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MOLECULAR QUANTIFICATION AND DETECTION OF MICROORGANISMS ON BUILDING MATERIALS AND MATERIALS OF WORKS OF ART

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Abstract

It is well-known that microorganisms play a decisive role in the biological decay of building materials and works of art. Physical and chemical processes and various effects induced by microorganisms – including archaea, bacteria and fungi – cause the degradation of building materials. Furthermore, microbes, especially fungi and their metabolic products represent a serious health hazard for humans. These properties, the frequent occurrence and the effects of microbial growth on building materials, in indoor environments and on Cultural Heritage demand for more and detailed studies in order to identify the inhabiting microbiota and to elucidate the risks for humans and materials.

In the first study of this cumulative doctoral thesis - performed in collaboration with the Technical University of Graz, molecular techniques were developed and optimized for building biological investigations. We evaluated five indoor insulation systems for their bio-susceptibility against fungal growth. Sprayed cellulose, bloated Perlite board, bloated Perlite plaster, wooden soft-board and reed board with loam – all based on ecological and historical materials, can be applied for improving the thermal performance of historical buildings. The systems were tested both under laboratory- and under natural conditions, after two years of installation in a historical house. In the laboratory, small test items of the insulation systems were subjected to three commonly indoors occurring fungal strains. After six months of incubation in a climate chamber at optimal growth conditions, small samples were taken for assessing the bio-susceptibility by classical cultivation- and by molecular techniques. Therefore, 1) the colony forming units (CFU) of each insulation system were determined, 2) the concentration of the extracted DNA was measured using a Nano Drop spectrophotometer, and 3) finally the fungal abundance was assessed by quantitative real time analysis targeting the β-actin gene. The same procedure was performed with samples from the historical building taken after one and two years of installation.

In order to apply the most suitable DNA extraction method for the different insulation systems we precedent evaluated up to thirteen direct – *in situ* DNA extraction protocols with three typical building materials - common plaster, red brick and gypsum card-board. Three different sample amounts of each material were tested. The following parameters were decisive for the comparison of the isolation methods: 1) the quantity and 2) the quality of the extracted DNA (both assessed by Nano Drop measurements), 3) visualization of the isolated DNA by electrophoresis on agarose gels, 4) the ability of the extracted DNA to be amplified in PCR reactions using one bacterial 16S rRNA primer pair and three fungal ITS primer sets, and 5) analysis of the amplified PCR products by Denaturing Gradient Gel Electrophoresis (DGGE) to compare the obtained community fingerprints.

The evaluation of the different DNA isolation protocols showed that the FastDNA Spin Kit for soil (MP Biomedicals) revealed the overall best performance for all parameters of the three tested building materials. This extraction method was further successfully applied for the samples of the indoor insulation systems. Summing up the results from the classical cultivation- and the molecular analysis of the indoor insulation systems from the historical house and the test items, we state, from the microbiological and hygienically point of view, the most appropriate thermal insulation system was presented by the bloated Perlite plaster. From this material only a few fungal colonies could be cultured, low DNA concentrations as well as the lowest fungal abundance could be observed. The sprayed cellulose, bloated Perlite board and the reed board with loam could also reveal good results in this evaluation, whereas the wooden soft-board system showed to be not recommendable, due to the highest CFU values as well as the highest fungal abundance detected in this evaluation.

The second study of this thesis was performed in collaboration with the Spanish working group of Prof. Dr. Cesareo Saiz-Jimenez from the University of Sevilla. Together, we investigated the microbiota responsible for the rosy discoloration of mural paintings in three historical Austrian buildings. Culture-dependent techniques were used to isolate the cultivable fraction of the inhabiting microorganisms. Additionally, a molecular strategy comprising of DNA extraction, PCR amplification of archaeal and bacterial 16S rRNA sequences, construction of clone libraries and fingerprinting by DGGE followed by sequence analysis of selected clones was applied for the identification of the microbes causing this phenomenon. Cloned 16S rDNA sequences revealed the dominant occurrence of halophilic members of *Actinobacteria*, mainly of the genus *Rubrobacter*. Representatives of the *Archaea* with the predominating genera *Halobacterium*, *Halococcus* and *Halalkalicoccus* could be detected on all three buildings. By using special culture media, several halophilic bacterial strains that had a rosy color appearance were isolated from two historical monuments. The majority of these isolates could be associated with members of the *Firmicutes*. A high similarity was observed among the bacterial and archaeal microbiota detected in the three investigated buildings as well as to other reported historical sites in Europe. Furthermore, two selected strains were inoculated on limestone and gypsum plaster. The unaesthetic discoloration effect could be reproduced under laboratory conditions showing that halotolerant and halophilic archaea and bacteria are responsible for the rosy pigmentation and biodeterioration on the ancient paintings.

Kurzfassung

Es ist wohlbekannt, dass Mikroorganismen eine entscheidende Rolle bei der Zerstörung von Baumaterialien und Kunstgegenständen spielen. Physikalische und chemische, sowie durch Mikroorganismen (Pilze, Bakterien und Archaea) induzierte biologische Prozesse sind für den Abbau von Baumaterialien verantwortlich. Zusätzlich stellen Mikroorganismen, vor allem Pilze und deren Stoffwechselprodukte, auch eine Gefahr für die menschliche Gesundheit dar. Diese Eigenschaften, das häufige Auftreten und die Auswirkungen mikrobiellen Wachstums auf Baumaterialien, in Innenräumen und auf unseren Kulturgütern verlangen nach detaillierteren Untersuchungen um die vorhandenen Mikroben zu identifizieren und um die Risiken für Mensch und Material einschätzen zu können.

In Zusammenarbeit mit der Technischen Universität Graz, wurden in dem ersten Projekt dieser kumulativen Doktorarbeit molekulare Techniken für baubiologische Untersuchungen entwickelt und optimiert. Wir untersuchten verschiedene Innendämmmaterialien auf deren biologische Affinität zu Pilz-Wachstum. Aufspritzzellulose, Perliteplatten, Perliteputz, Holzweichfaserplatten und Schilfplatten mit Lehmputz sind ökologische und historische Materialien, die für die Dämmung von denkmalgeschützten Gebäuden verwendet werden können um die Energiebilanz zu verbessern. Die verschiedenen Systeme wurden sowohl unter Labor- als auch unter natürlichen Bedingungen - durch 2-jährige Installation in einem historischen Haus, getestet. Im Labor wurden kleine Probekörper jedes Dämmmaterials mit drei häufig in Innenräumen auftretenden Pilzen beimpft. Nach 6-monatiger Inkubation unter optimalen Wachstumsbedingungen wurden Proben entnommen um den biologischen Befall mit Hilfe von Kultivierungs- und molekularen Methoden zu untersuchen. Hierfür wurde 1) die Kolonie-Bildende Einheit (KBE) jedes Materials bestimmt; 2) die DNA-Konzentration spektrophotometrisch gemessen, und 3) die Menge der Pilze mit quantitativer Real-Time PCR bestimmt. Dieselbe Vorgehensweise wurde mit Proben aus dem Versuchshaus, die nach ein und zwei Jahren entnommen wurden, durchgeführt.

Um eine optimale Methode für die DNA-Isolierung aus den Dämmmaterialen anzuwenden, wurden vorab bis zu 13 direkt – *in situ* – DNA-Extraktionsprotokolle an drei typischen Baumaterialien (konventioneller Wandputz, roter Ziegelstein und Gipskartonplatten) verglichen. Bei je drei unterschiedlichen Probenmengen jedes Materials wurden die folgenden Parameter für die Evaluierung der Isolierungsmethoden herangezogen: 1) die Menge und 2) die Qualität der extrahierten DNA (beides mittels Nano Drop Messungen bestimmt), 3) die elektrophoretische Visualisierung der DNA auf Agarosegelen, 4) die Möglichkeit die DNA in vier verschiedenen PCR Reaktionen mittels einem bakteriellen 16S rRNA und drei Pilz-ITS-Primer Paaren zu vervielfältigen, und 5) die Analyse der PCR-

Produkte mit Hilfe von Denaturierender Gradienten Gel Elektrophorese (DGGE) um die erhaltenen Fingerabdrücke der mikrobiellen Gemeinschaften zu vergleichen.

Bei der Evaluierung der verschiedenen DNA-Isolierungsmethoden zeigte der FastDNA Spin Kit for soil (MP Biomedicals) das beste Gesamtergebnis für alle Parameter bei den drei getesteten Baumaterialien. Diese Extraktionsmethode wurde im Anschluss erfolgreich für die Innendämmmaterialien angewendet. Die Ergebnisse der Kultivierungs- und der molekularen Untersuchungen zeigten, dass aus mikrobiologisch-hygienischer Sicht, der Perliteputz das am besten geeignete Innendämmmaterial darstellte. Von diesem Material wurden nur wenige Kolonien isoliert, geringe DNA Mengen detektiert und die geringste Zellzahl festgestellt. Die Aufspritzzellulose, die Perliteplatte und die Schilfplatten mit Lehmputz konnten ebenfalls gute Gesamtergebnisse erzielen; wohingegen die Holzweichfaserplatte, aufgrund der höchsten KBE und Pilzmenge im Test, nicht empfehlenswert war.

Das zweite Projekt dieser Doktorarbeit wurde unter Mitwirkung der spanischen Forschungsgruppe von Prof. Dr. Cesareo Saiz-Jimenez der Universität von Sevilla, durchgeführt. Zusammen wurden die mikrobiellen Gemeinschaften untersucht, die für die rosa Verfärbungen an Wandmalereien in drei denkmalgeschützten österreichischen Gebäuden verantwortlich sind. Kulturabhängige Verfahren wurden angewendet um den Anteil der kultivierbaren Mikroorganismen zu isolieren. Zusätzlich wurde eine molekulare Strategie, bestehend aus DNA-Extraktion, PCR-Vervielfältigung von Fragmenten der ribosomalen 16S RNA von Archaea und Bakterien, Konstruktion und Analyse von Klon-Bibliotheken durch DGGE, gefolgt von der Sequenzanalyse ausgewählter Klone für die Identifizierung der für dieses Phänomen verantwortlichen Mikroben, angewendet. Durch die vergleichende Sequenzanalyse der bakteriellen 16S rDNA konnte das gehäufte Auftreten von halophilen *Aktinobakterien*, vor allem der Gattung *Rubrobacter*, festgestellt werden. Vertreter der Archaea, die zu den Gattungen *Halobacterium*, *Halococcus* und *Halalkalicoccus* verwandt waren, konnten in allen drei Gebäuden detektiert werden. Auf speziellen Nährmedien wurden mehrere halophile Bakterien, die alle eine rosa Koloniefarbe aufwiesen, von zwei Gebäuden isoliert. Die Mehrheit der Isolate konnte mit Vertretern der *Firmicutes* assoziiert werden. Sehr hohe Gemeinsamkeiten konnten unter den mikrobiellen Gemeinschaften (sowohl Bakterien als auch Archaea) der drei untersuchten Gebäude festgestellt werden; sowie zu historischen Gebäuden in ganz Europa. Zusätzlich wurden zwei ausgewählte Bakterienisolate auf Kalkstein und Gipsputz inokuliert und die unästhetischen rosa Verfärbungen unter Laborbedingungen reproduziert. Dadurch konnte gezeigt werden, dass Salz-tolerierende und Salz-liebende Archaea und Bakterien für die rosa Pigmentierung und die biologische Schädigung von historischen Wandmalereien verantwortlich sind.

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1 General introduction

1.1 Microbes and biodegradation of building materials and works of art

The alteration and weathering of all materials are natural ongoing processes on earth. Evolution of life would not have been possible without the degradation and erosion of rocks to soil material. The materials exposure to natural, environmental parameters – including wind, rain, snow, sunlight, temperature and moisture - and also anthropogenic impacts are the dominant weathering agents of building materials, stone and our works of art. The physical weathering processes have a great influence on the stability of the rock matrix. Additionally, chemical factors strongly affect the material-matrix, as the corrosion of minerals is triggered through hydration- and oxidation reactions, as well as solubilization and dissolution of certain elements (Keller, 1957). Mankind contributes and even accelerates the natural decay of exposed materials. The release of high amounts of organic- and inorganic compounds into the atmosphere results in a more aggressive environment for building materials and works of art. The increasing air pollution and the deposition of particulate matter on the material surfaces significantly enhance the degradation of exposed stone materials (Arnold, 1981, 1993; Baedecker and Reddy, 1993; Koestler, 2000; Warscheid and Braams, 2000).

For a long time physical and chemical processes were assumed to be the major causes responsible for the decay of materials. Since the middle of the 20th century scientists have begun to analyze the role of biological agents (microbes, animals, plants) in the degradation of stone and building materials (Paine et al., 1933). In 1996, Bunyard stated that through the evolution of life, microbes have adapted to inhabit all substrates on earth, in indoor as well as outdoor environments, on historic and on modern buildings. In fact, a great variety of organisms including archaea, bacteria, fungi, cyanobacteria, algae, lichens, mosses, plants and higher organisms (insects, birds, etc.) were found to be successful invaders of all types of materials (see Figure 1; Ettenauer et al., 2010, 2011, 2012, 2014; Heyrman et al., 1999; Piñar et al., 2001a, 2001b; Schabereiter-Gurtner, 2000; Schabereiter-Gurtner et al., 2001a, 2001b, 2001c). The great importance of biological agents in the decay of building materials and works of art is nowadays well-established knowledge in science and has also increasingly drawn the attention of restorers and residents. Microbial growth and biodeterioration - defined as “any undesirable change in a material brought about by the vital activities of organisms” (Allsopp, 2011), is responsible for different destruction phenomena ranging from the mere aesthetically unacceptable staining of surfaces and spoilage by biogenic pigments to structural damages and significant material losses (Urzi et al., 1992).

Figure 1. Different types of biological colonization on stone materials. A) Stone-cross colonized by various lichens (Muxia, Spain). B) Abandoned house conquered by higher plants, algae, lichens, fungi, etc. (Finisterre, Spain). C) Statue in a buildings niche harbors doves (Santiago de Compostella, Spain). All pictures were taken by Jörg Ettenauer.

1.1.1 Bioreceptivity and biodeterioration phenomena

The bioreceptivity of materials as well as the type, intensity and extend of microbial growth are highly dependent on various factors: 1) environmental factors, like the water availability, climatic conditions, light and the materials exposure, 2) the physical structure of the surface – its porosity and permeability, mineral composition, and 3) the chemical nature of the material (pH, chemical composition and nutrient resources that can be utilized by the microbes) (Warscheid and Braams, 2000).

Building materials and works of art provide numerous ecological niches for the settlement of microbes. Autotrophic microorganisms are commonly the first invaders of inorganic substrates. However, on materials, especially if they are subjected to open air, organic substances can easily be accumulated by 1) atmospheric pollution and through airborne organic contaminations (pollen, dust, dripping water, etc.), 2) residuals from precedent biological colonizers and their metabolites, 3) various old and/or new treatments of the material surface (glues, oils, waxes, restoration works, coatings, consolidants, etc.), 4) the contribution of humans and/or animals (insects, birds, bats, animal feces, etc.), and/or 5) the materials themselves contain organic substances. As a consequence, the availability of organic substances on inorganic substrates allows the establishment of a heterotrophic microflora.

Nowadays, a great variety of microbial-induced deterioration phenomena are known. Microbial colonizers of building materials and works of art produce substrate-specific enzymes, biogenic organic and inorganic acids as well as different excretion products and

chelating agents. These substances induce biological corrosion processes; lead to the dissolution and solubilization of chemical compounds, cause changes in the pH of the material and also attack the material surfaces. Furthermore, the bacterial production of crystals (biomineralisation) on and in the material, the release of gases and the degradation of organic substances and consolidants contribute to the decay of the material.

Microbial growth can cause staining of the material due to the production of a great variety of colorful pigments (carotenes, chlorophyll, phenols, melanin, etc.) ranging from white, yellow, orange, pink, rosy, purple, green, brown and black. Many halophilic archaea and bacteria are known to cause yellow, orange, pink and rosy discolorations due to the production of characteristic carotenes. Melanins encrusting the cell walls of different fungi are often responsible for dark spots and rigid crusts on materials. Biofilms or patinas of different thickness, color and consistency are often formed by epilithic microorganisms that colonize the surface of the material (see Figures 1 and 2). Microbial growth as biofilms on and within the materials together with the production of acids leads to substantial material losses (Piñar and Sterflinger, 2009). Furthermore, bacterial cells in biofilms are embedded with extracellular polymeric substances (EPS) that store the available water and, therefore, alter the natural water retention and diffusion in the material. As a result, additional mechanical stress is exerted on the materials due to shrinking and swelling cycles (Dornieden et al., 2000). On the other hand, some endolithic fungi penetrate with their mycelia and hyphae into fissures and pores of the substrate or inside the plaster of building materials. This endolithic growth into the interior of the material can cause high mechanical pressures which can further lead to detachment and material loss (Sterflinger and Krumbein, 1997).

Figure 2. Different stages of stone decay. A) Picture of a new statue without microbial colonization. B) Picture of a small statue that was exposed to weathering processes for approximately 12 years. C) Picture of a statue that was exposed for several hundred years (at the front entry of the Cathedral of Santiago de Compostella; Spain). D) Direct comparison of deteriorated and restored facade covered with figures (side entry of the Cathedral of Santiago de Compostella, Spain). All pictures were taken by Jörg Ettenauer.

The physical, chemical and biological processes that alter and deteriorate all materials co-interact with each other. Furthermore, the complex, synergistic and antagonistic combinations of these agents usually do not allow a clear separation (Koestler et al., 1994, 1997; Valentin, 1993). The role of microorganisms in the degradation of materials has been shown in various studies (Bock and Sand, 1993; Urzi and Krumbein, 1994). Microbial actions lead to modifications of the mechanisms and rates of physical and chemical weathering processes. In order to prevent and avoid further decay of building materials and to preserve works of art of our Cultural Heritage, it is absolutely necessary to understand all mechanisms of biodeterioration and, therefore, precise investigations of the inhabiting microbiota are indispensable (Piñar and Sterflinger, 2009).

1.2 Fungi in indoor environments and on building materials

Since thousands of years in history, fungi – including yeasts, mushrooms, moulds, and toadstools – have been very important for humans (Boddy and Coleman, 2010). Beside the numerous positive properties and useful applications of fungi, their metabolic pathways and products, this group of organisms also has a “dark side”. Fungi can cause food spoilage, degrade organic materials, damage inorganic materials by produced acids and cause spoilage due to biogenic pigments. Many members of this phylogenetic group are known to produce mycotoxins and cause allergies; some are even pathogen and fungi are able to deteriorate all kind of building materials (Sterflinger and Ettenauer, 2012). Humans hassle

with moulds and wood-destroying fungi in buildings is not a newly occurring problem (Sterflinger and Piñar, 2013). Already in the bible, the biodeterioration of buildings by fungi was firstly mentioned as a white, red or green “leprosy” or “fretting” on wood, brick and clay (Old Testament, Third Book of Moses, chapter 14, verses 33-57).

Fungi play a considerable role in the deterioration of building materials and of Cultural Heritage. The weathering of materials is highly increased due to their ability to grow at relatively low water activity values and their strong enzymatic activities. Fungal growth on the outside of buildings and especially in indoor environments has more and more become an evident problem in the recent years (de Hoog and Guarro, 2000; Samson et al., 2010; Sterflinger, 2010).

The natural habitats for molds are soils, forests and dead organic materials (Crook and Burton, 2010), but they can also occur in other places where sufficient organic material and moisture can be found. Fungal spores, as means for outlasting non-optimal dry conditions, but also for the distribution of the fungal species, are always present in the air. Therefore, fungal spores can easily reach into our homes. Once these spores have entered our houses, they settle down on any material (furniture, house dust, etc.) and are able to survive there for several years. A sudden change in the climatic conditions - with an increase of the relative humidity in the air and/or the material - can lead to the germination and growth of fungal colonies within a few hours. This generalized scenario shows how easily mold growth and a fungal contamination can occur in indoor environments (Sterflinger and Ettenauer, 2012).

1.2.1 Fungal growth requirements

The ability and the amount of fungal growth in buildings are determined by numerous factors, whereby the four most decisive parameters are the humidity, the nutrient availability, the pH and the temperature. Thereby, the combination of temperature and humidity has to be taken into account, because the minimal and optimal humidity changes with the temperature (Fitz et al., 2006). Additionally, for the germination of the fungal spore and for mycelium growth a solid or porous material surface is necessary.

Humidity and the availability of water are the most important parameters for fungal growth. The water activity number (a_w) indicates the possibility of fungal growth on a certain material. Compared to bacteria (a_w>0.95-0.98), fungi can grow at much lower water activities (a_w>0.60-0.65). For nearly all indoor fungi a relative humidity above 70% (a_w>0.7; UBA, 2005) is necessary for growth, which can easily be reached after a water damage, flood, etc. Therefore, building materials that strongly absorb and store large amounts of water, favor the

development of microorganisms (bacteria and fungi) as well as a higher diversity of microbes can be detected compared to fast drying and breathable materials that hamper microbial growth.

Fungi are able to grow on a broad range of different temperatures from 0°C to 50°C (Sedlbauer, 2001). Every species has its preferences concerning the optimal temperature for growth and, therefore, the occurrence of different fungal species shows great variations in buildings. In heated rooms different fungal species can be found than in not/or not permanently heated parts of the building (like basements, storerooms, non-heated bedrooms, etc.).

Concerning the nutrient availability, fungi can utilize a great variety of substrates for their growth. Organic compounds as nutrients can be found in wood or other cellulose containing materials (gypsum card-board, packages, wallpapers, paper, etc.), biogenic contaminations in house dust, leftovers in the kitchen or other organic substances and additives in wall paintings or building materials (Sterflinger and Ettenauer, 2012).

The pH value is also a decisive parameter for fungal colonization on the material surface. Optimal pH values are ranging from 5 to 7, whereby some species also tolerate acidic pH values down to 2, or up to 11 in the alkaline region. Paint coatings and wallpapers are usually in the optimal pH range, whereas lime plaster and -washes and silicate coatings do not represent a favorable habitat for fungi. Nevertheless, organic contaminations on these materials also allow fungal colonization (Tappler et al., 2012).

1.2.2 Causes of fungal growth

Plentiful of different causes can favor the development of fungal growth in indoor environments. The investigation of the factors leading to mold contaminations is of great importance in order to apply preventative actions, to avoid health implications for the residents and to perform proper restoration strategies to remove fungal contaminations. The main reasons for increased moisture contents and associated mold growth in buildings are constructional problems, improper usage behavior of humans and water damages. In most cases a combination of different factors is responsible for the appearance of molds and a distinct correlation to one factor is often not possible.

The factors that can cause and enhance fungal growth in indoors can be grouped in five major categories (Hankammer and Lorenz, 2003; LGA, 2004; Tappler et al., 2012):

1. *Structural factors and improper completion*: improper thermal insulation and/or thermal bridges, lack of ventilation possibilities, problems in the waterproofing of the building, direct or indirect water infiltrations, cracks in walls or roof, deficient drainages, failures in the air-conditioning system, material faults, etc.
2. *Causes related to usage behavior:* poor heating and improper ventilation of buildings, high moisture production, permanently tilted windows, positioning of furniture, etc.
3. *Consequences after constructional measures:* improper ventilation of newly built houses and residual moisture in the materials, installation of wet construction materials, thermal modernization and improvements of residual and historical buildings by installation of new windows and/or doors with improper ventilation of the building, etc.
4. *Water damages and leakage:* broken dish washer or washing machine, damaged pipes of the buildings fresh water system or heating system, etc.
5. *Exceptional causes:* floods, fire water, wet years with changing climatic conditions, etc.

All these factors can lead to an increased relative humidity in indoor rooms and/or materials and further to the establishment of fungal growth.

1.2.3 Fungi – a risk for human health

Beside molds, in inner rooms also bacteria, *Actinomycetes* and wood-destroying fungi can be found. From the medical-hygienically point of view, molds play the major role in our homes and it is well-known that they represent a serious health hazard for the residents (Gutarowska and Piotrowska, 2007; Samson et al., 1994). Fungi have various implications on human health (Hutter et al., 2010):

1. *Allergic effects:* Spores that are released from molds can lead to allergic reactions. With a diameter of 2 to 5 µm the spores can easily pass the mucous membranes, reach the bronchi and cause asthma. In some cases, permanent damages to the respiratory organs were observed. Typical disease patterns are: conjunctivitis, rhinitis or bronchitis.
2. *Toxic effects:* Metabolic products and toxins (mycotoxins) as well as cell wall components from fungi (mycelium) can cause local inflammations and toxic effects. Toxin-related disease patterns are eye irritations and cold-symptoms. Some typical toxin-producing fungi are: *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Cladosporium* sp. and *Stachybotrys* sp.

3. *Infectious effects:* Fungi are opportunistic organisms and can rarely be infectious. Especially on people with chronically respiratory diseases, immune-compromised or immune-deficient people - AIDS or cancer patients as well as after organ transplantations - fungal infections can also be life-threatening and lead to death.
4. *Aesthetic- and odor nuisance:* Molds produce and release characteristic volatile compounds to the environment - microbial volatile organic compounds (MVOC). In higher concentrations these chemical substances (alcohols, ketones, alkanes, aldehyde, aromatic compounds, etc.) as well as fungal growth itself represent an aesthetic- and an odor nuisance for the residents.

For these reasons the terms “Sick building Syndrome” (SBS) and “Building related illness” (BRI) have been more and more used in the recent years. The World Health Organization has confirmed the “sick building syndrome” – SBS (Sykes, 1988) as a noticeable disease (Akimenko et al., 1986). SBS and BRI are complex combinations of different non-specific symptoms and disorders, like eye irritations, mucosal irritations, accumulating infections of the upper respiratory tract, attentiveness disorder, fatigue and others. These symptoms occur after residence in a certain building, working place or specific rooms without the discovery of any other possible cause. Beside fungal growth, including the release of spores and MVOC, numerous other harmful agents are possible; e.g. pollen, viruses, acarians, chemical compounds (carbon monoxide, nitrogen oxides, ozone, etc.), electromagnetic fields, and emissions and varnish vapors from building materials and furniture.

All these properties, the biodeteriorative effects, the human health risks together with the common occurrence of fungi call for more emphasis in building biological investigations and also for better integration of scientific research in order to gain more insight and to understand the mechanisms of microbial growth, community structures and identities of the fungal species on and in our living environment.

1.3 Salt efflorescences, microbes and the rosy discoloration phenomenon

The composition and diversity of the microbial communities on building materials and works of art are depending on the nature and chemical composition of the materials itself and the influencing climatic conditions. As a consequence, these factors also determine the different appearances of biodeterioration that can be found on the materials. A very special biodeterioration phenomenon is represented by the rosy discolouration of monuments, building materials, stone, wall paintings, etc. The affected and hitherto investigated buildings are located in different geographical sites of central and south Europe and beside diverse

climatic conditions and UV irradiations; all these buildings suffer from water infiltrations due to constructional problems. The co-migration of salts with the infiltrating water through the walls can further lead to the formation of salt deposits that represent optimal growth conditions for pigmented salt-loving microorganisms.

1.3.1 Salt efflorescences

Building materials, like stone, brick, concrete, slurries, stuccos, mortars, plasters, frescoes, coatings, paintings, and many other materials provide a large variety of ecological niches for microbial growth. In practically all porous building materials that are subjected to different types of water inputs a variety of hygroscopic salts (such as chlorides, carbonates, sulphates and nitrates) can be found. These salts may be present in the material itself, are available from biological processes (like ammonium salts) or are the result from different water impacts on the materials The within the material dispersed or locally concentrated salts are solubilized and migrate with the capillary water in and out of the material. Changes in physical parameters (e.g. humidity decreases and temperature increases) lead to the drying out, precipitation and crystallization of the salts on the material surface. These salt deposits – commonly known as salt efflorescences (Amoroso and Fassina, 1983) - occur usually inhomogeneous on the materials surfaces. The great variations of the distribution of different salinities are depending on the water input and the resulting fluctuations in the water circulation within the material, desiccation- and wetting processes.

Many buildings are exposed to harsh and wet climatic conditions, due to their geographical location and/or their local site with high annual amounts of rain, snow and raising damp. The different types of indirect water inputs, through the migration of seepage water from the ground, or rain water from the outside of the building into the walls, and also direct water intakes through open windows, leaky roofs and walls, etc., can favor the formation of salt efflorescences. The water impacts and salt crystallizations cause destructive effects for the materials. Some salts may crystallize to different hydrates that occupy a larger space within the surface layers and exert high physical pressures on the material. These physical stresses can eventually lead to cracking, powdering and flanking of the surface structures and finally to detachment and material loss (see Figure 3; Piñar et al., 2009, 2013; Saiz-Jimenez and Laiz, 2000).

Figure 3. Salt efflorescences: A) detailed view on a salt formation and B) a salt-covered wall in the St. Virgil chapel (Vienna), C) detaching plaster material at the altar in the Saint Rupert chapel in Weißpriach (Austria), D) detailed view of salt efflorescences in the castle Rappottenstein (Austria), E) private garage with infiltrating water and salt formations, E) salt crusts in a private house (basement). All pictures were taken by Jörg Ettenauer.

1.3.2 Salt-loving microorganisms and the rosy discolouration

Salt efflorescences on material surfaces represent an extremely halophilic environment, ideal for the colonization with salt-tolerating and salt-loving microorganisms (Saiz-Jimenez and

Laiz, 2000). In the last century this group of microorganisms was often overlooked in microbial ecology studies on Cultural Heritage. No detailed search for these specialists was implemented because halophilic organisms were not isolated from the used culture media. Several reasons can explain this fact: 1) the application of unsuitable culture media with too low salt concentrations, 2) too short and insufficient incubation time, and 3) the non-culturability of these microbes under laboratory conditions (Giovannoni et al., 1990; Head et al., 1998; Hugenholtz et al., 1998; Rappé and Giovannoni, 2003).

Additionally to the decay of building materials through the formation of salt efflorescences, the growth of halotolerant and halophilic microbes leads to unaesthetic color changes due to the formation of colored biofilms (see Figure 4). Many halophilic microorganisms produce a variety of characteristic carotenoids, like β-carotene, α-bacterioruberin, monoanhydrobacterioruberin, and their derivatives (Oren, 2006, 2009). The synthesis of the carotenoid pigments is as means of protection of the cells: protection against photo-oxidative damage through high UV-irradiation and also as membrane stabilizers against different types of chemical-, salt- and/or desiccation stresses (Agnanostidis et al., 1992; Köcher and Müller, 2011; Oren 2009). These biogenic pigments lead to colony morphologies with colors ranging from light yellow to light pink, orange to rosy, red or brown (see Figures 4 and 5). The colorful pigments produced by the microorganisms are usually very stable on the material surfaces even if the causative microorganisms are already dead. Furthermore, the pigments can be incorporated into biominerals, such as oxalates, carbonates and gypsum-, iron-, and manganese oxides leading to the formation of stable biopatinas (Piñar and Sterflinger, 2009).

Figure 4. The rosy discolouration phenomenon: A) Entry arc and B) rosy stained inner west wall of the Johannes chapel in Pürgg (Austria), C) Inner courtyard and D) arcade of the castle Rappottenstein (Austria), E) doorway on the first floor of the castle, F) detailed view on rosy stained plaster material. All pictures were taken by Jörg Ettenauer.

Since years the rosy discoloration phenomenon of salty micro-niches on stone works, on historical buildings and mural paintings has drawn the attention of scientists. Several buildings located in different parts of Europe, like the St. Virgil chapel (Vienna), the church of Saint Anna im Feld (Germany), the Roman Necropolis of Carmona (Spain), the Vilar de

Frades Church (Portugal), the Tomb of the Monkeys (Italy), the Capuchin catacombs of Palermo (Italy), the Crypt of the Original Sin (Italy) and others, were already investigated by using culture-dependent and -independent techniques (Gurtner et al., 2000; Imperi et al., 2007; Piñar et al. 2013; Schabereiter-Gurtner et al., 2001c). Due to the deficiencies in culturing these halophilic microorganisms, molecular methods helped to gain more insight in the microbial ecology of these extremely salty habitats. This approach has strongly increased the knowledge about the diversity and complexity of the inhabiting microbes that are responsible for the unaesthetic rosy effect which is not only limited to salt-attacked historical buildings (Ettenauer et al., 2010; Incerti et al., 1997; Piñar et al., 2001b, 2009; Ripka et al., 2006; Rölleke et al., 1996). A great diversity of bacterial strains could be cultured from samples taken from salt efflorescences, for example members of the genera *Bacillus*, *Halomonas, Halobacillus, Acinetobacter*, *Nesterenkonia*, *Paucisalibacillus*, *Paenibacillus*, *Staphylococcus, Exiguobacterium, Oceanobacillus, Virgibacillus, Marinococcus, Lysinibacillus, Idiomarina, Chromohalobacter, Brevibacterium* and *Arthrobacter*. Additionally, by using molecular techniques for the identification of the bacterial inhabitants members of the genera *Rubrobacter, Idiomarina*, *Salinisphaera, Planococcus*, *Planobacterium*, *Kocuria*, *Paracoccus*, *Janibacter*, *Natribacillus* and *Saccharopolyspora* could be detected (Ettenauer et al., 2010, 2014; Jurado et al., 2012; Laiz et al., 2009; Piñar et al. 2001b, Ripka et al., 2006; Schabereiter-Gurtner et al., 2001c).

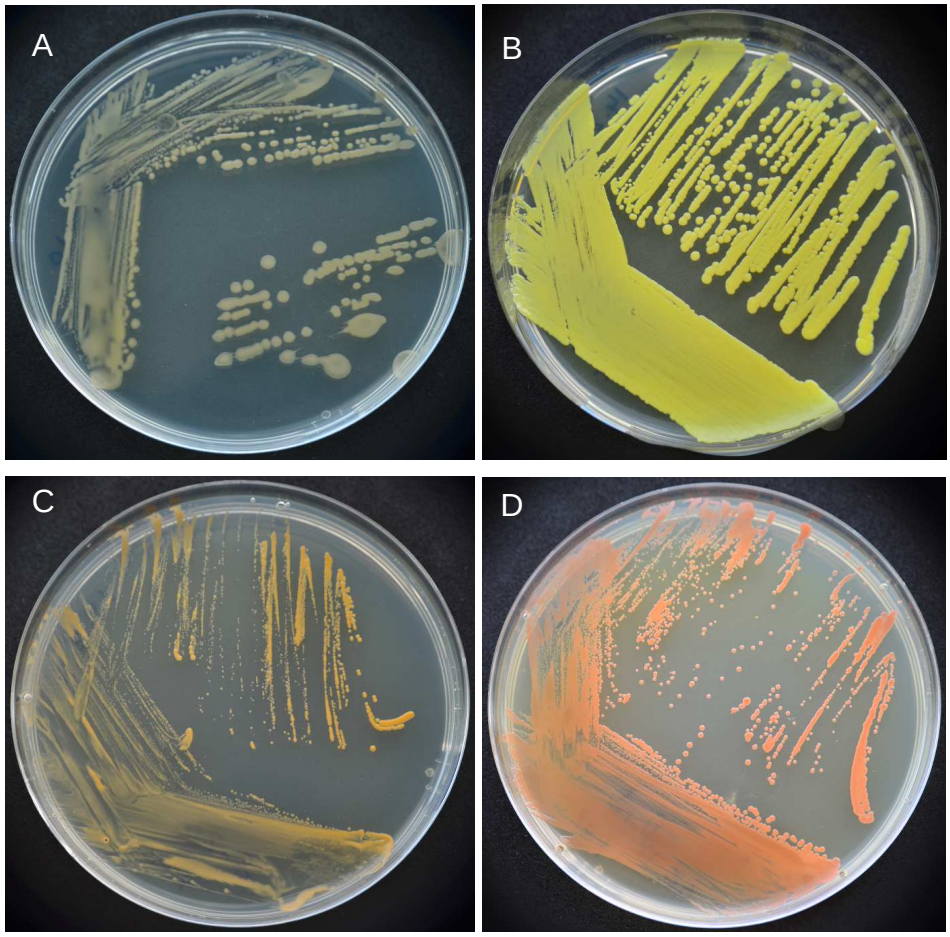

Figure 5. Pigmented halophilic bacteria isolated by Pinar et al. (2013) from cultivation media containing 3-20% NaCl. The colorful pigmentation of the strains ranged from light-yellow A), bright yellow B), orange C) to pink D). All pictures were taken by Jörg Ettenauer.

Furthermore, the application of molecular techniques allowed the detection of members of the domain *Archaea* (Ettenauer et al., 2010; Pinar et al., 2001a; Rölleke et al., 1998). Haloarchaea were previously only considered as inhabitants of other saline environments than salt-attacked building materials and works of art, like salterns, salt lakes, evaporating ponds of seawater or subsurface salt formations (Litchfield and Gillevet, 2002; Oren, 2002a, 2002b; Rothschild and Mancinelli, 2001; Sass et al., 2001; Vreeland et al., 1998). Members of the archaea were often overlooked, because only eubacteria specific primers were used for molecular analysis as well as unsuitable cultivation conditions were applied in cultivation experiments. Therefore, the findings of Haloarchaea in historical buildings and wall paintings represented a great surprise, also because these organisms require high salinities for their survival and can grow at salt concentrations up to 36% (w/v). Similar to the halophilic bacteria the salt-loving archaea have a colourful appearance and produce pigments as protective means. Typical pigmented representatives are members of the genera

Halococcus, *Halobacterium*, *Halalkalicoccus* and *Natronorubrum* (Ettenauer et al., 2010, 2014; Piñar et al., 2001a, 2001c; Rölleke et al., 1998).

2 Aims of the thesis

Microorganisms – including fungi, bacteria and archaea – represent an important agent in the natural decay of all types of building materials and works of art. Nowadays, the standard procedure for building biological investigations – for the isolation and further characterization of the inhabiting microbes – implies classical cultivation techniques. Beside many advantages of this traditional methodology, some of its drawbacks can be overcome by the application of modern molecular techniques. These methods are widely used in environmental microbiology studies to detect and identify the fungal communities on a great variety of sample materials.

For that reason, the objective of the first project shown in this doctoral thesis was the development and the optimization of molecular biological methods for building biological investigations. We tried to detect fungal growth on five ecological indoor insulation materials via the DNA of the organisms. The great advantage of this culture-independent approach is that cultivable as well as non-cultivable fungi can be detected. Furthermore, the molecular approach is far less time intensive then the so far used culture-based methods in this field. The required sample amounts are much smaller and allow a nearly non-destructive sampling from the building materials. Last but not least, the drawbacks of the traditional cultivation analysis, as certain detection limits and the selectivity by using only standard cultivation media can be overcome with the DNA-based methodology. Bio-susceptibility of the five insulation materials was studied under laboratory conditions – using test items of each material, as well as under “real life” conditions – after installation in a historical house for 2 years. The obtained colony forming units (CFU), the amount of extracted DNA, and the copynumber of the actin gene assessed by quantitative real time analysis were the decisive parameter for the evaluation.

Additionally, we performed a precedent evaluation of up to 13 direct *in-situ* DNA extraction protocols. The isolation of microbial DNA from a certain material is the first crucial step in a successful and complete investigation of the inhabiting microbiota. In order to find the most appropriate nucleic acid isolation method, we evaluated three commercial DNA extraction kits as well as four standard isolation protocols on three typically used building materials. Different modifications were introduced in every method to increase the extraction efficiency. The method showing the overall best results for the chosen parameters - DNA amount, -quality, visualization on agarose gels, PCR-amplification with four primer sets and DGGE analysis – should be further applied for the ecological insulation systems. Furthermore, this optimized DNA extraction and purification method would be a great benefit toward the

establishment of molecular techniques in building microbiology. A universally adaptable protocol that can be applied for all commonly used construction materials would be the first step in the development of automated and standardized procedures in building biological investigations that would further allow the comparison of results from different laboratories.

In the second project we studied the microbial communities responsible for the rosy discoloration of three historical buildings in Austria. Previous studies on other monuments located in different geographical places of Europe could already observe some similarities in the microbial communities but the origin and nature of the microbiota is still not completely understood. The goal of this work was the isolation and identification of the colored microorganisms – including bacteria and archaea - present in the red to pink pigmented sites in order to complete the picture of this biodeteriorative process. Therefore, culture-dependent and –independent techniques were applied for studying the microbiota. The obtained results were compared among the three buildings and further with those data obtained in previous investigations. In order to reproduce and prove the rosy effect as well as the deteriorative potential of the microorganisms, two colored isolates were inoculated on different building materials. Finally, the gained results from this study should help conservators to find meaningful counter-measurements and to protect the historical buildings from further biodeterioration and the unaesthetic rosy pigmentation.

3 Methods for detection and analysis of microorganisms

Plentiful of investigations were performed in order to study the phenomenon of biodeterioration and its causes (Benzzi et al., 2008; De Felice et al., 2010; Kolo et al., 2007). Due to of the complexity of the chemical and physical changes induced by microorganisms, it is difficult to understand the mechanisms of biodeterioration of building materials and works of art, and to develop countermeasures against the decay. Research has provided a great number of different techniques that can be applied for studying biodeterioration, its phenomena, the complexity and diversity of the inhabiting microorganisms ranging from the identification of specific members to whole microbial communities studies. Knowledge about the individual microbes colonizing on a certain habitat is not only important to understand the mechanisms of deterioration, but also to predict how these organisms respond to climatic and environmental changes and as a consequence allow researches and restorers to develop preventative actions to minimize the risk for undesirable growth of microorganisms on building materials and artworks (Dakal and Arora, 2012).

In the following sections a general introduction of different methods for the detection and identification of microorganisms is given. Special focus is drawn to the techniques that were implemented in this doctoral thesis.

3.1 Sample collection

A microbial ecology study starts with the correct application of an appropriate sampling technique. Furthermore, accurate sampling spots and –sizes have to be chosen in order to get representative results of the conditions at a place at a certain point of time or of a specific damage. Each sample has to be labeled in a traceable way and a short description of the sampling location with photographic images including a scale is recommended. The sampling amounts vary depending on the material and its value, the subsequent analysis and the kind of damage that should be investigated.

Generally, aseptic working procedures with sterile instruments, refrigerated and sterile storage (plastic bags or storage containers) as well as an immediate transport to the laboratory for further sample processing are essential basic prerequisites for a successful analysis.

Different sampling procedures are commonly used: 1) Scraping off sample material using a sterile scalpel, needle, glass-fiber brush, 2) different types of swabs, 3) adhesive tapes and

contact plates that are pressed against the surface of the material, 4) forceps for taking up detached material, etc. (BDA, 2012).

Furthermore, if greater amounts of sampling material are available, it is generally recommended to combine the material, carefully homogenize it and further use small subsamples from this homogenate to perform microbial community analysis (Ettenauer et al., 2012; Litchfield et al., 1975; Terry et al., 1981).

3.2 Culture-based approaches

For a long time classical cultivation methods have been used for studying microbial biodeterioration on building materials and works of art. The identification and, if possible, the quantification of the inhabiting microorganisms, are essential for evaluating the danger of deterioration to the materials and also the associated risks for humans. A successful cultivation study is highly dependent on the choice of appropriate cultivation media for the organisms that can be detected. Numerous compositions of different media, both solid and liquid, have been established to specifically cultivate certain types of microorganisms or by using standard media a general overview of the microbiota can be obtained. Likewise the cultivation conditions (temperature, liquid or solid media, agitation, time, light, etc.) have influence on the success to isolate microbes. Culture-based methods have the great advantage that living organisms can be obtained. Physiological and metabolic studies can be further performed to understand the potential of the isolated microbes. Therefore, the development of new cultivation media and –conditions is still encouraged in order to benefit from the advantages of this approach and to extend microbial culture collections.

However, there are also many drawbacks associated with this methodology. Cultivation techniques do not necessarily provide a comprehensive overview of the composition and complexity of the investigated microbial communities. Only a very small proportion of the total inhabiting microbiota can be recovered using this approach. Several studies have shown that less than 1% of the bacterial- and about 10% of the fungal community can be obtained (Pace, 1996; Ward et al., 1990). On the one hand cultivation usually yields relatively low cell numbers, due to suboptimal culture media and culture conditions, the methodological limitations, and the presence of inhibitory and predatory microorganisms. On the other hand some groups of microorganisms (e.g. spore-forming bacteria) favor the chosen cultivation conditions and may be overestimated by plating techniques leading to variations in the experimental results (Laiz et al., 2003a). Additionally, a huge number of microorganisms remain uncultured due to the following reasons:

1) Obligate-symbiotic and/or parasitic microorganisms lacking their symbiotic partner or host, respectively, fail to grow under laboratory conditions.
2) So far unknown species that have not been isolated as well as the non-culturability of microbes due to the lack of appropriate methods.
3) Known species that demand very special cultivation conditions or organisms that have entered a non-culturable state.
4) Insufficient incubation time for certain microbes that need several weeks for growing.

The general application of unsuitable/standard culture media leads to a certain selectivity and detection limits of the assay. Cultivation methods also require large amounts of sampling material, which rise severe problems when samples from very valuable cultural objects are concerned. Revolutionary progresses have been made by the application of phylogenetic markers and molecular techniques for the investigation of microbial communities in environmental samples without running into problems associated with selective cultivation and isolation of organisms. However, several drawbacks of this methodological approach have been reported. Each physical, chemical and biological step in a molecular ecology study (e.g. sampling, DNA-extraction, PCR-amplification, cloning, DGGE analysis, etc.) represents a source of a pitfall that can further lead to a distorted description of the diversity in the analyzed sample.

As a result many researchers have used a polyphasic approach and combined culture-dependent and –independent techniques. This methodology helps to increase the knowledge on the microbial diversity and the culture characteristics of the isolated microbes in environmental samples. An overall more complete picture of the microbial communities can be obtained rather than using only one of those techniques (Ettenauer et al., 2010, 2014). The combination of the two methodologies generally delivers divergent results (Laiz et al., 2003b). While culture-dependent methods only show the small fraction of living microorganisms from the environmental sample that are able to grow at the chosen isolation conditions, the molecular approach gives an estimate of the nucleic acid sequences extracted and amplified without any additional information about the microorganisms physiological stage (Amann et al., 1995; Roszak and Colwell, 1987). Each methodological approach has its pitfalls that can lead to an under- or overestimation of certain groups of microorganisms (Piñar et al., 2009). Nevertheless, the phylogenetic information of so far uncultured microorganisms obtained by culture-independent methods can provide additional hints to design new culture media and apply specific incubation conditions for the isolation of the desired organisms (Piñar et al., 2001b). Figure 6 gives an overview of a polyphasic

microbial community study where culture-dependent and –independent techniques are combined.

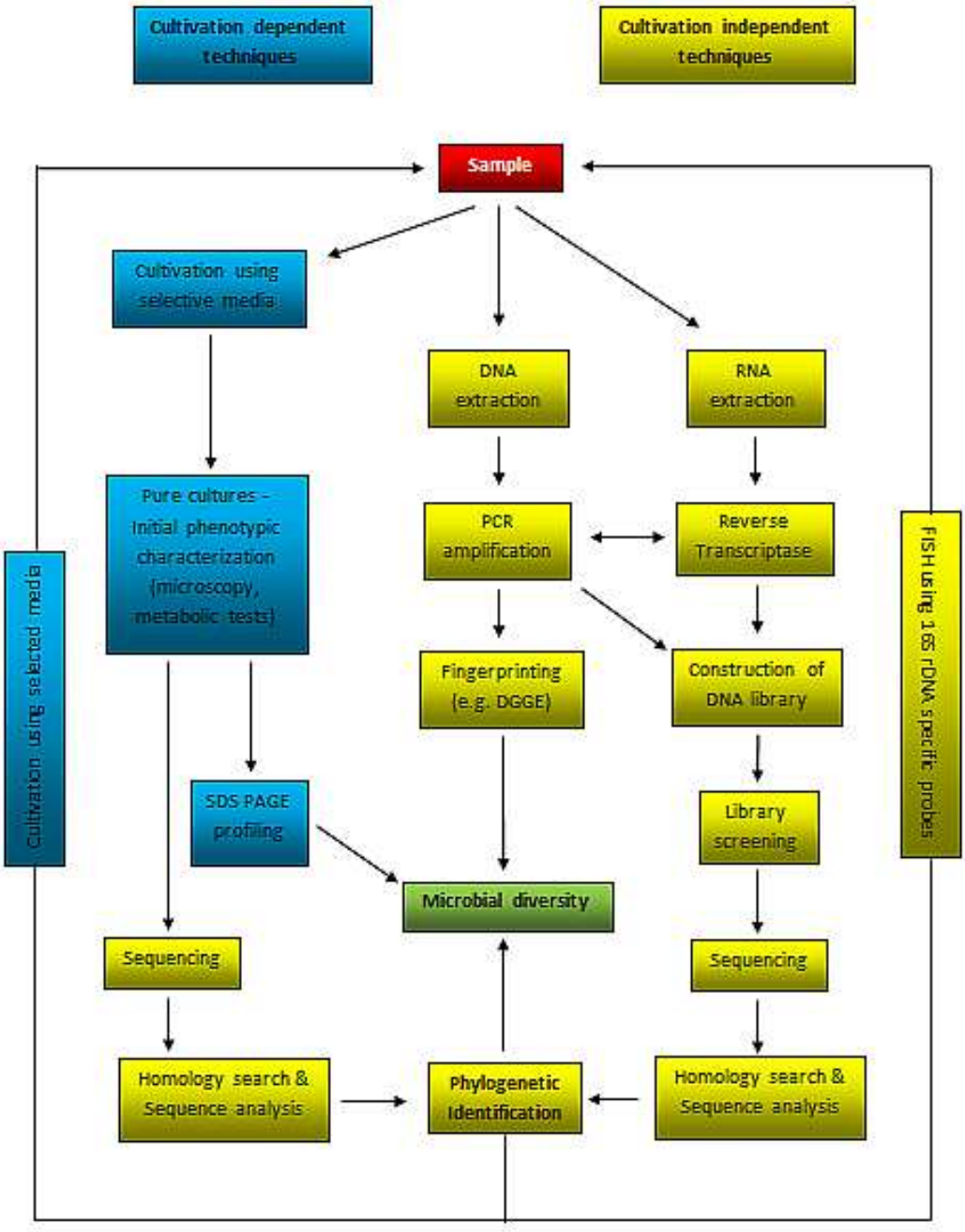

Figure 6. Polyphasic approach combining culture-dependent and –independent methods (modified from Ettenauer, 2010).

3.3 Molecular techniques – the culture-independent approach

Numerous experimental approaches using molecular techniques for culture-independent analysis have opened new doors for the identification, characterization and description of microorganisms, as well as for studying microbial community diversities and –complexities on building materials and works of art. Compared to culture-dependent analysis, these relatively rapid and reliable techniques deliver more comprehensive information on the actual proportion of the inhabiting organisms in an environmental sample (Laiz et al., 2003a, 2003b). Furthermore, the newly identified microbes give more insight into the complex causes and mechanisms of biodeterioration. The ongoing development of biotechnological tools, working procedures and instruments has discovered so far unknown microorganisms in a great variety of unusual habitats (Muyzer et al., 1993; Ortega-Moralez et al., 2004). Highly sensitive and specific methods allow the detection, visualization and identification of a great variety of microorganisms in even very small sample amounts; often less than 1 mg (Ettenauer et al., 2012, 2013; Gonzalez and Saiz-Jimenez, 2005; Gurtner et al., 2000; Sterflinger et al., 2013). An overview of the essential working steps of the molecular approach implemented in this doctoral thesis is given in Figure 7; whereas the detailed descriptions can be found in the Materials and Methods sections of each publication.

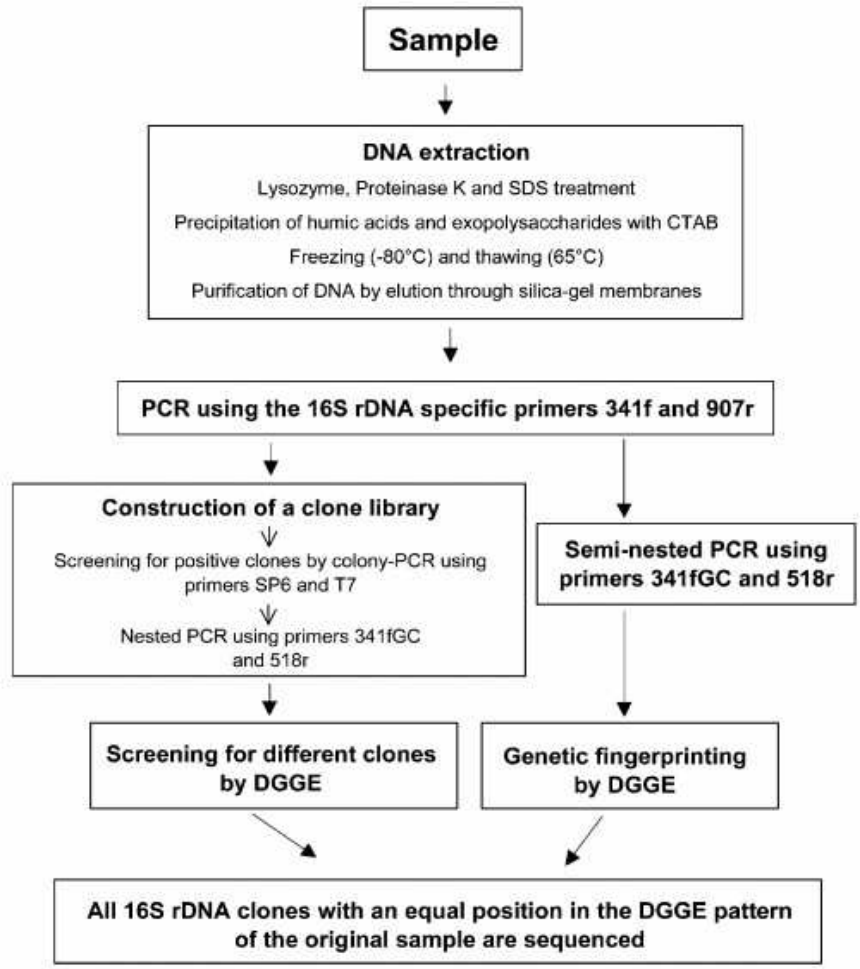

Figure 7. Schematic diagram giving an overview and showing the experimental procedure of the molecular approach implemented in this doctoral thesis. The general working steps from sampling, DNA extraction, PCR-amplification and community fingerprinting, as well as cloning, screening and finally the phylogenetic characterization of individual members, are shown (Schabereiter-Gurtner et al., 2001b).

3.3.1 Extraction of nucleic acids

Generally, the extraction of nucleic acids, either DNA or RNA, is the first crucial step in the molecular analysis of microorganisms from environmental samples. A great variety of protocols for all kind of materials has been established by researches as well as commercial extraction kits are available from biotechnology companies. Depending on the type of sampling material the protocol for the isolation of DNA or RNA, respectively, has to be adapted and modified in order to optimize and to enhance the extraction efficiency. A complete and successful study already starts with the choice of the appropriate nucleic acid isolation technique for the given sampling material (Martin-Laurent et al., 2001; Niemi et al., 2001; Stach et al., 2001). This important first step in the analysis of the inhabiting microbiota of complex environmental samples, like building materials, works of art, etc., requires

efficient unbiased extraction procedures which have to be performed very carefully. Most protocols for isolating nucleic acids from a certain sample include the following working steps (Ettenauer et al., 2012; Zhou et al., 1996):

1) Pre-washing of the sample with a phosphate buffer in order to reduce the amount of extracellular DNA and soluble organic contaminants. However, this optional working step is not included in direct *in-situ* extraction methods for several reasons: this step is time-consuming; the yielded DNA concentrations are lower; less bias are introduced and the risk for contaminations is minimized than in isolation protocols which in advance include the separation of the cells from the sample matrix.
2) An efficient lysis should affect all types of microbial cells, including spores as well as other structures with resistant cell walls. Different lysing procedures are commonly applied for this working step.
    a. For the chemical lysis of the cells CTAB, EDTA, SDS, high salt concentrations, β-Mercaptoethanol, etc. are included in the extraction buffers.
    b. Additionally, enzymatic lysis agents like Proteinase K or lysozyme can be incorporated.
    c. The mechanical break-up of the cells is usually done by grinding in liquid nitrogen, bead beating/ribolyzing with different types of beads (in various sizes and materials, like glass, ceramic, etc.), vortexing, sonication, microwave heating or through thermal shocking of the cells by repeated freeze and thawing cycles.
    d. A combination of mechanical, chemical and also enzymatic lysing procedures can significantly increase the efficiency of the cell breakage.
3) In the extraction step the upper, aqueous and DNA-containing layer is separated from the lower, organic layer with cell residues, proteins and other contaminants. The separation is usually done by the addition of phenol/chloroform/isoamyl alcohol.
4) Afterwards, the DNA is precipitated using either ethanol (96%), isopropanol or polyethylene glycol (PEG).
5) The subsequent step is the purification of the extracted DNA, which is a prerequisite for an efficient PCR amplification and many other molecular techniques. Cleaning of the nucleic acid can be performed through chromatography, agarose gel electrophoresis, cesium chloride or different commercial purification kits using spin filters or carrier RNA.

Modifications of the chemical composition, concentrations and volumes of the used extraction buffers, insertion and variation of cooling and incubation times and additional purification steps can lead to an improved nucleic acid extraction from a certain sample

material (Ettenauer et al., 2012). The quantity and quality of the isolated DNA or RNA, respectively, can be assessed by spectrophotometrical measurements (e.g. with a NanoDrop® ND-1000 Spectrophotometer; peqLab Biotechnologie GmbH, Linz, Austria) or by electrophoretical separation methods on agarose gels.

3.3.2 Molecular markers and PCR amplification

Molecular approaches based on the sequences of the small subunit ribosomal RNA genes (SSU: 16S in prokaryotes and the 18S in eukaryotes) have opened a new door for the identification of microorganisms in microbial phylogeny and -ecology studies (Amann et al., 1995). These SSU ribosomal genes are universally present in all organisms and due to the fundamental function in gene expression the sequence variations are very low. This results in highly conserved and also variable regions in the ribosomal sequences, which makes them suitable to be used as molecular markers (Woese, 1987). The Internal Transcribed Spacers region (ITS region) (Peterson, 1996), consisting of the ITS1, the 5.8S rDNA and ITS2, has become a reliable diagnostic marker for investigating the fungal diversity and for identifying fungal species. For all marker regions that allow the identification and discrimination between microbes on all phylogenetic levels, a great variety of different site specific oligonucleotides have been published to be used as primers for the Polymerase Chain Reaction (PCR).

In the PCR reaction the SSU ribosomal gene or ITS sequences, respectively, in the extracted DNA from the sampling material are amplified using two specific primers targeting those microorganisms. After 25-35 thermal cycles of DNA denaturation, primer annealing and extension of the newly synthesized DNA fragment, a large number of copies from the desired gene fragment can be obtained from originally even very small sample amounts (Schabereiter-Gurtner et al., 2001b). Today, the number of available primers is continuously increasing with the methodology of specific primer design (Daly et al., 2000). Not only members of the major phylogenetic classes - bacteria, archaea, fungi – can be detected in a sample, but also specific groups of microbes or single species can be targeted.

After the amplification of SSU ribosomal gene fragments from the extracted DNA, the amplified PCR products are further electrophorized on agarose gel to verify the success of the PCR reaction (positive control and samples, respectively), to check the correct size of the amplicons (PCR products) and the negative control (where no DNA template was added - to exclude the chance of contaminations).

3.3.3 Genotyping techniques - microbial community fingerprinting

The amplified DNA fragments can further be processed to obtain a microbial community fingerprint from the sample. The PCR products from a mixture of microorganisms show the same length but have different sequences and a differing electrophoretic mobility. Therefore, the microbiota can be visualized and differentiated by its electrophoretic profile. The so-called microbial community profile or fingerprint represents a banding pattern of nucleic acid fragments resolved by gel electrophoresis. The different migration of the PCR fragments from a certain sample is unique for the sample site and the time of sampling, and can be compared with fingerprints from other locations or at different points of time - allowing an evaluation and monitoring of sites (Gonzalez and Saiz-Jimenez, 2004).

A good fingerprinting technique should have a high discriminatory power that allows differentiating highly similar DNA fragments with even very little sequence variations. Some of the fingerprinting techniques that have been very successfully applied for the investigation of microbial communities in various samples are listed below.

1) **Denaturing Gradient Gel Electrophoresis (DGGE)**
2) Temperature Gradient Gel Electrophoresis (TGGE)
3) Terminal restriction fragment length polymorphisms (t-RFLP)
4) Amplified ribosomal DNA restriction analyses (ARDRA)
5) Ribosomal intergenic spacers analyses (RISA) – Automated RISA (ARISA)
6) Single strand conformation polymorphisms (SSCP)
7) Random amplification of polymorphic DNA (RAPD)

3.3.4 Denaturing Gradient Gel Electrophoresis – DGGE

Separation of PCR amplified fragments and visualization of the microbial community fingerprint from an environmental sample can be done by using DGGE. This profiling method allows the electrophoretic separation of PCR-generated DNA fragments with the same length but different sequences. The sequence differences with variations in the AT- and GC-content lead to different melting behaviors in a denaturing gel. As a result, in a polyacryamid gel containing a linear gradient of the DNA-denaturants urea and formamide, the mixed PCR fragments from a microbial community can be separated according to the characteristic sequence composition of each single species (see Figure 8; Muyzer et al., 1993; Muyzer and Smalla, 1998).

This simple principle has been successfully applied in many studies for different applications: for visualization of microbial community structures and screening of clone libraries to identify individual microbes, for monitoring of changes in the microorganisms diversity, for comparison of methods, etc. (Ettenauer et al., 2010, 2012; Miller et al., 2008; Piñar et al., 2001a, 2001b, 2001c, 2009; Portillo et al., 2008; Rölleke et al., 1996, 1998; Schabereiter-Gurtner et al., 2001a, 2001b). In order to perform DGGE analysis with environmental samples a small fragment within the primordial amplified PCR products from the ribosomal SSU genes or ITS regions, respectively, has to be re-amplified in a nested PCR. One of the primers contains a GC-clamp of about 40 bases at its 5' end, which helps to stabilize the melting behavior of the nested PCR fragments during the DGGE analysis (Muyzer et al., 1993). After completion of the electrophoresis (for 3.5 h at 60°C and 200 V) the biodiversity in the sample can be visualized by staining the DNA.

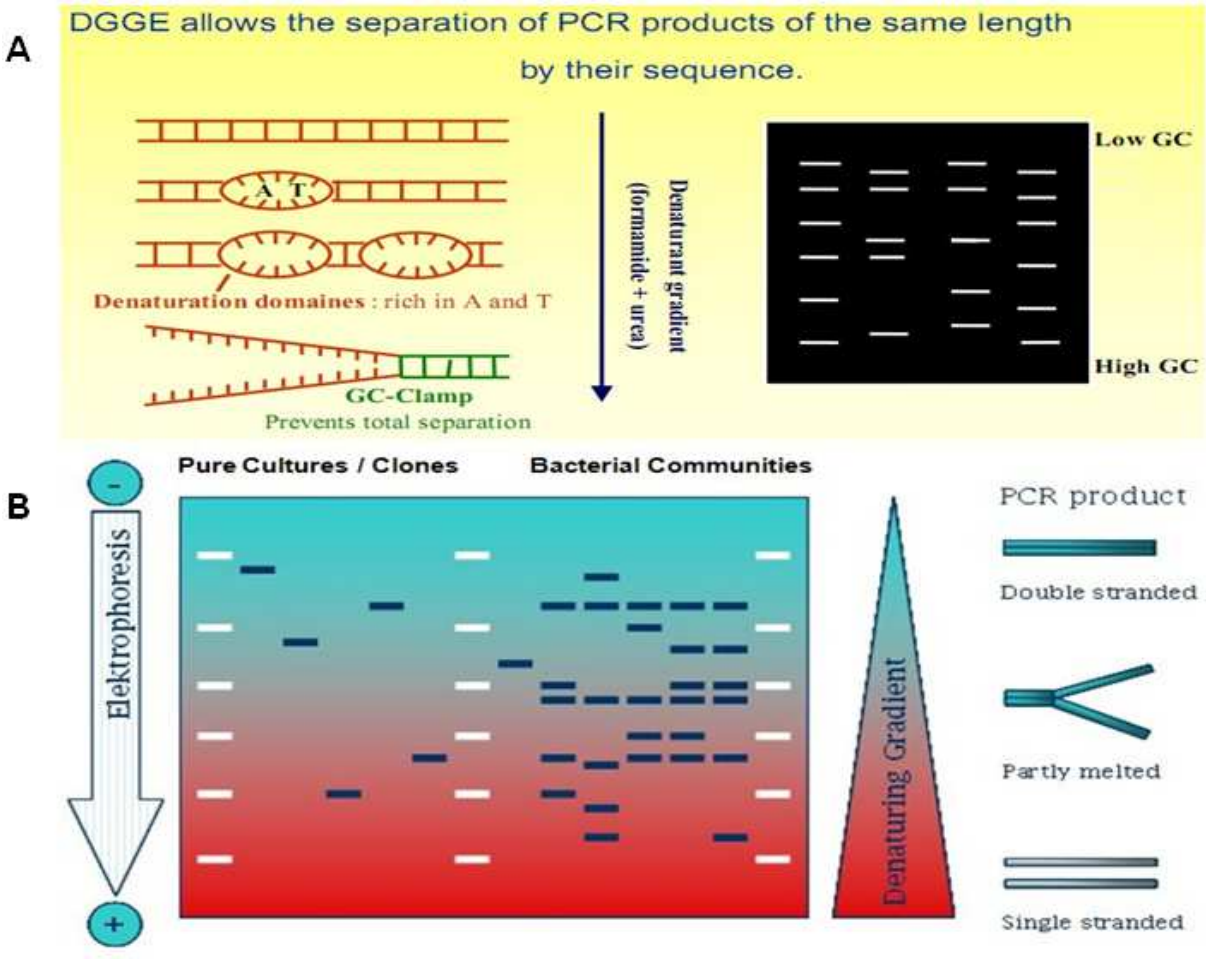

Figure 8. Schematic overview of Denaturing Gradient Gel Electrophoresis (DGGE). A) and B) Urea and formamide in a polyacrylamide gel denaturate the double stranded amplicons according to their nucleotide sequence composition (AT- and GC content). The 5' GC-clamp stabilizes the melting PCR fragments. DNA sequences with a low GC content show a very short migration in the gel, whereas 16S rDNA fragments with a higher degree of GC basepairs have a greater motility (Ripka, 2005; Ettenauer, 2010).

3.3.5 Creation of clone libraries, screening and sequence analysis

The individual members of the visualized microbiota in the DGGE fingerprint have to be further phylogenetically identified. This can be done by cutting out the single bands from the arcylamide gel and sequence analyses. The obtained nucleotide sequence is further compared with those deposited in online databases to gain phylogenetic information about the identity. However, this approach often does not allow a reliable characterization due to the co-migration of other sequences that have a similar melting behavior and the same position in the gel, and also due to the short sequence length of the excised band (Schabereiter-Gurtner et al., 2001a, 2001b, 2001c).

A more precise but relatively time-consuming methodology is presented by the construction of clone libraries. Therefore, the amplified marker regions (either ribosomal SSU genes or ITS-fragments, respectively) are purified and further ligated into a chosen vector system. Transformation of the vectors into competent cells results in transformed bacterial cells, each harbouring as an insert an individual marker region from the original sample. Re-amplification of the insert and screening of the sequence by DGGE allows the comparison with the original fingerprint of the sample. By this way, clones that match dominant or faint bands in the sample profile can be selected for sequencing and further compared with databases to be identified. By using this approach, the length of the cloned marker region is long enough to allow a reliable and unambiguous phylogenetic identification. The search programs FASTA and BLAST (Altschul et al., 1997; Pearson, 1994) can be used to compare the cloned sequence with online databases, like NCBI (National Centre for Biotechnology Information), EMBL (European Molecular Biology Laboratory), RDP (Ribosomal Database Project) or the database of type strains EZtaxon (Chun et al., 2007) with validly published prokaryotic names.

3.3.6 Quantitative real-time PCR

The amplification and detection of nucleic acid are among the most valuable techniques used in modern biological research. Scientists from all different fields apply these methods for a great variety of applications. Qualitative analysis (conventional PCR) as well as quantitative analysis (real-time PCR) can be performed by using this technology. Similar to the conventional Polymerase Chain Reaction, the real-time PCR, also known as qPCR, is based on the amplification of specific DNA-regions targeted by oligonucleotide primers. In qPCR the

amplicons are detected by including a fluorescent molecule (DNA-binding dyes, fluorescently labeled sequence-specific primers or probes) that reports the increase of the amplified DNA amount with a proportional increase in the emitted fluorescence signal. The fluorescence is monitored by fluorescence modules on the thermal cyclers and represents the amount of PCR products in each cycle. By using qPCR the starting template copy number can be determined through comparison with defined standards with known copy number concentrations. Similar to the conventional PCR, qualitative- but also quantitative statements can be made. The great advantages of qPCR are: 1) its quantitative properties; 2) the high sensitivity, specificity and accuracy; 3) the rapid and easy performance; and 4) since it is based on DNA, there is no need for cultivation of microorganisms.

For these reasons, qPCR has also been applied for quantitative assessments of fungi and bacteria in different sampling materials, like house dust and air samples, building materials, wall paintings, black stains in the Lascaux Cave in France, old parchments, and other environments (Ettenauer et al., 2014; Haugland et al., 1999, 2004; Imperi et al., 2007; Kaarakainen et al., 2009; Martin-Sanchez et al., 2013; Pietarinen et al., 2008; Smith and Osborn, 2009; Vesper et al., 2008; Zhang and Fang, 2006).

3.3.7 Newly emerging techniques

The above described general methodology for microbial community analysis comprising of DNA extraction, PCR amplification using universal primers, fingerprinting by DGGE, construction and screening of clone libraries and the final phylogenetic comparison and identification from selected clone sequences with various databases has been used for several decades by researches and has highly increased the knowledge about the occurrence, distribution and diversity of microbial life in different habitats. Although this molecular approach is still very helpful in identifying the causes and mechanisms of biodeterioration, it is also tedious, relatively time-consuming, cost-intensive, and it is no longer up to date.

The use of molecular methods for studies with samples derived from building materials and works of art has usually followed the technological inventions made in other scientific fields (e.g. medical research) or were imported and adapted from other sciences. The ongoing technological progress coupled with the continuously increasing scientific knowledge has led to the era of whole genome analysis on the DNA-, RNA- as well as on the protein level. By using metagenomics-, transcriptomics- and proteomics technologies a complete overview of the present microorganisms, their activity and the expressed proteins can be obtained. The

new state of the art methodologies for analysis of microorganisms in environmental samples are high throughput next generation sequencing technologies combined with bioinformatics tools (Ansorge, 2009; Ronaghi, 2001).

Similar to the explained methodology this technique involves DNA extraction, amplification using PCR followed by sequencing. Cloning steps are not needed for next generation sequencing and, therefore, some of biases (cloning related artifacts, selective cloning) can be avoided. Depending on the chosen method, one million up to one billion sequences can be analyzed in one run. With the newest instruments on the market the sequence length from previously only about 100 bp was elongated to nearly 1000 nucleotides. Additionally, by using primer bar coding it has become possible to analyze various samples at the same time. However, the huge amount of collected data also has to be sorted, analyzed and processed by using highly sophisticated bioinformatics tools. This state of the art methodology has already been applied by Edwards et al. (2006) for investigating the deep mine microbes in metagenomic studies. Amend et al. (2010) used next generation sequencing for studying the fungi in house dust from 72 sampling sites dispersed all over the world.

The drawback of this advanced technology is the requirement of very special, expensive instruments as well as highly trained personnel, which, to date makes next generation sequencing only affordable for a few laboratories on the world. Nevertheless, the ongoing research and technological development progress will possibly allow each laboratory to apply this technique for microbial ecology studies.

4 Results and Conclusions

Thermal insulation of houses has become self-evident in colder climate zones in order to reduce the costs and the amount of energy for heating. On modern and newly built houses exterior insulation systems are most frequently installed. Nowadays, the most popular exterior insulation system is Styrofoam. However, a high percentage of our living houses are historical buildings under preservation order that are now also included in country specific ordinances to improve the “energy efficiency”. Due to monument protection regulations an exterior insulation system is not allowed for historical buildings, and, therefore, alternative indoor insulation techniques have to be applied. In the last years, historical, organically and ecological materials such as bloated Perlite, cellulose, loam, reed, weed, straw, wood, etc. evolved on the market, but all of these “natural” materials bear the risk of a possible microbial contamination through bacteria and fungi.

In the first project of this doctoral thesis together with the Technical University of Graz five ecological interior insulation systems were tested for their affinity to microbial growth – especially their biosusceptibility to fungi. The goal was to show that molecular techniques can be successfully applied for building biological investigations (Ettenauer et al., 2012, 2013, 2014): Nowadays, building biological investigations commonly use classical cultivation techniques for the detection and identification of fungi. The associated drawbacks of this methodology can be overcome by molecular approaches that allow a more detailed view on the actual inhabiting fungal microbiota. Beside the possibility to identify the microorganisms, molecular techniques easily allow a monitoring of the microbial communities of certain environments and of indoor contaminations. Such a monitoring is especially important for the development and testing of different applications as preventative measures, cleanings or antimicrobial treatments like biocide actions and for evaluating the health risks for humans in indoor environments. Therefore, the molecular approach represents a much faster and more sensitive instrument for building biological investigations and assessments of material deterioration. Qualitative and especially quantitative measurements by using real time PCR allow a very precise view on the ongoing microbial dynamics in our living environments. Not only the whole microbiota can be investigated by using DNA-based approaches but also the living and actively growing fraction of the microbial community (by RNA analysis) can be detected.

The most decisive step in each molecular study is the extraction of nucleic acids from the sample material. Prior to the evaluation of the different insulation systems we wanted to find the most appropriate DNA isolation and purification protocol that can be universally applied

for the most commonly used building materials (Ettenauer et al., 2012). Mistakes and inefficient lysis of microbial cells during this first working step cannot be compensated in the further analysis and so an optimal isolation protocol has to be applied. Therefore, on three building materials (common plaster, red brick and gypsum cardboard) thirteen DNA extraction protocols were tested that comprise combinations of different mechanical, chemical and enzymatic lysing procedures. The results of this study showed great differences in the extraction methods: DNA could be isolated with all evaluated methods from all tested building materials. However, the DNA concentrations obtained from 1 g building material varied up to three orders of magnitude; independent of the used sample amount (50, 100 and 250 mg) and building material. Thus, these data evidenced the necessity to introduce standardized methods. The three commercial extraction kits yielded relatively low DNA concentrations, but in contrary the extracts of all standard extraction methods including an ethanol precipitation step had very high DNA yields. However, these protocols also had impurities that co-precipitated with the extracted nucleic acid and further led to negative results for the electrophoretic and PCR analysis. Additionally, our study showed that the ratio sample amount versus added buffer volume for the extraction is a very important factor for the obtained DNA yield: Normally one would expect to gain more DNA from larger sample amounts, but we could show that more often the smallest sample amount (50 mg) yielded the highest DNA amounts. The finely grounded sample powder absorbed the buffer and as a consequence the mixture did not represent a homogenous liquid suspension. Moreover, sample amounts of more than 100 mg overextended the capacities of some extraction protocols and the enclosed DNA in the material could not be extracted completely. The DGGE analysis of the bacterial and fungal fingerprints further showed that the smallest sample amounts represented the whole inhabiting community. Therefore, we recommend, homogenizing greater sample amounts of the investigated material and further using small subsamples for molecular analysis. In general, protocols including an additional purification step using columns, spin filters or commercial kits yielded drastically lower DNA amounts, whereas a great amount of contaminants could be eliminated that would further disturb molecular analysis.

Out of the thirteen evaluated methods, the FastDNA Spin kit for soil revealed the overall best results for all three building materials. The use of this kit enabled the processing of several samples at the same time and allowed the isolation of DNA from each tested material. The relatively low DNA yields showed due to the included purification step a very high quality compared to the other methods and further allowed a clear visualization on agarose gels. The ribolyzing step of this kit lead to an effective lysis of all target cells and, as a result, only this kit allowed the amplification with all used primer pairs. Furthermore, the DGGE

fingerprints represented the complete microbial diversity in the samples with no missing DNA bands. Finally, the risk for contaminations is minimized and it represents a relatively quick DNA extraction protocol that yields extracts applicable for all standard laboratory techniques. Therefore, we highly recommend using this DNA isolation and purification protocol as a standard method for microbial community studies of construction materials in order to allow inter-laboratory comparison of results.

In order to investigate the biosusceptibility of the five ecological thermal insulation systems classical culture dependent as well as modern molecular techniques were applied (Ettenauer et al., 2013, 2014). The materials were investigated under natural conditions - after installation in an historical building - and under laboratory conditions. Therefore, test items from all insulation materials were subjected to three commonly indoors occurring fungi and incubated in a climate chamber with high levels of relative humidity for half a year. Afterwards, small sample amounts were taken for cultivation and molecular analysis. The same procedure was performed with samples taken from the historical house 18 and 32 months after installation.

Fungal colonies growing on MEA plates derived from the insulation materials (test items as well as *in-situ* samples) were counted and the colony forming units (CFU values per gram material) were calculated. After 6 months of incubation under optimal growing conditions, fungal colonies were detected on all test items. The CFU values showed a great variation ranging from 10^2 to 10^6 cells per gram. The distinct strongest fungal growth was found on the wooden cardboard samples. On reed boards with loam, bloated Perlite boards and sprayed cellulose, germination numbers of about 2 orders of magnitude lower were calculated. The best results, with the lowest CFU values were found on the bloated Perlite plaster. From the *in-situ* samples only on the wooden soft-board and the reed board with loam a few fungal colonies germinated. The CFU values ranged from 10^3 to 10^4 cells per gram. No fungal growth was observed on the other materials. The control samples of all materials, taken before the inoculation with fungal spores did not show any fungal growth at all.

With the previous evaluation of isolation protocols, we were able to find the most appropriate method for the extraction of DNA from building materials. This protocol was further successfully applied for the isolation of DNA from the five tested indoor insulation systems. For the molecular analysis we developed a real time PCR method as a simple, rapid and reliable tool to detect and quantify fungi. The advantage of this assay relies in the quantification of the β-actin gene that occurs as a single gene copy per haploid genome in fungi. This fact allows a more precise quantification of the abundance of fungal cells in the

five insulation materials. The amount of newly synthesized target-DNA was measured continually and the emitted fluorescence from SYBR-green binding was detected in real time. The software allowed the comparison of the copy numbers in the samples with the known concentrations of standards. The values obtained from each insulation material were further extrapolated per ng of extracted DNA.

The results derived from qPCR analysis performed with the different interior insulation systems showed that fungal cells occurred in all samples. Compared to the classical cultivation methodology with qPCR it was possible to detect fungi in the control items. The detected copy numbers per ng of extracted DNA from the different test items ranged from approximately 6 to 5481. The highest fungal abundance was observed in the wooden soft-board samples, followed by the bloated Perlite board and the sprayed cellulose. The lowest β-actin gene numbers were found in the bloated Perlite plaster and the reed board with loam. Samples from the historical building showed copy numbers ranging from 1 to 92 per ng of DNA after 18 months, and from 4 to 317 copies/ng after 32 months. From the wooden soft-board samples, the highest β-actin gene numbers were retrieved. The detected copy numbers for nearly all *in-situ* samples were 1-2 orders of magnitude lower than the values obtained from the incubated test items. Only the reed board with loam from the historical building had slightly higher copy numbers, which could be explained by the higher relative humidity in the rooms. For the other materials, lower copy numbers – close or below the detection limit of the assay (31 copies/ng) – were measured. Generally, the obtained β-actin gene numbers were very low and close to, or even below, the limit of detection of the assay. Therefore, the fungal contaminations in the samples could be assumed very low. Furthermore, these results showed that the tested insulation materials did not represent optimal growth habitats for fungal colonization.

The results derived from the developed qPCR method (Ettenauer et al., 2014) were compared with those obtained in our previous investigations of the different insulation systems using classical cultivation analysis (Sterflinger et al., 2013; Ettenauer et al., 2013). Results derived from both methodologies showed that growing fungi were present in all inoculated test items, and further, that bloated Perlite plaster and wooden soft-board had the lowest and the highest fungal abundance, respectively. A statistical comparison of the CFU and qPCR metrics showed a significant correlation.

Taking together the results obtained from both strategies, we conclude that, from the microbiological point of view, the most appropriate interior insulation system was the bloated Perlite plaster. This material achieved the best results: the lowest fungal abundance was detected using the developed qPCR assay and only very few fungal colonies were cultivated from this material. On the contrary, the wooden soft-board system showed to be the most

unsuitable material for interior insulation, due to the highest fungal cell numbers detected on this material and the highest CFU values. Nevertheless, we think that nearly all tested materials (except the wooden soft-board) can be successfully installed in historical buildings for improving the thermal performance. As a prerequisite, we recommend an evaluation of possible constructional failures, water impacts, etc. to exclude the possibility of high humidity which could lead to fungal growth. Furthermore, residual mistakes have to be avoided that are also a major cause for fungal growth.

Additionally, the presented qPCR methodology is a fast, sensitive, direct (without the need of cultivation), and reliable assay for accurately quantifying fungi in different insulation materials. The approach described can be used to provide new information about fungal abundance in building biological investigations and of microbial habitats on works of art and cultural heritage. Compared to classical cultivation techniques only small sample volumes are necessary which allow a minimal invasive sampling procedure, that is of great importance in the case of objects of cultural heritage. Furthermore, the time effort for qPCR analysis is much lower and the drawbacks of cultivation assays, as selectivity and certain detection limits with the use of standard cultivation media, are avoided.

In the second project of the doctoral thesis culture-based and molecular methods were combined to get a complete overview of the microbial communities responsible for the rosy pigmentation on the walls and mural paintings in the three historical Austrian buildings. The classical cultivation techniques offer the possibility to isolate and visualize the microorganisms responsible for the phenomenon and to reproduce it in the laboratory. Nevertheless, it is well-known that by using only standard cultivation techniques with conventional laboratory media, only a small proportion of the total inhabiting bacterial population can be cultivated. Therefore, molecular techniques using PCR amplification of ribosomal 16S RNA genes, creation of clone libraries and the screening by DGGE and identification via sequence analysis, were additionally used in this project. This combination of different microbiological methods allowed the coverage of a wider spectrum of the microbial ecosystem present in the rosy discolored wall materials.

Cultivation analysis, performed by the Spanish working group of Prof. Dr. Cesareo Saiz-Jimenez from the University of Sevilla, allowed the isolation of twenty-nine bacterial strains with yellow to orange or pink appearance from Pürgg and Rappottenstein. No cultivable bacteria were found on samples from Weißpriach, as well as no archaea could be isolated from none of the samples. The bacterial strains could be grouped to cultured members of three different bacterial phyla, namely the *Firmicutes* (90% of all isolated strains), the *Proteobacteria* (7%) and the *Actinobacteria* (4%). For the molecular analysis of the bacterial

microbiota a total of 107 clones were selected based on the DGGE patterns. Clones were sequenced and grouped into members of the super-kingdom Bacteria without any further classification (28% of all selected clones) and representatives of the following three phyla: *Actinobacteria* (67%), *Firmicutes* (3%) and *Proteobacteria* (2%). The majority of the *Actinobacteria* were related to the subclass Rubrobacteridae (41%). From the archaeal fraction a total of 53 archaeal clones were chosen for sequence analysis. The results allowed a grouping into general members of the super-kingdom Archaea without any further classification (26% of all selected clones) and representatives of the phylum *Euryarchaeota*, namely to the order Halobacteriales (74%). Thereof, 38 were grouped to the family Halobacteriaceae (72%) and further to the genera Halococcus (40%), Halobacterium (17%) and Halalkalicoccus (13%).

Compared to the culture dependent approach, molecular analysis showed a higher biodiversity, with bacteria belonging to different genera. Generally, 58% of all 16S rDNA sequences were originally detected in historical buildings or paintings from which 63% corresponded to bacterial- and 47% to archaeal clone sequences. Also 55% of the bacterial isolates were previously found in historical buildings. The detected sequences were related to samples taken from the Saint Catherine chapel in castle Herberstein (Austria), the subterranean Saint Virgil chapel in Vienna, the Capuchin catacombs of Palermo (Italy), the Crypt of the Original Sin (Matera, Italy), the Roman tombs (Carmona, Spain), the Vilar de Frades Church (Barcelos, Portugal), the church of Saint Anna im Feld, Germany, the Roman Necropolis of Carmona, Spain, the Tomb of the Monkeys, Italy, an old mould-damaged building and different saline or soil environments. Bacterial strains that showed a yellow, orange or pink colour when cultivated on different media were selected for this study. Additionally, some of the microorganisms that were detected with molecular methods are also known for their pigmented colonies ranging from light yellow to light pink, orange, rosy to red or brown due to the production of the characteristic carotenoids bacterioruberin and monoanhydrobacterioruberin. All of those strains are moderately to extremely halophilic bacteria that can grow up to a maximum NaCl concentration of 7-23% (w/v). Similar to the bacterial clone sequences, the majority of the archaeal 16S rDNA sequences showed to be related to mural stonework or ancient paintings (47%). The identified archaeal sequences were previously detected either in the Saint Virgil chapel, the Saint Catherine chapel or the Capuchin catacombs, in saline environments, or have diverse origins (soil, groundwater, etc.). Some of the identified archaeal species also produce colourful pigments as means of protection against exposure to UV light and chemicals. These haloarchaea are able to grow on even higher salt concentrations than the detected halophilic bacteria – up to 30% NaCl (w/v).

Additionally, a laboratory-based colonization experiment was performed by the Spanish working group. Two isolated strains were inoculated on gypsum plaster and Hontoria limestone. Visual inspection of the stone samples revealed the development of rosy coatings over the surface of the probes, particularly on the gypsum plaster. Although less apparent, rosy discoloration was also observed on the limestone probes mainly within the pores. FESEM images of the surface of the inoculated stone samples displayed dense microbial mats spread all over the probe surfaces. Great amounts of coccoid cells were found on both types of materials. Additionally, the interaction between microorganisms and mineral substrata was studied by FESEM in order to investigate the real action of the isolated strains on the two stone probes and certain biodeterioration phenomena. The FESEM images showed that the development and activity of these microorganisms on both substrata were responsible for the rosy discoloration and might also cause dissolution features. The dissolution features observed on the inoculated probes were a clear evidence of the microbial activity present on these mineral substrata inducing biodeterioration. The data shows that the rosy discoloration phenomenon, in addition to an unaesthetic effect, produces also a biodeterioration through biogeochemical processes.

In this work we could show that the rosy biofilms on the walls of three different buildings harbour very similar halotolerant and halophilic bacterial and archaeal communities. Similar climatic conditions with relatively low UV irradiations and lowered annual temperatures, constructional problems with water infiltrations into the walls, the migration and further crystallisation of salts on the surface, lead to the formation of extreme saline environments that offer optimal growth conditions for halophilic microorganisms with brilliant rosy to purple colorations. The inhabiting members of the *Firmicutes* and *Actinobacteria*, mainly representatives of the subclass Rubrobacteridae, as well as Halobacteriales members are the main cause for the rosy coloured biofilms on the walls. These microorganisms were already detected in other historical buildings from different locations in Europe. Further investigations should address their goals in the design of special cultivation media to isolate the so far unidentified members of the *Rubrobacter* genus and the Halobacteriales order, which were also involved in this phenomenon. The intensity of the stains often is a serious aesthetical damage of wall paintings. Therefore, restorers often wish to carry out treatments to remove this microbiota from the surfaces. Since desalination is a general tool to decrease the salt crystallization, this method could also help to stop the growth of halophilic and halotolerant microorganisms. However, a desalination of the walls is only reasonable in combination with structural measures. Otherwise the salt-tolerant microorganisms would be replaced by more harmful and deteriorative microbes including fungi. If such measures are impossible, it should be taken into account to accept the coloured microbiota rather than

disturbing or changing the microbial community by treatments like desalination or application of biocides that, if not ineffective, can cause more fatal damage to the paintings.

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6 Publications and scientific manuscripts

6.1 Microbes on building materials - evaluation of DNA extraction protocols as common basis for molecular analysis

Jörg D. Ettenauer, Guadalupe Piñar, Ksenija Lopandic, Bernhard Spangl, Günther Ellersdorfer, Christian Voitl & Katja Sterflinger

Published in: Science of the Total Environment (Elsevier) 439: pp. 44-53.

6.2 Bio-susceptibility of thermal insulation systems used for historical buildings

Jörg D. Ettenauer, Guadalupe Piñar, Peter Kautsch & Katja Sterflinger

Published in: M. A. Rogerio-Candelera, M. Lazzari and E. Cano (eds.). Science and Technology for the Conservation of Cultural Heritage; 446; CRC Press Taylor & Francis Group; ISBN 9781138000094.

6.3 Quantification of fungal abundance on cultural heritage using real time PCR targeting the β-actin gene

Jörg D. Ettenauer, Guadalupe Piñar, Hakim Tafer & Katja Sterflinger

Published in: Frontiers in Microbiology – Section Microbiotechnology, Ecotoxicology and Bioremediation, 27. May 2014; doi: 10.3389/fmicb.2014.00262.

6.4 Halophilic microorganisms are responsible for the rosy discolouration of saline environments in three historical buildings with mural paintings

Jörg D. Ettenauer, Valme Jurado, Guadalupe Piñar, Ana Zélia Miller, Markus Santner, Cesareo Saiz-Jimenez & Katja Sterflinger

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Microbes on building materials — Evaluation of DNA extraction protocols as common basis for molecular analysis

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HIGHLIGHTS

- Up to thirteen extraction methods were evaluated with three building materials.
- Plaster, red brick and gypsum cardboard were chosen as representative materials.
- DNA yield, DNA purity and PCR amplifiability were the decisive parameters.
- The results from the DNA yield showed fluctuations of up to 3 orders of magnitude.
- The FastDNA Spin kit is the best DNA isolation method for building materials.

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ABSTRACT

The study of microbial life in building materials is an emerging topic concerning biodeterioration of materials as well as health risks in houses and at working places. Biodegradation and potential health implications associated with microbial growth in our residues claim for more precise methods for quantification and identification. To date, cultivation experiments are commonly used to gain insight into the microbial diversity. Nowadays, molecular techniques for the identification of microorganisms provide efficient methods that can be applied in this field. The efficiency of DNA extraction is decisive in order to perform a reliable and reproducible quantification of the microorganisms by qPCR or to characterize the structure of the microbial community. In this study we tested thirteen DNA extraction methods and evaluated their efficiency for identifying (1) the quantity of DNA, (2) the quality and purity of DNA and (3) the ability of the DNA to be amplified in a PCR reaction using three universal primer sets for the ITS region of fungi as well as one primer pair targeting the 16S rRNA of bacteria with three typical building materials — common plaster, red brick and gypsum cardboard. DNA concentration measurements showed strong variations among the tested methods and materials. Measurement of the DNA yield showed up to three orders of magnitude variation from the same samples, whereas A260/A280 ratios often prognosticated biases in the PCR amplifications. Visualization of the crude DNA extracts and the comparison of DGGE fingerprints showed additional drawbacks of some methods. The FastDNA Spin kit for soil showed to be the best DNA extraction method and could provide positive results for all tests with the three building materials. Therefore, we suggest this method as a gold standard for quantification of indoor fungi and bacteria in building materials.

1. Introduction

"Everything is everywhere, but the environment selects" is a statement that arose out of the microbiological work from Beijerinck and van de Knoniklijke (1913) and Baas Becking (1934) at the edge of the 19th to the 20th century. Microbial growth on building materials is a problem that has been known for a long time, but in the recent years it has drawn more attention. Floods, wet years, thermal modernization of residential buildings, air-conditioning systems, construction or material faults, and poor and improper ventilation are the major reasons for an increase of the relative air humidity and dampness of surfaces (Samson et al., 2010). These climatic conditions foster microbial growth in our living environment, on building materials and increase the risks for mold contaminations (Piñar and Sterflinger, 2009; Samson et al., 2010; Sterflinger, 2010, 2012). The properties and the common occurrence of bacteria and fungi contribute to the fact that these microorganisms

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represent the most frequent cause of biodeterioration of building materials (May et al., 1993; Piñar and Sterflinger, 2009). Biodegradation of buildings is caused by physical processes that affect the stability of the materials and also by chemical processes through biocorrosion such as oxidation, hydration reactions and dissolution and solubilization of material components. Degradation of construction material occurs through microbially produced substrate-specific enzymes or biogenic acids. Microbial excretion products can result in biopitting, and chemical changes in the material surface can moreover lead to the formation of crusts and the release of gases (Warscheid and Braams, 2000). Additionally microorganisms represent a serious health hazard for the residents (Gutarowska and Piotrowska, 2007; Samson et al., 1994). A worldwide phenomenon called sick building syndrome – SBS (Sykes, 1988) – has been confirmed as a recognizable disease by the World Health Organization (Akimenko et al., 1986). The sick building syndrome is a complex combination of nonspecific ailments associated with an individual's working place or residence that has become contaminated with any number of harmful agents. The causes for this syndrome are manifold but we focus in this study on the serious contaminants presented by microbial growth, especially fungal contamination — commonly referred to as mold (Crook and Burton, 2010). Microorganisms can affect human health in different ways: they can lead to allergic reactions due to allergens and spores released from fungi; can cause toxic effects by microbial toxins or cell components; can rarely have an infectious impact, especially on immune-suppressed people; and microorganisms can also represent an odor nuisance and esthetical annoyance. All these properties and effects of microbial growth call for the need to gain more insight into the microbial communities inhabiting the different construction materials.

Nowadays, the isolation and identification of microorganisms, especially of fungi, still stick to the use of traditional culture-based methods to estimate microbial contamination in buildings. These classical cultivation techniques allow a quantitative and qualitative assessment of the investigated environment and represent an important methodology in this field. Nevertheless, the dramatical changes in microbiology over the past 20 years have developed new technologies that can be applied for studying microbial communities. Typically, by using standard techniques with laboratory media, it is estimated that only a small minority of about 1% of the total bacterial and up to 10% of the fungal population observable in nature can be cultured (Amann et al., 1995). Therefore, molecular DNA and phylogenetic techniques have provided means that allow the identification of organisms without the need for cultivation (Hill et al., 2000; Hugenholtz and Pace, 1996; Hugenholtz et al., 1998; Pace et al., 1986). Fast and sensitive alternatives to classical cultivation techniques are polymerase chain reaction (PCR)-based techniques that offer an opportunity to analyze the full diversity of microbial communities. The first step for a successful and complete analysis of the inhabiting microbiota of a certain environment is the choice of an appropriate nucleic-acid isolation method (Martin-Laurent et al., 2001; Niemi et al., 2001; Stach et al., 2001). The efficiency of cell lysis, the quality and quantity of the extracted nucleic acid, its degree of purification and its size are crucial for the success of further molecular analysis. Building materials are, to a certain extent, comparable to soils and sediments because of their complex compositions. They represent systems containing various substances that co-extract with DNA, such as heavy metals, pigments, humic acids and chemical additives (Holben et al., 1988; Möhlenhoff et al., 2001; Rochelle et al., 1992; Wilson, 1997). The removal of these substances is one out of many critical steps in the extraction of nucleic acids that can lead to great biases during the PCR amplification. The heterogeneity of soils and sediments led to the establishment and publication of a huge number of different methods for the extraction of total microbial community DNA (Holben, 1994; Ogram et al., 1987; Picard et al., 1992; Sayler et al., 1992; Smalla et al., 1993; Steffan et al., 1988; Zhou et al., 1996).

To our knowledge, DNA extraction from construction materials was to date never the goal of any microbial ecology study. Therefore, in this study we evaluated up to thirteen direct-in situ DNA extraction methods. As basis, three commercial DNA extraction kits for soils and four standard DNA extraction protocols were chosen. These techniques incorporate a combination of mechanical, chemical and also enzymatic lysis of the cells. Additionally, modifications were introduced in some protocols. The extraction methods were applied to three different sample aliquots of three typically used building materials — common plaster, red brick and gypsum cardboard. Attention at the evaluation of these protocols was focused on three different parameters: 1) the quantity of the isolated DNA; 2) the quality of the extracted nucleic acid; and 3) the ability of the DNA to be amplified in different PCR reactions using one universal bacterial primer pair and three fungal ITS-primer sets. Additionally, the amplified PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) in order to compare the community fingerprints obtained from the different isolation methods. According to the results obtained from these criteria we identified the methods that worked better and continually eliminated others that did not work as well in order to find a standard DNA extraction method for molecular analysis which should be applied for construction materials.

2. Materials and methods

2.1. Sampling and sample preparation

In this study three commonly used building materials, plaster, red brick and gypsum cardboard, were investigated. Samples of the building materials were taken from a private house and the adjacent wine cellar in Baumgarten (Lower Austria). All samples showed a heavy fungal contamination. Sampling was done using a sterile scalpel or an ethanol-flamed hammer and a chisel to remove the material from the walls and collected it in sterile plastic bags. The transport and storage of the sampling material till further processing were done at room temperature.

In the laboratory, samples were ground for 2 min in liquid nitrogen using a sterile mortar and pestle, collected in a sterile 50 ml falcon tube and homogenized by manual shaking. Three different sample amounts of each material, 50 mg, 100 mg and 250 mg (each in triplicate), were weighed in a Sartorius precision scale for each extraction method. From that point on, to set an equal starting point for all aliquots of all extraction methods, the tubes were either immediately processed or stored at −20 °C. The resulting nine samples for each method were further subjected to the different DNA extraction methods as described below.

2.2. DNA extraction methods

In this study all evaluated methods are based on the direct in situ lysis of microbes in the material. Three different DNA extraction kits and four commonly used standard DNA extraction protocols were each applied and compared with each other. In order to maximize the yield, increase the purity and enhance the ability to amplify the extracted DNA, some modifications were introduced and evaluated with at least one of the three building materials. For the plaster samples all methods described below were applied. Out of these 13 methods the most promising 7 extraction protocols, marked with a bold number in Table 1, were further used for the other two building materials, red brick and gypsum cardboard. The extracted DNA from all methods was stored till further analysis at −20 °C.

1. The FastDNA® SPIN Kit for Soil from MP Biomedicals, (Illkrich, France) was applied according to the manufacturer's instructions. The kit combines a mechanical, using bead beating, and chemical lysis of the cells. After removal of the soil residues, cell debris and lysing matrix, the DNA is purified with a silica-based GENECLEAN® procedure using spin filters. After a washing step the DNA is finally eluted in DNase/pyrogen-free water.
2. The UltraClean® Soil DNA Isolation Kit from MO BIO Laboratories, Inc., (Carlsbad, CA) was used according to the manufacturer's

Table 1
Overview of the different extraction methods used for the three building materials. For details of the extraction protocols see the Materials and methods section. Methods indicated with bold numbers were applied for all building materials.

Method	Short description of the used protocols
1	FastDNA® SPIN Kit for Soil from MP Biomedicals: according to the manufacturer's instructions
2	UltraClean® Soil DNA Isolation Kit from MO BIO Laboratories, Inc.: according to the manufacturer's instructions
3	PowerSoil® DNA Isolation Kit from MO BIO Laboratories, Inc.: according to the manufacturer's instructions
4	Modified PowerSoil® DNA Isolation Kit protocol I as recommended by Mag. Günther Ellersdorfer and the kit manual.
5	Modified PowerSoil® DNA Isolation Kit protocol II as recommended by Mag. Günther Ellersdorfer and the kit manual.
6	Protocol of Ausubel et al. (1991).
7	Protocol of Claudia Schabereiter-Gurtner et al. (2001) with a purification step using the QIAamp Viral RNA Mini Kit with the same modifications as introduced by Schabereiter-Gurtner et al. (2001).
8	Modification I of protocol 7: without the QIAamp Viral RNA Mini Kit purification. Instead the DNA was precipitated in ethanol (96%).
9	Modification II of protocol 7: instead of the QIAamp Viral RNA Mini Kit purification, the DNA was purified using the QIAamp PCR Purification Kit.
10	Modified protocol of Sert and Sterflinger (2010).
11	Modification of protocol 10: protocol 10 was performed using the following buffer with an increased concentration of sodium chloride: extraction buffer II: 50 mM Tris–HCl, 250 mM NaCl, 50 mM EDTA and 0.3% SDS (w/v), pH 8.0.
12	Modified CTAB protocol (van den Ende and de Hoog, 1999).
13	Modification of protocol 12: protocol 12 was applied with a similar CTAB buffer II, that has the double Tris and a 10 fold higher EDTA concentration: 200 mM Tris–HCl, 200 mM EDTA, 8.2% NaCl (w/v), 2% CTAB, 0.8% β-mercaptoethanol (v/v), pH 8.0.

instructions. The general principal of the kit is to lyse the microorganisms by a combination of heat, detergent and also mechanical action against beads using a vortex. DNA released from the lysed cells is further bound to a silica membrane in a spin filter format. The filter is washed and the DNA is recovered in a supplied buffer. The kit contains a special inhibitor removal solution for the removal of humic acids and other disturbing substances.

3. The PowerSoil® DNA Isolation Kit from MO BIO Laboratories, Inc., was applied according to the manufacturer's instructions. The general principle resembles the UltraClean kit (method 2). A special solution is included which allows the removal of humic substances, respectively brown color from even the most difficult soil types.
4. Modified PowerSoil® DNA Isolation Kit protocol I: the sample material in the tubes with the supplied buffer was first heated up to 65 °C for 10 min without shaking. After a bead beating step for 45 s at a speed of 5 (meters per second) the protocol was followed according to the manufacturer's manual till point 7. Further at each step the whole supernatant was transferred and so not the fully recommended amount of the next kit solution could be added.
5. Modified PowerSoil® DNA Isolation Kit protocol II: To the sample material 60 μl of the C1 kit-solution, containing SDS and other disruption agents required for the complete cell lysis, were added and the tubes with the supplied buffer were incubated for 10 min at 65 °C with agitation (800 rpm). The further DNA extraction was done as described in method 4.
6. The protocol of Ausubel et al. (1991) was used as the sixth extraction method. It was originally designed for the extraction of DNA from pure bacterial cultures and was now tested for its ability to gain mixed genomic DNA from building materials.
7. The protocol of Schabereiter-Gurtner et al. (2001) was originally used to extract the DNA from objects of art and stone material. The extracted DNA was also purified using the QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) with the same modifications as introduced by Schabereiter-Gurtner et al. (2001).
8. Modification I of protocol 7: method 7 was applied without the QIAamp Viral RNA Mini Kit. Instead the DNA was precipitated overnight at 4 °C in 2 volumes (~300–700 μl) of ethanol (96%). The next day, the sample was centrifuged for 5 min at 13,200 rpm, the ethanol was discarded and the DNA pellet was dried at 40–50 °C. The DNA was re-suspended in 100 μl ultra-pure water (Sigma).
9. Modification II of protocol 7: another variation of method 7 was tested: Instead of the purification with the QIAamp Viral RNA Mini Kit the DNA was purified with the QIAamp PCR Purification Kit (QIAGEN GmbH, Hilden, Germany).
10. Modified protocol of Sert and Sterflinger (2010): this method which was originally used as a routine method for DNA extraction from pure fungal strains was also tested. To the weighted sample amount in a 1.5 ml bead beater tube, 0.4 g glass beads (0.75–1 mm, Carl Roth GmbH Co.KG, Karlsruhe, Germany) and 500 μl extraction buffer I [50 mM Tris–HCl, 150 mM NaCl, 50 mM EDTA and 0.3% SDS (w/v), pH 8.0] were added. After vortexing, the sample was processed two times in the Fast Prep FP120 Ribolyzer for 40 s at a speed of 6 (m/s). Between these ribolyzing steps the sample was incubated at 65 °C for 1 h at 800 rpm. After centrifugation for 10 min at 10,400 rpm (all centrifugation steps at room temperature), the supernatant was transferred into a new microfuge tube. Further DNA extraction was done with 1:1 vol (500 μl) chloroform–isoamyl alcohol (24:1 v/v; Roth). During vortexing a white interface formed and after centrifugation for 5 min at 13,200 rpm the aqueous supernatant was transferred into a new tube. This step was repeated using the same volume (1:1 vol) of phenol/chloroform/isoamyl alcohol (25:24:1, v/v; Roth). After a centrifugation step (5 min at 13,000 rpm), the supernatant was transferred into a new microfuge tube. Two volumes (~1000 μl) of ethanol (96%; −20 °C) were added and the DNA was precipitated overnight at 4 °C. The next day the sample was centrifuged for 5 min at 13,200 rpm, the ethanol was discarded and the pellet was dried at 40–50 °C. The DNA was re-suspended in 100 μl autoclaved ultra pure water from Sigma.
11. Modification of protocol 10: additionally the extraction protocol 10 was performed using the following buffer with an increased concentration of sodium chloride: extraction buffer II: 50 mM Tris–HCl, 250 mM NaCl, 50 mM EDTA and 0.3% SDS (w/v), pH 8.0.
12. Modified CTAB protocol (van den Ende and de Hoog, 1999). This method was also applied for the building materials. To the sample material 0.4 g glass beads (0.75–1 mm) and 500 μl CTAB buffer I [100 mM Tris–HCl, 20 mM EDTA, 8.2% NaCl (w/v), 2% CTAB, 0.8% β-mercaptoethanol (v/v), pH 8.0] were added. A short vortexing step was followed by bead beating in the Fast Prep FP 120 Ribolyzer for 45 s at a speed of 5 (m/s). Afterward the sample was heated on a thermoblock for 10 min at 65 °C without shaking. 500 μl chloroform–isoamyl alcohol (24:1 v/v) was added. The sample solution was vortexed for 2 s and further centrifuged for 5 min at 13,200 rpm at room temperature. The aqueous supernatant was transferred into a new microfuge tube, 2 volumes (~800–1000 μl) of ethanol (96%; −20 °C) were added and the DNA was precipitated overnight at 4 °C. After a centrifugation step (5 min at 13,200 rpm) the ethanol was discarded. The DNA pellet was washed with 500 μl ice-cold ethanol (96%) and dried on the thermoblock at ~50 °C. The DNA pellet was re-suspended in 100 μl ultra-pure water from Sigma.
13. Modification of protocol 12: method 12 was applied with a similar CTAB buffer II, that had the double Tris- and a 10 fold higher EDTA concentration: 200 mM Tris–HCl, 200 mM EDTA, 8.2% NaCl (w/v), 2% CTAB, and 0.8% β-mercaptoethanol (v/v), pH 8.0. This buffer, without β-mercaptoethanol, was already used by van den Ende and de Hoog (1999).

2.3. Estimation of DNA concentration and purity

The concentration and quality of the DNA preparations were estimated by a NanoDrop spectrophotometer and by electrophoresis on 1.5% agarose gels. Measurements were assessed using a NanoDrop® ND-1000 spectrophotometer (peqLab Biotechnologie GmbH, Linz, Austria).

The analyses were performed according to the manufacturers' protocol and the extracted DNA of all triplicate sample amounts from all methods was measured thrice. Mean values were calculated for each sample amount. The purity of the extracted DNA was assessed by measuring the A260/A280 absorbance ratio which indicates the presence of impurities as protein, phenol, humic acids or other contaminants that strongly absorb at or near 280 nm. From each triplicate of each sample amount, 20 μl of the extracted DNA was pooled and transferred into a new microfuge tube. Additional spectrophotometric concentration measurements were performed with the pooled samples (data not shown) that were further used for the electrophoresis and for the PCR amplifications. Eight microliters of the pooled extracted DNA was run on 1.5% (w/v) agarose gels at 110 V for 40 min, stained in an ethidium bromide solution [1 μg/ml; stock: 10 mg/ml] for 20 min and visualized with an UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

2.4. Polymerase chain reaction and electrophoresis

2.4.1. PCR amplification of bacterial 16S rRNA fragments

For all PCR reactions 2× PCR Master Mix from Promega [50 units/ml of TaqDNA polymerase supplied in an appropriate reaction buffer (pH 8.5), 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP, 3 mM $MgCl_2$] was diluted 1× and 12.5 pmol/μl of each primer (stock: 50 pmol/μl) was added. To amplify the eubacterial 16S rRNA fragments, a PCR amplifying the nucleotide positions 341–926 of the *Escherichia coli* 16S rRNA gene, was carried out with the forward primer 341f (Muyzer et al., 1993) and the reverse primer 907r (Teske et al., 1996). In a total volume of 25 μl, 400 μg/ml BSA (stock: 20 mg/ml; Roche, Diagnostics Gmbh, Germany) and 2.5 μl DNA template were added. The PCR was performed in a MJ Research PTC-200 Peltier thermal cycler with the thermocycling program described by Schabereiter-Gurtner et al. (2001). Eight microliters of each PCR product was run on a 2% (w/v) agarose gel as described above. For each Master Mix, positive (DNA of *Bacillus pumilus* added as template) and negative controls (no DNA added) were carried out to ensure the proper amplification of bacterial DNA in the PCR reaction and to exclude the possibility of false-positive results through cross-contaminations.

All PCR products were assessed by visualization of the DNA on agarose gels. A eubacterial PCR product was assigned positive if a DNA band corresponding to an approximately 585-bp long amplicon was detected.

2.4.2. PCR amplification of fungal ITS region fragments

As for the amplification of bacterial 16S rRNA the 2× PCR Master Mix from Promega and the same primer concentration (12.5 pmol/μl) were used. Three different primer combinations were selected to amplify the ITS1, ITS2 or both regions. Primer pairs ITS1f/ITS2r and ITS3f/ITS4r were chosen to amplify smaller fragments (~300 bp), corresponding to either the ITS1 or ITS2 region, which is usually used for DGGE analysis. Fragments of about 450–600 bp in size, corresponding to the ITS1 and the ITS2 region and the 5.8S rRNA gene between them, were amplified with the primers ITS1f and ITS4r (White et al., 1990). The 25 μl PCR reactions were carried out with 400 μg/ml BSA (stock: 20 mg/ml) and 2.5 μl DNA template. All PCR reactions were conducted with the following thermocycling program: 5 minute denaturation at 95 °C, followed by 35 cycles of 1 minute denaturation at 95 °C, 1 minute primer annealing at 55 °C and 1 minute extension at 72 °C. Five minutes at 72 °C was used as a final extension step. As described for the amplification of bacterial DNA, 8 μl of the PCR products was analyzed by agarose gel electrophoresis and each PCR reaction included a positive (DNA of *Alternaria alternata*) and a negative control.

2.5. DGGE analysis

For DGGE analysis a semi-nested PCR was performed in a total volume of 100 μl (2×50 μl reaction size), each with 3 μl of PCR products from the first amplification as template (from PCR performed with primers ITS1 and ITS4 for fungi). Genetic fingerprinting was done as described by Ettenauer et al. (2010). Briefly, a semi-nested PCR was performed with primers having a GC-clamp attached (Muyzer et al., 1993). Primers 341GC (Muyzer et al., 1993) and 518r (Neefs et al., 1990), as well as primers ITS1-GC and ITS2 (Michaelsen et al., 2006) were used for the bacterial and fungal DNA amplification, respectively. The same thermocycling program as described for the first round was used for the amplification of the 16S rDNA fragments. For the amplification of the fungal DNA the thermocycling program described by Michaelsen et al. (2006) was used. The fingerprinting of the bacterial community was performed in a linear chemical gradient ranging from 30 to 55% (100% denaturant contains 7 M urea and 40% v/v formamide). For the fungal DGGE analysis an acrylamide gradient from 25 to 50% was applied. DGGE was carried out as described by Muyzer et al. (1993), using a DCODE™-System (Bio-Rad) in 0.5×TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 8.0). The gels were run at a constant temperature of 60 °C, at 200 V for 3.5 h with the bacterial samples and for 6 h with the fungal DNA. The visual comparison of the different DGGE profiles from different sample amounts and extraction methods was done on the computer.

2.6. Statistical analysis

A 3-way ANOVA model was used to test for the material, amount and method effects, written in the form

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + e_{ijkl}$$

with the grand mean μ, and the main effects α_i, β_j, and γ_k, reflecting changes in the response variable due to material, amount, method and interactions $(\alpha\beta)_{ij}$, $(\alpha\gamma)_{ik}$, and $(\beta\gamma)_{jk}$, and e_{ijkl} as the error term. The model was calculated for measurement variables 'DNA yield' and 'DNA purity'.

For comparisons of significant differences Tukey's 'honest significant difference' post-hoc method (Miller, 1981) was applied.

p-Values smaller than 0.05 were regarded as statistically significant. All statistical analyses were carried out with R (R Development Core Team, 2011).

3. Results

The suitability of the extraction methods was evaluated based on the following parameters: 1) DNA quantity; 2) DNA purity; and 3) DNA amplifiability.

3.1. The plaster material

All 13 DNA extraction methods were conducted with the plaster material in order to make a pre-selection prior to analyzing the other two building materials. The results shown in Table 2 reveal that the amount of DNA extracted from the plaster material greatly varied between the investigated sample amounts. Surprisingly, from a 50 mg sample, the highest DNA yield was obtained (2.7×10^6 ng DNA per gram sample material with method 10), whereas, from 250 mg, the lowest DNA concentration was isolated (2.4×10^3 ng/g with methods 3 and 9). The three commercial DNA extraction kits (methods 1, 2 and 3) allowed a DNA recovery ranging from 2.4×10^3 to 2.3×10^4 ng/g. The modifications, heating and shaking with the buffer, introduced to the PowerSoil DNA isolation kit (methods 4 and 5) did not lead to distinct changes in the DNA yield, nor in the PCR amplification tests. Only the A260/A280 ratios were slightly decreased and in the 250 mg samples an appreciable increase of the DNA amount, compared to the manufacturers' manual (method 3) could be achieved. Therefore, we decided to continue working with the standard protocol of this kit (method 3). Method 6, the DNA extraction protocol of Ausubel et al. (1991), yielded moderate DNA

Table 2
Summary of the results obtained from the thirteen methods evaluated with the plaster samples. The given numbers for the DNA yield (ng/g) and DNA purity (A260/A280 ratio) are rounded mean values of triplicate measurements with the NanoDrop spectrophotometer and rounded values for the standard deviation. For the visualization of the DNA on agarose gels and the PCR products the table shows if all three sample amounts or only certain sample lots (50, 100 and 250 mg) were detectable after electrophoresis and ethidium bromide staining.

Plaster	Sample lot	1	2	3	4	5	6	7	8	9	10	11	12	13
DNA yield	50 mg	$1.9\times10^4\pm$	$2.3\times10^4\pm$	$9.1\times10^3\pm$	$7.9\times10^3\pm$	$7.2\times10^3\pm$	$1.5\times10^5\pm$	$1.3\times10^5\pm$	$1\times10^6\pm$	$1.1\times10^4\pm$	$2.7\times10^6\pm$	$2.5\times10^6\pm$	$3.5\times10^5\pm$	$2.4\times10^6\pm$
		3.9×10^3	2.5×10^3	2.1×10^3	2.5×10^3	2×10^3	4.7×10^4	6.7×10^3	9.1×10^4	1.1×10^3	2.7×10^5	7×10^5	1×10^5	5.4×10^5
	100 mg	$1.2\times10^4\pm$	$9.9\times10^3\pm$	$5.1\times10^3\pm$	$5.9\times10^3\pm$	$3.9\times10^3\pm$	$3\times10^5\pm$	$6.5\times10^4\pm$	$7.3\times10^5\pm$	$9\times10^3\pm$	$1.6\times10^6\pm$	$1.9\times10^6\pm$	$5.5\times10^5\pm$	$1.5\times10^6\pm$
		1.5×10^3	1.5×10^3	7.5×10^2	1.4×10^3	1.3×10^3	4.4×10^4	3.2×10^3	4.2×10^4	2×10^3	3.8×10^5	3.3×10^5	9.8×10^4	9.9×10^4
	250 mg	$7.5\times10^3\pm$	$3.8\times10^3\pm$	$2.4\times10^3\pm$	$3.6\times10^3\pm$	$3.7\times10^3\pm$	$4.6\times10^5\pm$	$2.4\times10^4\pm$	$3.3\times10^5\pm$	$2.4\times10^3\pm$	$1.2\times10^6\pm$	$8.5\times10^5\pm$	$4.3\times10^5\pm$	$9.8\times10^5\pm$
		2.8×10^2	4.5×10^2	2.6×10^2	3.2×10^2	1.1×10^3	5.7×10^4	7.2×10^2	1.3×10^4	1.2×10^3	5.9×10^4	1.2×10^5	3.4×10^4	1.1×10^5
DNA purity	50 mg	1.73 ± 0.4	1.83 ± 0.1	2.48 ± 0.72	1.4 ± 0.41	1.95 ± 0.8	0.77 ± 0.17	2.95 ± 0.2	1.05 ± 0.02	2.29 ± 0.43	1.24 ± 0.11	1.02 ± 0.07	1.32 ± 0.51	1.4 ± 0.06
	100 mg	1.78 ± 0.14	1.8 ± 0.27	2.14 ± 0.59	1.19 ± 0.19	2.25 ± 1.32	0.97 ± 0.12	2.97 ± 0.18	1.15 ± 0.02	1.82 ± 0.18	1.19 ± 0.08	1.09 ± 0.15	1.32 ± 0.54	1.42 ± 0.07
	250 mg	1.8 ± 0.1	1.76 ± 0.26	2.04 ± 0.87	1.52 ± 0.37	1.86 ± 0.29	1.06 ± 0.21	3.04 ± 0.16	1.18 ± 0.04	1.72 ± 0.45	1.36 ± 0.03	1.26 ± 0.05	1.31 ± 0.1	1.39 ± 0.04
Visualization PCR		All	None	All	All	250	None	None	All	None	None	None	None	None
341f/907r		All	All	All	All	All	50, 100	All	None	All	50, 100	50, 100	50, 100	250
ITS1/ITS4		All	All	All	All	All	50	All	None	All	50	50, 100	None	250
ITS1/ITS2		All	All	All	All	All	None	All	None	All	50	50	None	250
ITS3/ITS4		All	None	100, 250	250	250	None	50	None	All	None	None	None	None

concentrations (1.5×10^5–4.6×10^5 ng/g). The assessment of the DNA purity by the A260/A280 ratio (0.77 to 1.06) revealed impurities that led to biases in the amplification analysis where only bacterial 16S rDNA and ITS1/ITS4 fragments were amplifiable. The protocol of Schabereiter-Gurtner et al. (2001) was applied in three different versions (methods 7–9): 1) the original version with the additional QIAamp Viral RNA Mini Kit purification (method 7); 2) without any purification (method 8) and 3) using the QIAamp PCR Purification Kit (method 9). The resulting DNA content of the extracts from the three methods showed strong variations. Without any purification, the samples had the highest DNA amount (3.3×10^5–1×10^6 ng/g), whereas the eluents after the QIAamp Viral RNA Mini Kit only contained 2.4×10^4–1.3×10^5 ng/g of DNA and the yield further decreased when the QIAamp PCR Purification Kit was applied (2.4×10^3–1.1×10^4 ng/g). Methods 7 and 9, that included an additional purification of the DNA did not display any bands after visualization, but improved the quality of the DNA and further provided positive amplification results with all tested primer pairs. However, the extracts from the original protocol (method 7) showed A260/280 ratios of almost 3, whereas in samples from method 8 the ratios were around 1.15. The high DNA concentrations from method 8 allowed the visualization of DNA on agarose gels, whereas the impurities strongly inhibited all further amplification trails. Method 7, the original protocol of Schabereiter-Gurtner et al. (2001), was selected for further evaluation with the other materials, because of the good DNA yields and positive PCR results with all primers tested. Methods 10 and 11 are both modified versions of the protocol of Sert and Sterflinger (2010). Plaster samples treated with protocols 10 and 11 yielded on average the highest DNA concentrations of all tested methods (Table 2). Method 10 showed values from 1.2×10^6 to 2.7×10^6 ng/g and method 11 yielded 8.5×10^5–2.5×10^6 ng/g. No significant difference was observed in the quality of the DNA from these two methods. Protocols 12 and 13 include modifications of the method described by van den Ende and de Hoog (1999) and they differed in the chemical composition of the used extraction buffers. DNA yields from method 12 ranged from 3.5×10^5 to 5.5×10^5 ng/g, whereas with method 13 9.8×10^5–2.4×10^6 ng/g of DNA was recovered. Additionally, the last method showed a higher quality of DNA and allowed the amplification with more primer pairs than method 12.

The DGGE fingerprints of the bacterial and fungal micro-biota were carried out in order to evaluate if the community profiles, including the quantity and the quality of the bands, were influenced by the extraction methods used and by the amount of sample material used for extraction. The results of our study – examples of DGGE fingerprints are shown in Fig. 1 – clearly demonstrated that the fingerprints were not influenced by the extraction methods, provided that the extraction allowed successful PCR amplification.

3.2. The red brick material

Based on the results derived from the plaster extractions, we selected methods 1–3, 6, 7, and 10–13 for the isolation of DNA from red brick. Results are shown in Table 3. For this material the three commercial kits (methods 1–3) resulted in DNA yields ranging from 1.2×10^3 to 1.9×10^4 ng/g. From the commercial kits, the extracts from the FastDNA kit (method 1) showed the highest DNA yields (7×10^3–1.5×10^4 ng/g) and the best quality – A260/A280 ratios ranging from 1.74 to 1.91 – and further allowed the amplification with all primer sets. By contrast, the two kits from MO BIO (methods 2 and 3) did not generate satisfactory results in all evaluated parameters (Table 3). Astonishingly, with protocol 6 of Ausubel et al. (1991), 1.6×10^6–6.5×10^6 ng/g of DNA was obtained from the same sample amounts, that is up to three decimal powers more DNA than from methods 1–3. However, the DNA extracts contained impurities that strongly inhibited the PCR tests. Positive results were only obtained from the 50 mg samples tested with the ITS1/ITS2 primer pair. With protocol 7 of Schabereiter-Gurtner et al. (2001) moderate DNA amounts (1.8×10^4–9.4×10^4 ng/g) with higher A260/A280 ratios (around 2.9) were obtained, as observed for the

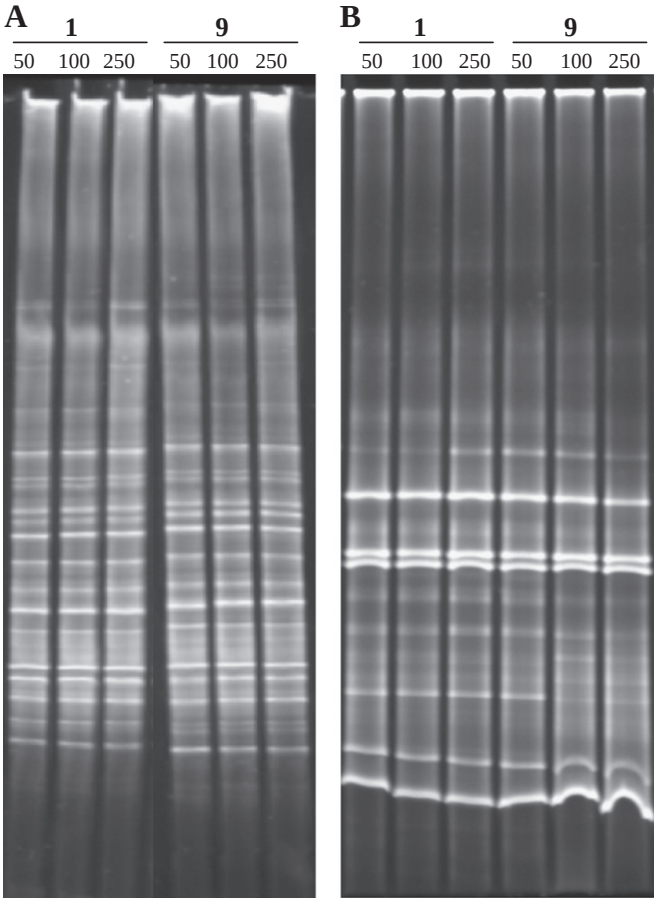

Fig. 1. Representative examples of bacterial and fungal fingerprints obtained from the plaster material. The banding patterns of the three tested sample amounts (50, 100 and 250 mg) from the FastDNA Spin kit for soil (method 1) and the protocol from Schabereiter-Gurtner et al. (2001) with the additional QIAamp PCR purification kit (method 9) are shown. Both methods revealed positive PCR amplification results for 16S rDNA and ITS1/ITS4 fragments. A. Bacterial community fingerprints. B. Fungal fingerprints.

plaster material. However, these high values did not lead to any disadvantages in the amplification analysis. The DNA quality and quantity from the extracts obtained with methods 10 and 11 were similar to those of method 6. The low quality of DNA of these methods (ranging from 1.6×10^6 to 5.3×10^6 ng/g for both methods, with A260/A280 ratios from 1.45 to 1.5) did not have such drawbacks in the polymerase chain reaction as in the case of method 6 and they showed positive amplification results (Table 3). Finally, the results obtained from the modified protocols (methods 12 and 13) of Sert and Sterflinger (2010) showed similarities to those obtained from the plaster material. Method 13 allowed the recovery of nearly one decimal power more DNA than method 12. The A260/A280 ratios of the extracts from both methods were not significantly different with the exception of the 50 mg sample (1.33 versus 1.86 for methods 12 and 13, respectively). Nevertheless, both methods showed negative results with the four tested primer pairs. Therefore, for the last construction material only method 13 was further evaluated.

The DGGE analysis of the red brick samples led to the same conclusions as for the plaster material. Similarly, the extraction protocols that provided positive amplification results were able to display a complete community fingerprint with all three sample amounts (data not shown).

3.3. The gypsum cardboard material

Our previous results reduced the number of methods to only seven, which were applied for the gypsum cardboard samples (Table 4). The three commercial kits (methods 1–3) just delivered DNA yields ranging from 1.1×10^3 to 9.1×10^3 ng/g and A260/A280 ratios from 1.36 to 1.76. With the FastDNA kit PCR products with all four primer pairs were obtained. However, by using the UltraClean and PowerSoil kits positive results were obtained from PCR reactions with the bacterial and fungal ITS1/ITS4 primers. NanoDrop measurements with protocol 6 (Ausubel et al., 1991) showed high DNA concentrations ranging from 1.2×10^6 to 5.9×10^6 ng/g, but the impurities (A260/A280 ratios 1.39–1.44) did not allow an amplification with any tested primer set. Method 7 showed moderate DNA yields (1.8×10^4–9.1×10^4 ng/g) with very high A260/A280 ratios (3.17–3.22), as previously observed in the other two materials. These impurities resulted in drawbacks in the PCR analysis, where only with bacterial primers could PCR products be obtained. Method 10 gained the highest DNA amounts from the gypsum cardboard. The DNA concentration of 1.4×10^6–8.9×10^6 ng/g represents up to three decimal powers more DNA than from the commercial kits. The A260/A280 ratio revealed some contaminations in the extracts that strongly influenced the PCR assays. Finally, method 13 displayed relatively high DNA yields (7.7×10^5–1.7×10^6 ng/g). The A260/A280 ratios resembled those of method 10. However, negative PCR results were obtained with all four primer pairs tested.

DGGE results derived from the gypsum cardboard samples were in agreement with those obtained from the first two building materials. Methods producing positive results for the amplification with the

Table 3
Summary of the results obtained from the nine methods evaluated with the red brick samples. The given numbers for the DNA yield (ng/g) and DNA purity (A260/A280 ratio) are rounded mean values of triplicate measurements with the NanoDrop spectrophotometer and rounded values for the standard deviation. For the visualization of the DNA on agarose gels and the PCR products the table shows if all three sample amounts or only certain sample lots (50, 100 and 250 mg) were detectable after electrophoresis and ethidium bromide staining.

Red brick	Sample lot	1	2	3	6	7	10	11	12	13
DNA yield	50 mg	$1.5\times10^4\pm2.9\times10^3$	$1.9\times10^4\pm2\times10^3$	$7.6\times10^3\pm1.4\times10^3$	$6.5\times10^6\pm9.7\times10^5$	$9.4\times10^4\pm2.1\times10^4$	$5\times10^6\pm3.9\times10^5$	$5.3\times10^6\pm3.1\times10^5$	$6.1\times10^5\pm1.3\times10^5$	$1.1\times10^6\pm1.7\times10^5$
	100 mg	$1\times10^4\pm1.1\times10^3$	$4.6\times10^3\pm1.3\times10^3$	$4.8\times10^3\pm6.3\times10^2$	$2.2\times10^6\pm3.9\times10^5$	$4.8\times10^4\pm1.1\times10^4$	$2.5\times10^6\pm3.3\times10^5$	$3.3\times10^6\pm2.9\times10^5$	$4.2\times10^5\pm5.2\times10^4$	$1.1\times10^6\pm2.6\times10^5$
	250 mg	$7\times10^3\pm1\times10^3$	$2\times10^3\pm6.5\times10^2$	$1.2\times10^3\pm4.6\times10^2$	$1.6\times10^6\pm1.2\times10^5$	$1.8\times10^4\pm3.8\times10^3$	$1.7\times10^6\pm5.5\times10^5$	$1.6\times10^6\pm2.7\times10^4$	$3.1\times10^5\pm4.9\times10^4$	$5.9\times10^5\pm6\times10^4$
DNA purity	50 mg	1.9 ± 0.35	2.41 ± 0.71	1.86 ± 0.84	1.45 ± 0.06	2.92 ± 0.29	1.47 ± 0.05	1.47 ± 0.06	1.33 ± 0.17	1.86 ± 0.16
	100 mg	1.91 ± 0.25	2.04 ± 0.52	1.59 ± 0.57	1.4 ± 0.08	2.94 ± 0.29	1.45 ± 0.03	1.48 ± 0.03	1.35 ± 0.18	1.42 ± 0.07
	250 mg	1.74 ± 0.26	2.3 ± 0.62	1.63 ± 0.61	1.4 ± 0.07	2.97 ± 0.47	1.5 ± 0.07	1.48 ± 0.02	1.38 ± 0.05	1.41 ± 0.05
Visualization PCR		All	None	50, 100	None	None	None	None	None	None
341f/907r		All	All	All	None	All	All	All	None	None
ITS1/ITS4		All	All	All	None	All	250	None	None	None
ITS1/ITS2		All	All	All	50	All	All	All	None	None
ITS3/ITS4		All	None	100	None	All	None	None	None	None

Table 4
Summary of the results obtained from the seven methods evaluated with the gypsum cardboard samples. The given numbers for the DNA yield (ng/g) and DNA purity (A260/A280 ratio) are rounded mean values of triplicate measurements with the NanoDrop spectrophotometer and rounded values for the standard deviation. For the visualization of the DNA on agarose gels and the PCR products the table shows if all three sample amounts or only certain sample lots (50, 100 and 250 mg) were detectable after electrophoresis and ethidium bromide staining.

Gypsum cardboard	Sample lot	1	2	3	6	7	10	13
DNA yield	50 mg	$8.4\times10^3\pm4.7\times10^3$	$9.1\times10^3\pm2.4\times10^3$	$7.5\times10^3\pm3\times10^3$	$5.9\times10^6\pm1.1\times10^5$	$9.1\times10^4\pm6.7\times10^3$	$8.9\times10^6\pm1.7\times10^6$	$1.7\times10^6\pm1.6\times10^5$
	100 mg	$3\times10^3\pm9.4\times10^2$	$4.1\times10^3\pm1\times10^3$	$3.1\times10^3\pm1.3\times10^3$	$2.8\times10^6\pm1.1\times10^5$	$4.5\times10^4\pm1.3\times10^3$	$3.2\times10^6\pm4.2\times10^5$	$1.2\times10^6\pm3.4\times10^4$
	250 mg	$1.3\times10^3\pm3.9\times10^2$	$1.6\times10^3\pm4.6\times10^2$	$1.1\times10^3\pm5.8\times10^2$	$1.2\times10^6\pm2.8\times10^4$	$1.8\times10^4\pm8.5\times10^2$	$1.4\times10^6\pm4.6\times10^5$	$7.7\times10^5\pm5.1\times10^4$
DNA purity	50 mg	1.43 ± 0.35	1.5 ± 0.24	1.43 ± 0.31	1.44 ± 0.01	3.22 ± 0.46	1.55 ± 0.04	1.63 ± 0.11
	100 mg	1.76 ± 0.49	1.5 ± 0.64	1.7 ± 0.62	1.41 ± 0.02	3.17 ± 0.23	1.48 ± 0.03	1.6 ± 0.03
	250 mg	1.65 ± 0.5	1.36 ± 0.32	1.56 ± 0.84	1.39 ± 0.01	3.22 ± 0.37	1.45 ± 0.06	1.55 ± 0.04
Visualization PCR		50	None	None	None	None	None	None
341f/907r		All	All	250	None	All	None	None
ITS1/ITS4		All	All	250	None	None	None	None
ITS1/ITS2		All	None	None	None	None	None	None
ITS3/ITS4		All	None	None	None	None	None	None

different primer sets further delivered reliable fingerprints of the inhabiting bacterial and fungal communities (data not shown).

3.4. Statistical results

The statistical analysis using a 3-way ANOVA model showed that regarding the measurement variable 'yield' all treatment factors and all interactions showed significant effects (all p-values smaller than 0.0001). The homogeneous subgroups in Table 5 are indicated by small letters.

Regarding the measurement variable 'purity' the treatment factors 'material' and 'method' showed significant effects (both p-values smaller than 0.0001), whereas the treatment factor 'amount' was not significant. The interactions containing the factor 'material', i.e., interactions between 'material' and 'amount' and between 'material' and 'method', also showed significant effects ($p = 0.0342$ and $p < 0.0001$, respectively). The homogeneous subgroups may also be found in Table 5.

4. Discussion

Analyzing microbial communities in building materials is the basis for a series of implications like: 1) assessment of material deterioration; 2) development and testing of anti-microbial treatments e.g. biocides and 3) evaluation of health risks for humans in indoor environments (Piñar and Sterflinger, 2009). The first crucial step in the DNA based assessment of microbial communities is a quantitative and reproducible extraction of nucleic acid. Variations in the efficiency of cell lysis, DNA yield and purity can fundamentally affect the success of analytical techniques such as PCR, hybridization, cloning or analysis of a metagenome (Bertrand et al., 2005; Picard et al., 1992; Straub et al., 1994; Young et al., 1993). In this way, biases can be introduced, which dramatically affect the success of microbial community analysis and hinder inter-laboratory comparisons (Kang and Mills, 2006; Krsek and Wellington, 1999; Rainley et al., 1994; Ranjard et al., 2003; von Wintzingerode et al., 1997).

The DNA extraction protocols used in this study generally comprise combinations of different mechanical, chemical and also enzymatic lysing procedures. Generally, a variation of the commonly used working steps for DNA extraction is included in these methods. Mechanical breakage of the cells – additionally to the grinding with liquid nitrogen – is done by bead beating, vortexing or freezing and thawing. The chemical composition of the extraction buffers includes SDS, CTAB, high salt concentrations, EDTA, β-mercaptoethanol and also enzymes like lysozyme or proteinase K. Purification steps included in the protocols usually comprise phenol–chloroform precipitation or different kit purifications using spin filters or carrier RNA. DNA-precipitation is done using 96%-ethanol.

Table 5
Homogeneous subgroups generated by the statistical program R. The small letters a, b, c and d represent homogeneous groups. ab is another homogeneous group that cannot be grouped to either group a or to group b.

Method	1	2	3	6	7	10	13
DNA yield	a	a	a	c	a	d	b
DNA purity	c	c	c	a	d	ab	b

The commercial kits were evaluated with all three building materials, due to their worldwide availability and the guaranteed constant composition of the supplied solutions without the possibility of contaminations. The other extraction protocols where reaction buffers are prepared individually always imply the risk for operator specific mistakes. On the other hand they represent a good possibility to reduce costs in everyday laboratory work.

The order of the three evaluated building materials was determined by the occurrence of these materials, wherein plaster covers most masonries, red brick is a historically old and very commonly used building material and gypsum cardboard is more commonly used in modern buildings.

The results of our study show that the differences in extraction methods are extreme: although DNA was extracted with all the methods tested, the DNA yields of 1 g building material varied from 1.1×10^3 to 8.9×10^6 ng. As an example, from a 50 mg plaster material extracted with method 5 only 7.2×10^3 ng/g of DNA was obtained, whereas from the same amount of plaster material method 10 allowed the recovery of 2.7×10^6 ng/g of DNA, which is nearly 3 orders of magnitude more. Similar results were observable with the 100 mg and 250 mg samples and also with the other materials. Thus, these data evidence the necessity to introduce standardized methods.

Methods 1–3, 7 and 13 resulted in the highest DNA concentrations from the plaster material, whereas methods 6 and 10 gained more DNA from the red brick and gypsum cardboard samples (Fig. 2A, $p < 0.0001$). The quantity of DNA extracted from the samples of one building material strongly varied by using the different tested methods. Especially the commercial extraction kits resulted in relatively low DNA yields that were close to the detection limit (2 ng/μl) of the NanoDrop instrument (Tables 2–4). Protocols 6–13 delivered up to 3 orders of magnitude more DNA from the same sample amount than the commercial kits. For instance, the highest DNA concentration obtained with a commercial kit from the 50 mg gypsum cardboard samples was 9.1×10^3 ng/g using the UltraClean DNA Isolation Kit (method 2). On the other hand with the extraction protocol 10 it was possible to gain 8.9×10^6 ng/g of DNA from the same sample lot. These fluctuations can be explained by drawbacks occurring with NanoDrop measurements, when contaminations like protein, phenol, humic acid impurities, and other contaminants from the building materials or buffer residues, also strongly absorb at

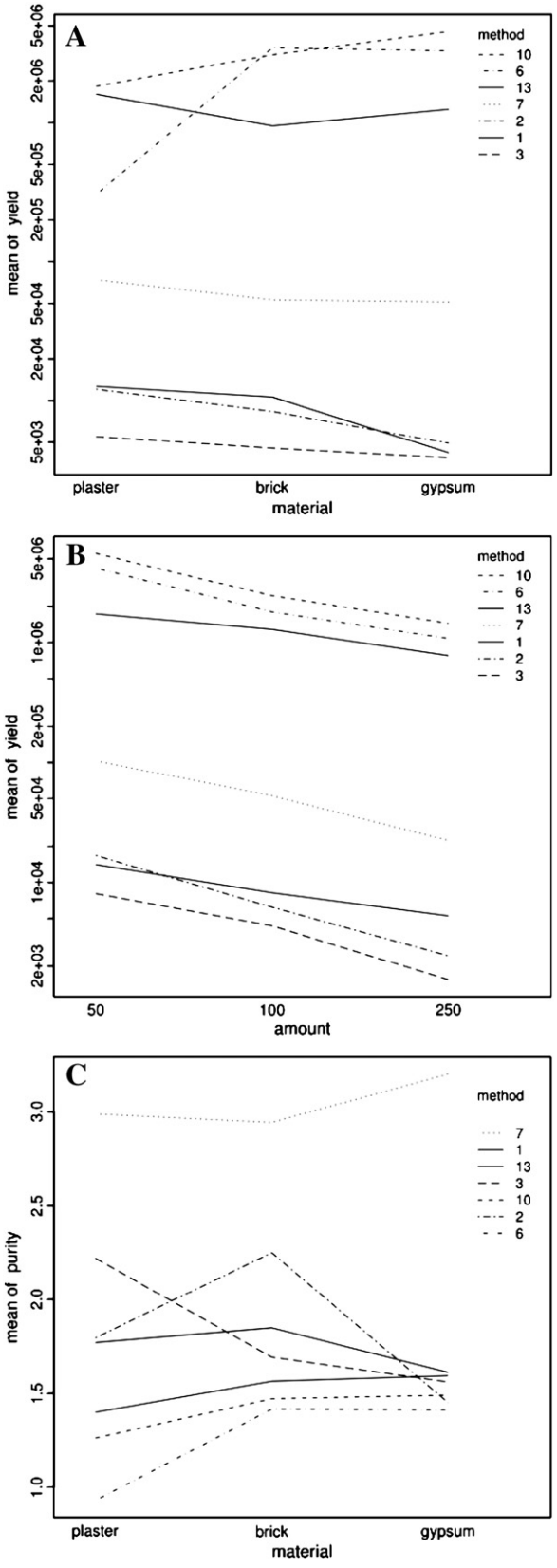

Fig. 2. Interaction plots of the mean values for measurement variable yield and purity of corresponding factor combinations are displayed and linked by profile lines for each method. A. Mean values for the yield of the factor combination material and method. B. Mean values for the yield of the factor combination sample amount and method. C. Mean values for purity of the factor combination material and method.

260 nm (Fig. 2C, $p<0.0001$). Furthermore, our data showed that the extracts of all standard extraction protocols that included a precipitation step using ethanol had very high DNA concentrations. However, they also had lowered A260/A280 ratios (1.3–1.6) revealing impurities that co-precipitated with the extracted nucleic acid. These findings were additionally confirmed by negative results obtained by electrophoresis and PCR analysis. Therefore, it is very doubtful if the measured DNA concentration represented the real DNA content in the solution. The very high A260/280 ratios obtained with the protocol of Schabereiter-Gurtner et al. (2001; method 7), from all three building materials could be due to high salt residues as remains from the high salt concentrations in the extraction buffer used. These salt impurities did not lead to biases in the PCR analysis of the plaster and red brick samples. Only the amplification tests from the gypsum cardboard material were negative using the fungal primer pairs. Interestingly, similar A260/A280 ratios from different methods led to different PCR amplification results, which can be explained by the presence of PCR inhibitors (e.g. methods 8 and 11; 10, 12 and 13 from the plaster material; methods 6, 10–13 for the red brick samples; methods 2, 3 and 13 from the gypsum cardboard samples).

Concerning the material amounts tested (50, 100 and 250 mg) one could expect to gain the higher DNA yield from the larger sample amounts. This theory did not hold true for any of the tested methods (Fig. 2B, $p<0.0001$). More often the smallest sample amount (50 mg) yielded the highest DNA concentration. This can be explained by the ratio of the used sample amount versus the added buffer volume used for the extraction of the DNA. For instance, in protocol 7, which was developed for small samples (1–2 mg) obtained from objects of art (Schabereiter-Gurtner et al., 2001), a very small buffer volume (~100 µl extraction buffer, according to the author's instructions) is added at the beginning of the extraction procedure. The small volume is not enough to reach the whole sample material, when the sample material exceeds >100 mg. Generally, protocol 7 delivered very good results, but the abilities of this method were overextended by the sample volumes tested in this study. Especially finely grounded sample powder from building materials strongly absorbs the buffer and the mixture does not represent a homogenous liquid suspension. Additionally, shaking on an incubator or vortexing is not an enough rigorous strategy to completely homogenize the material. According to the manuals, the commercial kits are suitable for ≤250 mg (PowerSoil DNA Isolation kit), ≤500 mg (FastDNA Spin kit) and 250–1000 mg (UltraClean Soil DNA Isolation kit) of sample material. Thus, the tested sample volumes did not really challenge the kit-capacities. Just to mention, it is not possible to increase the buffer volumes, due to the limiting size of the tubes (1.5–2 ml) used for the extraction. In general, our study shows that the ratio sample amount versus the added buffer volume is an important factor for the DNA yield obtained. Sample amounts >100 mg overextend the capacities of some of the extraction procedures tested. As a consequence the enclosed DNA in the material cannot be extracted completely, which represents a great bias of the approach.

The DGGE analysis performed in this study further showed that microbial fingerprints obtained from the smallest sample amounts represented the whole inhabiting community. Therefore, when greater amounts of sample material are available, we recommend the careful homogenization of the samples and the further use of small subsamples from this homogenate for DNA extraction and community analysis. Similar procedures were suggested by Litchfield et al. (1975) and Terry et al. (1981) for marine sediments and forest soil, respectively.

In general, protocols which include an additional purification step using columns or commercial kits yielded drastically lower DNA amounts than the standard extraction methods using a phenol/chloroform purification of the DNA. DNA is lost during the application of these spin filters, whereas purity measurements, visualization and PCR analysis showed that a great amount of contaminants could be eliminated through the purification with these columns. Zhou et al. (1996) and Miller et al. (1999) already demonstrated for many soil and sediment types that

high-throughput DNA purification procedures that imply mini-columns, spin-filters or gel-extraction kits are able to sufficiently reduce or eliminate PCR inhibitors from DNA extracts. The results obtained from the plaster material with the protocol of Schabereiter-Gurtner et al. (2001) and its two modified versions (methods 7–9) corroborated these findings.

The goal of this study was the development of a universally adaptable DNA extraction and purification method for all commonly used building materials. This extraction technique would be a great step forward to establish molecular techniques for assessing the microbial ecology of construction materials. Such a standard protocol would be a great benefit toward the introduction of automated procedures in building microbiology that further allow a subsequent inter-laboratory comparison of results. This standard protocol should 1) be equally efficient for all building materials; 2) effectively lyse all target organisms; 3) allow the processing of multiple samples simultaneously in a short time (Ogram, 2000); 4) generate a sufficient amount of high-molecular weight DNA, respectively RNA, out of very small sample amounts; 5) include a purification procedure that removes any contaminating substances that could disturb further molecular applications; 6) and be appropriate for all standard laboratory techniques.

Out of the thirteen evaluated methods, the FastDNA Spin kit for soil revealed the best results for all three building materials. The use of this kit enabled the isolation of DNA from each tested material. The DNA obtained showed a very high quality compared to that isolated with the other methods. Contrary to other protocols that showed higher DNA concentrations (e.g. methods 6–13) this method allowed a clear visualization on agarose gels. Furthermore, the FastDNA Spin kit for soil delivered positive amplification results from all sample amounts from each construction material. The DGGE fingerprints obtained with this method represented the complete microbial diversity present in the sample material and comparison with the banding patterns from other methods showed that no DNA bands were missing. The incorporated ribolyzing step of the FastDNA Spin kit led to the complete lysis of all bacterial and fungal cells and, therefore, only this kit allowed the amplification with bacterial and all fungal primers (Tables 2–4). Finally, the usage of this commercial extraction kit minimizes the risk for contaminations and it represents a relatively quick DNA extraction procedure.

5. Conclusions

The results of this study clearly show that the FastDNA Spin kit for soil from MP Biomedicals is the method of choice for DNA extraction from construction materials. We recommend the standard application of this commercial kit for molecular ecology analysis of building materials in order to set standards in the assessment of microbial community analysis and to allow comparisons of results between different laboratories.

We declare that this study was not influenced by financial or commercial interests and that we are in no relationship with any of the companies mentioned above.

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Bio-susceptibility of thermal insulation systems used for historical buildings

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ABSTRACT: In historical buildings of Northern countries high levels of energy are necessary for reaching comfortable temperatures. For this reason several indoor insulation systems based on historical and ecological materials are on the market that should improve the thermic performance. However, using organic materials bears the risk of fungal growth. Therefore, five ecological indoor insulations systems were tested for their bio-susceptibility against various fungi both under natural- and under laboratory conditions. Fungal growth was evaluated by cultivation as well as by molecular methods. The materials turned out to have a different susceptibility towards fungal contamination. Whereas insulations made of bloated Perlite (plaster and board) did not show any fungal growth under natural exposition, loam and weed had high cell counts. In laboratory experiments, wooden soft-board represented the best environment for fungal growth. As a result, bloated Perlite is the most appropriate material for thermal insulation from the microbiological point of view.

1 INTRODUCTION

The ongoing climate change calls for necessary changes of human behavior and -living. In colder European climate zones, thermal insulation of houses is self-evident to reduce the amount of energy for heating. Exterior insulations systems are frequently applied on modern, newly built houses and Styrofoam is the most common exterior insulation system. However, a high percentage of our living houses are historical buildings under preservation order. These buildings are now included in country specific regulations and ordinances to enhance the "energy efficiency". Since an exterior insulation is incompatible with monument protection, alternative insulation techniques have to be applied Historical, organically and ecological insulation materials, such as bloated Perlite, cellulose, loam, reed, weed or wood are on the market but the risk of these materials is a possible microbial contamination through bacteria and fungi. Microbial growth on building materials is a problem that has been known for a long time, but floods, wet years, thermal modernization, air-conditioning systems, construction or material faults, poor and improper ventilation are major reasons for an increase of the relative air humidity and dampness of surfaces (Samson *et al*., 2010). These climatic conditions foster microbial growth (Sterflinger 2010). The properties and the common occurrence of microorganisms contribute to the fact that they represent the most frequent cause of biodeterioration of building materials (Piñar & Sterflinger 2009). Furthermore, a worldwide

phenomenon called sick building syndrome – SBS - (Sykes, 1988) has been confirmed as a recognizable disease by the World Health Organization (Akimenko *et al*., 1986). All these properties and effects of microbial growth call for the need to gain more insight into the microbiota inhabiting the different construction materials.

Nowadays, the isolation and identification of microorganisms still sticks to classical culture-based methods to estimate microbial contamination in buildings. Cultivation allows a quantitative and qualitative assessment of the environment. Nevertheless, the dramatically changes in microbiology over the past 20 years have developed fast and sensitive technologies that can be applied for studying microbial communities without the need for cultivation (Hill *et al*., 2000). The first crucial step for a successful and complete analysis of the micro-biota is the choice of an appropriate DNA isolation method (Martin-Laurent *et al*., 2001).

In this study 5 ecological indoor insulation materials were tested for their bio-susceptibility. The materials were investigated both under natural conditions - after 2 years of installation in an historical building - and under laboratory conditions. Therefore, samples items of all materials were inoculated with 3 commonly indoors occurring fungi and treated in a climate chamber for half a year. After this incubation time small samples were taken for cultivation- and molecular analyses. The colony forming units of each material were determined as well as DNA was extracted and evaluated by Nano Drop measurements. The same procedure was performed with samples taken from the historical house. In order to apply an appropriate DNA extraction method for the insulation materials we evaluated up to 13 direct - *in situ* DNA extraction methods with 3 typically used building materials. Attention at the evaluation of these protocols was focused on the parameters: 1) the quantity and 2) the quality of the DNA; 3) visualization of the extracted DNA; and 4) the ability of the DNA to be amplified in different PCR reactions. Additionally, the amplified PCR products were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) in order to compare the community fingerprints obtained from the different isolation methods and sample amounts.

2 METHODOLOGY

2.1 *Classical Cultivation studies*

Five indoor insulation materials – bloated Perlite (plaster and board), loam and reed, wooden softboard and sprayed cellulose – were evaluated. Therefore, small areas (5x5 cm) of the test items were inoculated with each 1 ml of 4 spore solutions from 3 commonly indoors occurring fungi (ACBR-Culture Collection, www.acbr-database.at: *Cladosporium cladosporioides*, *Aspergillus niger* and *Penicillium chrysogenum*, and a mixture of all 3). For 6 months, the samples were incubated in a climate chamber at 28°C and 90% relative humidity. Afterwards, samples from the surface were removed for cultivation and molecular analysis. Similar, 2 years after installation of the insulation systems in the historical building, samples were taken and 1 g of each material was shaken in 100 ml Erlenmeyer flasks, filled with 50 ml Tween 80 for 1 hour at room temperature on a rotary shaker (170 rpm). Hundred Microliter of each solution of a dilution series (10^{0}-10^{-3}) were plated on each two Malt-extract-agar plates (MEA) supplemented with 20 µl Streptomycin (Stock: 25 mg/ml) to inhibit bacterial growth. The pla-

tes were incubated at room temperature and fungal growth was checked every day. Colonies on the plates were counted to finally calculate the colony forming units (CFU) for each material.

2.2 *Molecular analyses*

In order to extract DNA from the insulation material an appropriate isolation method had to be found to overcome the biases commonly occurring during DNA extraction from building materials. Therefore, 3 commercial DNA extraction kits for soils and four standard DNA extraction protocols were tested with common plaster, red brick and gypsum cardboard (Ettenauer *et al*., 2012). Each material was ground, homogenized and 3 different samples amounts (50, 100 and 250 mg; each in triplicate) were weighed for each extraction protocol. After DNA extraction, the DNA yield and –purity (A260/A280 ratio) were assessed using the Nano Drop spectrophotometer. Therefore, from all triplicates the DNA concentration and –purity was measured thrice and mean values and standard deviations were calculated. Afterwards, the triplicates were pooled and electrophoresed on 1.5 % agarose gels. Further, the pooled DNA was used for PCR reactions with 3 fungal ITS primer pairs (ITS1/ITS4, ITS1/ITS2 and ITS3/ITS4) and 1 bacterial 16S rRNA primer set (341f/907r). Amplification products were assessed by visualization on 2% agarose gels. Genetic fingerprinting using DGGE analysis was performed with fungal and bacterial PCR fragments. According to the results obtained from these criteria (DNA-quantity, -quality, agarose gel electrophoresis, PCR- and DGGE results) the methods that worked better were identified and continually eliminated others that did not work as well.

According to the results from the comparative extraction study the most suitable method was used for the isolation of DNA from all samples of this study. Hundred milligram of each insulation material were used for the DNA extraction.

3 RESULTS AND DISCUSSION

3.1 *Classical Cultivation studies*

Fungal colonies growing on MEA plates derived from the insulation materials were counted and the CFU values were calculated. Figure 1a shows the calculated CFU-values.

After 6 months incubation under optimal growing conditions, fungal colonies were detected on all test items. The CFU values ranged from 5.0 $x10^2$ to 1.97 $x10^6$. The distinct strongest fungal growth was found on the wooden cardboard samples (CFU: 9.83 $x10^5$ to 1.97 $x10^6$). On reed boards with loam, bloated Perlite boards and sprayed cellulose, germination numbers of about 2 orders of magnitude lower were calculated. The best results, with the lowest CFU values (2.5 $x10^3$ to 2.1 $x10^4$) were found on the bloated Perlite plaster.

From the *in-situ* samples only on the wooden soft-board and reed board with loam a few fungal colonies germinated. The CFU values ranged from 1.5 $x10^3$ to 1.63 $x10^4$. No fungal

growth was observed on the other materials. The control samples of all materials, taken before the inoculation with fungal spores did not show any fungal growth at all.

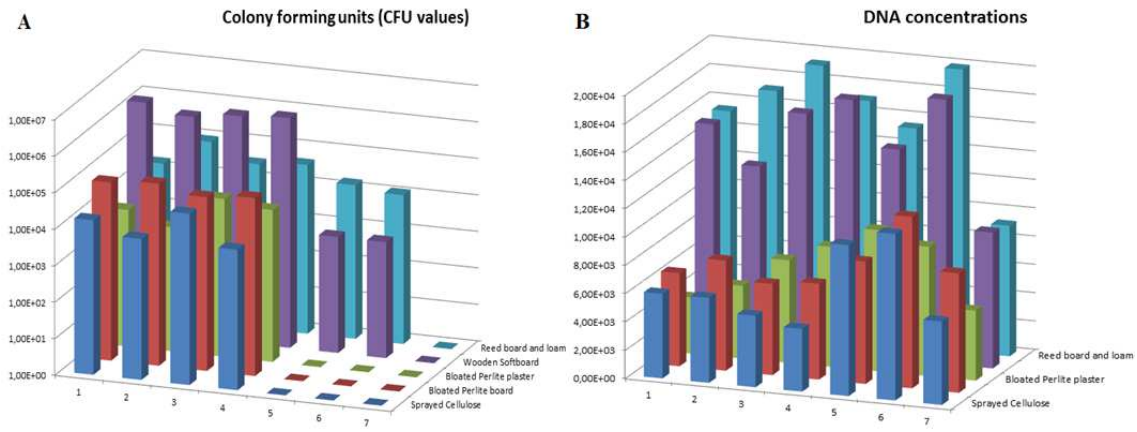

Figure 1. 3D diagrams showing (a) the CFU values on a logarithmic scale and (b) the DNA yields. Sample items incubated with *Cladosporium cladosporioides* (1), with *Aspergillus niger* (2), with *Penicillium chrysogenum* (3), with a mixture spores (4), samples from the 1st floor (5), and the 2nd floor of the building (6); not incubated control items (7).

3.2 *Molecular analyses*

All 13 extraction methods allowed the isolation of DNA from all sample amounts of all 3 building materials. However, Nano Drop measurement for the DNA concentration showed very divergent values. All 3 kits delivered the lowest DNA yields (Fig. 2a). Protocols including a purification step using columns or spin filters yielded drastically lower DNA amounts than standard methods using phenol/chloroform precipitation. A lot of DNA is lost, whereas purity measurements, visualization and PCR analysis showed that many contaminants could be eliminated. The standard protocols showed the highest DNA yields. Up to 3 orders of magnitude more DNA could be isolated from the same sample amounts. However, they also had lowered A260/A280 ratios revealing impurities that co-precipitated with the nucleic acid (Fig. 2c). These findings were confirmed by negative results obtained from electrophoresis and PCR analysis.

The DNA yield did not correlate to the sample amounts. All methods gained more DNA from the 50 mg samples than from the 100 or 250 mg samples (Fig. 2b). This is due to the ratio: used sample amount versus added buffer volume for the extraction. Small buffer volumes are not enough to reach the whole sample material, when the sample material exceeds >100 mg. As a consequence the enclosed DNA in the material cannot be extracted completely. The DGGE results clearly demonstrated that the fingerprints were not influenced by the extraction methods, provided that the extraction allowed successful PCR amplification. The results further showed that fingerprints obtained from the 50 mg subsamples of homogenized sampling material represented the whole inhabiting microbial community.

The FastDNA Spin kit was the only method that revealed positive results for all parameters of all 3 building materials. Therefore, this method was applied for the samples and allowed the isolation of DNA from all tested insulation materials (Fig. 1b).

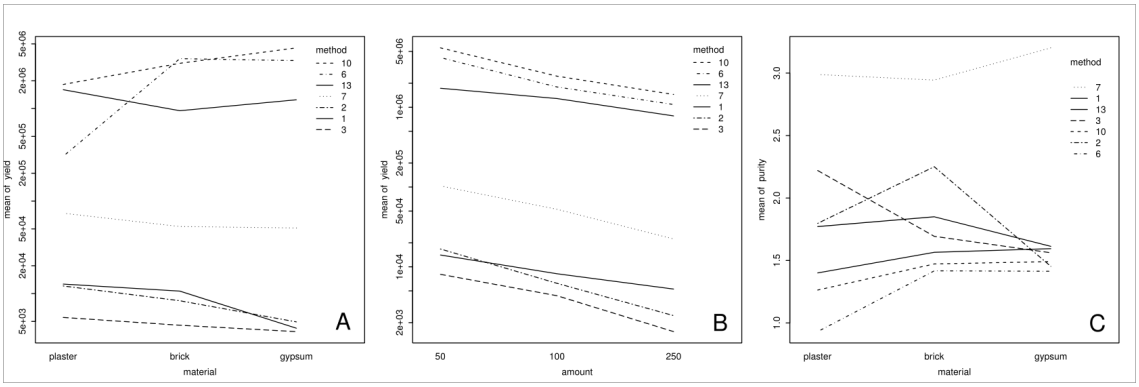

Figure 2. Interaction plots of the mean values for measurement variables yield and purity of corresponding factor combinations are displayed and linked by profile lines for each method. (a) Mean values for yield of the factor combination material and method. (b) Mean values for yield of the factor combination sample amount and method. (c) Mean values for purity of the factor combination material and method.

CONCLUSIONS

From the microbiological and hygienically point of view, plaster and board made of bloated Perlite are presented as being the most appropriate materials for thermal indoor insulation. The FastDNA Spin kit for soil (MP Biomedicals) is the method of choice for DNA extraction from construction materials. We recommend the standard application of this kit for molecular ecology analysis of building materials in order to set standards in the assessment of microbial community analysis and to allow comparisons of results between different laboratories.

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Quantification of fungal abundance on cultural heritage using real time PCR targeting the β-actin gene

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The traditional methodology used for the identification of microbes colonizing our cultural heritage was the application of cultivation methods and/or microscopy. This approach has many advantages, as living microorganisms may be obtained for physiological investigations. In addition, these techniques allow the quantitative and qualitative assessment of the investigated environment. Quantitative analyses are done by plate count and the determination of abundance by the colony forming unit (CFU). Nevertheless, these techniques have many drawbacks that lead to an underestimation of the cell numbers and do not provide a comprehensive overview of the composition of the inhabiting microbiota. In the last decades, several molecular techniques have been developed enabling many advantages over the cultivation approach. Mainly PCR-based, fingerprinting techniques allow a qualitative detection and identification of the microbiota. In this study, we developed a real time PCR method as a simple, rapid and reliable tool to detect and quantify fungal abundance using the β-actin gene, which is known to appear as a single-copy gene in fungi. To this end, five different indoor thermal insulation materials applied for historical buildings that were previously tested for their bio-susceptibility against various fungi were subjected to qPCR analyses. The obtained results were compared with those obtained from a previous study investigating the bio-susceptibility of the insulation materials using classical cultivation experiments. Both results correlated well, revealing that Perlite plaster was the most suitable insulation material, showing the lowest fungal CFU and qPCR values. In contrast, insulations made of wood showed to be not recommendable from the microbiological point of view. In addition, the potential of qPCR was tested in other materials of cultural heritage, as old parchments, showing to be a suitable method for measuring fungal abundance in these delicate materials.

Keywords: fungi, abundance, real-time qPCR, β-actin gene

INTRODUCTION

Our cultural heritage is continuously exposed to the effects of physical, chemical, and biological factors. The latter including biodeterioration caused by microorganisms (Sterflinger and Piñar, 2013). Therefore, the identification of the microorganisms involved in biodeterioration is the first necessary step for understanding the effects of microorganisms on cultural assets. The second step is to elucidate the actual amount, activity and functional stage of these microorganisms and their role in biodeterioration. Finally, the third step is to use the obtained information to develop strategies for the conservation and protection of monuments and art-works (González and Saiz-Jimenez, 2005).

Traditionally, the methodology used for the isolation and identification of microorganisms from different types of materials of our cultural heritage was the application of cultivation methods and/or microscopy. The classical cultivation of microorganisms has offered many advantages, as living microorganisms could be obtained for further physiological investigations. Furthermore, these techniques allowed the quantitative and qualitative assessment of the investigated environment. By using this methodology, quantitative analyses have been done by plate count and the determination of activity by the colony forming unit (CFU), both being analyses based on the growth of microorganisms on selective media. Nevertheless, nowadays these techniques are known to have many drawbacks (e.g., need of considerable sample amounts, great time effort, only a small proportion of cultivable microorganisms present on samples, etc.) that lead to an underestimation of the cell numbers and further do not provide a comprehensive overview of the composition of the inhabiting micro-biota (Ward et al., 1990).

In the last decades, several culture-independent, molecular DNA and phylogenetic techniques have been developed supplying many advantages over the traditional cultivation approach (Amann et al., 1995; Hugenholtz and Pace, 1996; Hugenholtz et al., 1998). Molecular techniques take advantage of the specificity provided by nucleic acid sequences for the identification of microorganisms and their independence of culturing microorganisms. Different, mainly PCR-based, genotyping techniques have been developed and adapted for the fingerprinting of microbial communities on biodeteriorated cultural heritage (Piñar et al., 2001a; Schabereiter-Gurtner et al., 2001; González, 2003; González and Saiz-Jimenez, 2004, 2005; Michaelsen et al., 2006).

These techniques have enabled a reliable study and monitoring of the microbial communities associated with different materials, such as stone, prehistoric caves, wall paintings, oil paintings, historical glasses, paper, parchment, human remains, etc. (Piñar et al., 2001b, 2010, 2011, 2013a,b; Schabereiter-Gurtner et al., 2001, 2002, 2004; Carmona et al., 2006; Michaelsen et al., 2006, 2010, 2013; Bastian et al., 2010; Ettenauer et al., 2010, 2011; Portillo and González, 2011; López-Miras et al., 2013a,b). To date, molecular techniques are very well established and are complementing to more classical microbiological methods in the study of microorganisms and their role in cultural heritage. The fusion of these two different strategies have delivered complementary results which allow a much better understanding of the identity and diversity of the microorganisms inhabiting our cultural heritage (Laiz et al., 2003; Ettenauer et al., 2010; López-Miras et al., 2013a,b).

In this context, recent investigations have focused on the development of quantitative molecular tools that are broad-coverage, sensitive, and specific. One of these methods is based on real-time quantitative polymerase chain reaction (qPCR), which is a well-known method for microbial detection (Zhang and Fang, 2006). It is based on amplification of specific DNA-regions, monitoring the amplification continuously by using fluorescent dyes, and quantification of the target based on standards. Since the detection is based on DNA, it is not dependent on the cultivability of the microbes. The main advantages of real-time PCR are its quantitative property and high specificity. It is also rapid and easy to perform, after the assay has been set up and validated properly. Quantitative PCR analyses have been widely applied for studying the levels of individual species and assay groups in indoor samples (Haugland et al., 1999, 2004; Meklin et al., 2004; Vesper et al., 2008; Kaarakainen et al., 2009) and in less cases, in building materials (Pietarinen et al., 2008; Pitkäranta et al., 2011). However, few studies have explored the total mycobiota using DNA-based universal community characterization methods, like ribosomal DNA amplicon sequencing or metagenome analysis (Pitkäranta et al., 2008; Tringe et al., 2008; Liu et al., 2012). Nevertheless, it is worth noting that by using universal rRNA primers, it is difficult to calculate single fungal cells in a certain environmental sample. The great variation of the number of rRNA gene clusters in a genome and in a species (Herrera et al., 2009) makes it difficult to estimate the number of fungal individuals.

Therefore, in this study, we have developed a real time PCR method as a simple, rapid and reliable tool to detect and quantify fungi using the β-actin gene. Fungi appear to have a tendency toward a single actin gene copy per haploid genome (Gallwitz and Seidel, 1980; Ng and Abelson, 1980; Mertins and Gallwitz, 1987; Fidel et al., 1988; Voigt and Wostemeyer, 2000), thus enabling a precise quantification of fungal cells. Moreover, comparative sequence analysis of actin information, both at the nucleotide and at the amino acid level, is developing into a highly appreciated tool for long-range phylogenetic studies (Voigt and Wostemeyer, 2000).

To this end, five different indoor thermal insulation materials, based on ecological materials that can be applied for historical buildings, were tested for their bio-susceptibility against various fungi under natural and laboratory conditions (Sterflinger et al., 2013) based on the qPCR targeting the β-actin and the CFU method. In addition, the potential of qPCR for the detection of the β-actin gene was tested in other materials of cultural heritage, as old parchments, which were already investigated and known to be colonized by fungi (Piñar et al., 2011, 2014).

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURE

Five different indoor insulation materials—bloated Perlite plaster, bloated Perlite board, reed board and loam, wooden soft-board and sprayed cellulose—were evaluated (see **Table 1**). Therefore, small areas (4 × 4 cm) of the test items (∼10 × 10 cm in size) were inoculated with each 1 ml (concentration 10^5 spores ml^{-1}) of 4 different spore solutions from 3 commonly indoors occurring fungi: *Cladosporium cladosporioides* (MA 1610—further named a), *Aspergillus niger* (MA 1615—b) and *Penicillium chrysogenum* (MA 1701—c), and a mixture of all three (d), by plating and spreading the spore solutions with a spatula on the materials surface. These samples were incubated in a climate chamber (Weiss-Klimakammer WKL 100) at 28°C and 90% relative humidity for a period of 6 months. Afterwards, the surface area (to a depth of ∼0.5 cm) was removed for cultivation and molecular analysis.

Similar investigations were performed with samples of each insulation material collected after 18 months (1st sampling) and 32 months (2nd sampling) after installation from the tentative historical building and were investigated in the laboratory. From the sprayed cellulose, only samples from the 2nd floor could be taken after 32 months.

DNA EXTRACTION

DNA extracted from insulation materials

The FastDNA Spin kit for soil from MP Biomedicals (Illkrich, France) was the method of choice for DNA extraction from construction materials (Ettenauer et al., 2012). The kit combines a mechanical lysis, using bead beating, and chemical lysis of the cells. Samples from each material were ground in liquid nitrogen using a sterile mortar and pestle, homogenized in Falcon tubes and, thereof, 100 mg were weighed for DNA extraction.

After DNA extraction, the DNA yield and –purity (A260/A280 ratio) were assessed using the NanoDrop® ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH, Linz, Austria). Afterwards, 7 µl of the extracted DNA were visualized on 1.5% agarose gels by electrophoresis. Further, the DNA was used as template for PCR reactions.

Table 1 | Overview of the investigated insulation materials and the fungal strains used for inoculation in this study.

Insulation materials	Fungal strains (short cut)
Sprayed cellulose	*Cladosporium cladosporioides* (a)
Bloated Perlite board	*Aspergillus niger* (b)
Bloated Perlite plaster	*Penicillium chrysogenum* (c)
Wooden soft-board	Mixture of all three fungi (d)
Reed board with loam	

DNA extracted from parchment samples

DNA extraction was performed directly from seven parchment samples, dating from the 13th–19th century (Piñar et al., 2014), by using the FastDNA Spin Kit for Soil from MP Biomedicals, as well. The protocol of the manufacturer was slightly modified. About 10–20 mg (2–3 mm^2) of parchment were placed in the Lysing Matrix E Tubes with the appropriate buffers, and then processed twice in the Fast Prep FP120 Ribolyzer (Thermo Savant; Holbrook, USA) for 30 s at speed 5.5 ($m\ s^{-1}$), with 5 min intervals on ice. The Lysing Matrix E Tubes were centrifuged at 14.000 × g for 15 min, and the supernatants were transferred to clean 2 ml tubes. The PPS reagent and the binding matrix solution were applied to the supernatant; the suspension was transferred to the provided spin filter and centrifuged at 14.000 × g for 1 min, following the instructions of the manufacturer. DNA was washed twice with 500 ml of the SEWS-M solution and eluted from the binding matrix with 100 ml DNase/Pyrogen Free Water. The DNA crude extracts were further purified prior to PCR amplification with the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) with modifications as follows: the washing step was performed twice with 750 µl buffer AW1/AW, rolling the column to allow more contact with the cartridge and leaving the tubes to stand for 2 min with the buffer, prior to centrifugation. The final elution step was repeated twice with 100 µl of 80°C preheated ddH_2O (Sigma Aldrich, St. Louis, USA) letting the tubes stand for 2 min before centrifugation. After the DNA extraction and purification steps, the concentration and quality of the DNA extracts was assessed using a NanoDrop® ND-1000 Spectrophotometer. The analyses were performed according to the manufacturer's protocol and the extracted DNA was analyzed in duplicate. Finally, the purified DNA was used directly for PCR amplification.

QUANTITATIVE REAL-TIME PCR ANALYSES (qPCR)

Quantitative real-time PCR was performed in a BioRad CFX96™ real-time PCR by using the SensiMix Plus™ SYBR-Kit (Bioline). Each 20 µl reaction contained 10 µl SensiMix-Plus, 1 µl 50 mM $MgCl_2$ (final conc. 2.5 mM), 0.25 µl of a 10 pmol/µl primer solution using the β-actin primers: ACT 512-F (5′ ATG TGC AAG GCC GGT TTC GC 3′) and ACT 783-R (5′ TAC GAG TCC TTC TGG CCC AT 3′) (Carbone and Kohn, 1999), 6.5 µl H_2O and 2 µl of DNA template. The amplification conditions were 95°C for 10 min and then 40 cycles of 95°C 15 s, 61°C 20 s and 72°C 15 s. Fluorescence measurements were made at the end of each annealing cycle and an additional measuring point at 80°C (for 1 s) to detect the formation of primer dimers during amplification. A melt curve analysis was made by raising the temperature from 65 to 95°C in 0.5°C steps for 5 s each.

STANDARD CURVES

To enable the quantification of PCR products, standard curves based on threshold cycles were produced by re-amplifying 10-fold dilution series of PCR products from genomic DNA. An aliquot of each dilution (0.035 fg −0.035 ng, equivalent to $1 \times 10^2 - 1 \times 10^8$ β-actin copies) in 3 replicates were used as templates in real time PCR.

The DNA standards were generated with the β-actin primers, mentioned above, with the following PCR program: 95°C for 3 min and the 30 cycles of 95°C 30 s, 55°C 30 s, 72°C 30 s and a final elongation step at 72°C for 1 min. PCR was done with a BioRad C1000 thermal cycler using the PCR Master Mix (Promega, Mannheim, Germany) [50 units/ml of TaqDNA Polymerase in a supplied reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$]. Each 100 µl reaction contained 50 µl 2x PCR Master Mix, each 1 µl of forward and reverse primer (stock: 10 pmol/µl), 43 µl ultra-pure water and 5 µl template of genomic DNA of *Aspergillus niger*. The PCR products were cleaned using the QIAquick PCR Purification kit (QIAGEN) and checked for purity on agarose gels and by sequence analysis with database comparison. Concentration of the PCR product was measured spectrophotometrically at 260 nm with a NanoDrop® ND-1000. The resulting PCR products were used to construct standard curves for absolute quantification. The numbers of copies in the standards were calculated using the formula from Le Calvez et al. (2009) and various online-tools, like from the URI Genomics and Sequencing Center (http://cels.uri.edu/gsc/cndna.html). Standard curves were automatically generated by the BioRad Precision Melt Analysis™ software.

STATISTICAL ANALYSIS

A Pearson correlation coefficient was done to compare the results derived from qPCR analyses and those derived from classical cultivation (CFU method) determined in one of our previous studies (Sterflinger et al., 2013). Further ANOVA and pairwise Wilcoxon tests were applied to look at the statistical significance of the differences in fungal abundance between the different materials inspected. All statistics were done with R (R Core Team, 2014).

RESULTS

QUANTIFICATION OF THE β-ACTIN GENE IN INSULATION MATERIALS BY qPCR ANALYSES

The five selected insulation materials were subjected to qPCR analyses targeting the β-actin gene to quantify the fungal abundance present in such materials. The amount of newly synthesized target-DNA during the ongoing PCR reaction-cycles was measured continually and the emitted fluorescence from SYBR-green binding was detected in real time by the instrument used. The BioRad Precision Melt Analysis™ software allowed the comparison of the β-actin gene copy numbers in the samples with the known concentrations of the standards. The values obtained from each insulation material sample were further extrapolated per ng of extracted DNA from each material, and are shown in **Table 2**. Representative primer-specific quantification- and standard curves, as well as melt peak charts are shown in **Figures S1A–D** (Supplementary Data).

The quantitative real time PCR allowed the detection of fungi in all analyzed samples (see **Table 2**). Compared to the classical cultivation methodology it was possible to detect fungi in the control items (3.6–25.13 copies per ng of extracted DNA from the sample material) using qPCR.

The detected copy numbers per ng of extracted DNA from the different test items ranged from 5.76 to 5480.56. The highest fungal abundance was observed in the wooden soft-board samples (906.79–5480.56 copies/ng), followed by the bloated Perlite board (82.83–167.19 copies/ng) and the sprayed cellulose

Table 2 | Results of the qPCR analysis for the insulation materials: calculated copy numbers per ng extracted DNA for the test items and the samples taken from the historical building after 18 and 32 months of installation.

Material	Climate chamber					Historical building			
	Inoculated strains—6 months					18 months		32 months	
	a	b	c	d	Control	1st floor	2nd floor	1st floor	2nd floor
Sprayed cellulose	55.47 ± 20.62	216.93 ± 25.83	8.732 ± 32.33	*21.68 ± 14.10*	*8.88 ± 5.39*	*1.88 ± 0.95*	*1.84 ± 1.27*	No sample	*3.39 ± 1.06*
Bloated Perlite board	95.26 ± 14.28	167.19 ± 17.04	156.22 ± 109.96	82.83 ± 12.53	*798 ± 0.95*	*0.88 ± 0.33*	*2.85 ± 1.99*	*8.75 ± 2.10*	*11.82 ± 10.51*
Bloated Perlite plaster	*5.76 ± 0.14*	*20.38 ± 6.61*	*6.04 ± 5.64*	*27.47 ± 13.18*	*15.93 ± 4.17*	*5.75 ± 5.35*	*4.41 ± 1.21*	*3.85 ± 0.92*	*15.70 ± 1.05*
Wooden soft-board	4174.16 ± 170.36	5480.56 ± 569.09	1092.86 ± 118.93	906.79 ± 139.36	*25.13 ± 11.28*	36.95 ± 12.89	73.10 ± 23.79	*19.94 ± 1.40*	317.33 ± 39.12
Reed board with loam	*29.19 ± 3.78*	35.48 ± 19.46	*25.80 ± 11.55*	*21.59 ± 3.01*	*3.60 ± 1.91*	*19.59 ± 3.04*	91.90 ± 11.65	92.51 ± 22.90	112.34 ± 30.54

Copy number values written in italics are below the detection limit (30.57 copies/ng extracted DNA) of the qPCR assay. (a) MA 1610 Cladosporium cladosporioides, (b) MA 1615 Aspergillus niger, (c) MA 1701 Penicillium chrysogenum, (d) mixture of all three fungal spore suspensions.

(21.68–216.93 copies/ng). The lowest β-actin gene numbers were found in the bloated Perlite plaster (5.76–27.47 copies/ng) and the reed board with loam (21.59–35.48 copies/ng) (see **Figure 1**). The growth differences observed for the different materials were significant (One-Way ANOVA p-value: 4.44 × 10–11). In addition, the growth difference between each pair of materials was computed with a Wilcoxon tests, and showed that all but one pair exhibited a $p < 0.05$ (see **Table 3**). Bloated Perlite plaster and wooden soft-board showed the lowest and the highest fungal abundance, respectively (see **Table 2** and **Figure 1**).

FIGURE 1 | Distribution of fungal abundance for different growth materials as measured by CFU (l.h.s) and qPCR (r.h.s). For both metrics the bloated Perlite plaster and wooden soft-board showed the lowest and highest fungal abundance, respectively. The only inconsistence between CFU and qPCR was seen between the reed board and the sprayed cellulose, where the latter showed a reduced fungal abundance compared to sprayed cellulose based on the qPCR methodology, but a higher fungal abundance based on the CFU approach.

Table 3 | *P*-value returned by the Wilcoxon test for the pairwise comparison of fungal abundance for different materials.

Material	Bloated Perlite board	Bloated Perlite plaster	Wooden soft-board	Reed board with loam
Sprayed cellulose	0.19	7.17×10^{-5}	7.40×10^{-7}	0.0045
Bloated Perlite board		7.40×10^{-7}	7.40×10^{-7}	1.48×10^{-6}
Bloated Perlite plaster			7.40×10^{-7}	0.0036
Wooden soft-board				7.40×10^{-7}

Samples obtained from the insulation materials installed in the historical building showed qPCR values ranging from 0.88 to 91.90 copy numbers per ng of DNA after 18 months, and from 3.85 to 317.33 copies/ng after the second sampling (see **Table 2**). From the wooden soft-board samples, the highest β-actin gene numbers were retrieved (19.94–317.33 copies/ng). The detected copy numbers for nearly all *in situ* samples were 1-2 orders of magnitude lower than the values obtained from the incubated test items. Only the reed board with loam material from the historical building had slightly higher copy numbers, which could be explained by the higher relative humidity in the rooms (19.59–112.34 copies/ng). For the other materials, lower copy numbers–close or below the detection limit of the assay (30.57 copies/ng)—were measured: bloated Perlite board (0.88–11.82 copies/ng), sprayed cellulose (1.84–3.39 copies/ng) and bloated Perlite plaster (3.85–15.7 copies/ng).

QUANTIFICATION OF THE β-ACTIN GENE IN PARCHMENT SAMPLES BY qPCR ANALYSES

To determine if the quantification of the β-actin gene by qPCR could be used as cellular abundance indicator in other materials of cultural heritage, the DNAs extracted from valuable parchment samples were subjected to qPCR analyses using the same protocol described for insulation materials. The quantitative real time PCR allowed the detection of fungal cells in all parchment samples (see **Table 4**). The detected β-actin copies were referred to the total amount of DNA extracted from each parchment sample. The resulting values were very similar for all samples and ranged from 168.01–680.09 copies/ng of extracted DNA.

DISCUSSION

Molecular techniques employing a quantitative real-time PCR have been used for microbial quantification in a variety of environments (Zhang and Fang, 2006; Smith and Osborn, 2009). The method avoids sample limitations and therefore, is particularly suitable for cultural heritage studies. This method has been widely developed for detection of indoor microbes and used for determination of microbes in indoor samples, e.g., house dust, building materials and air (Haugland et al., 1999, 2004; Pietarinen et al., 2008; Vesper et al., 2008; Kaarakainen et al., 2009). However, to date, qPCR has been successfully applied in only very few cultural assets studies. Imperi et al. (2007) used qPCR to investigate the relative abundance of eubacterial and archaeal populations in different wall painting areas suffering from rosy discoloration. Piñar et al. (2010) used qPCR for the specific and sensitive detection and quantification of a *Myxococcus xanthus* strain in a mixed culture used for biological consolidation of ornamental limestone. Martin-Sanchez et al. (2013) developed a qPCR to detect, quantify and monitor *Ochroconis lascauxensis* in the Lascaux Cave in France, being this fungus the principal causal agent of the black stains threatening the Paleolithic paintings of this UNESCO World Heritage Site.

Table 4 | Quantification results of the single-copy β-actin gene for the detection of fungal cells on parchment samples by qPCR.

Sample	Copies/ng extracted DNA
P1	168.01 ± 5.09
P2	573.84 ± 71.45
P3	337.12 ± 21.15
P5	178.66 ± 1.41
P6	380.55 ± 15.79
P7	680.09 ± 53.23
P8	187.55 ± 23.03

In this study, a qPCR method targeting the β-actin gene was developed for the quantitative assessment of fungi on different insulation materials. The advantage of this method relies in the quantification of a gene that has been proved to appear as a single actin gene copy per haploid genome in fungi (Gallwitz and Seidel, 1980; Ng and Abelson, 1980; Mertins and Gallwitz, 1987; Fidel et al., 1988; Voigt and Wostemeyer, 2000). This fact enables a more precise quantification of the actual amount of fungal cells in an environmental sample than when using universal rRNA primers, due to the great variation of the number of rRNA gene clusters in a genome and among species (Herrera et al., 2009). The results derived from qPCR analysis performed with the different interior insulation systems showed that fungal cells occurred in all samples. The β-actin gene copy number was in nearly all test items higher than the gene copy numbers detected in the samples installed *in situ* in a historical building. Only for the reed board with loam samples, a higher amount of fungal cells was measured after 18 and 32 months of the installation of this material in the building. These findings can be explained by a higher relative humidity in the room during the course of the experiment. The higher humidity further led to increased copy numbers detected on the bloated Perlite plaster and wooden soft-board, whereas the sprayed cellulose and the bloated Perlite board did not show increased cell counts. In these two last materials, the obtained β-actin gene numbers were generally very low and close to, or even below, the limit of detection of the assay. Therefore, the fungal contaminations in the samples can be assumed very low. Furthermore, these results show that the tested insulation materials do not represent optimal growth habitats for fungal colonization.

FUNGAL ABUNDANCE IN INSULATION MATERIALS: qPCR vs. CULTIVATION ANALYSES (CFU)

In parallel to the qPCR analyses, classical cultivation analyses were performed with these ecological interior insulation materials (Sterflinger et al., 2013). Results derived from cultivation analyses proved that actively growing fungi were present in all inoculated test items (see **Table S1**).

As seen in **Figure 1**, both methods showed that bloated Perlite plaster and wooden soft-board have the lowest and the highest fungal abundance, respectively. The only inconsistence between CFU and qPCR was seen for the sprayed cellulose. With the qPCR method, the sprayed cellulose showed a higher fungal abundance than the reed board, while with the CFU method the opposite was observed. The CFU and qPCR metrics showed a significant correlation ($p = 1.123 \times 10^{-9}$, cor = 0.70).

In summary, the results derived from the developed qPCR method correlated well with those obtained in our previous investigations of the different insulation systems using classical

cultivation analysis. Taking together the results obtained from both strategies, we conclude that, from the microbiological point of view, the most appropriate interior insulation system is the bloated Perlite plaster. This material achieved the best results: the lowest fungal abundance was detected using the developed qPCR assay and only very few fungal colonies were cultivated from this material. On the contrary, the wooden soft-board system showed to be the most unsuitable material for interior insulation, due to the highest fungal cell numbers detected on this material and the highest CFU values.

APPLICABILITY OF THE DEVELOPED qPCR PROTOCOL TO OTHER MATERIALS OF CULTURAL HERITAGE

To investigate the application range of the developed qPCR for the detection of cellular abundance in other materials of cultural heritage, the potential of this qPCR was tested on samples retrieved from old parchment manuscripts. These parchment samples were previously investigated due to their heavy damage, consisting of dark purple stains, holes and an unusual powdery consistency of pages. All investigated samples were proved to be colonized by fungi in a previous molecular survey. However, the isolation of fungi from these samples showed negative results (Piñar et al., 2011, 2014). In this study, the results derived from the qPCR analyses showed that this technique was enough sensitive to detect fungi in such valuable artifacts, from which usually a very tiny amount of sample can be obtained for microbiological and/or molecular analyses. This opens the possibility to apply this technique for assessing fungal abundance in other cultural assets, from which always sampling is a limiting step.

In conclusion this study shows that the presented qPCR methodology is a fast, sensitive, direct (without the need of cultivation), and reliable assay for accurately quantifying fungi in different insulation materials and samples of cultural heritage. The approach described can be used to provide new information about fungal abundance in building biological investigations and on microbial habitats on works of art and cultural heritage. Compared to classical cultivation techniques only small sample volumes are necessary which allow a minimal invasive sampling procedure, that is of great importance in the case of object of cultural heritage. Furthermore, the time effort for qPCR analysis is much lower and the drawbacks of cultivation assays, as selectivity and certain detection limits with the use of standard cultivation media, are avoided. Finally, this method enables long range phylogenetic studies at the nucleotide and amino acid level thanks to the sequence information gained from the qPCR, something that is not possible with the traditional CFU method.

AUTHOR CONTRIBUTIONS

Jörg Ettenauer took samples of the insulation materials in the historical building, did the incubation of test items in the climate chamber, the complete cultivation analysis and the real time experiments. Guadalupe Piñar did the DNA extraction of the parchment samples. Katja Sterflinger designed and managed the project on insulation materials, she is the project leader of the FFG project providing the financial basis of the work and she supervised the lab work. Hakim Tafer did the statistical analysis. Guadalupe Piñar and Jörg Ettenauer wrote the manuscript. Jörg Ettenauer, Guadalupe Piñar, Hakim Tafer, and Katja Sterflinger proof-read the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00262/abstract

Figure S1 | Representative curves showing the primer-specific quantification curves (A), standard curves (B), melt peak curve (C) and melt curve (D).

Table S1 | Summary of the cultivation results from our previous study (Sterflinger et al., 2013) for the insulation materials: calculated colony forming units (CFU) per gram material for the test items and the samples taken from the historical building after 18 and 32 months of installation.

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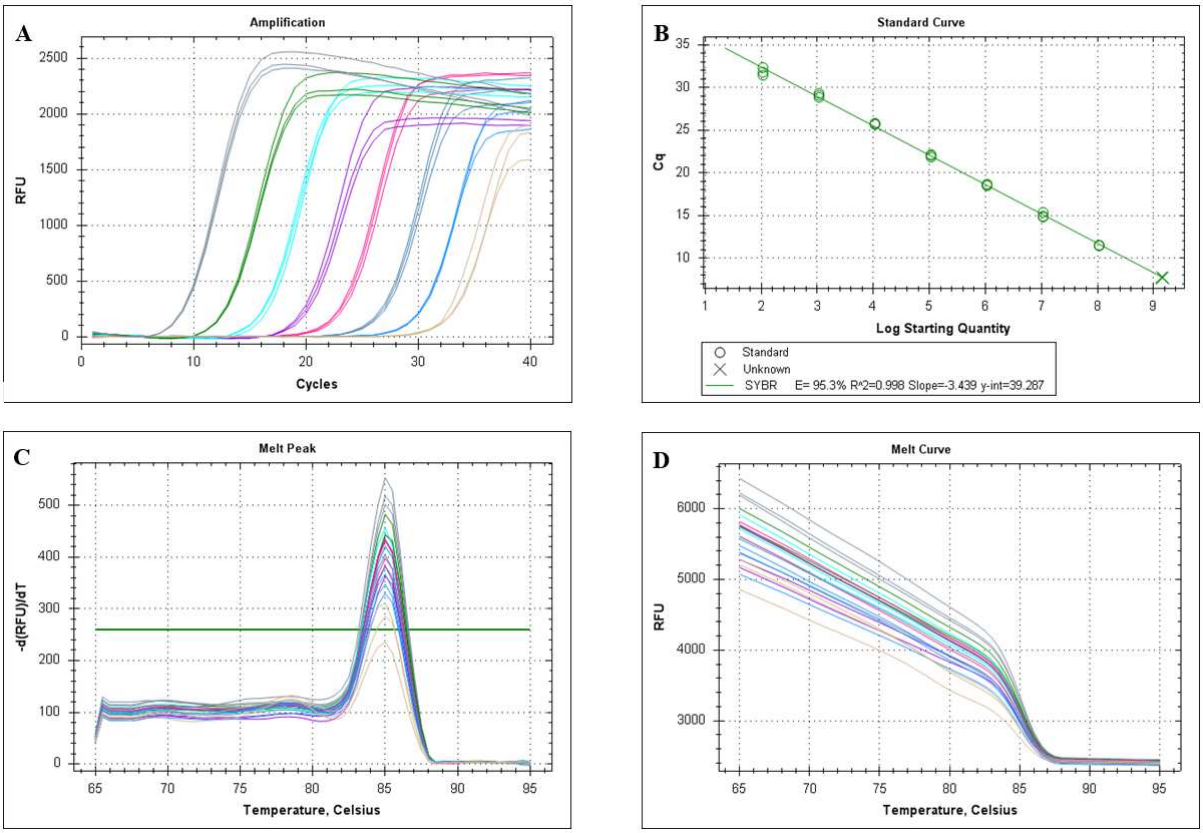
Supplementary Figure S1. Representative curves showing the primer-specific quantification curves (**A**), standard curves (**B**), melt peak curve (**C**) and melt curve (**D**).



Supplementary Table 1. Summary of the cultivation results from our previous study (Sterflinger *et al.*, 2013) for the insulation materials: Calculated colony forming units (CFU) per gram material for the test items and the samples taken from the historical building after 18 and 32 months of installation. Samples that did not show fungal growth are indicated with $< 10^2$. (a) MA 1610 *Cladosporium cladosporioides*, (b) MA 1615 *Aspergillus niger*, (c) MA 1701 *Penicillium chrysogenum*, (d) mixture of all three fungal spore suspensions.

Material	**Climate chamber**					**Historical building**			
	Inoculated strains - 6 months					**18 months**		**32 months**	
	a	**b**	**c**	**d**	**control**	**1st floor**	**2nd floor**	**1st floor**	**2nd floor**
Sprayed Cellulose	$1.70 \times 10^4 \pm 1.50 \times 10^3$	$7.25 \times 10^3 \pm 2.25 \times 10^3$	$4.93 \times 10^4 \pm 4.25 \times 10^3$	$7.00 \times 10^3 \pm 1.00 \times 10^3$	$< 10^2$	$< 10^2$	$< 10^2$	No sample	$< 10^2$
Bloated Perlite board	$7.58 \times 10^4 \pm 5.75 \times 10^3$	$9.68 \times 10^4 \pm 4.25 \times 10^3$	$5.70 \times 10^4 \pm 6.00 \times 10^3$	$7.50 \times 10^4 \pm 2.50 \times 10^3$	$< 10^2$	$< 10^2$	$< 10^2$	$2.50 \times 10^2 \pm 2.50 \times 10^2$	$< 10^2$
Bloated Perlite plaster	$5.50 \times 10^3 \pm 1.00 \times 10^3$	$2.50 \times 10^3 \pm 5.00 \times 10^2$	$2.10 \times 10^4 \pm 0$	$1.45 \times 10^4 \pm 2.50 \times 10^3$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
Wooden soft-board	$1.97 \times 10^6 \pm 1.00 \times 10^5$	$1.10 \times 10^6 \pm 7.50 \times 10^3$	$1.60 \times 10^6 \pm 4.50 \times 10^4$	$1.95 \times 10^6 \pm 5.00 \times 10^4$	$< 10^2$	$1.50 \times 10^3 \pm 0$	$1.50 \times 10^3 \pm 0$	$4.45 \times 10^4 \pm 2.00 \times 10^3$	$4.70 \times 10^4 \pm 2.00 \times 10^3$
Reed board with loam	$1.73 \times 10^4 \pm 2.75 \times 10^3$	$9.40 \times 10^4 \pm 2.00 \times 10^3$	$3.18 \times 10^4 \pm 1.25 \times 10^3$	$4.20 \times 10^4 \pm 0$	$< 10^2$	$1.63 \times 10^4 \pm 1.25 \times 10^3$	$1.20 \times 10^4 \pm 5.00 \times 10^2$	$6.00 \times 10^3 \pm 5.00 \times 10^2$	$6.75 \times 10^3 \pm 2.50 \times 10^2$

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Halophilic Microorganisms Are Responsible for the Rosy Discolouration of Saline Environments in Three Historical Buildings with Mural Paintings

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Abstract

A number of mural paintings and building materials from monuments located in central and south Europe are characterized by the presence of an intriguing rosy discolouration phenomenon. Although some similarities were observed among the bacterial and archaeal microbiota detected in these monuments, their origin and nature is still unknown. In order to get a complete overview of this biodeterioration process, we investigated the microbial communities in saline environments causing the rosy discolouration of mural paintings in three Austrian historical buildings using a combination of culture-dependent and -independent techniques as well as microscopic techniques. The bacterial communities were dominated by halophilic members of Actinobacteria, mainly of the genus Rubrobacter. Representatives of the Archaea were also detected with the predominating genera Halobacterium, Halococcus and Halalkalicoccus. Furthermore, halophilic bacterial strains, mainly of the phylum Firmicutes, could be retrieved from two monuments using special culture media. Inoculation of building materials (limestone and gypsum plaster) with selected isolates reproduced the unaesthetic rosy effect and biodeterioration in the laboratory.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files. The ribosomal sequences of the bacterial- and archaeal clones and the bacterial isolates have been deposited at the NCBI GenBank database under the accession numbers (KF692550–KF692709 for the cloned sequences and HG515390–HG515401 for the bacterial isolates).

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Introduction

It is well-known that microorganisms play a crucial role in the degradation and deterioration of mural paintings and building materials. Stone materials and wall paintings provide a great variety of ecological niches for all types of microorganisms that can induce biodeterioration. Biodegradation is caused by biochemical processes, through bio-corrosion, dissolution and solubilization of material components, however aesthetical effects are often more evident in some biodeterioration processes. The aesthetical changes are triggered by the deterioration of painting pigments on walls and/or by the formation of coloured biofilms or excretion of extracellular pigments. Fungi, algae, different bacteria and archaea produce a wide variety of biogenic pigments such as chlorophyll, carotenes, phenols, anthraquinones and melanin with colours ranging from light yellow, orange, pink, purple, violet, green, grey, dark brown to black [1–5]. The formation of orange to red pigments is due to the production of carotenes as a means of protecting the cells against high UV-radiation, chemical- and/or salt stress [6]. On salty walls the inhabiting halophilic bacteria and haloarchaea usually form pink to purple or violet stains. Orange pigmentations on sandstone or marble often resemble iron oxide and therefore it has to be clarified if the discolouration is due to microbial growth [7,8]. The biogenic pigments are usually very stable on the materials even if the causative microorganisms are already dead.

In this study we investigated the pink to rosy discolouration phenomenon presented by two historical chapels and a medieval castle located in Austria (Figure S1). The Johannes Chapel in Pürgg (Styria) was built in Romanesque style and dates back to the 12th century. The frescos inside with the famous and mysterious motive of the ''cats-mice-war'' was one of the most prominent and well-preserved Romanesque paintings in Europe, dated to 1160 (Figure 1A). After constructional changes on the whole west wall a rosy biofilm established which further spread across the whole chapel (Figure 1B).

The castle of Rappottenstein (Lower Austria) is a medieval castle, which was never conquered and is, therefore, one of the most well-preserved castles in Austria. It dates back to 12th century and combines three constructional ages with correspon-

Figure 1. The three historical buildings. The Johannes Chapel in Pürgg (A, B), the castle Rappottenstein (C) and the Saint Rupert Chapel in Weißpriach (D): A) The famous romanesque fresco "Cats-mice-war". B) Interior view onto the West-wall in the Johannes chapel in Pürgg which was totally covered with a rosy biofilm. C) Differently coloured biofilms cover the inner courtyard of the castle Rappottenstein with rosy strains spreading upwards. D) Pink discolouration of the wall in the tower-room of the Saint Rupert chapel in Weißpriach. All photographs were taken by Jörg D. Ettenauer.
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dent wall paintings: Romanesque, Gothic and Renaissance. The walls on the ground floor and the inner courtyard are covered with green to black biofilms but also a strong rosy discolouration of the walls can be observed that spreads to the inside of the rooms on the ground- and further up till the second floor (Figure 1C).

The Saint Rupert chapel of Weißpriach, in Salzburg is the oldest of the three buildings and dates back to 750 AD. The walls contain Romanesque decorative stones and many outstanding frescos that date back from the 10th to 13th century. Due to a leaky roof, water caused the formation of salt efflorescences and a strong rosy pigmentation can be found in the tower room (Figure 1D). During the last years the pigmentation extended into the actual chapel.

All three buildings are exposed to harsh climatic conditions due to their geographical location in alpine regions and/or their local site. Hence, all of them suffer from different types of water impact: i) water mostly enters the buildings from outside through the ground into the walls and ii) additionally water from rain and snow directly enters the buildings through a leaky roof, open windows or directly into the inner courtyard, respectively. As a result, a variety of hygroscopic, soluble salts in the porous buildings materials and wall paintings are solubilized and further migrate with the capillary water through the stone. The changes in the physical parameters lead to crystallization of the salts at the surfaces, the so-called salt efflorescences. Furthermore, the water impact and crystallization of salts cause a physical stress for the materials leading to cracking, powdering and flaking of the surface material and material loss [9].

Several reports have shown that ancient stone works and mural paintings represent a common habitat for extremely salt tolerant and moderate halophilic bacteria [10–13] and archaea [10,14]. The rosy discolouration of stone works in different historical buildings located in various parts of Europe was also already subject of different studies [3,15–17].

The objective of this work was the identification of the microorganisms present in the red to pink pigmented sites of the three buildings, in order to compare the data with those obtained in previous investigations on other monuments situated in different geographical locations of Europe. Finally, the results should be a basis to advice conservators in order to protect the buildings from further biodeterioration.

To analyse the microbial communities inhabiting the wall surface of the three buildings, samples were collected from different sites of each building where a rosy discolouration could be observed. Thereafter, to evaluate the whole structure of the microbial communities inhabiting the walls, three strategies were applied: Classical cultivation techniques, to identify the cultivable members of the microbial communities, molecular techniques - comprising direct DNA extraction, PCR amplification of ribosomal 16S rRNA gene sequences, DGGE analysis and construction and screening of clone libraries - to identify the non-cultivable fraction of the communities, and reproduction of the phenomenon in the laboratory using selected isolates and building materials.

Materials and Methods

Sampling

Stone samples with rosy biofilms (about 0.16 to 14.52 g) were taken from 4–16 different locations of each building, hereafter referred to as 'P'-samples from Pürgg, 'W'-samples from Weißpriach and 'R'-samples from Rappottenstein, respectively (Table S1). The samples were collected with sterile scalpels by scraping off wall material to a depth of 1–3 mm or by taking partly detached plaster pieces of 1–4 cm^2 from the walls using sterile forceps, respectively. Each aseptically collected sample was immediately stored in sterile plastic tubes or containers, respectively, and transported under cooled conditions to the laboratory for further analysis. The samplings were performed under the supervision of different conservators due to the high value of the objects. Permissions for sampling at each location were issued by the Ministry of Care of Monuments in Austria (Bundesdenkmalamt) as well as the local municipal office and church, respectively. Stone samples from similar locations of each building were mixed and divided into two parts. In the case of Pürgg the pooled samples were called P1, P2 and P3. Regarding the Saint Rupert chapel of Weißpriach, the mingled samples were named W1, W2 and W3 and R1, R2 and R3 for the castle of Rappottenstein, respectively (Table S1). The larger proportion of each sample (1.4–10 g) was

sent under cooled conditions to Spain for cultivation analyses and the smaller part was used for molecular analyses.

Cultivation analysis

All samples were inoculated at 28°C for 30 days on different culture media: trypticase soy agar supplemented with Na and Mg (TSA Na-Mg [18], TSA Na-Mg supplemented with 15% NaCl (w/v) instead of 3% NaCl (w/v), nutrient agar (Difco, Becton Dickinson, Sparks MD, USA) diluted 1:100, nutrient agar supplemented with NaCl (3%, w/v), marine agar 2216 (Difco, Becton Dickinson, Sparks MD, USA), DSMZ media 372 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium372.pdf) and 1018 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1018.pdf). The last two culture media were specific for Archaea. All orange or pink pigmented colonies that might be responsible for the aesthetical damage of the objects were picked up and transferred to fresh medium.

Molecular characterization of the isolated strains

Bacterial DNA was extracted following the method described by Marmur [19]. The 16S rRNA gene was amplified by PCR using the conserved primers 27F [20] and 1522R [21] with the following PCR thermal conditions: 95°C for 1 min; 35 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 2 min; and a final extension cycle at 72°C for 10 min. Forward and reverse strands of the amplified DNA fragment were sequenced in an ABI 3700 sequencer (Applied Biosystems). The identification of phylogenetic neighbours was carried out by the BLAST program [22] against the NCBI database and the database of type strains EZtaxon [23] with validly published prokaryotic names.

Molecular analysis - DNA extraction from the walls

The mingled samples of each building were ground for 2 minutes in liquid nitrogen using a sterile mortar and pestle. From the homogenized material each 100 mg were weighed in a Sartorius precision scale for DNA extraction. The complete microbial DNA was directly isolated using the FastDNA SPIN Kit for soil from MP Biomedicals (Illkrich, France). The DNA concentration, -quality and -purity, respectively, was measured using a NanoDrop ND-1000 spectrophotometer (peqLabBiotechnologie GmbH, Linz, Austria). Additionally, the extracted DNA was visualized on 1.5 (w/v) agarose gels at 110V for 40 minutes, stained in an ethidium bromide solution [1 $\mu g\ ml^{-1}$; stock: 10 mg ml^{-1}] for 20 minutes and documented using an UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

PCR amplification of bacterial 16S rDNA fragments

All PCR reactions were executed in a BioRad C1000 Thermal Cycler. The 2× PCR Master Mix (Promega, Mannheim, Germany) [50 units ml^{-1} of TaqDNA Polymerase in a supplied reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$] was diluted to 1×, 12.5 pmol of each primer and 2.5 µl of template DNA were added to 25 µl total reaction volumes.

For genetic fingerprinting of the eubacterial 16S rDNA fragments by DGGE two different PCR reactions were performed. For the first round the universal primers 341f [24] and 907r [25] were used. The second round, a semi-nested PCR for genetic fingerprints, was done using the primers 341GC and 518r [26]. The forward primer possesses a 40-base Guanine-Cytosine (GC) clamp at its 5′ end that stabilizes the melting behaviour of the DNA fragments in DGGE analysis [24]. The semi-nested PCR was executed in 100 µl volumes, separated into two tubes to which each 50 µl mastermix, 25 pmol of each primer and 3.5 µl of template were added. The thermocycling conditions described by Schabereiter-Gurtner et al. [27] were used for genetic fingerprinting. Seven microliter of each PCR product was electrophoresed on a 2% (w/v) agarose gel as described above. In each PCR reaction a negative control was included, where no DNA template was added, to exclude the possibility of cross-contamination.

PCR amplification of archaeal 16S rDNA fragments

The amplification of archaeal 16S rDNA fragments was carried out similar to the bacterial PCR analysis with the addition of BSA (25 pmol in 25 µl reaction volume) to the mastermix. For the first round the primers ARC344 and ARC915 [28] were applied, using the thermocycling program described by Piñar et al. [29]. To obtain genetic fingerprints a semi-nested PCR was performed with primers 518r carrying a GC clamp at its 5′ end [24] and the archaea specific primer ARC344. The same cycling conditions were used as described for the amplification of the bacterial 16S rDNA.

Fingerprint analysis by DGGE – Denaturing Gradient Gel Electrophoresis

For DGGE fingerprinting 100 µl PCR products from the semi-nested PCR were pooled, precipitated overnight with 96% ethanol at −20°C and re-suspended in 20 µl ultra-pure water (Qiagen GmbH, Hilden, Germany). The concentrated PCR products supplemented with 5 µl 6× Loading Dye Solution (Thermo Scientific) were separated on gels in 0.5× TAE buffer [20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 8.0] for 3.5 hours at 200 V and 60°C in a Bio-Rad-DCode – Universal Mutation Detection System [24]. A linear chemical gradient ranging from 35 to 55% of urea and formamide in an 8% (w/v) polyacrylamide gel (Bio-Rad, Munich, Germany) for screening of bacterial communities and from 35 to 50% for separation of bands of the archaeal population was used. After completion of electrophoresis staining of the gels was done in an ethidium bromide solution for 20 minutes and afterwards visualized by a UVP documentation system.

Construction of 16S rDNA clone libraries and screening by DGGE

In order to obtain phylogenetic identification data on the inhabiting microorganisms, two clone libraries of each sample containing the bacterial or the archaeal 16S rDNA fragments, respectively, were created. Therefore, 2×3.5 µl DNA templates of each sample were amplified in 2×50 µl reaction volumes using the primers for the first round as mentioned above. Aliquots of the PCR products were electrophoresed, purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and re-suspended in 30 µl ultra-pure water. The purified DNA was again analysed by gel electrophoresis and 5.5 µl were used as a ligation template for the pGEM-T easy Vector system (Promega). The ligation products were transformed into One Shot TOP10 cells (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Recombinant cells (white colonies) could be identified on indicator LB medium containing ampicilline (100 $\mu g\ ml^{-1}$), streptomycine (25 $\mu g\ ml^{-1}$) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 0.1 mM) [30].

About 50–150 white colonies from each clone library were harvested and screened by DGGE as described by Schabereiter-Gurtner et al. [27]. The band positions of the clones were

compared with the DGGE fingerprint of the original sample and inserts of clones matching dominant- and faint bands of the banding profile of the original sample were selected for sequencing.

16S rDNA sequencing and sequence analysis

In 2×50 µl reaction volumes with each 3 µl template DNA of the clone inserts were amplified using the vector specific primers SP6 and T7 (Promega, Mannheim, Germany) [27]. After visualization on agarose gels and purification of the pooled PCR products, 25 µl aliquots were sent to GATC Biotech sequencing service (www.gatc-biotech.com). Comparative sequence analysis was done by comparing pair-wise insert sequences with those available in the online databases provided by the NCBI (National Centre for Biotechnology Information), and RDP (Ribosomal Database Project), respectively, using the search program BLAST [22]. The ribosomal sequences of the bacterial- and archaeal clones and the bacterial isolates have been deposited at the NCBI GenBank database under the accession numbers (KF692550–KF692709 for the cloned sequences and HG515390–HG515401 for the bacterial isolates) listed in Tables S2, S3 and S4, for each 16S rDNA sequence.

Laboratory-based colonization experiment of building materials

Strains of *Halobacillus naozhouensis* and *Kocuria polaris* were used for inoculation and reproduction of biodeterioration processes in the laboratory. Gypsum plasterand Hontoria limestone [31] were sliced into squares of 3×3×0.5 cm, sterilized in an autoclave under fluent vapor and inoculated with suspensions of each bacterium. Probes of gypsum plaster and limestone were inoculated with three different suspensions of cells (A: *Halobacillus naozhouensis*, B: *Kocuria polaris* and C: mixture of both strains) at concentrations of 1.5×10^9 cells ml−1. All probes were inoculated with 150 µL of suspension and incubated at 30°C for one month.

Field Emission Scanning Electron Microscopy (FESEM)

FESEM was used to accurately assess surface topography, microbial growth and biodeterioration phenomena on the inoculated gypsum plaster and limestone probes. Bulk fragments were directly mounted on a sample stub and sputter coated with a thin gold/palladium film. Subsequently, samples were examined on a Jeol JSM-7001F microscope equipped with an Oxford X-ray energy dispersive spectroscopy (EDS) detector. FESEM examinations were operated in secondary electron (SE) detection mode with an acceleration potential of 15 kV.

Results

Phylogenetic identification of the cultivated microorganisms

During the first 48 hours of incubation up to a maximum of 12 days, twenty-nine bacterial strains with yellow to orange or pink appearance were isolated. Coloured bacterial strains isolated from sample P2 of the Johannes Chapel in Pürgg represented 41.4% of all isolated strains, and, on average, from all three samples of the castle Rappottenstein 19.5%. No cultivable bacteria were found on samples from Weißpriach, which might be due to the known difficulties in culturing these pigmented, halophilic microorganisms and perhaps also due to the low number of samples as well as very small sample amounts that could be taken from this location (Table S1). Similarly, no archaea could be isolated from none of the samples. The molecular identification of the isolated strains is shown in Table 1 and also in Table S2 where the detailed information about each isolate, its closest related neighbour, the isolation source as well as the accession number for the submitted 16S rDNA sequence are given. According to the NCBI database, the bacterial strains showed similarity values ranging from 98 to 100% and could be grouped to cultured members of three different bacterial phyla, namely the Firmicutes (89.7% of all isolated strains), the Proteobacteria (6.9%) and the Actinobacteria (3.5%).

Out of the twelve isolates obtained from sample P2, two isolates from Pürgg were closely affiliated with *Planococcus salinarum* (98%) and the other bacterial strains were highly related to *Halobacillus herbersteinensis* (99%) - all belonging to the phylum Firmicutes (Table S2).

From the castle of Rappottenstein seventeen isolates could be grown on the used culture media: ten bacteria from sample R1, six from sample R2 and one from sample R3. Five isolates from sample R1 showed the best match in the NCBI database search with *Halobacillus herbersteinensis* (99%). Further, three bacteria were affiliated with *Marinococcus luteus* (99%) and one bacterium with *Paracoccus marcusii* (100%), - being all representatives of the phylum Firmicutes. Only one strain showed to be related to members of the Proteobacteria, namely to *Halomonas muralis* (98%). Out of the six cultured bacteria from sample R2, five isolates affiliated also with members of the Firmicutes. Two of them were related to cultivable *Planomicrobium flavidum* (98%), another one to *Planococcus psychrotoleratus* (99%), one to *Planococcus antarcticus* (99%) and one to *Planococcus donghaensis* (99%). The last strain of sample R2 was the only member of the Actinobacteria, which could be identified as *Kocuria rosea* (99%). The only isolate obtained from sample R3 was similar to *Planococcus psychrotoleratus* (99%) belonging to the Firmicutes phylum (Table S2).

Alternatively, using EZtaxon (a 16S rRNA gene sequence database of type strains), NCBI affiliations changed and *Halobacillus herbersteinensis* was identified as *Halobacillus naozhouensis*, *Marinococcus luteus* as *Marinococcus tarijensis*, *Planococcus psychrotoleratus* either as *Planococcus okeanokoites* or *Planococcus donghaensis*, *Planococcus antarcticus* as *Planococcus donghaensis*, and *Kocuria rosea* as *Kocuria polaris* (Table S2).

Phylogenetic identification of the microbial communities using molecular techniques

The second part of each mixed sample was subjected to direct DNA extraction to further elucidate the non-cultivable microbiota. The bacterial 16S rDNA could be amplified by PCR using universal primers from all investigated samples. However, the amplification of archaeal ribosomal DNA was possible for nearly all samples except from sample P1 of Pürgg. DGGE fingerprint analyses were conducted with the amplified 16S rDNA to obtain information on the diversity present on the walls. The received fingerprints of the inhabiting bacterial and archaeal communities of all investigated samples from the three buildings are shown in Figures 2 and 3, respectively. The band numbers of all identified clones are indicated in Tables S3 and S4 and in Figures 2 and 3 to allow an easy tracking of the corresponding band in the community profile of each sample.

The bacterial fingerprints showed to be rather complex with three to seven dominant bands and many faint bands (Figure 2). The DGGE profiles from the different samples of each building and among the different buildings were very similar. The archaeal DGGE analysis showed rather simple banding profiles with two to four dominant bands and only a few faint bands (Figure 3). As already observed on the DGGE profiles derived from the bacteria,

Table 1. Phylogenetic analysis.

Culture-independent analysis						
Bacteria		**Samples - Building**	**Pürgg**	**Rappottenstein**	**Weißpriach**	**Total**
Phylum	Phylogenetic group	Genus				
Firmicutes	Bacillales					**3 (2.8%)**
		Natribacillus		1 (2.9%)		*1 (0.9%)*
		Planococcus		1 (2.9%)		*1 (0.9%)*
		Bacillus		1 (2.9%)		*1 (0.9%)*
Proteobacteria						**2 (1.9%)**
	β-Proteobacteria	*Ralstonia*		1 (2.9%)		*1 (0.9%)*
	γ-Proteobacteria	Uncultured Proteobacteria		1 (2.9%)		*1 (0.9%)*
Actinobacteria						**72 (67.3%)**
	Rubrobacteridae					*44 (41.1%)*
		Rubrobacteracea/*Rubrobacter*	8 (19.5%)	7 (20%)	8 (25.8%)	*23 (21.5%)*
		Uncultured Rubrobacteridae	13 (31.7%)	5 (14.3%)	3 (9.7%)	*21 (19.6%)*
		Uncultured Actinobacteria	8 (19.5%)	4 (11.4%)	6 (19.4%)	*18 (16.8%)*
	Actinobacteridae	*Actinomycetospora*	1 (2.4%)			*1 (0.9%)*
		Saccharopolyspora		1 (2.9%)	1 (3.2%)	*2 (1.9%)*
		Thermocrispum		1 (2.9%)		*1 (0.9%)*
		Amycolatopsis	1 (2.4%)			*1 (0.9%)*
		Pseudonocardia		1 (2.9%)		*1 (0.9%)*
		Nocardioides	1 (2.4%)			*1 (0.9%)*
		Jiangella		1 (2.9%)		*1 (0.9%)*
		Nesterenkonia	1 (2.4%)			*1 (0.9%)*
		Janibacter		1 (2.9%)		*1 (0.9%)*
Unclassified bacterium clone			8 (19.5%)	9 (25.7%)	13 (41.9%)	30 (28%)
Total no. of clones			**41 (100%)**	**35 (100%)**	**31 (100%)**	**107 (100%)**
Archaea		**Samples - Building**	**Pürgg**	**Rappottenstein**	**Weißpriach**	**Total**
Phylum	Family	Genus				
Euryarchaeota						**39 (73.6%)**
	Halobacteriaceae					38 (71.7%)
		Halococcus	9 (42.9%)	6 (33.3%)	6 (42.9%)	*21 (39.6%)*
		Halobacterium	5 (23.8%)	2 (11.1%)	2 (14.3%)	*9 (17%)*
		Halalkalicoccus		5 (27.8%)	2 (14.3%)	*7 (13.2%)*
		Natronorubrum		1 (5.6%)		*1 (1.9%)*
	Order Halobacteriales	*Haloarchaeon Nie 13*	1 (4.8%)			1 (1.9%)
Unclassified archaeon clone			6 (28.6%)	4 (22.2%)	4 (28.6%)	14 (26.4%)
Total no. of clones			**21 (100%)**	**18 (100%)**	**14 (100%)**	**53 (100%)**
Culture-dependent analysis						
Bacteria		**Samples - Building**	**Pürgg**	**Rappottenstein**	**Weißpriach**	**Total**
Phylum	Family	Genus				
Firmicutes	Bacillales					**26 (89.7%)**
		Halobacillus	10 (83.3%)	5 (29.4%)		*15 (51.7%)*
		Planococcus	2 (16.7%)	4 (23.5%)		*6 (20.7%)*
		Marinococcus		3 (17.6%)		*3 (10.3%)*
		Planomicrobium		2 (11.8%)		*2 (6.9%)*
Proteobacteria						**2 (6.9%)**
	α-Proteobacteria	*Paracoccus*		1 (5.9%)		*1 (3.5%)*
	γ-Proteobacteria	*Halomonas*		1 (5.9%)		*1 (3.5%)*

Table 1. Cont.

Culture-independent analysis						
Bacteria		**Samples - Building**	**Pürgg**	**Rappottenstein**	**Weißpriach**	**Total**
Actinobacteria						**1 (3.5%)**
	Actinomycetales	*Kocuria*		1 (5.9%)		*1 (3.5%)*
Total no. of isolates			**12 (100%)**	**17 (100%)**		**29 (100%)**

The results from the culture-independent and -dependent analysis of the samples of the three buildings are shown according the NCBI database search. Number of clones/isolates and calculated percentages (rounded values) indicate the amounts of the 16S rDNA sequences related to the corresponding phylum, phylogenetic group and genera, respectively.
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generally, the archaeal DGGE fingerprints showed a high homology among the different samples investigated from all three buildings.

Phylogenetic identification of the bacterial clone sequences

A total of 107 clones were selected based on the DGGE patterns shown in Figure 2. Clones were sequenced and identified as unclassified Bacterium clone sequences (28% of all selected clones) and grouped to representatives of the following three phyla: Actinobacteria (67.3%), Firmicutes (2.8%) and Proteobacteria (1.9%). Generally, the bacterial 16S rDNA sequences from clone inserts showed similarities ranging from 92 to 100% to known sequences in the used databases (Table S3).

Generally, most identified 16S rDNA clone sequences affiliated with uncultured cloned sequences (75.7% of all selected clones) but also with some cultured bacterial strains (24.3%). Three cloned sequences, all belonging to the order Bacillales of the Firmicutes, were identified as the species *Natribacillus halophilus*, *Bacillus agaradhaerens* and as a *Planococcus* sp. Only two 16S rDNA sequences were related to members of the classes Beta- and Gammaproteobacteria, namely to *Ralstonia insidiosa* and an uncultured gammaproteobacterium. The majority of the detected clone sequences were affiliated with members of the phylum Actinobacteria. Thereof, 44 clones were related to the subclass Rubrobacteridae, six clones to the suborder Pseudonocardineae, two clones to the Micrococcineae and one clone each to Nocardioidaceae and Jiangellaceae, respectively. In Table 1 the distribution to the different phyla and genera is given for the bacterial clone sequences obtained from the samples of all three historical buildings.

Phylogenetic identification of the non-cultivable archaeal community

The DGGE patterns of the archaeal community are shown in Figure 3. A total of 53 clones were chosen for sequence analysis. The results allowed a grouping into unclassified archaeon clones (26.4% of all selected clones) and representatives of the phylum Euryarchaeota, namely to the order Halobacteriales (73.6%). Thereof, 38 were grouped to the family Halobacteriaceae (71.7%) and further to the genera *Halococcus* (39.6%), *Halobacterium* (17%) and *Halalkalicoccus* (13.2%). In general, the comparative sequence analysis revealed similarity values ranging from 95 to 99% with known sequences from the NCBI database (Table S4).

As already observed for the bacterial cloned sequences, the identified archaeal 16S rDNA inserts generally affiliated with uncultured cloned sequences (62.3% of all selected clones) and to 20 cultured archaeal species (37.7%) in the database. Table 1 shows the distribution of the cloned archaeal 16S rDNA sequences to the different phyla and genera.

Biodeterioration of building materials by halophilic microorganisms under laboratory conditions

Visual inspection of the gypsum plaster and Hontoria limestone probes inoculated with Halobacillus naozhouensis and Kocuria

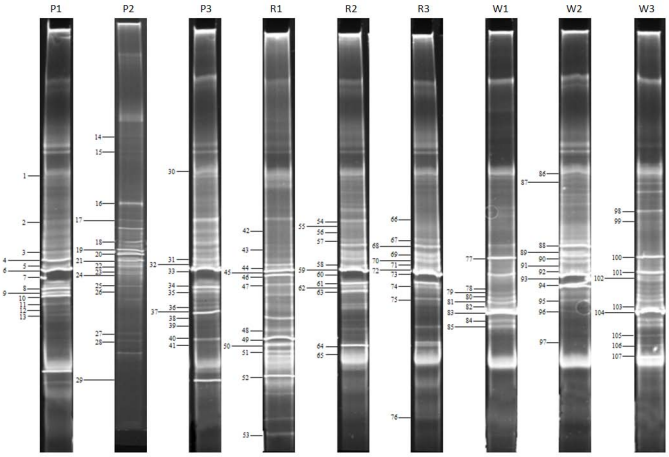

Figure 2. DGGE analysis of the bacterial communities present in the samples from the three buildings. The selected clone sequences are marked with bars, numbered consecutively and are detailed explained in Table S3.
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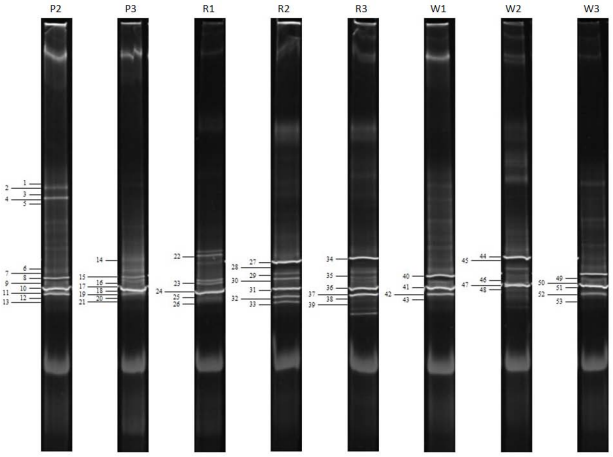

Figure 3. DGGE analysis of the archaeal communities present in the samples from the three buildings. The selected clone sequences are marked with bars, numbered consecutively and are detailed explained in Table S4.
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polaris revealed that the laboratory-based colonization experiment led to the development of rosy coatings over the surface of the probes, particularly on the gypsum plaster (Figure 4A–D). Although less apparent, rosy discolouration was also observed on the limestone probes mainly within the pores of this lithotype due to its high open porosity and cell penetration into the limestone pores (Figure 5A–D).

FESEM images of the surface of the inoculated gypsum plaster and Hontoria limestone probes displayed the crystal morphology of gypsum and calcite minerals, respectively, as well as dense microbial mats spread all over the probe surfaces (Figure 4 and Figure 5). Great amount of coccoid cells, corresponding to Kocuria polaris, embedded in extracellular polymeric substances (EPS) were found on both type of materials (Figure 4F, G and Figure 5F, G). Rod-shaped cells were less frequently observed on the probes inoculated with both strains (Figure 5G).

In addition, gypsum mineral grains were found extensively etched, evidencing microbe-mineral interactions. The etched mineral surfaces presented imprints of microbial cells, rough texture and rounded edges (Figure 4F, G). Active dissolution is evidenced by the penetration of coccoid cells into the mineral substratum, producing etching features in the form of shallow imprints or cavities on the mineral substratum (Figure 4F, G, H arrows). In Figures 4G and 4H it is also clearly noticeable the signs of dissolution on the crystal surfaces, which were not observed on the non-inoculated gypsum plaster probes (Figure 4E). These dissolution features were much lesser noticed on the surface of calcite crystals than on the gypsum substratum (Figure 5F, H arrows).

Discussion

In this study culture-based and molecular methods were combined to get a complete overview of the microbial communities responsible for the rosy pigmentation in the buildings. The classical cultivation techniques offer the possibility to isolate the microorganisms responsible of the phenomenon and to reproduce it in the laboratory. Moreover, cultivation offers the ability to visualize the pigmentation of the grown strains as an important agent of aesthetical damage on the objects. Different culture media that were already successfully used for the isolation of microorganisms from historical buildings [18] were applied. Additionally, the composition of the used media was adapted to the natural conditions by adding different salts in various concentrations. Nevertheless, it is well-known that by using only standard cultivation techniques with conventional laboratory media, only a small proportion of the total inhabiting bacterial population can be cultivated [32]. Therefore, molecular techniques using PCR amplification of ribosomal 16S RNA genes, creation of clone libraries and the screening by DGGE and identification via sequence analysis, were additionally used in this project. This combination of different microbiological methods allowed the coverage of a wider spectrum of the microbial ecosystem present in the rosy discoloured wall materials.

Compared to the culture dependent approach, molecular analysis showed a higher biodiversity, with bacteria belonging to different genera. Generally, 57.5% of all 16S rDNA sequences were originally detected in historical buildings or paintings from which 62.6% corresponded to bacterial- and 47.2% to archaeal clone sequences (Table 2). Also 55.2% of the bacterial isolates were previously found in this type of buildings. The bacterial clone sequences showed a great dominance of members of the Actinobacteria, where more than 67% of all analysed sequences affiliated with this phylum (Table 1). Thereof, 41.1% grouped to the subclass Rubrobacteridae. About 9 to 19% of all identified clone sequences were also detected on one or both of the other buildings investigated in this study (subsets in Table 2).

Forty-four sequences (27.5%) were related to samples taken from the Saint Catherine chapel in castle Herberstein (Austria). The walls of this chapel also showed a strong rosy discolouration due to microbial colonization. Nineteen sequences (11.9%) were related to sequences retrieved from different studies performed at the subterranean Saint Virgil chapel in Vienna. The subterranean chapel suffered from water-infiltrations through the walls and as a result widespread salt efflorescences were visible by naked eye in the whole building [10–15,17,33].

Fifteen sequences (9.4%) were related to sequences detected in a study performed by Piñar et al. [16] about the microbiota in the Capuchin catacombs of Palermo (Italy). Also there - and similar to the Saint Virgil chapel - water was migrating horizontally into the walls, thus leading to extensive rosy discolourations and the precipitation of salts on the walls. Another fifteen sequences (9.4%) showed to be related with cultured strains isolated from rosy discoloured ancient wall paintings of the Crypt of the Original Sin (Matera, Italy) which was performed by Imperi et al. [3].

Jurado et al. [18] and Laiz et al. [34] identified a *Rubrobacter* community in Roman tombs (Carmona, Spain) and the interior walls of the Vilar de Frades Church (Barcelos, Portugal). In this study seven (4.4%) 16S rDNA sequences that were related to the mentioned works were found. Finally eight sequences (5%) were related to four different historical locations, which partly also showed the pink pigmentation phenomenon: the church of Saint Anna im Feld, Germany [17], the Roman Necropolis of Carmona, Spain [14,29,34], the Tomb of the Monkeys, Italy [35] and an old mould-damaged building [36].

Members of the genus *Rubrobacter* represented around 20–26% of clones retrieved from the three monuments studied. The species of this genus are difficult to isolate, required culture media specifically designed for such purpose and some of the strains obtained represented new species [18]. A novel *Rubrobacter* species isolated from Roman tombs [34] is waiting for description. *Rubrobacter* seems to be associated to phototrophic microorganisms as illustrate Figure 1C and reported by Laiz et al. [34]. These authors stated that *Rubrobacter* strains play an active role in

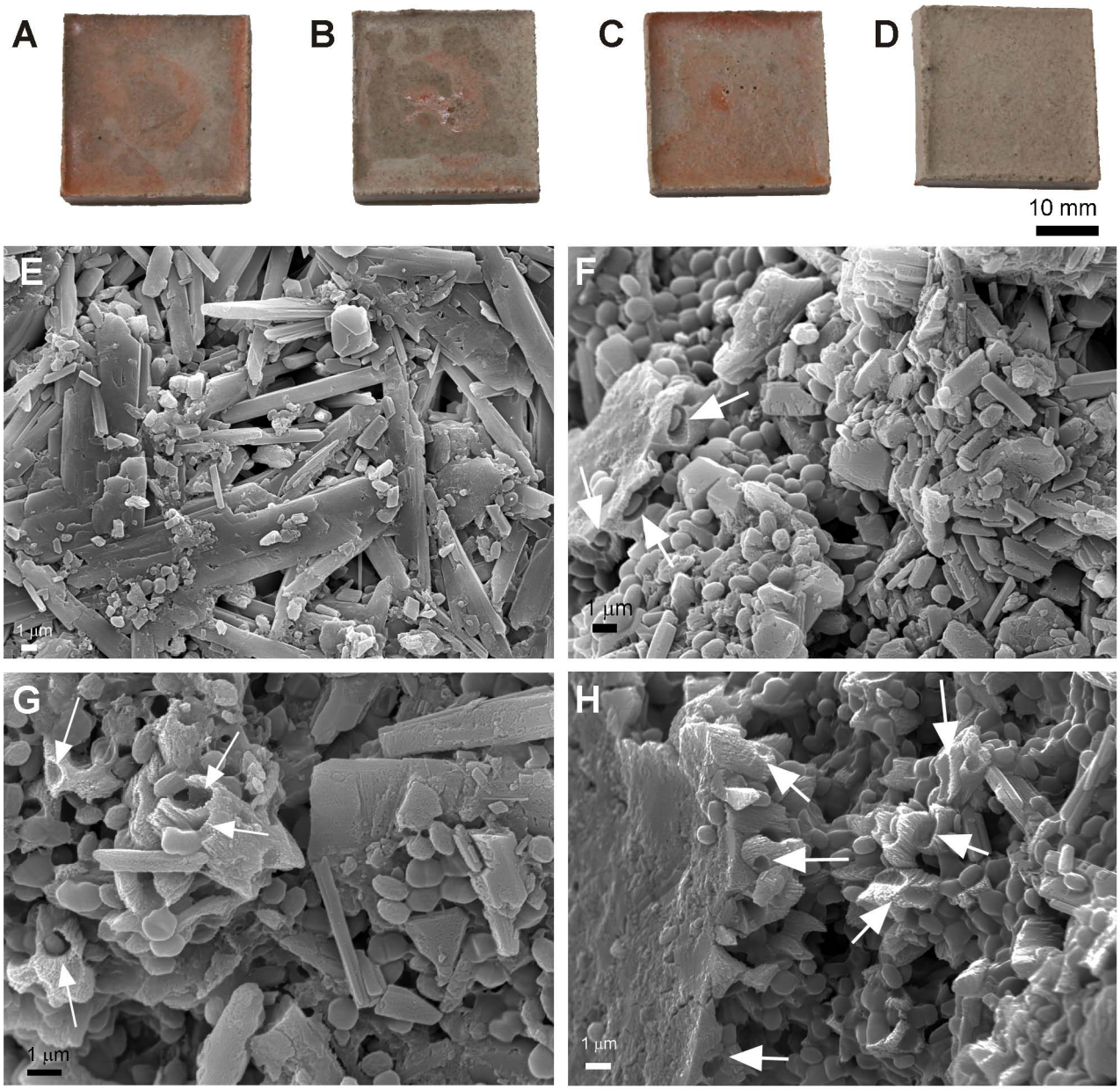

Figure 4. Colonization on gypsum plaster and FESEM images. Upper view of one representative gypsum plaster probe after one month of incubation: A) probe inoculated with *Halobacillus naozhouensis*, B) probe inoculated with *Kocuria polaris*, C) probe inoculated with the mixture of both strains, and D) non-inoculated gypsum plaster probe; and corresponding FESEM images depicting: E) gypsum mineral grains of a non-inoculated probe, F) microbial cells spread all over the surface and coccoid cells actively penetrating into the gypsum substratum (arrows), G) Gypsum minerals extensively etched (arrows, H) dissolution cavities or imprints of bacterial cells on calcium sulfate mineral grains. All photographs were taken by Cesareo Saiz-Jimenez and Ana Z. Miller, respectively.
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efflorescence niches and in mineral precipitation, and contribute to biodeterioration processes.

Classical cultivation experiments allowed the isolation of *Halomonas muralis* which was previously cultivated by Heyrman et al. [33] from the walls of the Saint Catherine chapel from castle Herberstein. Four years later Ripka et al. [13] were able to cultivate different *Halobacillus* species (*Halobacillus herbersteinensis*) from the same location. We were able to isolate a similar strain from samples of the Johannes chapel in Pürgg as well as from the castle Rappottenstein, showing that 51.7% of all isolates were detected on both buildings (subsets in Table 2).

Further eight isolates (27.6%) were originally found in saline environments, like marine solar salterns (*Planococcus salinarum* and *Planomicrobium flavidum*) [37,38], marine sediments (*Kocuria rosea*) [39] or a salt mine (*Marinococcus luteus*) [40], respectively. *Planococcus donghaensis*, *Paracoccus marcusii* and *Planococcus antarcticus* were previously detected in soil material from the Antarctic, from coastal regions in South Korea [41] and in soil of Issyk Kul region in Kyrgyzstan, respectively.

Bacterial strains that showed a yellow, orange or pink colour when cultivated on different media were selected for this study. Additionally, some of the microorganisms that were detected with

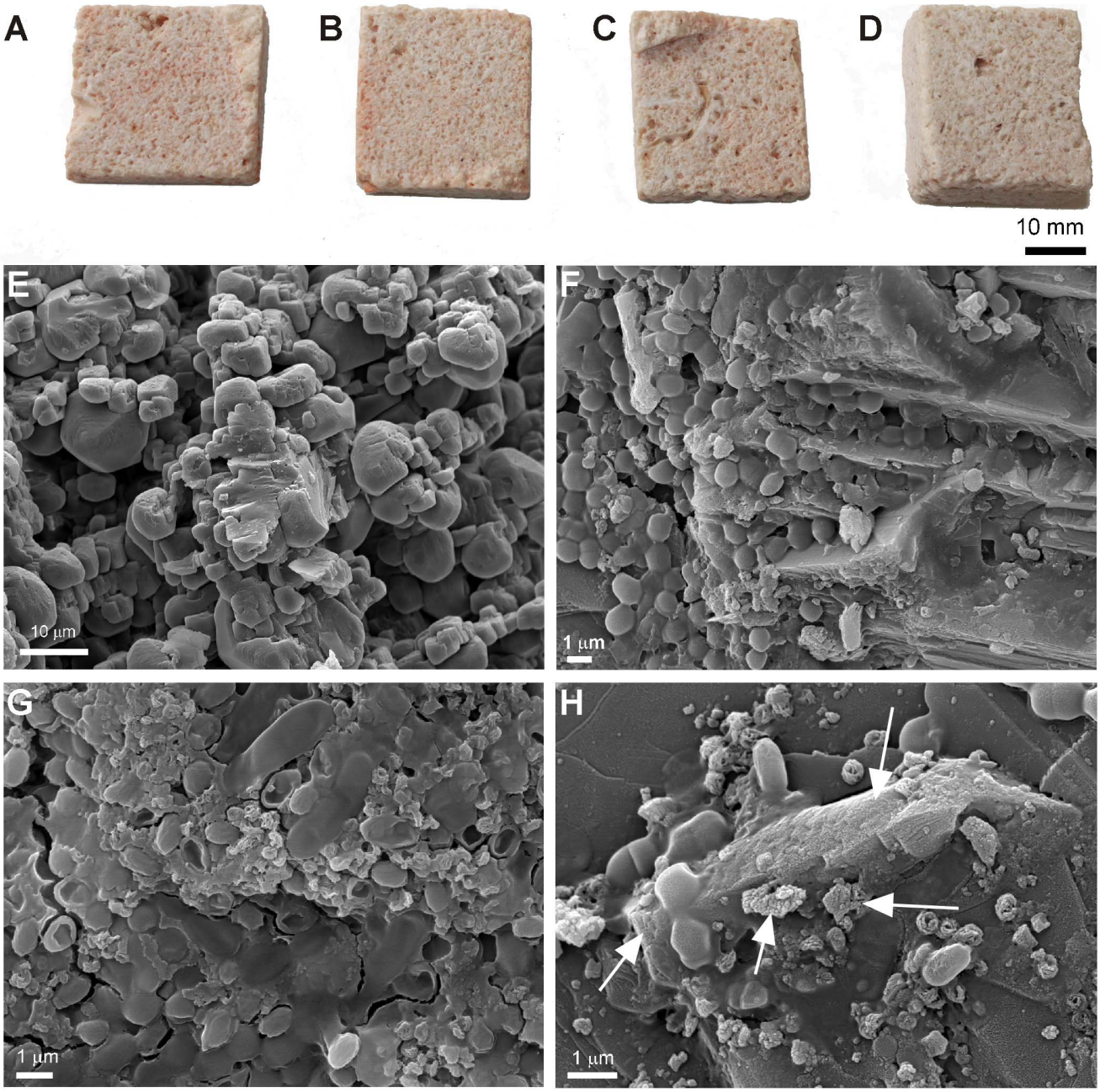

Figure 5. Colonization on limestone and FESEM images. Upper view of one representative Hontoria limestone probe after one month of incubation: A) probe inoculated with *Halobacillus naozhouensis*, B) probe inoculated with *Kocuria polaris*, C) probe inoculated with the mixture of both strains, and D) non-inoculated limestone probe; and FESEM images of the inoculated limestone probes depicting: E) calcium carbonate grains of a non-inoculated stone probe, F) microbial cells spread all over the surface and within stone cavities embedded in EPS, G) coccoid and rod-shaped cells on a limestone probe inoculated with both strains, H) calcium carbonate mineral grain showing signs of dissolution (arrows). All photographs were taken by Cesareo Saiz-Jimenez and Ana Z. Miller, respectively.
doi:10.1371/journal.pone.0103844.g005

molecular methods are also known for their pigmented colonies ranging from light yellow to light pink, orange, rosy to red or brown due to the production of the characteristic carotenoids bacterioruberin and monoanhydrobacterioruberin such as *Nesterenkonia xinjiangensis*, *Janibacter corallicola*, *Natribacillus halophilus*, *Saccharopolyspora salina* and *Rubrobacter* sp. [18,34,42,43]. All of those strains are moderately to extremely halophilic bacteria that can grow up to a maximum NaCl concentration of 7–23% (w/v).

By using culture dependent techniques members of the Firmicutes phylum were predominantly found in the samples (89.7%), whereas the actinobacterial fraction represented the smallest part of the isolated strains (3.5%). Conversely, the application of molecular methods for the identification of the bacteria showed primarily representatives of the Actinobacteria (67.3%) and the Firmicutes accounted only for 2.8% of the cloned sequences.

Table 2. Sequence affiliations.

16S rDNA clones

Bacteria	**Affiliation with sequence from**				**Clones without a match**			**Clones matching each other from different buildings**			
	historical building/ painting	**salt environment**	**soil**	**other environments**	**P**	**R**	**W**	**P⊆R**	**P⊆W**	**R⊆W**	**P⊆R⊆W**
P-clones (41)	25 (61%)	3 (7.3%)	12 (29.3%)	1 (2.4%)	13 (31.7%)			10 (24.4%)	9 (22%)		9 (22%)
R-clones (35)	20 (57.1%)	6 (17.1%)	9 (25.7%)			20 (57.1%)		5 (14.3%)		5 (14.3%)	5 (14.3%)
W-clones (31)	22 (71%)	2 (6.5%)	6 (19.4%)	1 (3.2%)			9 (29%)		11 (35.5%)	5 (16.1%)	6 (19.4%)
Total no. (107)	*67 (62.6%)*	*11 (10.3%)*	*27 (25.2%)*	*2 (1.9%)*	13 (12.1%)	20 (18.7%)	9 (8.4%)	15 (14%)	20 (18.7%)	10 (9.3%)	20 (18.7%)
Archaea	**Affiliation with sequence from**				**Clones without a match**			**Clones matching each other from different buildings**			
	historical building/ painting	**salt environment**	**soil**	**other environment**	**P**	**R**	**W**	**P⊆R**	**P⊆W**	**R⊆W**	**P⊆R⊆W**
P-clones (21)	11 (52.4%)	5 (23.8%)	4 (19%)	1 (4.8%)	10 (47.6%)			3 (14.3%)	6 (28.6%)		2 (9.5%)
R-clones (18)	6 (33.3%)	9 (50%)	1 (5.6%)	2 (11.1%)		9 (50%)		3 (16.7%)		4 (22.2%)	2 (11.1%)
W-clones (14)	8 (57.1%)	4 (28.6%)		2 (14.3%)			2 (14.3%)		5 (35.7%)	3 (21.4%)	4 (28.6%)
Total no. (53)	*25 (47.2%)*	*18 (34%)*	*5 (9.4%)*	*5 (9.4%)*	10 (18.9%)	9 (17%)	2 (3.8%)	6 (11.3%)	11 (20.8%)	7 (13.2%)	8 (15.1%)

Bacterial isolates

	Affiliation with sequence from				**Clones without a match**		**Clones matching each other from different buildings**
	historical building/ painting	**salt environment**	**soil**	**other environments**	**P**	**R**	**P⊆R**
P-samples (12)	10 (83.3%)	2 (16.7%)			2 (16.7%)		10 (83.3%)
R-samples (17)	6 (35.3%)	6 (35.3%)	3 (17.6%)	2 (11.8%)		12 (70.6%)	5 (29.4%)
Total no. (29)	*16 (55.2%)*	*8 (27.6%)*	*3 (10.3%)*	*2 (6.9%)*	2 (6.9%)	12 (41.4%)	15 (51.7%)

Overview of the 16S rDNA sequences from the bacterial- and archaeal clones sequences as well as the bacterial isolates from this study that affiliated with known sequences from the NCBI database, which were previously detected from different environments. Number of clones and calculated percentages indicate the relatedness to these habitats as well as the subsets of the sequences derived from different buildings to each other.

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In a previous study 47.1% of the isolated bacteria from the Saint Virgil Chapel belonged to the *Halobacillus* genus [10]. In this study even a higher percentage of the cultured strains (55.2%) were affiliated with this genus. However, it is worth noting that the culture-independent analysis of the samples did not yield any clones harbouring halobacilli sequences. A similar pitfall of molecular analysis was already observed by Piñar et al. [12] during the observation of the microbiota in the Saint Virgil chapel and were discussed by several authors [24,44–49]. The disparities in the results obtained by culture dependent and –independent techniques in this study once more show the drawbacks of each approach for an accurate description of the microbial community in a certain habitat [50,51].

We could again proof the co-existence of moderately halophilic bacteria and neutrophilic halophilic archaea on hypersaline environments represented by historical stone works, which was already shown before [10,12,52]. Similar to the bacterial clone sequences, the majority of the archaeal 16S rDNA sequences showed to be related to mural stonework or ancient paintings (47.2%; Table 2). The identified archaeal sequences were previously detected either in the Saint Virgil chapel, the Saint Catherine chapel or the Capuchin catacombs. Thirty-four percent of all analyzed archaeal clones were originally found in saline environments, e.g. in a solar saltern in Greece (*Halococcus* sp.) [53] or in different saline sediments (*Halalkalicoccus* sp., Haloarchaeon Nie 13) [54,55]. A further 18.8% have diverse origins (soil, groundwater, etc.).

Some of the identified archaeal species also produce colourful pigments as means of protection against exposure to UV light and chemicals. *Halalkalicoccus* sp. and *Natronorubrum* sp. show a pink-pigmented colour appearance [56,57], whereas bright orange to pink colonies are formed by *Halococcus hamelinensis* [58]. These haloarchaea are able to grow on even higher salt concentrations than the detected halophilic bacteria – up to 30% NaCl (w/v) [59].

The interaction between microorganisms and mineral substrata was studied by FESEM in order to address the real action of *Halobacillus naozhouensis* and *Kocuria polaris* on gypsum plaster and limestone probes, and certain biodeterioration phenomenon on these building materials. The distribution pattern of the rosy biofilms on the inoculated probes was different for both materials due to their petrophysic characteristics, mainly, stone surface roughness. The presence of larger pores on the Hontoria limestone allowed the development of the inoculated microorganisms within the stone probes, contrasting with the smoother surface of the gypsum plaster. The development and activity of these microorganisms on both substrata were responsible for the rosy discolouration and might also cause the dissolution features observed by FESEM, particularly on the gypsum mineral grains. Biogeochemical deterioration is the direct action caused by the metabolic processes of organisms on a substratum [60]. The biogenic release of corrosive acids is probably the best well-known and most commonly investigated biogeochemical damage mechanism in inorganic materials. The process known as biocorrosion, involves the release of organic acids which can etch or solubilize stone minerals [61]. The dissolution features observed on the inoculated gypsum plaster probes is a clear evidence of the microbial activity present on these mineral substrata inducing biodeterioration. Microorganisms may also induce biodeterioration though actively dissolution of carbonates and other minerals to enable penetration into the substratum enhancing stone porosity [62,63]. These biogeochemical processes give rise to changes on the lithic substratum, as particularly observed on the inoculated gypsum plaster probes (Figure 4). The data shows that the rosy discolouration phenomenon, in addition to an unaesthetic effect, induces also biogeochemical deterioration.

Conclusions

In this work we could show that the rosy biofilms on the walls of three different buildings harbour very similar bacterial and archaeal communities. Similar climatic conditions with relatively low UV irradiations and lowered annual temperatures, constructional problems with water infiltrations into the walls, the migration and further crystallisation of salts on the surface lead to the formation of extreme saline environments that offer optimal growth conditions for halophilic microorganisms. The inhabiting members of the Firmicutes and Actinobacteria, mainly representatives of the subclass Rubrobacteridae, as well as Halobacteriales members are the main cause for the rosy coloured biofilms on the walls. These microorganisms were already detected in other historical buildings from different locations in Europe. Further investigations should address their goals in the design of special cultivation media to isolate the so far unidentified members of the *Rubrobacter* genus and the Halobacteriales order, which were also involved in this phenomenon.

The results of this study show that halotolerant and halophilic microbes with brilliant rosy to purple colourations are the most important biodeteriogens of walls and wall paintings in salt-burdened historical sites. The intensity of the stains often is a serious aesthetical damage of wall paintings and in some cases it might even lead to an illegibility of the painting. For this reason, restorers often wish to carry out a treatment to remove this microbiota from the surfaces. Since desalination – by use of compresses – is a general tool in order to decrease the salt crystallization and the mechanical damage related to this, this method could also help to stop the growth of halophilic and halotolerant microorganisms. However, without any accompanying measures that decrease the humidity, the habitat would then be open for a wide variety of less salt-tolerant microbes including fungi [12]. Since especially fungi are very potent producers of organic acids and also decomposers of organic binders in wall paintings, such a microbiota might be even more harmful for the object than the predominantly aesthetical damage caused by pigmented bacteria and archaea. Therefore, a desalination of the walls is only reasonable in combination with structural measures that decrease the humidity down to a level that does not allow microbial growth. Such measures could be drainage and repair of constructional damages or better ventilation to avoid condensation. The same holds true for the application of biocides. None of the biocide compounds that are currently used in restoration – including quaternary ammonium compounds, ethanol or formaldehyde-releasers – have a preventive effect against re-colonization. Thus, application of a biocide can only be recommended in parallel to climate control measures. If climate control is impossible, it should be considered to accept the coloured microbiota rather than disturbing or changing the microbial community by treatments like desalination or application of biocides that, if not ineffective, can cause more fatal damage to the paintings [5,64].

Supporting Information

Figure S1 Additional information about the buildings. The Johannes Chapel in Pürgg (A, B), the castle Rappottenstein

(C, D) and the Saint Rupert Chapel in Weißpriach (E, F). A) The Johannes chapel in Pürgg was first restored between 1889 and 1894 in the sense of historicism, which was again removed between 1939 and 1949 where all original paintings were restored. B) The chapel is built on top of a hill and due to the strong exposure to the weather, in the 1960s a shingles wall was installed outside of the west wall to protect it against rain, wind and snow. Since 1996 a few additional actions to protect the chapel were made: The whole chapel received an outside exterior rendering with lime plaster, the entrance was moved from the west side to the north with an additional small room as climatic sluice. These constructional changes led to a cooling of the west wall and the further establishment of a rosy colour on the whole wall. C) The castle Rappottenstein was built on a hill and therefore under strong atmospheric exposure. The walls in the inner courtyard are highly exposed to rain and snow as well as water migrates through the walls of the whole building leading to the formation of salt efflorescences. The castle contains arcaded sidewalks with Sgraffiti decoration over three floors and famous frescos with profane-paintings from the 16th century. D) The famous "Green room", also called "Brudermordzimmer" (brother-murderer-room), on the 2nd floor shows medieval scenes on green background and dates back to 1520-1480. E) Only 50 kilometres away from Pürgg is the Saint Rupert chapel of Weißpriach that, due to its geographical location in the Alps, is also exposed to alpine climatic conditions. The chapel consists of a north orientated tower and an inner hall with rectangular choir with a semicircle apse. F) The most outstanding murals were discovered in 1949 and also in 1977/78. The "Last Judgment" in two registers, the "Legend of Ägidius", hunting scenes of Visigoth kings and martyr were found underneath the plaster, laid open and subsequent restored. All photographs were taken by Jörg D. Ettenauer.
(TIF)

Table S1 Description of the pooled and further analysed samples from the three historical buildings. The compositions of the mixed samples with the original samples numbers, sample amounts (in gram) as well as the mixed sample amounts (in gram) are given that were further used for cultivation- and molecular analysis.
(DOCX)

Table S2 Phylogenetic affiliations of isolated strains. Phylogenetic affiliations of the 16S rRNA gene sequences obtained from the cultivated bacteria in the samples from Pürgg (P2) and Rappottenstein (R1, R2 and R3). The number of isolates, the colour appearance, the growth conditions and isolation time after incubation start (in hours and days), the sequence length of the 16Sr DNA for database comparison, the similarity of the closest relative from the NCBI- and EZtaxon- (marked with an asterisk*) database and the accession numbers are given. Accession codes: Sequences were deposited at the NCBI GenBank under the accession numbers HG515390–HG515401.
(DOCX)

Table S3 Phylogenetic affiliations of the bacterial sequences. Phylogenetic affiliations of the partial 16S rRNA gene sequences obtained from all bacterial clones of the samples from the three buildings. Accession codes: Sequences were deposited at the NCBI GenBank under the accession numbers KF692550–KF692709 for the cloned sequences.
(DOCX)

Table S4 Phylogenetic affiliations of the archaeal sequences. Phylogenetic affiliations of the partial 16S rRNA gene sequences obtained from all archaeal clones in the samples from the three buildings. Accession codes: Sequences were deposited at the NCBI GenBank under the accession numbers KF692550–KF692709 for the cloned sequences.
(DOCX)

Author Contributions

Conceived and designed the experiments: KS MS CSJ. Performed the experiments: JE VJ AZM. Analyzed the data: JE VJ AZM CSJ. Contributed reagents/materials/analysis tools: JE VJ GP AZM. Contributed to the writing of the manuscript: JE GP CSJ KS. Obtained permission for sampling: MS.

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Supporting Figure S1

Supporting Table S1. Description of the pooled and further analysed samples from the three historical buildings. The compositions of the mixed samples with the original samples numbers, sample amounts (in gram) as well as the mixed sample amounts (in gram) are given that were further used for cultivation- and molecular analysis.

Location	Mixed samples	Taken sample no.	Location	Sample amount	Mixed sample amount
Pürgg	**P1**	1	chapel entry (north wall)	3.39	5.16
		2	chapel entry (north wall)	0.5	
		3	chapel entry (north wall)	0.7	
		4	chapel entry (north wall)	0.57	
	P2	5	west wall	1.42	16.86
		6	west wall	0.92	
		7	west wall	14.52	
	P3	8	east wall	5.36	5.77
		9	east wall	0.25	
		10	east wall	0.16	
Weißpriach	**W1**	1	tower-room	1.8	3.37
		2	tower-room	1.57	
	W2	3	north wall	0.76	0.76
	W3	4	south wall	1.65	1.65
Rappottenstein	**R1**	1	ground floor	10.16	30.42
		2	ground floor	0.23	
		3	ground floor	2.67	

	4	ground floor	0.3	
	5	ground floor	0.57	
	6	ground floor	0.91	
	7	ground floor	14.46	
	8	ground floor	1.12	
R2	9	second floor	3.71	10.89
	10	second floor	0.3	
	11	second floor	1.18	
	12	second floor	5.7	
R3	13	first floor	8.82	11.08
	14	first floor	0.93	
	15	first floor	0.49	
	16	first floor	0.84	

Supporting Table S2. Phylogenetic affiliations of isolated strains. Phylogenetic affiliations of the 16S rRNA gene sequences obtained from the cultivated bacteria in the samples from Pürgg (P2) and Rappottenstein (R1, R2 and R3). The number of isolates, the colour appearance, the growth conditions and isolation time after incubation start (in hours and days), the sequence length of the 16Sr DNA for database comparison, the similarity of the closest relative from the NCBI- and EZtaxon- (marked with an asterisk*) database and the accession numbers are given. Accession codes: Sequences were deposited at the NCBI GenBank under the accession numbers HG515390-HG515401.

Sample	No. of isolates	Colour-appearance	Culture medium Incubation time	Sequence length (bp)	Nearest published relative and isolation source from NCBI- and from EZtaxon* database	Similarity (%)	Accession No.
P2	2	Orange	TSA-NaMg, 48h	1441	*Planococcus salinarum* strain ISL-16 [FJ765415.1] from a marine solar saltern	98 98.6*	HG515390
	10	Lightly pink - pink	TSA-NaMg15%, 6d M1018, 6d M372, 12d	1504	*Halobacillus herbersteinensis* strain I7 [AM161504.1] from medieval paintings and stone-works in Castle of Herberstein, Styria, Austria *Halobacillus naozhouensis* JSM 071068(T) [EU925615.1] from a sea anemone*	99 98.5*	HG515391
R1	1	Orange	TSA-NaMg, 7d	1382	*Paracoccus marcusii* isolate SCH0403 [AY881236.1] from east and south coast, South Korea *Paracoccus marcusii* DSM 11574(T) [Y12703.1] isolated as a contaminant on a nutrient agar plate*	100 100*	HG515392
	3	Orange – Yellow	TSA-NaMg15%, 7d M1018, 7d	1456	*Marinococcus luteus* strain KCTC 13214 [AB769483.1] from a salt mine located in Tarija, Bolivia	99	HG515394

		Lightly orange			*Marinococcus tarijensis* SR-1(T) [JQ413413.1] from a salt mine located in Tarija, Bolivia*	99.7*	
	5	Pink	M1018, 7d TSA-NaMg15%, 7d	1468	*Halobacillus herbersteinensis* strain I7 [AM161504.1] from medieval paintings and stone-works in Castle of Herberstein, Styria, Austria *Halobacillus naozhouensis* JSM 071068(T) [EU925615.1] from a sea anemone*	99 99.3*	HG515393
	1	Lightly orange	TSA-NaMg15%, 8d	1436	*Halomonas muralis* strain LMG 20969 [NR_025486.1] from microbial biofilms on walls and murals in the Saint-Catherine chapel, Castle Herberstein, Austria	98 97.8*	HG515395
R2	1	Orange	TSA-NaMg, 48h	1436	*Planococcus psychrotoleratus* [AY771711.1] *Planomicrobium okeanokoites* IFO 12536(T) [D55729.1] from a marine mud*	99 98.5*	HG515396
	2	Orange	Marine Agar, 48h, Marine Agar, 5d	1418	*Planomicrobium flavidum* strain ISL-41 [FJ265708.1] from a marine solar saltern	98 98.30*	HG515398
	1	Orange	Marine Agar, 48h	1439	*Planococcus donghaensis* strain L10.15 [KC479344.1] from soil in the Antarctic *Planococcus donghaensis* JH 1(T) [EF079063.1] from deep-sea sediment of the East Sea, South Korea*	99 99.1*	HG515397
	1	Pink - Orange	TSA-NaMg, 5d	1479	*Kocuria rosea* [DQ060382.1] from the Arctic Ocean marine sediments	99	HG515399

					Kocuria polaris CMS 76or(T) [AJ278868.1] from an Antarctic cyanobacterial mat sample*	99.5*	
	1	Orange	Marine Agar, 7d	1440	*Planococcus antarcticus* strain B-9 [KF318398.1] from soil of Issyk Kul region, Kyrgyzstan	99	HG515400
					Planococcus donghaensis JH 1(T) [EF079063.1] from deep-sea sediment of the East Sea, South Korea*	98.7*	
R3	1	Orange	Marine Agar, 48h	1440	*Planococcus psychrotoleratus* [AF324659.1]	99	HG515401
					Planococcus donghaensis JH 1(T) [EF079063.1] from deep-sea sediment of the East Sea, South Korea*	98.9*	

Supporting Table S3. Phylogenetic affiliations of the bacterial sequences. Phylogenetic affiliations of the partial 16S rRNA gene sequences obtained from all bacterial clones of the samples from the three buildings. Accession codes: Sequences were deposited at the NCBI GenBank under the accession numbers KF692550-KF692709 for the cloned sequences.

DGGE band	Clone number	Sequence length [bp]	Nearest published relative and isolation source from NCBI database	Similarity (%)	Accession number
1	P1-57	396	Uncultured *Rubrobacter* sp., clone K7 [AM161166.1] from mural paintings in the Chapel of St. Virgil, Vienna, Austria	100	KF692659
2	P1-81	588	*Rubrobacter* sp. CBF L56 [AB166956.1] from deep-sea sediment, Central Basin Fault, West Philippine Basin	98	KF692660
3	P1-75	588	Uncultured actinobacterium, clone E6 [KC442786.1] from soils of the Lake Wellman Area, Darwin Mountains, South Victoria Land, Antarctica	99	KF692704
4	P1-47	588	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692661
5	P1-82	588	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692662
6	P1-37	588	Uncultured *Rubrobacter* sp., clone B8-K12 [KC535155.1] from wall with purple stains in the Capuchin catacombs in Palermo, Italy	99	KF692663
7	P1-29	588	Uncultured actinobacterium, clone F15cmFL474 [JN002715.1] from serpentinized dunite of ultramafic	99	KF692703

			rocks in the Leka ophiolite complex		
8	P1-83	588	*Rubrobacter* sp. VF70612_S1 [EU512991.1] from biodeteriorated monuments, Vilar de Frades Church, Barcelos, Portugal	99	KF692664
9	P1-77	588	Uncultured actinobacterium, clone F15cmFL453 [JN002699.1] from serpentinized dunite of ultramafic rocks in the Leka ophiolite complex	99	KF692705
10	P1-96	588	Uncultured bacterium, clone A6 [AM746684.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692665
11	P1-46	588	Uncultured bacterium, clone A6 [AM746684.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692666
12	P1-68	587	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	97	KF692667
13	P1-17	588	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692611
14	P2-42	504	Uncultured *Rubrobacter* sp., clone K8 [AM161167.1] from mural paintings in the Chapel of St. Virgil, Vienna, Austria	99	KF692612
15	P2-14	572	*Nesterenkonia xinjiangensis*, strain YIM70097 [NR_029075.1] from saline soils in the west of China	94	KF692613
16	P2-71	588	Uncultured eubacterium MP1-K4 [AJ298571.1] from rosy discolouration of masonry and lime wall paintings	99	KF692614
17	P2-34	588	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692615

18	P2-15	588	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692616
19	P2-9	588	Uncultured *Rubrobacter* sp., clone B8-K12 [KC535155.1] from wall with purple stains in the Capuchin catacombs in Palermo, Italy	99	KF692617
20	P2-91	566	*Amycolatopsis* sp. 2-5 [GU132436.1] from Nalaikh coal mining site soil, Mongolia	97	KF692618
21	P2-18	590	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692619
22	P2-90	588	Uncultured actinobacterium, clone F15cmL146 [JN002733.1] from serpentinized dunite of ultramafic rocks in the Leka ophiolite complex	99	KF692706
23	P2-1	589	Uncultured bacterium, clone A7 [AM746685.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	95	KF692620
24	P2-61	588	Uncultured bacterium, clone A6 [AM746684.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692621
25	P2-22	530	Uncultured bacterium clone RamatNadiv03b02 [JF295260.1] from arid soils	93	KF692622
26	P2-52	588	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692623
27	P2-39	582	Uncultured bacterium, clone ncd2161f11c1 [JF185740.1] from skin, popliteal fossa of Homo sapiens	96	KF692624
28	P2-75	584	*Actinomycetospora chlora*, strain: TT07I-57 [AB514519.1] from a paddy soil on Iriomote Island, Okinawa, Japan	98	KF692625
29	P2-11	583	*Nocardioides* sp. Gsoil BX5-10 [GQ339904.1] from soil of a ginseng field	96	KF692626

30	P3-29	580	Uncultured actinobacterium, clone FBP483 [AY250887.1] from lichen-dominated Antarctic: Southern Victoria Land, McMurdo Dry Valleys	99	KF692702
31	P3-27	588	Uncultured actinobacterium, clone E6 [KC442786.1] from soils of the Lake Wellman Area, Darwin Mountains, South Victoria Land, Antarctica	99	KF692707
32	P3-51	588	Uncultured eubacterium MP1-K4 [AJ298571.1] from rosy discolouration of masonry and lime wall paintings	99	KF692627
33	P3-47	588	Uncultured actinobacterium, clone E6 [KC442786.1] from soils of the Lake Wellman Area, Darwin Mountains, South Victoria Land, Antarctica	99	KF692709
34	P3-38	588	*Rubrobacteraceae* bacterium, isolate VF70612_S4 [FR852391.1] from a green biofilm	99	KF692628
35	P3-81	588	Uncultured eubacterium MP1-K6 [AJ298572.1] from rosy discolouration of masonry and lime wall paintings	99	KF692629
36	P3-80	588	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692630
37	P3-41	588	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692631
38	P3-43	588	Uncultured actinobacterium, clone FBP483 [AY250887.1] from lichen-dominated Antarctic: Southern Victoria Land, McMurdo Dry Valleys	99	KF692708
39	P3-72	588	*Rubrobacter* sp. CBF L56 [AB166956.1] from deep-sea sediment, Central Basin Fault, West Philippine Basin	99	KF692632
40	P3-67	588	Uncultured bacterium, clone Hb6-K1 [AJ400551.1] from two different biodeteriorated wall paintings	99	KF692633

41	P3-84	588	Uncultured bacterium, clone A16 [AM746694.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	98	KF692634
42	R1-48	547	Uncultured bacterium, clone TX4CB_142 [FJ153011.1] from alkaline saline soils of the former lake Texcoco, Mexico	98	KF692635
43	R1-8	531	Uncultured *Rubrobacter* sp., clone K8 [AM161167.1] from mural paintings in the Chapel of St. Virgil, Vienna, Austria	100	KF692636
44	R1-11	586	*Jiangella* sp. 13658J [EU741189.1] from marine sediment, Cabo Blanco Absolute Natural, Costa Rica	97	KF692637
45	R1-43	588	*Rubrobacter bracarensis*, strain VFA70612_S5 [HE672088.1] from a green biofilm of a biodeteriorated monument	99	KF692638
46	R1-24	588	Uncultured bacterium, clone A7 [AM746685.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692639
47	R1-29	588	Uncultured gamma proteobacterium, clone B2-K12 [KC535205.1] from clothes from mummies in the Capuchin catacombs in Palermo, Italy	99	KF692640
48	R1-26	570	*Janibacter corallicola*, strain: 02PA-Ca-009 [AB286024.1] from a coral in Palau	98	KF692641
49	R1-27	584	Uncultured actinobacterium, clone SC6-RK112 [HF584649.1] from white spots on mural paintings from Etruscan tombs	96	KF692642
50	R1-6	569	Uncultured bacterium, clone TX4CB_142 [FJ153011.1] from alkaline saline soils of the former lake Texcoco, Mexico	98	KF692668
51	R1-42	583	Uncultured bacterium, clone P-11_B21 [HQ910321.1] from desert soil in the Mars Desert Research Station, Utah, USA.	97	KF692669

52	R1-28	572	Uncultured *Pseudonocardia* sp., clone 343G [AY571815.1] from hydrocarbon-contaminated soil around Scott Base, Southern Victoria Land, Antarctica	99	KF692670
53	R1-21	566	Uncultured bacterium, clone CAR-BSb-E9 [FN298047.1] from vertical calcarenite walls of underground tombs from Roman Necropolis of Carmona, Seville, Spain.	99	KF692671
54	R2-9	587	*Ralstonia insidiosa,* strain BGR27 [KC789786.1] from soil of Shule river, Gansu province, China	99	KF692672
55	R2-26	587	Uncultured bacterium, clone:13C-M8 [AB205724.1] from denitrifying activated sludge, Ibaraki, Japan	100	KF692673
56	R2-35	588	*Planococcus* sp. ljh-25 [GU217715.1] from mud volcano 1281 meters above sea, WuSu, Xinjiang, China	99	KF692674
57	R2-33	589	*Bacillus agaradhaerens*, strain IB-S7 [FN432808.1] from mud from salt lake, Buryatiya, Russia	99	KF692675
58	R2-14	588	*Rubrobacter bracarensis*, strain VFA70612_S4 [HE672087.1] from a green biofilm of a biodeteriorated monument	99	KF692676
59	R2-13	588	Uncultured *Rubrobacter* sp., clone B8-K12 [KC535155.1] from wall with purple stains in the Capuchin catacombs in Palermo, Italy	99	KF692677
60	R2-5	587	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692678
61	R2-34	588	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692679
62	R2-43	589	*Natribacillus halophilus* [AB449109.1] from soil in Saitama, Okabe, Japan	98	KF692680
63	R2-48	564	*Thermocrispum agreste*, strain DSM 44070 [NR_026171.1] from waste and mushroom composts	94	KF692681
64	R2-16	583	*Saccharopolyspora salina*, strain YIM 91168 [EF687715.1] from a salt lake in Xinjiang Province, North-West China	99	KF692682

65	R2-29	576	Uncultured actinobacterium, clone B6-K26 [KC535176.1] from rosy discolored wall in the Capuchin catacombs in Palermo, Italy	99	KF692683
66	R3-14	580	*Rubrobacteraceae* bacterium, isolate VF70612_S1 [FR852390.1] from green biofilm	99	KF692684
67	R3-35	580	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692685
68	R3-13	588	*Rubrobacter* sp. C05_TS_X24_S3 [EU512989.1] from biodeteriorated monuments: Necropolis of Carmona, Seville, Spain	98	KF692686
69	R3-40	588	Uncultured actinobacterium, clone FBP483 [AY250887.1] from lichen-dominated Antarctic: Southern Victoria Land, McMurdo Dry Valleys	99	KF692687
70	R3-12	588	Uncultured *Rubrobacter* sp., clone B7-K27 [KC535229.1] from salt efflorescence in the Capuchin catacombs in Palermo, Italy	99	KF692688
71	R3-17	588	Uncultured actinobacterium, clone B4-K48 [KC535199.1] from hairs of mummies in the Capuchin catacombs in Palermo, Italy	99	KF692689
72	R3-19	588	Uncultured bacterium, clone A6 [AM746684.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692690
73	R3-26	588	Uncultured eubacterium Hb7-K2 [AJ298575.1] from rosy discolouration of masonry and lime wall paintings	99	KF692691
74	R3-20	588	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692692
75	R3-16	588	Uncultured bacterium, clone A4 [AM746682.1] from rosy-discoloured mural wall painting of the Crypt	98	KF692693

			of the Original Sin, Matera, Italy		
76	R3-18	571	Uncultured bacterium, clone RamatNadiv03g07 [JF295210.1] from arid soils	92	KF692694
77	W1-95	390	Uncultured *Rubrobacter* sp., clone K8 [AM161167.1] from mural paintings in the Chapel of St. Virgil, Vienna, Austria	100	KF692643
78	W1-36	582	Uncultured eubacterium Hb7-K3 [AJ298577.1] from rosy discolouration of masonry and lime wall paintings	98	KF692644
79	W1-5	588	Uncultured actinobacterium, clone F15cmFL477 [JN002718.1] from serpentinized dunite of ultramafic rocks in the Leka ophiolite complex	98	KF692645
80	W1-37	588	*Rubrobacter* sp. C05_TS_X24_S3 [EU512989.1] from biodeteriorated monuments: Necropolis of Carmona, Seville, Spain	95	KF692646
81	W1-4	586	Uncultured bacterium, clone A16 [AM746694.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692647
82	W1-39	588	Uncultured bacterium, clone A3 [AM746681.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	98	KF692648
83	W1-92	586	Uncultured bacterium, clone A16 [AM746694.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692649
84	W1-55	588	*Rubrobacter xylanophilus*, strain DSM 9941 [NR_074552.1]	94	KF692650
85	W1-81	560	Uncultured bacterium, clone Hb6-K1 [AJ400551.1] from two different biodeteriorated wall paintings	100	KF692651
86	W2-53	588	Uncultured actinobacterium, clone F15cmL164 [JN002746.1] from serpentinized dunite of ultramafic rocks in the Leka ophiolite complex	99	KF692652

87	W2-14	587	Uncultured actinobacterium, clone E6 [KC442786.1] from soils of the Lake Wellman Area, Darwin Mountains, South Victoria Land, Antarctica	99	KF692653
88	W2-23	587	Uncultured actinobacterium, clone F15cmL169 [JN002750.1] from serpentinized dunite of ultramafic rocks in the Leka ophiolite complex	99	KF692654
89	W2-22	587	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692655
90	W2-80	588	*Rubrobacter bracarensis*, strain VFA70612_S4 [HE672087.1] from a green biofilm of a biodeteriorated monument	99	KF692656
91	W2-44	419	Uncultured *Rubrobacter* sp., clone K7 [AM161166.1] from mural paintings in the Chapel of St. Virgil, Vienna, Austria	100	KF692657
92	W2-6	588	Uncultured actinobacterium, clone F15cmFL453 [JN002699.1] from serpentinized dunite of ultramafic rocks in the Leka ophiolite complex	99	KF692658
93	W2-62	579	Uncultured bacterium, clone A6 [AM746684.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692603
94	W2-33	588	Uncultured bacterium, clone A7 [AM746685.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692604
95	W2-10	588	*Rubrobacter* sp. CBF L56 [AB166956.1] from deep-sea sediment, Central Basin Fault, West Philippine Basin	99	KF692605
96	W2-96	588	Uncultured *Rubrobacter* sp., clone K8 [AM161167.1] from mural paintings in the Chapel of St. Virgil, Vienna, Austria	100	KF692606

97	W2-64	588	Uncultured bacterium, clone Hb6-K1 [AJ400551.1] from two different biodeteriorated wall paintings	100	KF692607
98	W3-95	580	Uncultured bacterium, clone Hb6-K1 [AJ400551.1] from two different biodeteriorated wall paintings	100	KF692608
99	W3-76	587	Uncultured bacterium, clone WT42H53 [HE966153.1] from composting soil	100	KF692609
100	W3-58	586	Uncultured bacterium, clone A3 [AM746681.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	99	KF692610
101	W3-86	588	*Rubrobacter bracarensis*, strain VFA70612_S5 [HE672088.1] from a green biofilm of a biodeteriorated monument	99	KF692695
102	W3-62	588	Uncultured eubacterium Hb7-K1 [AJ298576.1] from rosy discolouration of masonry and lime wall paintings	99	KF692696
103	W3-89	588	Uncultured bacterium, clone Hb6-K1 [AJ400551.1] from two different biodeteriorated wall paintings	100	KF692697
104	W3-15	588	Uncultured bacterium, clone A16 [AM746694.1] from rosy-discoloured mural wall painting of the Crypt of the Original Sin, Matera, Italy	98	KF692698
105	W3-30	583	*Saccharopolyspora salina*, strain YIM 91168 [EF687715.1] from a salt lake in Xinjiang Province, North-West China	99	KF692699
106	W3-6	576	Uncultured actinobacterium, clone B6-K26 [KC535176.1] from rosy discolored wall in the Capuchin catacombs in Palermo, Italy	99	KF692700
107	W3-46	583	Uncultured bacterium, clone P11-P81 [GU574055.1] from mould-colonized water damaged building material	99	KF692701

Supporting Table S4. Phylogenetic affiliations of the archaeal sequences. Phylogenetic affiliations of the partial 16S rRNA gene sequences obtained from all archaeal clones in the samples from the three buildings. Accession codes: Sequences were deposited at the NCBI GenBank under the accession numbers KF692550-KF692709 for the cloned sequences.

DGGE band	Clone number	Sequence length [bp]	Nearest published relative and isolation source from NCBI database	Similarity (%)	Accession number
1	P2-16	552	Uncultured archaeon, clone TP-AM-A17 [HQ645127.1] from soil samples from high altitude meadow, China	99	KF692550
2	P2-9	552	Uncultured archaeon, clone PM37 [AJ608183.1] from soil, Netherlands	99	KF692551
3	P2-10	447	Uncultured *Halococcus* sp., clone 16 [FN435871.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	99	KF692552
4	P2-36	552	Uncultured archaeon, clone sscpArc_V5A5 [FM179191.1] from groundwater, Tyrol, Austria	98	KF692553
5	P2-5	552	Uncultured archaeon, clone DOL-A146 [JF737821.1] from dolomite rock from the Nanjiang Canyon, Guizhou Karst Region, China	98	KF692554
6	P2-15	555	Uncultured *Halobacterium* sp., clone A9-K51 [KC535277.1] from wall samples in the Capuchin catacombs in Palermo, Italy	99	KF692555
7	P2-17	555	Unidentified archaeon H6-K6 [AJ291422.1] from two disparate deteriorated ancient wall paintings	99	KF692556
8	P2-18	555	*Halococcus hamelinensis* 100A6 [NR_028168.1] from stromatolites of Shark Bay, Hamelin Pool, Western Australia	98	KF692557
9	P2-45	555	Uncultured *Halobacterium* sp., clone library K2, clone 79 [FN433757.1] from salt efflorescence on	99	KF692558

			medieval stonework in Chapel of St. Virgil, Vienna, Austria		
10	P2-19	555	Uncultured *Halobacterium* sp., clone K14 [AM159641.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	99	KF692559
11	P2-14	555	Unidentified archaeon, H6-K5 [AJ291421.1] from two disparate deteriorated ancient wall paintings	99	KF692560
12	P2-13	555	Uncultured *Halococcus* sp., clone A9-K64 [KC535280.1] from wall samples in the Capuchin catacombs in Palermo, Italy	98	KF692561
13	P2-48	555	Uncultured *Halococcus* sp., clone library K9, clone 2 [FN435859.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	99	KF692562
14	P3-21	555	*Halococcus* sp. FC211 [EU308208.1] from a solar saltern in Western Greece	98	KF692563
15	P3-48	555	Uncultured *Halobacterium* sp., clone A9-K41 [KC535276.1] from wall samples in the Capuchin catacombs in Palermo, Italy	99	KF692564
16	P3-37	555	Uncultured *Halococcus* sp., clone 3 [FN435860.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	98	KF692565
17	P3-14	555	Uncultured *Halobacterium* sp., clone K14 [AM159641.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	99	KF692566
18	P3-36	554	*Halococcus* sp. KeC-02 [AB534723.1] from a seawater aquarium, Nakano-ku, Minamidai, Tokyo, Japan	98	KF692567
19	P3-26	555	*Halococcus* sp. IARI-ABCL-7 [JX428954.1] from saline sediments and water, Chilka Lake, Orrisa, India	98	KF692568
20	P3-38	555	*Halococcus* sp. KeC-16 [AB534732.1] from a seawater aquarium, Nakano-ku, Minamidai, Tokyo, Japan	98	KF692569
21	P3-9	555	Haloarchaeon Nie 13 [AB291223.1] from salt field soil in Nie, Ishikawa, Japan	98	KF692570
22	R1-102	555	*Halalkalicoccus jeotgali B3* [NR_102920.1] from salt-fermented seafood, South Korea	99	KF692585

23	R1-99	555	Uncultured *Halobacterium* sp., clone A9-K41 [KC535276.1] from wall samples in the Capuchin catacombs in Palermo, Italy	99	KF692586
24	R1-98	555	Uncultured *Halococcus* sp., clone 3 [FN435860.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	98	KF692587
25	R1-104	554	*Halococcus* sp. IARI-ABCL-7 [JX428954.1] from saline sediments and water, Chilka Lake, Orrisa, India	98	KF692588
26	R1-43	555	Halococcus sp. KeC-08 [AB534726.1] from a seawater aquarium, Nakano-ku, Minamidai, Tokyo, Japan	98	KF692589
27	R2-6	555	*Halalkalicoccus* sp. C15 [DQ373058.1] from Salda Lake, Turkey	99	KF692590
28	R2-19	555	Uncultured archaeon, clone FR2_c49a [GU126495.1] from spacecraft assembly clean room, Germany	97	KF692591
29	R2-42	555	*Halalkalicoccus* sp. YIM 93701 [JF449426.1] from salt soil of Lup Nur region, Xinjiang Province, North-West China	99	KF692592
30	R2-1	589	Uncultured archaeon, clone FlD_6_EA [JX865727.1] from intensive care unit floor, Regensburg, Germany	99	KF692593
31	R2-17	554	Uncultured *Halococcus* sp., clone 35 [FN435865.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	95	KF692594
32	R2-20	555	*Natronorubrum* sp., strain CG-4 [FN376860.1] from sediment of saline Lake Chagannor in Inner Mongolia, China	99	KF692595
33	R2-22	555	*Halococcus* sp. CBA1101 [JX989265.1] from marine environment in South Korea	99	KF692596
34	R3-46	574	*Halalkalicoccus* sp. C15 [DQ373058.1] from Salda Lake, Turkey	98	KF692597
35	R3-28	555	*Halalkalicoccus* sp. YIM 93701 [JF449426.1] from salt soil of Lup Nur region, Xinjiang Province, North-West China	99	KF692598

36	R3-36	555	*Halococcus hamelinensis* 100A6 [NR_028168.1] from stromatolites of Shark Bay, Hamelin Pool, Western Australia	98	KF692599
37	R3-34	555	Unidentified archaeon H6-K5 [AJ291421.1] from two disparate deteriorated ancient wall paintings	98	KF692600
38	R3-45	555	Uncultured archaeon, clone 24 [FN435863.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	98	KF692601
39	R3-15	555	Uncultured *Halobacterium* sp., clone A9-K63 [KC535279.1] from wall samples in the Capuchin catacombs in Palermo, Italy	97	KF692602
40	W1-22	555	*Halococcus morrhuae*, clone K16 [AM159638.1]] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	98	KF692571
41	W1-5	555	Unidentified archaeon H6-K5 [AJ291421.1] from two disparate deteriorated ancient wall paintings	99	KF692572
42	W1-6	555	*Halococcus* sp. IARI-ABCL-7 [JX428954.1] from saline sediments and water, Chilka Lake, Orrisa, India	98	KF692573
43	W1-16	555	Uncultured *Halococcus* sp., clone A9-K64 [KC535280.1] from wall samples in the Capuchin catacombs in Palermo, Italy	97	KF692574
44	W2-71	555	Uncultured archaeon, clone FR2_c49a [GU126495.1] from spacecraft assembly clean room, Germany	99	KF692575
45	W2-30	555	*Halalkalicoccus* sp. C15 [DQ373058.1] from Salda Lake, Turkey	99	KF692576
46	W2-39	555	Uncultured archaeon, clone FlD_6_EA [JX865727.1] from intensive care unit floor, Regensburg, Germany	99	KF692577
47	W2-20	555	*Halococcus* sp. IARI-ABCL-7 [JX428954.1] from saline sediments and water, Chilka Lake, Orrisa, India	98	KF692578
48	W2-33	555	Uncultured *Halalkalicoccus* sp., clone 97 [FN433769.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	98	KF692579

49	W3-2	555	Uncultured *Halococcus* sp., clone library K9, clone 2 [FN435859.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	99	KF692580
50	W3-1	554	*Halococcus* sp. KeC-16 [AB534732.1] from a seawater aquarium, Nakano-ku, Minamidai, Tokyo, Japan	98	KF692581
51	W3-97	555	Uncultured *Halobacterium* sp., clone K14 [AM159641.1] from salt efflorescence on medieval stonework in Chapel of St. Virgil, Vienna, Austria	99	KF692582
52	W3-100	555	Uncultured *Halobacterium* sp., clone A9-K51 [KC535277.1] from wall samples in the Capuchin catacombs in Palermo, Italy	99	KF692583
53	W3-98	553	Unidentified archaeon H6-K5 [AJ291421.1] from two disparate deteriorated ancient wall paintings	99	KF692584

7 List of figures

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For my parents

9 Curriculum Vitae

Mag.rer.nat.

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PERSONAL DATA

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EDUCATION

University of Natural Resources and Life Sciences

07/2010 – 09/2014	PhD studies Doctoral programme in Engineering Sciences; Diploma programme: Food Chemistry and Biotechnology, University of Natural Resources and Life Sciences, Vienna

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06/2010	2nd Diploma examination of the microbiology studies – graduation with distinction
07/2008 – 06/2010	Diploma thesis in the subject Gene- and Biotechnology
01/2003	1st Diploma examination
10/2002 – 05/2010	Diploma studies: Genetics – Microbiology (Branch of study)
10/1998 – 10/2002	Continuation of the Diploma studies Biology
02/1998 – 09/1998	Austrian military service, Raab casern, Mautern
10/1997 – 02/1998	Diploma studies Biology, University of Vienna

LANGUAGE SKILLS

German	mother tongue
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French	basic knowledge

10 List of Publications, presentations, congresses, etc.

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Scientific journal articles

Piñar, G.; Sterflinger, K.; Ettenauer, J.; Quandt, A.; Pinzari, F. (2014): Archaeology of biodeterioration and modern contamination of Archimedes Palimpsest. Submitted to Microbial Ecology.

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8th Indoor Air Quality Meeting; IAQ 2008; Kunsthistorisches Museum, Wien; 17.–19. April 2008.

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Students

Enrica Vella, Master student at the University of Bologna, was supervised during her 3 months stay in the ACBR (May - Aug. 2012). After introducing her into molecular biotechnological methods for microbial ecology studies, she was directed to unassisted scientific work for her Master thesis.

Fikret Rifatbegovic, former Bachelor student at the University of Vienna, was co-supervised during his work (2011, three months stay).

Maria López-Miras, former PhD student at the University of Granada, Spain, and *Lucia Krakova*, former PhD student at the Slovak Academy of Science Bratislava, Slovakia, were co-supervised during their exchange in Vienna (2011 and 2012, each three months).