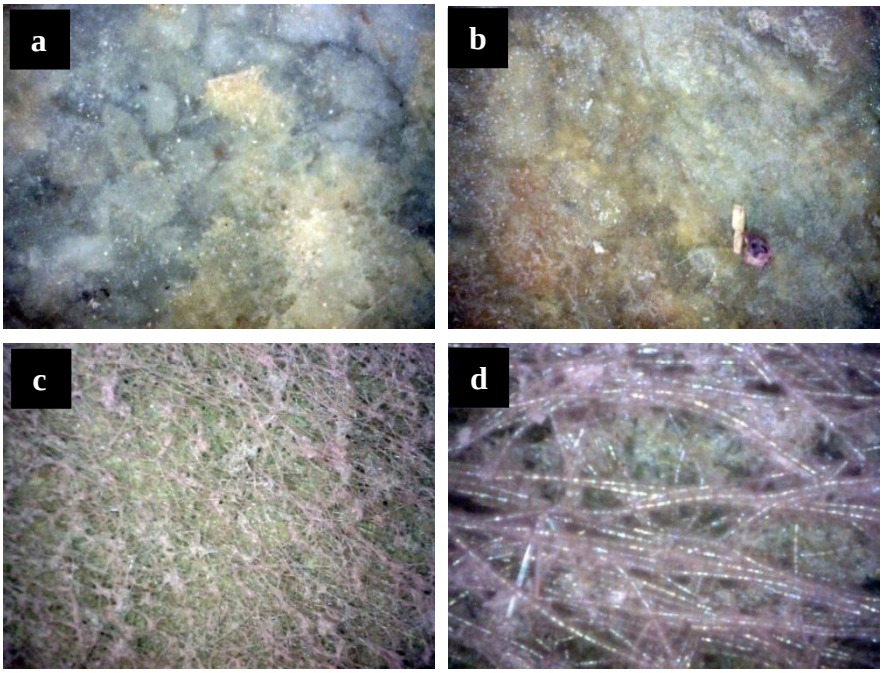


Fig. 62. The microscopical aspect of biological growth observed by USB portable microscope after 6 months of running water on Fountain from Patio de la Lindaraja (**a**) above the water level (20x), (**b**) at the air/water interface (20x) and (**c, d**) below the water level (20x and 40x respectivelly).

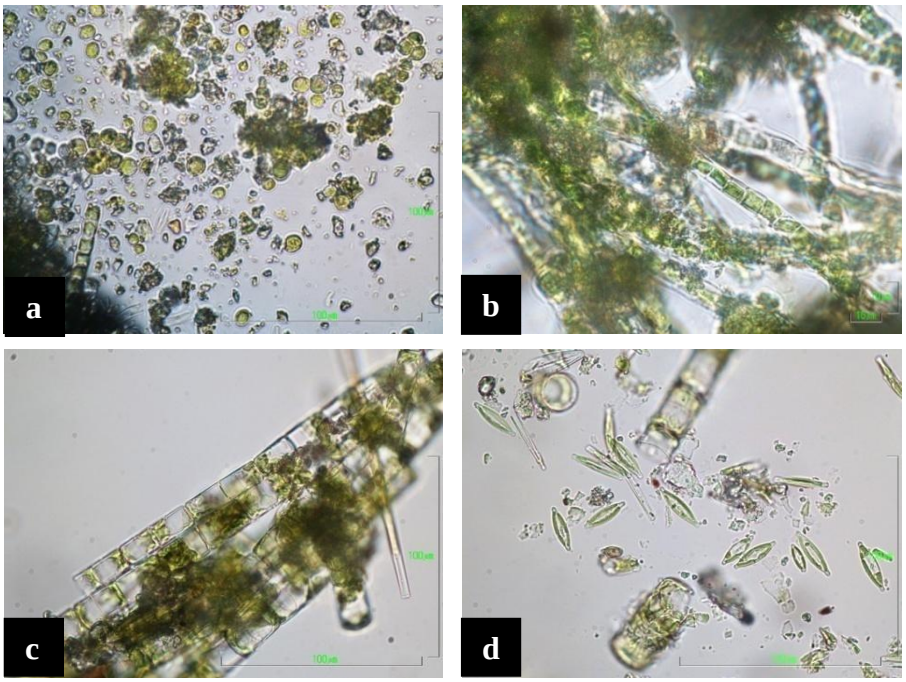


Fig. 63. The microbiological components of the biofilm developed on Fountain from Patio de la Lindaraja before the treatment (**a,b**) and after 6 months (**c,d**) from the reopening of water flux: (**a**) *Apatococcus* sp., diatoms.; (**b**) *Aulacoseira* sp.; (**c**) *Aulacoseira* sp., diatom; (**d**) *Aulacoseira* sp., *Navicula* spp.

IV.2.2.3.2. Molecular evaluation of treatments efficacy

ARISA fingerprint method, for the first time used as an evaluation tool of the treatments efficacy, provided useful and additional data about the diversity of cyanobacteria and eubacteria taxa present in the biofilms that developed on the selected areas (treated and untreated) of the investigated fountains, after 6 months of water immersion. In these two case studies the microscopic observations showed the abundant presence of diatoms, which are eukaryotes. Owing to the fact that, in both fountains, cyanobacteria were not dominant, the possibility that data obtained by ARISA fingerprint method include also other phototrophic fraction, such as diatoms, is very high, because the chloroplast sequences could be amplified. Further detailed investigations are required for improving this technique, in order to obtain the maximum information related with the composition and variation of microbial diversity of the main groups involved in the colonization of monumental stones.

The ARISA analysis showed variable community diversity of the six different investigated areas (treated and untreated) both for Fountain from Patio de la Lindaraja (Fig. 64. a, b) and Tacca's Fountain 2 (Fig. 65. a, b). Generally speaking, it seems the treatments had an influence on fountains diversity, by decreasing the number of the possible colonizing taxa. In what the eubacteria and cyanobacteria (including chloroplasts sequences) are concerned, it can be observed that there are one or two ribotypes dominant in the treated areas and they can colonize, being without competitors, all the surface. Therefore, the applied treatments, at concentrations used did not stop the biofilm formation, but they only influenced the microbial community composition and possibly slowed down the colonization process. More precise data related with the biodiversity variation after the treatments, in quality and quantity as well, can be surely obtained by choosing the most suitable primers to target and control the main microbial groups considered as first colonizers.

Eubacteria were dominant in Fountain from Patio de la Lindaraja, while cyanobacteria presented an high diversity in Tacca's Fountain 2 (Fig. 66). The biodiversity of untreated areas seems to be reverted at that one found before cleaning in Tacca's Fountain 2, after six months of water immersion. In the other fountain, the biodiversity of both eubacteria and cyanobacteria is still a bit lower with respect to the one before cleaning.

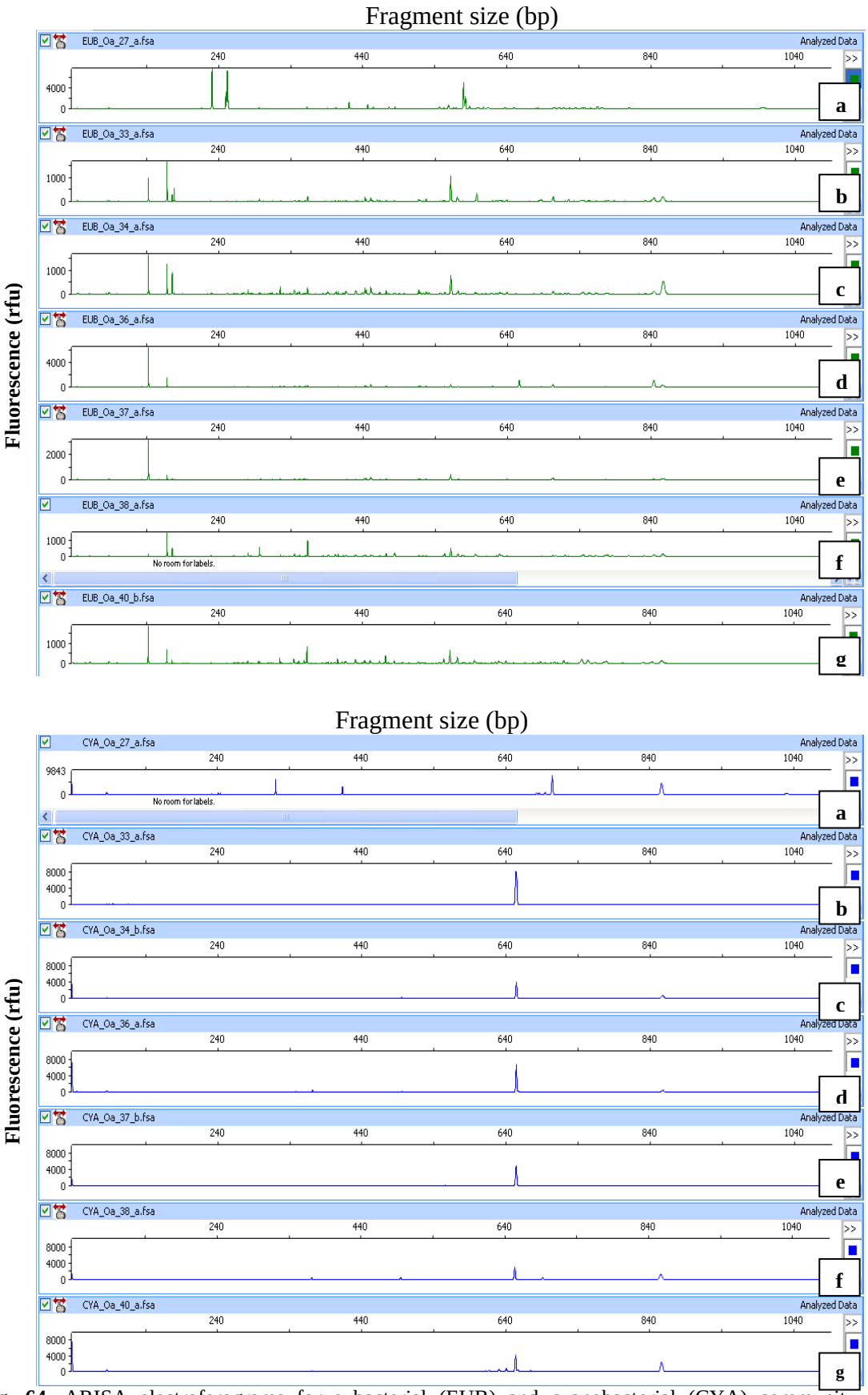


Fig. 64. ARISA electroferograms for eubacterial (EUB) and cyanobacterial (CYA) community in collected samples from treated and untreated areas on Fountain from Pation de la Lindaraja: (a) before treatment; (b) Blank; (c) W; (d) W+pAPS; (e) W+ZA; (f) W+CS; (g) W+A.

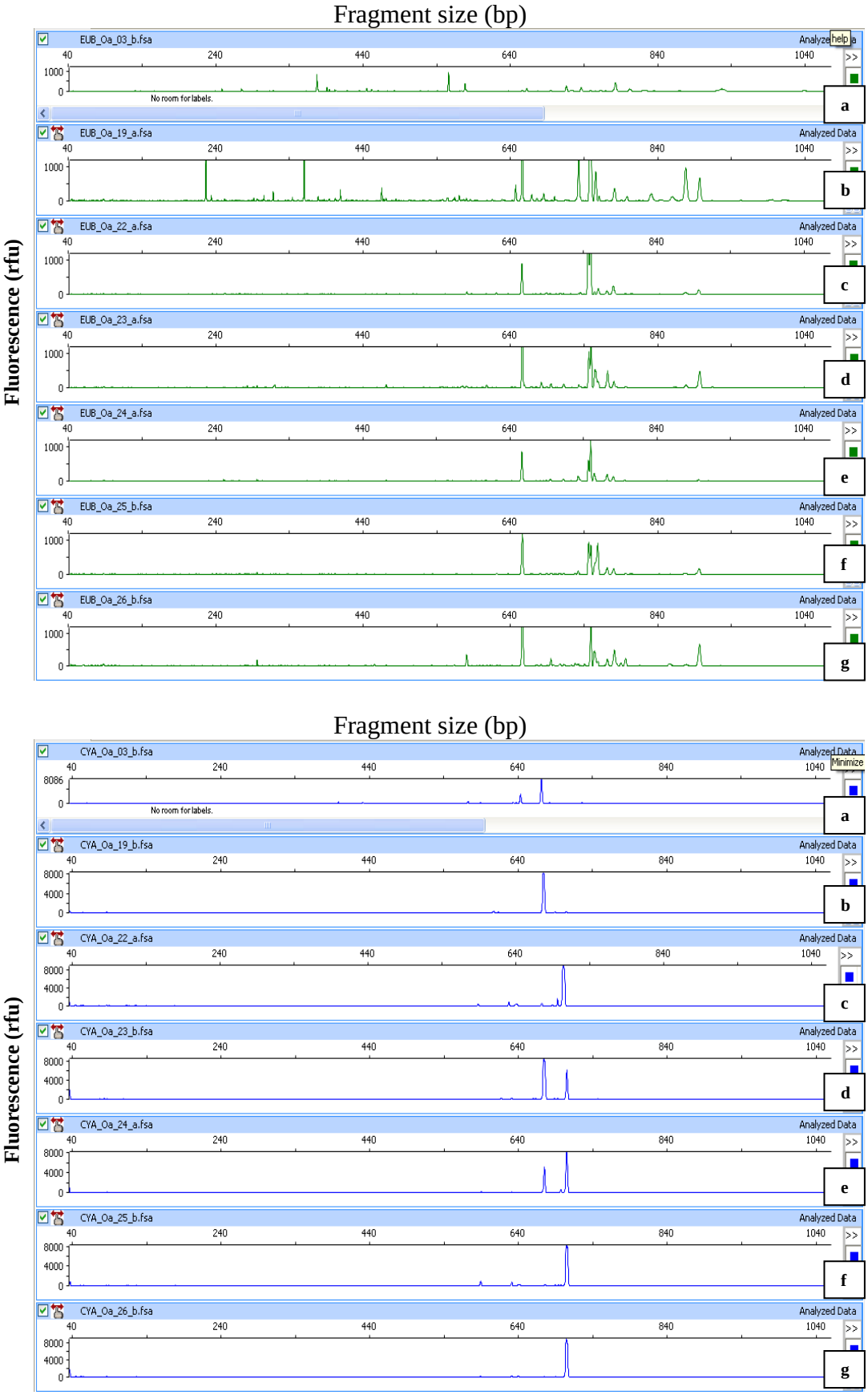


Fig. 65. ARISA electroferograms for eubacterial (EUB) and cyanobacterial (CYA) community in collected samples from treated and untreated areas on Tacca's Fountain 2: (a) before treatment; (b) Blank; (c) W; (d) W+pAPS; (e) W+ZA; (f) W+CS; (g) W+A.

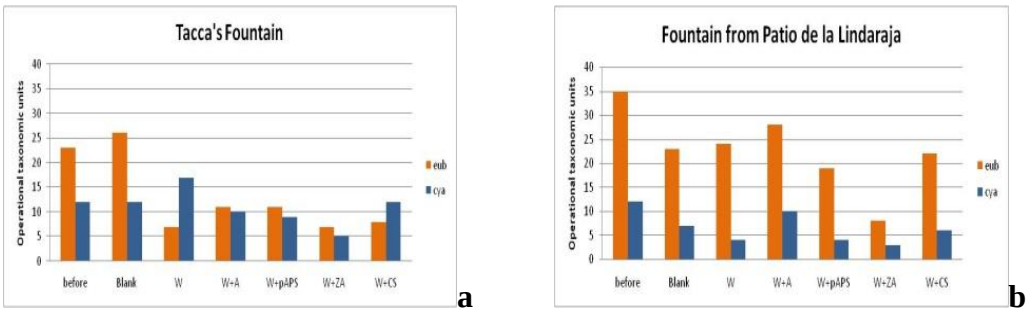


Fig. 66. Eubacterial (eub) and cyanobacterial (cya) microbial community present in Tacca's Fountain 2 (a) and Fountain from Patio de la Lindaraja (b), before and after application of treatments containing ABAs

In Tacca's Fountain the treatments showed a good efficiency against eubacteria and cyanobacteria. The areas treated with ABAs incorporated into W coating presented less cyanobacterial growth with respect to the reference and the area treated only with W.

In the Fountain from Patio de la Sultana the applied treatments showed a good efficiency against cyanobacterial attachment, while the eubacterial growth seemed to be favoured in some cases (W, W+A, W+CS). In fact, this result can be attributed to a different quality of water, climate and biofouling.

Due to the different microenvironmental conditions occurred in the two investigated fountains, it is difficult to understand which group is more sensitive to the ABAs presence. A general evaluation of ABAs efficiency was done by summing the number of eubacterial and cyanobacteria operational taxonomic units present in each fountain. ZA and pAPS incorporated into W induced a decreasing of microbial diversity for both "in situ" experiments (Fig. 67).

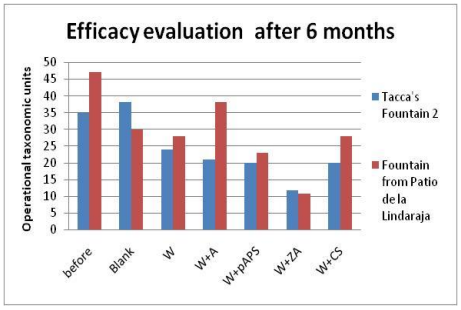


Fig. 67. Efficiency evaluation of in situ tested W treatments containing ABAs.

V. CONCLUSIONS

The phototrophic biofilm developed on monumental fountains induces esthetical damages associated with physical and chemical alterations of stone materials. In fact, biofilms are structures of high metabolic activity, where extracellular polymeric substances, produced by the cells, allow their adhesion to a given surface and protect itself against external stressing factors, facilitate entrapment of airborne particles, aerosols, minerals and organic compounds, increasing the dirty appearance of the substratum.

The results obtained studying the main microbial components of the phototrophic biofilms developed on monumental fountains, with both traditional and molecular methods (ARISA fingerprint), have evidenced a common composition. In fact, many isolated genera are ubiquitous and only few of them have been found in one fountain only. Moreover, microbial community dwelling on the monumental fountains is very similar with that one reported for other monumental assets and statues, the most frequent genera being *Aphanocapsa* sp., *Calothrix* sp., *Chroococcus* sp., *Gloeocapsa* sp., *Nostoc* sp., *Oscillatoria* sp., *Phormidium* sp., *Synechosystis* sp., *Synechococcus* sp., *Chlorella* sp., *Scenedesmus* sp., *Achnanthes* sp., *Navicula* sp., *Nitzschia* sp. and black fungi. Strains of microorganisms (algae, cyanobacteria, diatoms and fungi), dwelling on monumental fountains, were isolated and morphological characterized. They are maintained in specific cultural medium in the Culture Collection at the laboratories of ISE-CNR and IPP-CNR, Florence. Furthermore, 35 cyanobacterial strains were characterized by phylogenetic investigations.

The different microenvironmental conditions found in the fountains usually favor a quick and aggressive biofouling. The understanding of fountains' microbial ecology is the starting point for a scientific approach to evaluate the possible risk for stone conservation and for developing the most suitable control methods. It must be also taken into account that all treatments of stone have an influence on the bioreceptivity of the stone itself.

The patina structure and the mechanism of its development follow that one observed on other stone monuments. In the natural biofilm of monumental fountains it can be observed alternating layers of microorganisms and calcium carbonate due to the dry/wet conditions and/or to the ability of some microorganisms to precipitate calcium carbonate. The formation and evolution of phototrophic biofilms are dependent on

several factors. Light intensity has shown an influence on the type of dominant microorganism in the biofilm, cyanobacteria preferring low light intensity, while diatoms the higher one.

ARISA fingerprint method, for the first time used as an evaluation tool for treatments efficacy, provided useful and additional data about the diversity of cyanobacteria and eubacteria taxa present in the biofilms developed on the selected areas (treated and untreated) of the investigated fountains. The results obtained using molecular analysis are indicative of microbial community changes, with regard to diversity and abundance, this method being very sensitive to the identification of microbial presence. ARISA fingerprint method, correlated with protein measurements and optical analysis, can contribute with significant data to a better understanding of the protective treatments behaviour as regard the microbiological colonization.

The experiments performed for evaluating the efficacy of selected ABAs (CI - Cinnamaldehyde, CS - Capsaicin, CBE – *Ceramium botryocarpum* extract, pAPS – poly-Alkylpyridinium Salts, ZA – Zosteric Acid) under indoor and outdoor conditions, displayed, for almost all these active molecules, at the higher tested concentration, a certain inhibitory effect against phototrophic patina formation or against development of some microbial groups found in biofilms (e.g. cyanobacteria). Non-incorporated ABAs revealed inhibitory properties especially for pAPS and ZA, while CI and CS had rather a toxic effect.

The results obtained through laboratory experiments and outdoor fountains simulation are quite encouraging for the use of some Antibioufuling Agents (ABAs) as potential effective products (CI, pAPS and ZA) against biofilm formation. This study was focused on testing the ABAs efficiency especially against phototrophic fraction present in biofilms (cyanobacteria, diatoms and algae). The W (Wacker Silres BS 290) coating behaved better than S (Silres BS OH 100) coating as incorporating agent, but only when incompletely cured. The chosen cured period (5-15 days) allows the action of the ABAs inhibitors However this partial curing most probably favors its removal in water environment with the consequence of eliminate the ABAs active products. Furthermore, the results obtained through laboratory experiments and outdoor fountains have evidenced, a poor inhibition efficiency when too low ABAs concentration is used.

The complete curing of W coating containing ABAs (30 days) did not show any preventive action against biofilm formation. This can be due to: (i) the loosing/lacking of leaching properties for W to release ABAs and/or (ii) the occurrence

of a chemical reaction between the ABAs and coating, with subsequent changes of their inhibitory properties and hence, more suitable supporting product must be investigated.

The results achieved with the application of ABAs outline their environmental friendly alternative use for biofilm control. Tested ABAs inhibit the biofilm growth, in liquid media, when not mixed with any coatings and, on solid substrata, when the incorporating silicone agent was not completely cured. Therefore, a further detailed studies for defining the optimal concentration and the best condition for a constant releasing and long time lasting of incorporated ABAs are a key point for further similar researches. The use of biological anti-biofilm strategy for interfering with the signal molecules of phototrophic patina has never be attempted before in the cultural heritage field, and the possibility of ABAs application is extremely promising. Surface coatings containing ABAs can be applied as preventive technique against biofouling in monumental fountains and others situations where biofilms occur.

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Plate 1 - The monumental fountains investigated in this study: (a) Fountain from Patio de la Sultana, Generalife, Granada (Spain); (b) Tacca's Fountains - 1 and 2, Florence (Italy); (c) Fountain from Patio de la Lindaraja from Alhambra, Granada (Spain); (d) Second Fountain from Villa la Pietra, Florence (Italy).

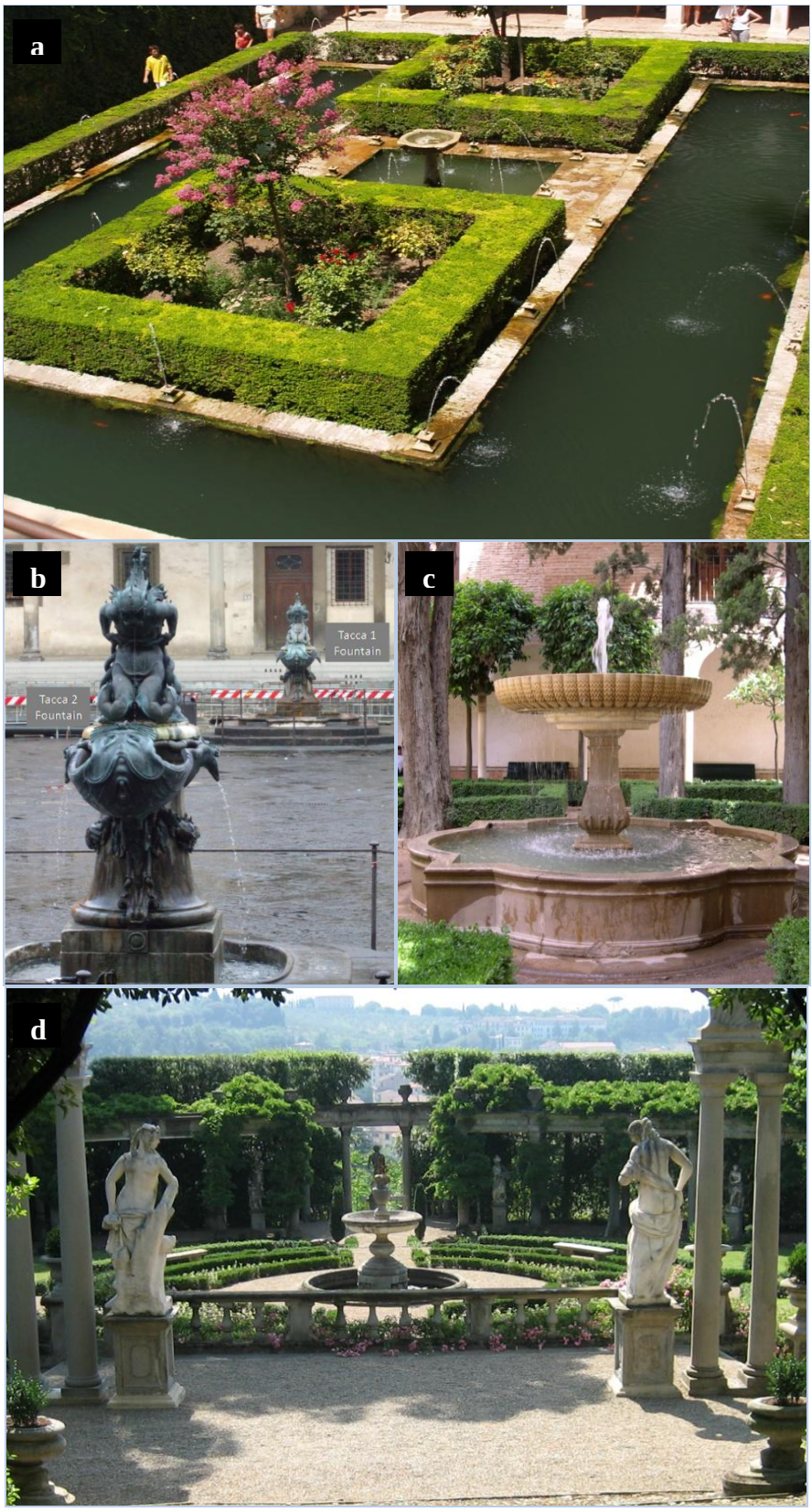


Plate 2 – General view of the sampling areas from Tacca's Fountain 1 (a), Tacca's Fountain 2 (d,e), Second Fountain from Villa la Pietra (f) and Fountain from Patio de la Lindaraja (k), with details: sample 1T (b); sample 2T (c); sample VP3 (g); sample VP2 (h); sample VP1 (i); samples VP5 and VP6 (j); sample 4L (l); sample 1L (m); sample 5L (n); sample 3L (o); sample 6L (p)

Plate 3 – Fountain from Patio de la Sultana, Generalife, Granada, Spain and sampling areas: (a) general view of the sampling site; (b) sample S-1a; (c) sample S-1e; (d) sample S-2; (e) sample S-5W; (f) sample S-6N; (g) sample S-6S; (h) sample S-9E; (i) sample S-9N; (j) sample S-9W.

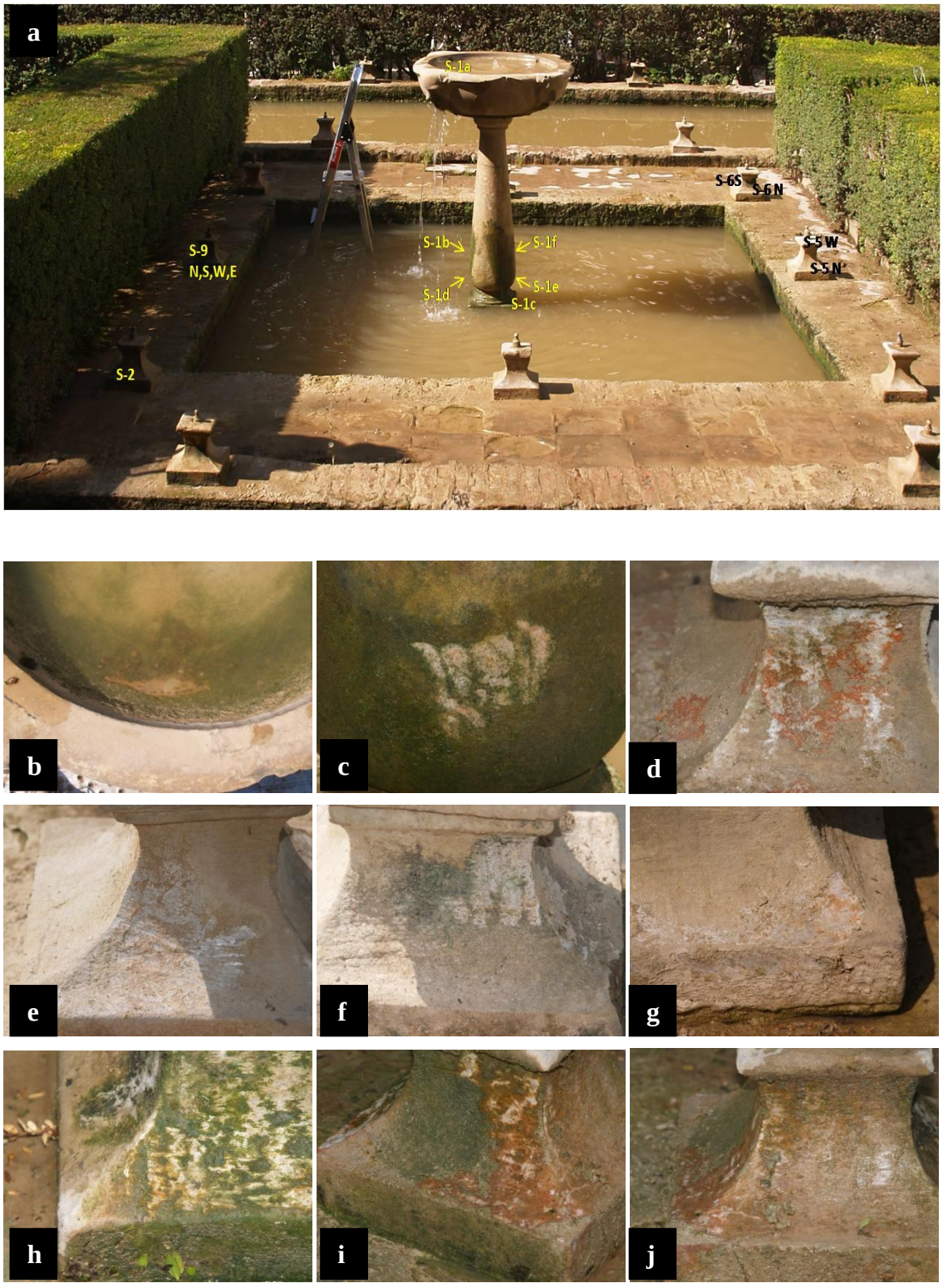
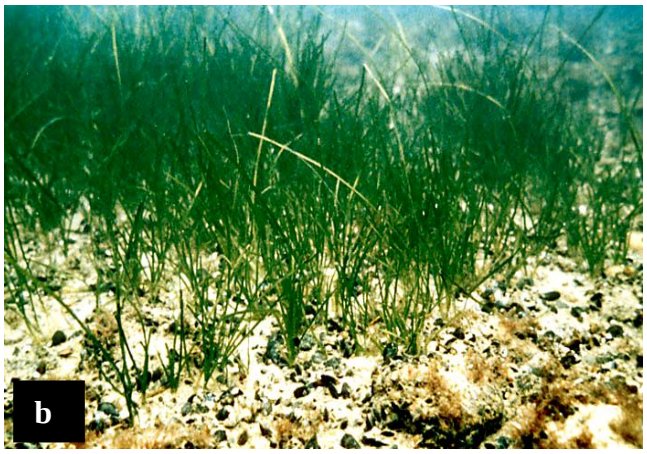


Plate 4 – Natural sources of selected antibiofouling agents (poly-alkylpyridinium salts, zosteric acid, Ceramium botryocarpum extract, capsaicin and cinnamaldehyde, respectively): (a) mediteranean sponge *Reniera sarai* (Porifera, Demospongiae); (b) marine plant *Zostera marina* (Spermatophyta, Potamogetonaceae); (c) marine rodophyta *Ceramium botryocarpum* (Rhodophyta, Ceramiales); (d) chili pepper - *Capsicum anuum* (Solanales, Solanaceae); (e) cinnamon – *Cinnamonum* sp. (Laurales, Lauraceae) with leaves and flower buds (detail).



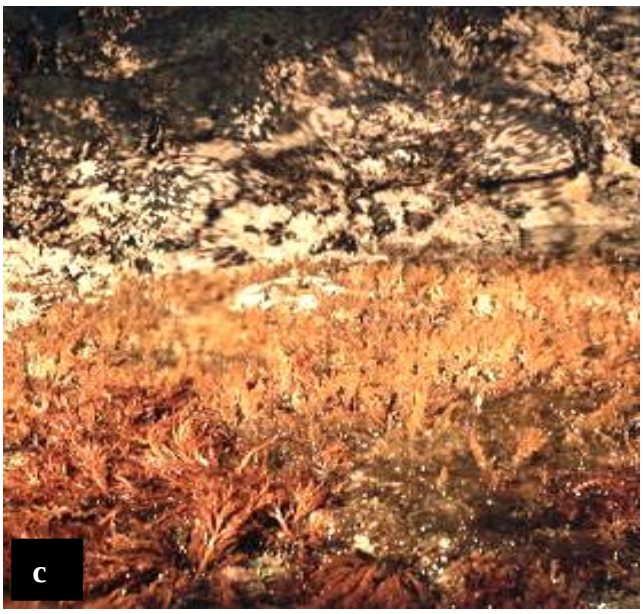


Plate 5 - Morphology under the optical microscope of different species of cyanobacteria (a-c, e, g-i, k-m), algae (j) and diatoms (d-f, m) observed in raw samples collected from Second Fountain from Villa la Pietra (a-b, g-i, k), Tacca's Fountain (e, l, m), Fountain from Patio de la Sultana (c, f, j) and Fountain from Patio de la Lindaraja (d). (a) *Schizothrix lacustris*; (b) *Calothrix parietina*; (c) *Lyngbya* sp.; (d) *Aulacoseira varians* and *Navicula* spp.; (e) *Achnantes affinis* and *Chroococcus turgidus*; (f) *Cymbella* sp.; (g) *Aphanocapsa grevillei*; (h) *Chroococcus turgidus*; (i) *Borzia trilocularis*; (j) *Apatococcus lobatus*; (k) *Aphanocapsa novacekii*; (l) *Synechocystis* sp.; (m) *Achnantes* sp. and *Leptolyngbya* sp. Scale bar 5µm (a), 10µm (b-m).

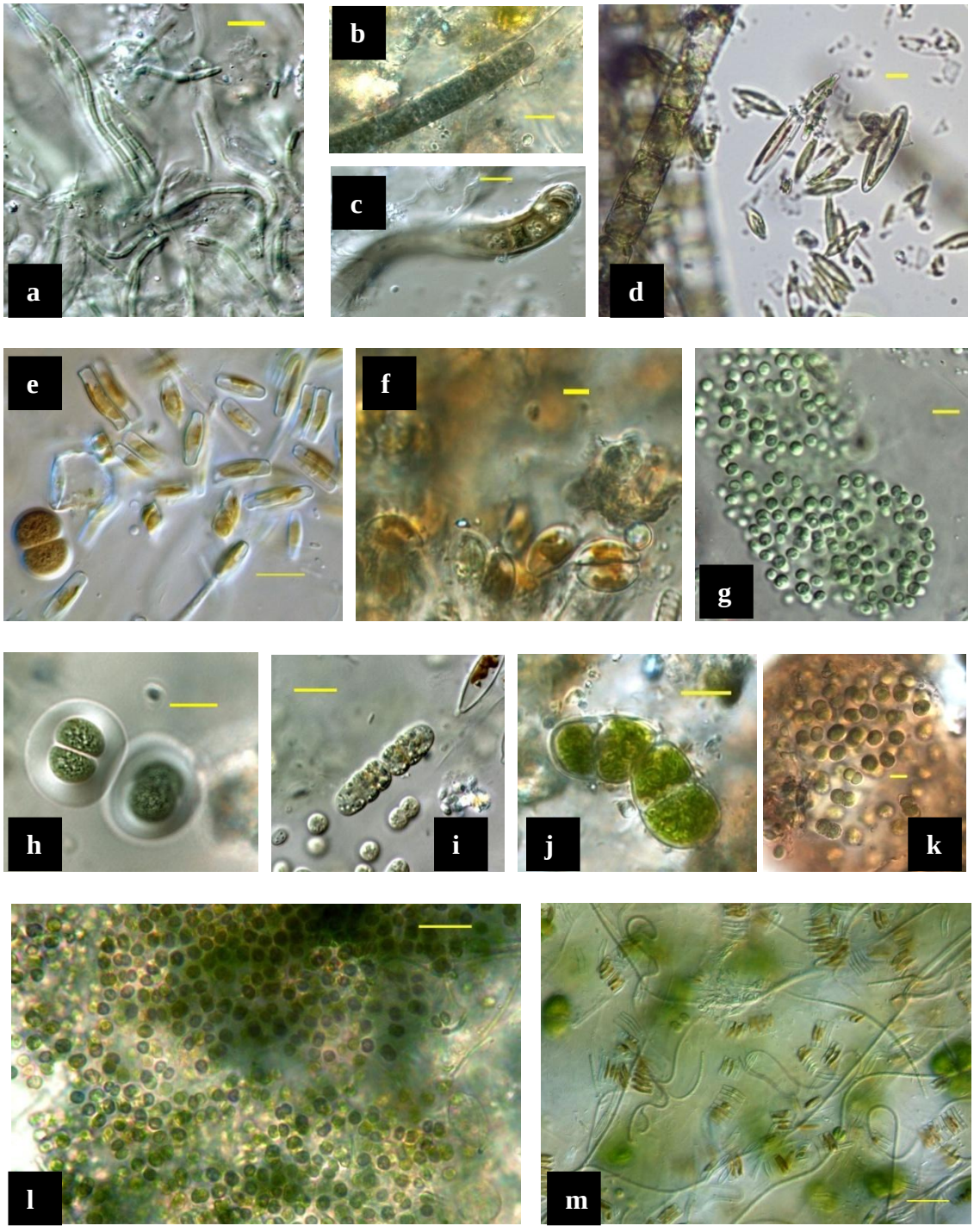


Plate 6 - Cyanobacteria strains isolated from the biofilms developed in artistic fountains: (a,b) *Pseudanabaena* spp.; (c, d, e) *Chroococcus* spp.; (f, g, h) *Gloeocapsa* spp.; (i, l) *Aphanocapsa* sp.; (j) *Aphanothece* sp.5h; (k) *Aphanothece stagnina*. Scale bar 5µm (b, e, f, h, j, l), 10µm (a, b, c, d, g, k), 30µm (i).

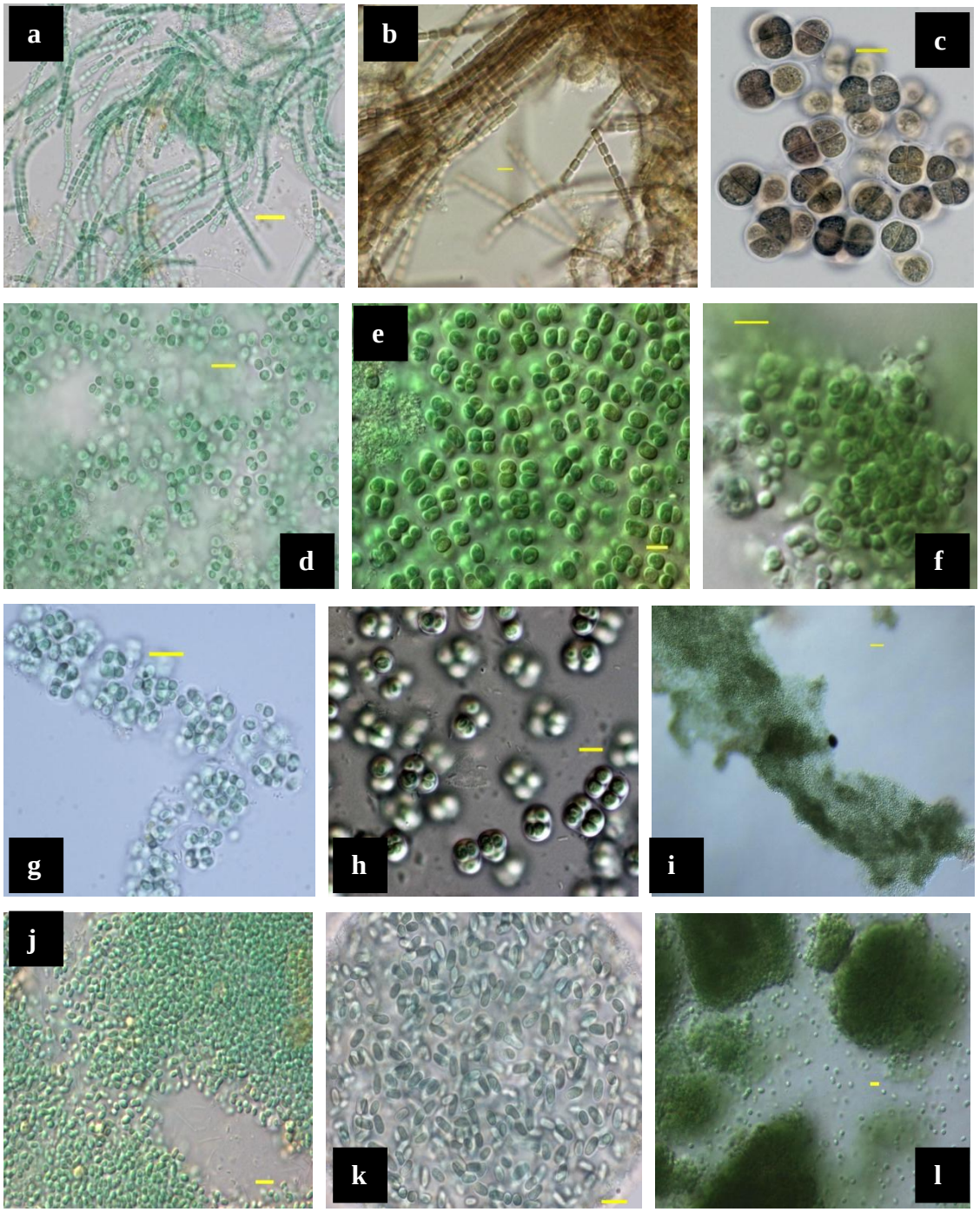


Plate 7 - Cyanobacteria strains isolated from the biofilms developed in artistic fountains: (a,b) *Leptolyngbya* spp.; (c) *Pseudophormidium* sp.; (d, e) *Calothrix* spp.; (f) *Staniera* sp.; (g, h) *Cyanosarcina* spp.; (i) *Gloeobacter violaceus*; (j, k) *Dermocarpa* spp.; (l, m, n, o) *Nostoc* spp. Scale bar 5µm (b), 10µm (a, c - o).

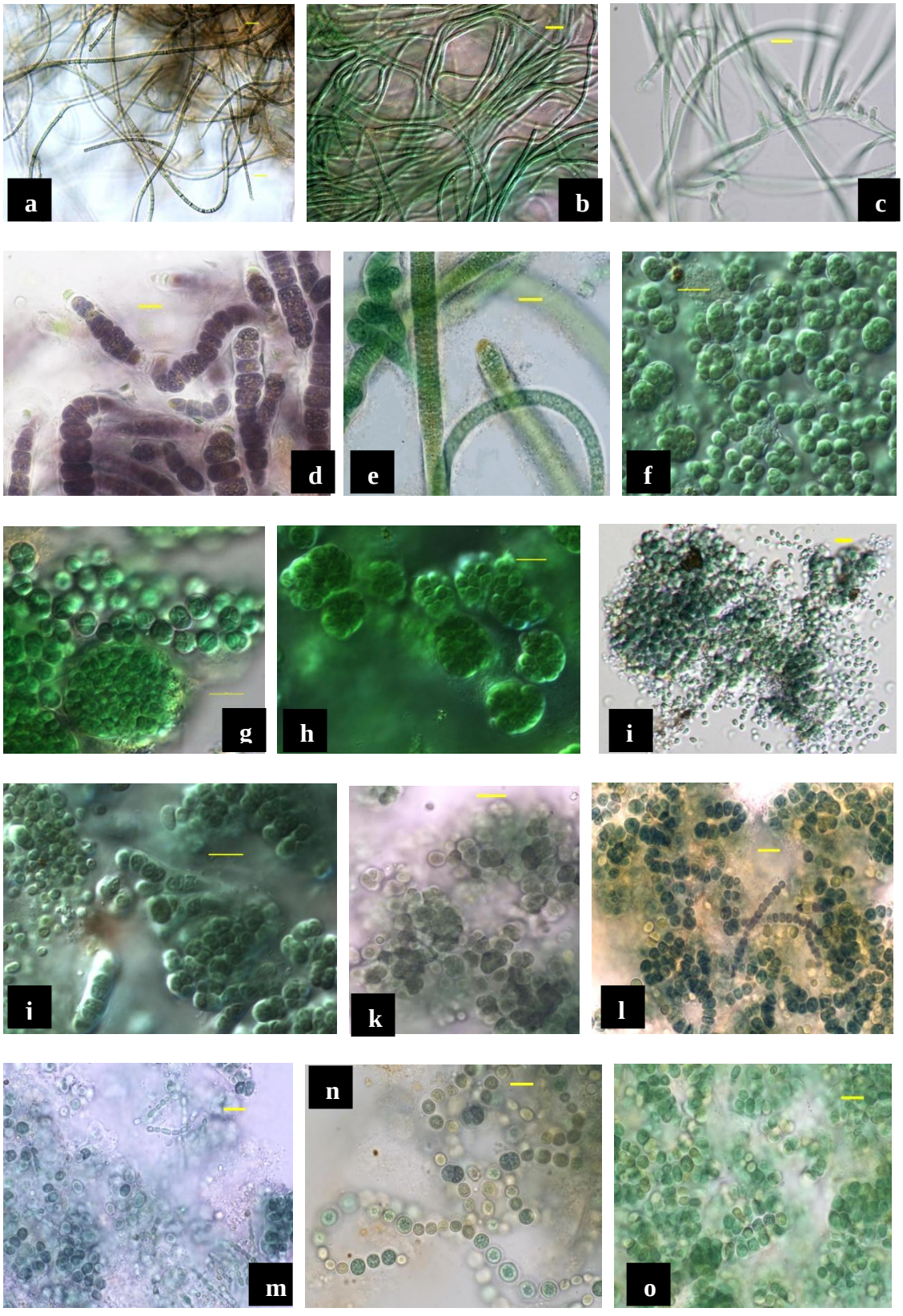


Plate 8 - Diatoms (a-c) and algae (d-l) strains isolated from the biofilms developed in artistic fountains: (a) *Nitzchia* sp.; (b) *Achnathes* sp.; (c) *Diatoma* sp.; (d-f) *Chlorella* spp.; (g) *Cosmarium* sp.; (h) *Scenedesmus quadricauda*; (i) *Scenedesmus bijugatus* forma *alternata*; (j) *Apatococcus* sp.; (k) *Monoraphidium contortum*; (l) *Monoraphidium* sp. Scale bar 5µm (a) and 10 µm (b-l).

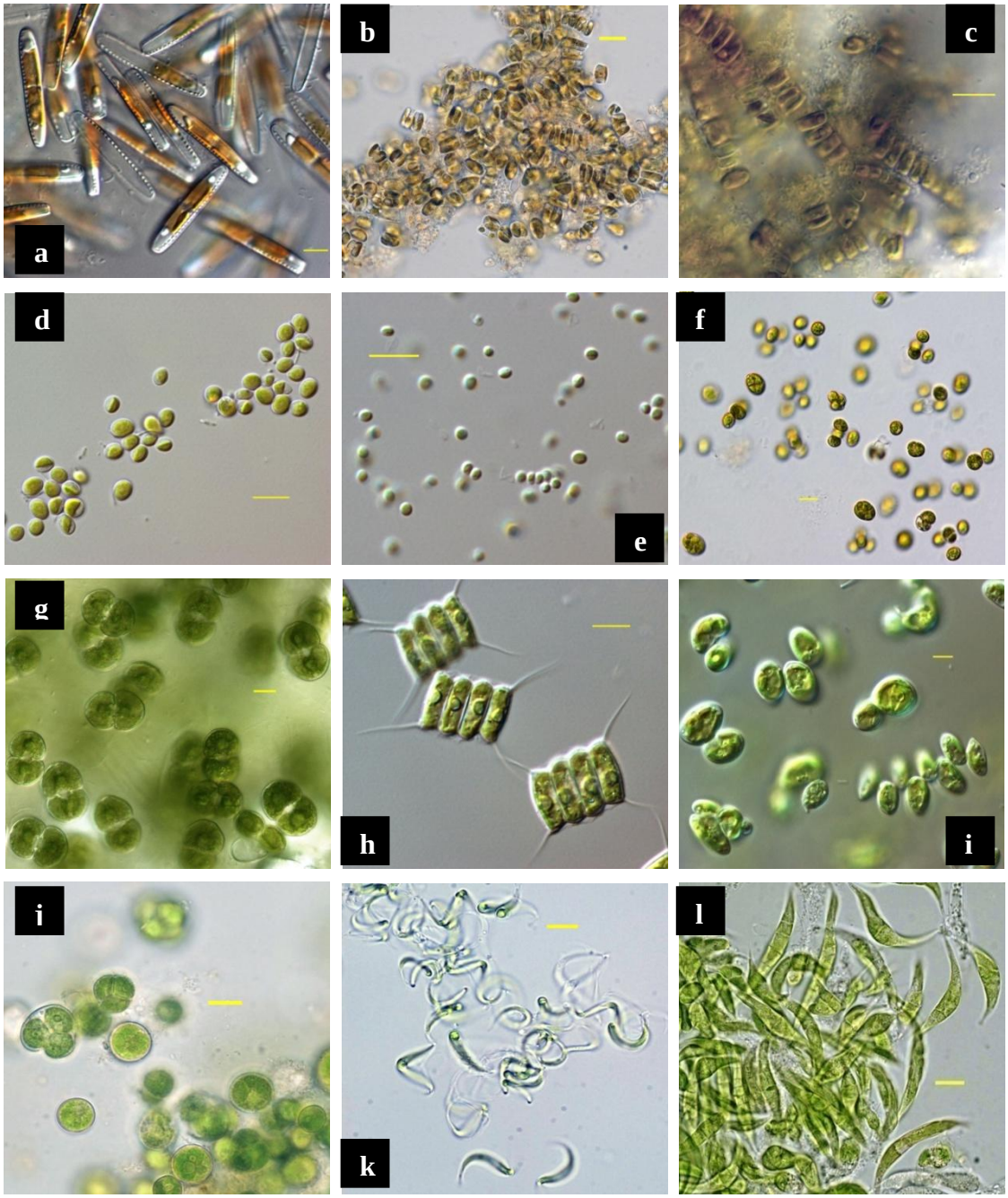


Plate 9 - Fungal strains isolated from the biofilms developed on monumental fountains: (a-c) *Acremonium* spp.; (d) *Arthrinium* sp.; (e,f) *Aspergillus* sp.; (g) *Torula* sp.; (h) *Penicillium* sp.; (i) *Epicoccum* sp.; (j) *Trichoderma* sp.; (k) *Ulocladium* sp.; (l) *Thielaviopsis* sp.; (m) *Phialophora* sp. Scale bar 10µm.