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Growth of phototrophic biofilms from limestone monuments under laboratory conditions

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ABSTRACT

In the current study, five phototrophic biofilms from different Southern Europe limestone monuments were characterised by molecular techniques and cultivated under laboratory conditions. Phototrophic biofilms were collected from Orologio Tower in Martano (Italy), Santa Clara-a-Velha Monastery and Ajuda National Palace, both in Portugal, and Seville and Granada Cathedrals from Spain. The biofilms were grown under laboratory conditions and periodically sampled in order to monitor their evolution over a three-month period. Prokaryotic communities from natural samples and cultivated biofilms were monitored using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments in conjunction with clone sequencing and phylogenetic analysis. DNA-based molecular analysis of 16S rRNA gene fragments from the natural green biofilms revealed complex and different communities composition with respect to phototrophic microorganisms. The biofilms from Orologio Tower (Martano, Italy) and Santa Clara-a-Velha Monastery (Coimbra, Portugal) were dominated by the microalga *Chlorella*. The cyanobacterium *Chroococcidiopsis* was the dominating genus from Ajuda National Palace biofilm (Lisbon, Portugal). The biofilms from Seville and Granada Cathedrals (Spain) were both dominated by the cyanobacterium *Pleurocapsa*. The DGGE analysis of the cultivated biofilms showed that the communities developed differently in terms of species establishment and community composition during the three-month incubation period. The biofilm culture from Coimbra (Portugal) showed a remarkable stability of the microbial components of the natural community in laboratory conditions. With this work, a multiple-species community assemblage was obtained for further stone colonisation experiments.

1. Introduction

Sedimentary rocks, especially limestones, have been extensively used in the construction of monuments and historical buildings. This cultural heritage is at risk of biodeterioration caused by diverse communities of microorganisms, being extremely susceptible to cyanobacteria and algae. A large number of studies have already assessed the occurrence of cyanobacteria and algae on sedimentary stone materials from cultural heritage (Dupuy et al., 1976; Ortega-Calvo et al., 1993; Altieri et al., 2000; Bellinzoni et al., 2003; Ascaso et al., 2004; Zurita et al., 2005). Currently the complex interactions between lithobionts and the mineral substrates are of interest due to their implications in the biodeterioration of stone monuments. Since the natural biofilm communities are often difficult to investigate *in situ*, the study of artificial phototrophic biofilms under laboratory conditions may increase our understanding of the relative effects of physical and chemical changes in the substrate. Several investigators have developed laboratory-based stone colonisations (Ortega-Calvo et al., 1991; Guillitte and Dreesen, 1995; Prieto and Silva, 2005; Miller et al., 2006). In most of these experiments the individual community members were studied separately impeding to understand the complex stone biodeterioration processes caused by phototrophic biofilms. Laboratory studies using multiple-species and stable communities will enhance the understanding of the microbial processes involved in stone cultural heritage assets.

In this study, phototrophic biofilms were collected from five different limestone monuments and compared using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments in conjunction with DNA sequencing and phylogenetic analysis. These five biofilms were cultivated under laboratory conditions over a three-month incubation period and

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monitored by DGGE in order to investigate the changes in community composition. The aim was to obtain a multiple-species phototrophic community stable under laboratory conditions to be used for further colonisation experiments on stone.

2. Materials and methods

2.1. Site description and sampling

Samples of microbial communities developing as green biofilms were collected from five Southern Europe monuments constructed with different types of limestone. The samples were obtained from the Orologio Tower in Martano (Italy), two Portuguese monuments, Santa Clara-a-Velha Monastery (Coimbra) and Ajuda National Palace (Lisbon), and two Southern Spanish monuments, the cathedrals of Seville and Granada.

The Orologio Tower is located in the city of Martano, province of Lecce, in the Salentine Peninsula (Italy). Like many historical buildings in Southern Italy, the Orologio Tower was built with *Lecce* stone, a very soft and malleable limestone dating from the Miocene period – Langhian age – widely used in the Late Baroque.

The Santa Clara-a-Velha Monastery is located in Coimbra (Central Portugal) and represents an emblematic monument from the Portuguese Gothic style. Built in the early 13th century on the left bank of Mondego River, the Monastery lied buried in riverine flood sediments for centuries. It was constructed with a dolomitic limestone of Sinemurian age and a fine-grained compact and homogeneous limestone of Bathonian–Bajocian age, known locally as *Ançã* limestone. The monastery is now under restoration works to rescue it from the swamp and its state of ruin.

The Ajuda National Palace is a neoclassical monument located in Lisbon. The Palace was built during the 19th century with *Lioz* limestone, a light-coloured microcrystalline limestone from Middle Turonian (Middle Cretaceous) age.

The Cathedral of Seville (Andalusia, Spain), built on the site of a mosque in the 15th century, is the largest Gothic building in Europe and is considered one of the most important monuments of Christendom. Stones from the quarries of Cadiz and Seville provinces and Portugal were used in its construction. The most abundant lithotype used was a yellowish fossiliferous calcarenitic limestone of Miocene age, extracted from El Puerto de Santa María quarry (Cadiz) and locally known as *San Cristóbal* stone.

The Cathedral of Granada is located in Andalusia (Spain) and represents the country's finest Renaissance church. It was built in the 16th century, with a very soft and porous biocalcarenite from the Tortonian age, *Escúzar* stone, extracted from Santa Pudia quarries, in the Escúzar region (Granada).

The differences in the petrographic, petrogenic and petrophysic characteristics of the five sampled lithotypes were studied in a previous work (Miller et al., in press).

Martano, Coimbra, Lisbon, Seville and Granada are located in similar climatic regions in Southern Europe. The climatic parameters are typical of the Mediterranean Basin: mild and rainy winters, warm and dry summers and, usually, extended periods of sunshine throughout most of the year. All cities have limestone monuments of very high importance as cultural heritage buildings, triggering a progressive deterioration process, where phototrophic communities cause significant aesthetic damage.

The samples from each monument were collected from the vertical outer surfaces of the North facade between 20 and 50 cm above the ground. Biofilm biomass was scraped in triplicate from surface areas with approximately 5 cm^2 using sterilised material and gathering it in sterile tubes. The surveys were conducted on sunny days during the autumn season of 2006 and were restricted to surfaces with green patina appearance; lichens were not sampled. Each sample was divided into subsamples for identification by molecular methods (stored at −80 °C), and for culturing procedures which were preserved at 4 °C until being processed.

2.2. Characterisation of natural phototrophic biofilms

The identification of the microbial components in the samples was achieved by molecular techniques. DNA was extracted using the Nucleospin Food DNA Extraction Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's recommendations. PCR amplifications of bacterial 16S rRNA gene fragments were performed using the primer pair 616F (5′- AGA GTT TGA TYM TGG CTC AG) (corresponding to position 27 of *Escherichia coli* 16S rRNA gene) and 907R (5′-CCC CGT CAA TTC ATT TGA GTTT) (Zimmermann et al., 2005), with PCR conditions comprising a denaturing step of 2 min at 95 °C, 35 cycles of denaturing (95 °C for 15 s), annealing (55 °C for 15 s), and elongation (72 °C for 2 min), following a terminal elongation step of 10 min at 72 °C. Cyanobacterial and chloroplasts 16S rRNA genes were amplified using the primer pair Cya106F (5′- CGG ACG GGT GAG TAA CGC GTG A) and Cya781R (5′- GAC TAC TGG GGT ATC TAA TCC CWT T) and PCR conditions as described by Nübel et al. (1997). PCR amplifications of 16S rRNA gene fragments were performed using ExTaq DNA polymerase (Takara, Tokyo, Japan). PCR products were inspected on 1% (w/v) agarose gels.

Amplification products were purified with the JetQuick PCR Purification Spin Kit (Genomed, Löhne, Germany) and cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Clone screening was carried out by DGGE as described by Gonzalez et al. (2003) and clones representative of DGGE types were sequenced by SECUGEN Sequencing Services (Madrid, Spain) to determine their phylogenetic affiliations. Sequence data was edited using the software Chromas, version 1.45 (Technelysium, Tewantin, Australia) and submitted for homology search using the Blast algorithm (Altschul et al., 1990) on the NCBI database (http://www.ncbi.nlm.nih.org/blast/).

Microbial community fingerprints were obtained by DGGE as previously described (Muyzer et al., 1993; Gonzalez and Saiz-Jimenez, 2004) using a GC-rich tailed primer (341F-GC) and the reverse primer 518R in the nested-PCR reaction. Amplification conditions were as described for bacterial 16S rRNA genes with the exception of an extension step of only 30 s. Four reference strains were used as migration markers for comparison of DGGE patterns: *Pseudomonas* sp., *E. coli*, *Paenibacillus* sp. and *Streptomyces* sp.

Nucleotide sequences derived from the biofilm that colonises *Ançã* limestone (Santa Clara-a-Velha Monastery in Coimbra, Portugal) and from its cultivation have been deposited in the GenBank database under accession number from EU333004–EU333018.

2.3. Biofilms growth conditions and monitoring

The methodology used for biofilms growth and monitoring the changes in the microbial community during 90 days were similar to those described by Miller et al. (2008). The aliquots for culturing procedures were inoculated into flasks containing 400 ml of BG11 liquid medium. The biofilms were grown under natural light, at room temperature (22 °C ± 2 °C) and with air circulation provided by an air pump. The composition and growth of the biofilms were monitored for 90 days in order to evaluate when the cultures reached a mature stage, representing an establishment of the microbial components of the communities. Every two weeks (days 15, 30, 45, 60, 75 and 90), 200 ml of each culture assemblage were aseptically removed into sterile containers and three aliquots were stored at −80 °C until further PCR-DGGE analysis. The sampled volume was replaced by fresh sterile BG11 medium. The change and

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development in the phototrophic communities during the incubation period were investigated by analysis of banding patterns in DGGE. DNA extraction and amplification were performed as described before (2.2 Section).

3. Results

3.1. Identification of natural phototrophic biofilms

In this study, prokaryotic microorganisms, within the samples taken from five limestone monuments, have been approached by DNA-based molecular analyses. The comparative analyses of the five different green biofilms revealed the presence of a variety of microorganisms belonging to different phyla and showed major differences among them. A total of twenty-three taxa have been identified, fifteen of which were Bacteria, six Cyanobacteria, one Chlorophyta and one Bacillariophyta.

The phyla Bacteria and Cyanobacteria detected in the analysed 16S rRNA gene library were found in the five locations. Chloroplasts from the phylum Chlorophyta were detected in two of the studied biofilms, Orologio Tower, in Martano, Italy (Fig. 1) and in Santa Clara-a-Velha Monastery, located in Coimbra, Portugal (Fig. 2).

Within the Cyanobacteria, only three genera were present in more than one monument. *Pleurocapsa* was the most widespread cyanobacterium occurring in the five biofilms (Table 1). A high proportion of clones corresponding to microorganisms of the genus *Chroococcidiopsis* was detected in the biofilm from Ajuda National Palace (Fig. 3, bands 3, 4, 7 and 10). This cyanobacterium was also identified in the biofilms from Cathedral of Seville (Fig. 4, band 14) and Cathedral of Granada (Fig. 5, bands 3 and 8). *Leptolyngbya* sp. was present in the Cathedral of Seville (Fig. 4, band 12) and also in the Santa Clara-a-Velha Monastery (Fig. 2, bands 10 and 12). In the latter, bands affiliated to *Leptolyngbya* were only detected in the biofilm liquid culture. According to band migration during DGGE analysis, this cyanobacterium was also identified in the natural sample profiles. *Cylindrospermopsis*, *Nostoc* and *Microcoleus* were the genera represented just in one of the investigated monuments, namely in Ajuda National Palace (Fig. 3, bands 2, 9 and 11). This last biofilm comprised the largest genera diversity within Cyanobacteria and the lower within Bacteria. In contrast, the Cathedral of Seville showed the largest genera diversity within Bacteria (Fig. 4). This monument showed the presence of microorganisms belonging to Alpha- and Gammaproteobacteria, Actinobacteria and Verrucomicrobia in a total of five bacterial phyla detected in this study. The major component of this bacterial community was Verrucomicrobia, with a high proportion of clones.

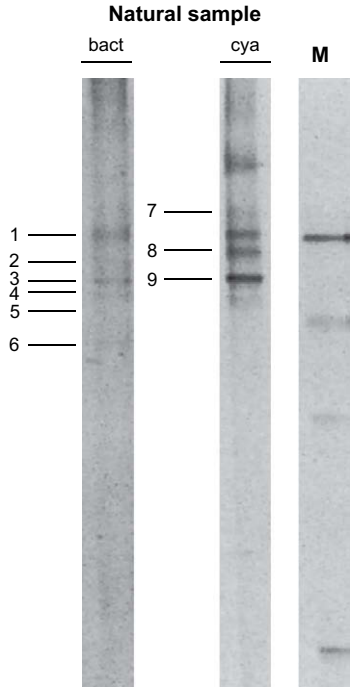

Fig. 1. DGGE profiles of 16S rRNA gene from the natural green biofilm collected from Orologio Tower, in Martano (natural sample), obtained after amplification using bacterial and cyanobacterial primers (Lanes "bact" and "cya", respectively). The band numbers refer to clones obtained from the natural sample. Lane "M" corresponds to DGGE markers, consisting of *Pseudomonas* sp., *E. coli*, *Paenibacillus* sp. and *Streptomyces* sp. Identification of bands based on comparison with closest relatives at NCBI databank (Acc. Number in parenthese): 1- *Pleurocapsa minor* (AJ344564), 2- *Variovorax* sp. (AB196432), 3- *Lysobacter* sp. (DQ191178), 4- Uncultured *Nitrobacteria* sp. (AM990004), 5- *Stenotrophomonas* sp. (DQ984206) 6- *Lysobacter* sp. (EF687714), 7- *Pleurocapsa minor* (AJ344564), 8- *Chlorella* sp. chloroplast (D11348), 9- *Chlorella* sp. chloroplast (X12742).

The bacterial composition of the five monuments showed only similarities in the phylum Proteobacteria, represented by members of the Alpha- Beta- and Gammaproteobacteria. Among the Alphaproteobacteria, members of the *Methylobacterium*, *Porphyrobacter*, *Sphingomonas* and *Nitrobacteria* genera were detected. *Saccharospirillum*, *Marinobacter*, *Stenotrophomonas* and *Lysobacter* were the most representative genera belonging to Gammaproteobacteria. *Variovorax* was the unique genus belonging to Betaproteobacteria detected in Orologio Tower, in Martano (Fig. 1). Phyla Bacteroidetes, Actinobacteria and Verrucomicrobia were detected in more than one biofilm and Acidobacteria were only found in the Lisbon biofilm (Fig. 3).

Chloroplasts of the phylum Chlorophyta were also detected in this study, which was represented by the genus *Chlorella*, detected in the Orologio Tower (Martano) and in the Santa Clara-a-Velha Monastery (Coimbra). Coimbra showed the highest proportion of clones affiliated to *Chlorella* (Fig. 2) the cyanobacterial 16S rRNA gene-DGGE profile from the natural sample consisted of two dominants bands and three faint bands, all affiliated to chloroplasts of *Chlorella* alga. Martano biofilm also revealed high proportion of *Chlorella*, as demonstrated by the DGGE pattern of 16S rRNA gene fragments obtained after amplification using cyanobacterial primers; the two dominant bands in this profile were affiliated to chloroplasts of this alga (Fig. 1, bands 8 and 9). Cathedral of Seville revealed chloroplasts of the Bacillariophyta *Gyrosigma* (Fig. 4, band 10).

Table 1 summarises the occurrence of the prokaryotic microorganisms detected in the green biofilms of the five monuments. It is clear that Cathedral of Seville showed the widest biodiversity, with six taxa of Bacteria and three of Cyanobacteria. Ajuda National Palace presented the highest biodiversity within Cyanobacteria, with five taxa identified. The microbial communities from Coimbra, Granada and Martano showed the lowest biodiversity.

3.2. Monitoring of photosynthetic cultures

The biofilms growth was macroscopically monitored by the increase of green colour and photosynthetic cells distribution. Visible growth of the phototrophic biofilms started one week after inoculation, presenting a brightly green colour, with the exception of the Orologio Tower biofilm culture. After one month, notable dense green colour and homogeneously distributed photosynthetic cells were observed for Coimbra and Granada biofilm cultures. Liquid cultures of Lisbon and Seville biofilms showed heterogeneous distribution of cells; as the cultures biomass increased, there was an increased tendency for algal aggregates precipitate and for the cells to stick to the glass and steel surfaces. No visible photosynthetic growth was observed for the green biofilm from Orologio Tower (Martano, Italy) during the three months of cultivation.

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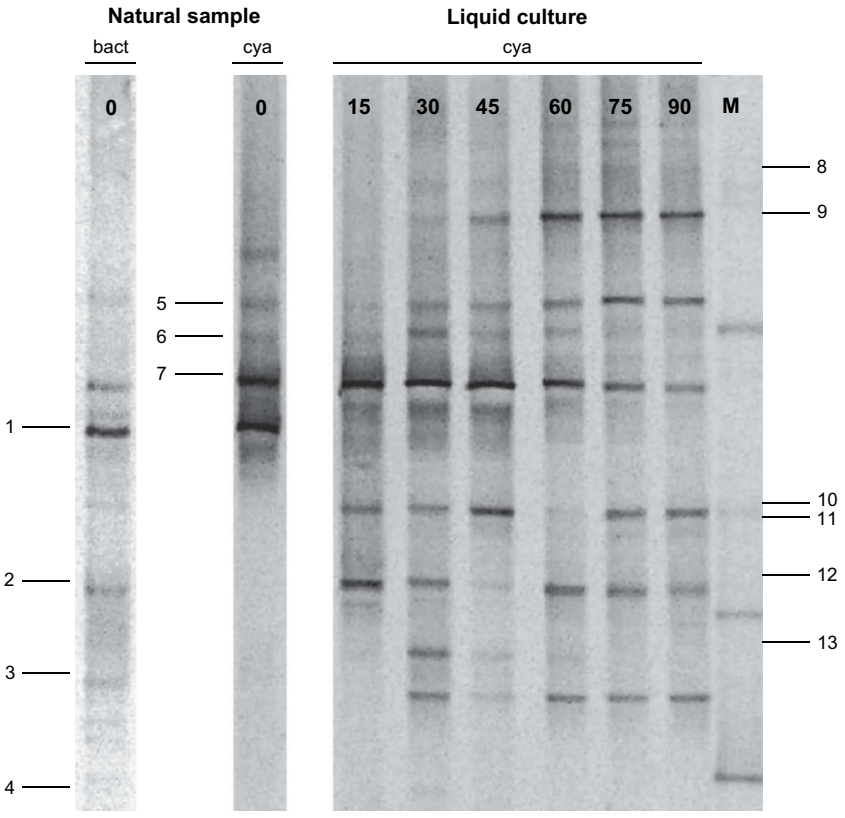

Fig. 2. DGGE profiles of 16S rRNA gene from the natural green biofilm collected from Santa Clara-a-Velha Monastery (natural sample) and its liquid culture monitored during three months (Lanes 15, 30, 45, 60, 75 and 90, corresponding to the sampling days). Lanes "0 bact" and "0 cya" correspond to the DGGE profiles of the inoculum obtained after amplification using bacterial and cyanobacterial primers, respectively. DGGE profiles of the liquid culture were obtained using cyanobacterial primers. The band numbers refer to clones obtained from the natural sample (bands 1–7) and from the sample 75 of the liquid culture (bands 8–13). Lane "M" corresponds to DGGE markers, consisting of *Pseudomonas* sp., *E. coli*, *Paenibacillus* sp. and *Streptomyces* sp. Identification of bands based on comparison with closest relatives at NCBI databank (Acc. Number in parenthese): 1- *Chlorella vulgaris* chloroplast (EU333015), 2- Bacteroidetes (EU333004), 3- Alphaproteobacteria (EU333006), 4- Actinobacteria (EU333005), 5- *Chlorella vulgaris* chloroplast (EU333016), 6- *Chlorella vulgaris* chloroplast (EU333018), 7- *Chlorella* sp. chloroplast (EU333008), 8- *Chlorella* sp. chloroplast (EU333007), 9- *Pleurocapsa* sp. (EU333017), 10- *Leptolyngbya* sp. (EU333012), 11- Verrucomicrobia (EU333009), 12- *Leptolyngbya* sp. (EU333013), 13- Verrucomicrobia (EU333010). Modified figure from Miller et al. (2008).

Table 1
Occurrence of prokaryotic microorganisms in the green biofilms of the studied monuments, detected by amplification using bacterial and cyanobacterial primers.

	Orologio Tower (Martano)	Santa Clara-a-Velha Monastery (Coimbra)	Ajuda National Palace (Lisbon)	Cathedral of Seville (Seville)	Cathedral of Granada (Granada)
Bacteria					
Acidobacteria			+		
Actinobacteria		+		+	
Bacteroidetes		+			+
Lysobacter	+				
Luteolibacter				+	
Marinobacter				+	
Methylobacterium			+	+	
Nitrobacteria	+				
Porphyrobacter					+
Saccharospirillum				+	
Sphingomonas					+
Stenotrophomonas	+				+
Variovorax	+				
Verrucomicrobia		+		+	
Cyanobacteria					
Chroococcidiopsis			+	+	+
Cylindrospermopsis			+		
Leptolyngbya		+		+	
Microcoleus			+		
Nostoc			+		
Pleurocapsa	+	+	+	+	+

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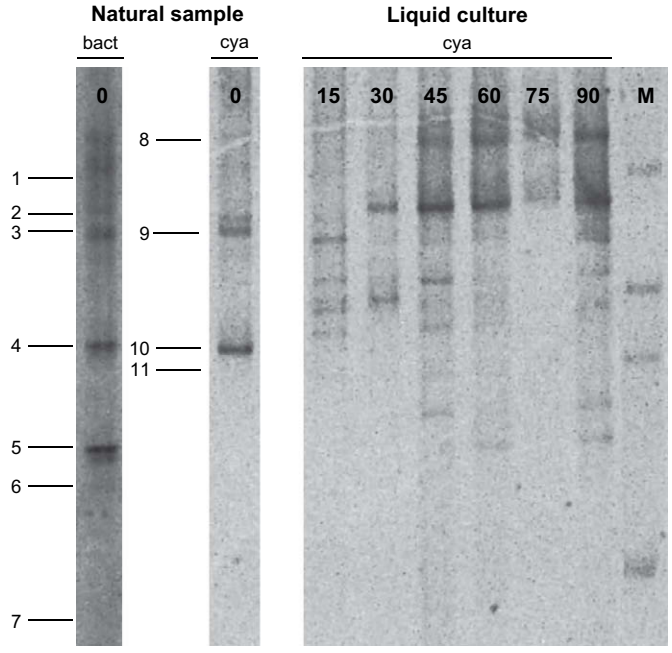

Fig. 3. DGGE profiles of 16S rRNA gene from the natural green biofilm collected from Ajuda National Palace (natural sample) and its liquid culture monitored during three months (Lanes 15, 30, 45, 60, 75 and 90, corresponding to the sampling days). Lanes "0 bact" and "0 cya" correspond to the DGGE profiles of the inoculum obtained after amplification using bacterial and cyanobacterial primers, respectively. DGGE profiles of the liquid culture were obtained using cyanobacterial primers. The band numbers refer to clones obtained from the natural sample. Lane "M" corresponds to DGGE markers, consisting of *Pseudomonas* sp., *E. coli*, *Paenibacillus* sp. and *Streptomyces* sp. Identification of bands based on comparison with closest relatives at NCBI databank (Acc. Number in parenthese): 1- Uncultured Acidobacteria (EU122726), 2- *Cylindrospermopsis* sp. (AF516732), 3- *Chroococcidiopsis* sp. (DQ914863), 4- *Chroococcidiopsis* sp. (EF150802), 5- *Methylobacterium* sp. (AY358007), 6- *Methylobacterium* sp. (AM237344), 7- *Chroococcidiopsis* sp. (DQ914865), 8- *Pleurocapsa* sp. (DQ293994), 9- *Nostoc* sp. (AY742448), 10- *Chroococcidiopsis* sp. (DQ914865), 11- *Microcoleus* sp. (AJ871987).

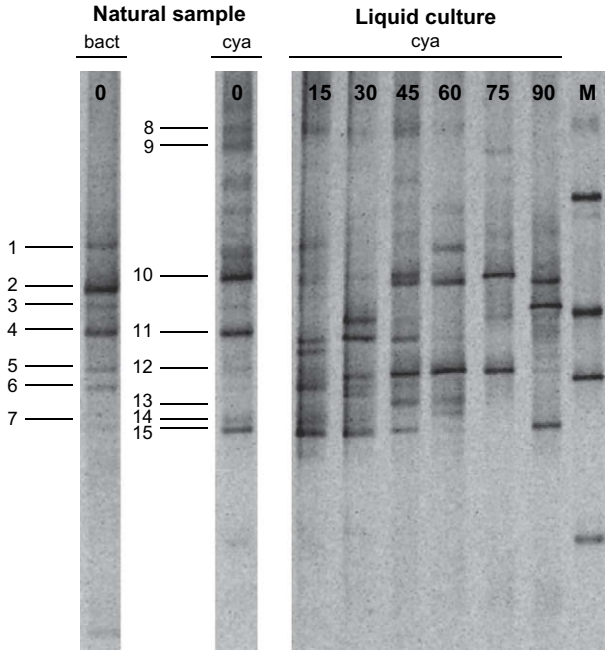

Fig. 4. DGGE profiles of 16S rRNA gene from the natural green biofilm collected from Cathedral of Seville (natural sample) and its liquid culture monitored during three months (Lanes 15, 30, 45, 60, 75 and 90, corresponding to the sampling days). Lanes "0 bact" and "0 cya" correspond to the DGGE profiles of the inoculum obtained after amplification using bacterial and cyanobacterial primers, respectively. DGGE profiles of the liquid culture were obtained using cyanobacterial primers. The band numbers refer to clones obtained from the natural sample. Lane "M" corresponds to DGGE markers, consisting of *Pseudomonas* sp., *E. coli*, *Paenibacillus* sp. and *Streptomyces* sp. Identification of bands based on comparison with closest relatives at NCBI databank (Acc. Number in parenthese): 1- *Methylobacterium* sp. (AB302928), 2- Uncultured bacterium (EU137447), 3- *Saccharospirillum* sp. (AJ315983), 4- *Marinobacter* sp. (DQ665805), 5- Uncultured actinobacterium (EU299252), 6- *Saccharospirillum* sp. (AJ315983), 7- *Marinobacter* sp. (CP000514), 8- *Pleurocapsa* sp. (DQ293994), 9- *Pleurocapsa* sp. (DQ293994), 10- *Gyrosigma fasciola* chloroplast (AF514847), 11- Uncultured Verrucomicrobia (AY770729), 12- *Leptolyngbya* sp. (X84809), 13- Uncultured Verrucomicrobia (AM690904), 14- *Chroococcidiopsis* sp. (DQ914863), 15- *Luteolibacter* sp. (AB331895).

The composition and development of the microbial communities' assemblages were monitored during different incubation times of the photosynthetic cultures by DGGE. Differences in richness over all cultures were observed in the DGGE profiles of the biofilms (Figs. 1–5).

The culture of Coimbra seemed very stable in composition, even over a time span of 90 days. According to band migration, the bands affiliated to chloroplasts of *Chlorella*, present in the cyanobacteria-specific inoculum profile, were dominant in the culture community (Fig. 2, bands 5–7), being detected in all sampling days as well as the bands affiliated to the cyanobacterium *Pleurocapsa* (band 9). *Leptolyngbya* (Fig. 2, bands 10 and 12) was also detected over the course of batch incubation of Coimbra culture. However, it was not detected in the inoculum profiles probably due to altered extractability of the DNA or due to the presence of other dominant microorganisms, such as the microalga *Chlorella*. Minor changes in the Coimbra liquid culture fingerprints consisted in the intensity of bands; the only variation concerned band 1 corresponding to *Chlorella vulgaris*, which was present in the inoculum fingerprint and absent in the liquid culture. In addition, two bands corresponding to Verrucomicrobia were detected in cyanobacteria-specific DGGE (Fig. 2, bands 11 and 13). The DGGE patterns of the liquid culture revealed that after 45 days the culture reached a mature stage, remaining constant until 90 days of incubation.

In all other biofilm cultures variations in the banding patterns occurred. A wide variability was observed in the cultures of Lisbon and Seville biofilms. The cyanobacterial 16S rRNA gene-DGGE profiles from the Lisbon biofilm cultivation revealed little similarity with the inoculum profiles (Fig. 3). The cyanobacteria-specific inoculum profile contained only three visible bands. These dominant bands, affiliated to cyanobacteria *Pleurocapsa*, *Nostoc* and *Chroococcidiopsis* (Fig. 3, bands 8–10), were not visible in the culture fingerprints. The DGGE profiles of the cultivated biofilm showed only two dominant bands and several faintly visible bands. The two top bands of the liquid culture were dominant over the course of batch incubation; according to band migration, the first band corresponded to *Pleurocapsa* sp. In the sample from day 90, the DGGE profile contained up to seven distinguishable bands, suggesting a higher biodiversity.

The widest variations in the DGGE profiles of the cultures were recorded in the case of the Seville biofilm culture. Their inoculum profiles showed greatest biodiversity between all the natural samples studied, which was revealed by the presence of several bands with different intensities (Fig. 4). This variability was also recorded for its liquid culture which DGGE profiles showed marked changes in the number and intensity of bands in the different sampling days. Bands faintly visible in samples taken during the incubation were identically positioned to bands 8 and 9 (Fig. 4), which corresponded to *Pleurocapsa* sp.

The cyanobacteria-specific inoculum DGGE profile from Granada biofilm (Fig. 5) also showed a very faintly visible band affiliated to the cyanobacterium *Pleurocapsa*. As occurred to the cultivation of Coimbra biofilm, Granada culture showed similarities with the initial inoculum. Only minor variations were seen in the sample from day

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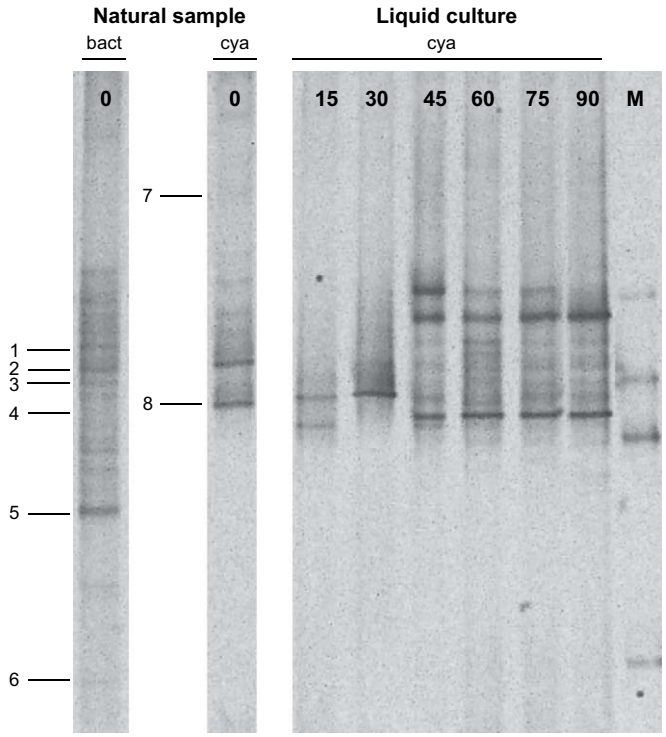

Fig. 5. DGGE profiles of 16S rRNA gene from the natural green biofilm collected from Cathedral of Granada (natural sample) and its liquid culture monitored during three months (Lanes 15, 30, 45, 60, 75 and 90, corresponding to the sampling days). Lanes "0 bact" and "0 cya" correspond to the DGGE profiles of the inoculum obtained after amplification using bacterial and cyanobacterial primers, respectively. DGGE profiles of the liquid culture were obtained using cyanobacterial primers. The band numbers refer to clones obtained from the natural sample. Lane "M" corresponds to DGGE markers, consisting of *Pseudomonas* sp., *E. coli*, *Paenibacillus* sp. and *Streptomyces* sp. Identification of bands based on comparison with closest relatives at NCBI databank (Acc. Number in parenthese): 1- Uncultured Bacteroidetes (AM168126), 2- *Porphyrobacter* sp. (AB033328), 3- *Chroococcidiopsis* sp. (DQ914863), 4- *Stenotrophomonas* sp. (DQ109037), 5- *Sphingomonas* sp. (EU814953), 6- Uncultured Bacteroidetes (EF522197), 7- *Pleurocapsa* sp. (DQ293994), 8- *Chroococcidiopsis* sp. (DQ914863).

30 which last band, strongly visible in the other sampling days, was faintly visible in this profile. Bands that migrated in the same position as band 8 (Fig. 5) were present in the first two sampling days and almost disappeared after 45 days of incubation. This is probably due to senescence of the microorganism.

The cyanobacterium *Pleurocapsa* was present in all biofilms cultivation, with one exception, the Martano biofilm cultivation which no photosynthetic growth was obtained during the batch incubation. Consequently, it was not possible to monitor by DGGE the composition and development of the phototrophic community assemblage. Nevertheless, bands affiliated to *Pleurocapsa* were also detected in the inoculum profiles (Fig. 1, bands 1 and 7).

4. Discussion

The analysed biofilms consisted of a phylogenetically diverse array of prokaryotes including cyanobacteria as well as bacteria belonging to Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia and Acidobacteria. The biofilms also included green algae and Bacillariophyta, as indicated by the detection of chloroplasts through molecular techniques. The cyanobacterial and proteobacterial sequences were the most common and accounted for the largest fractions of each community.

The diversity of species found in our study is similar to those observed in other studies on historic buildings and monuments (Ortega-Calvo et al., 1991; Lamenti et al., 2000; Tomaselli et al., 2000; Bellinzoni et al., 2003). Most detected microorganisms are found in soils and water habitats, not being characteristic of any particular region.

Comparative analyses of the sequences obtained in this work revealed clear differences between the microbial communities of the five green biofilms (Table 1). Differences in the bacterial and cyanobacterial diversity among the Martano, Coimbra and Granada samples were not as high as those between Lisbon and Seville samples. Lisbon biofilm presented the largest proportion of microorganisms belonging to Cyanobacteria, being dominated by the cyanobacterium *Chroococcidiopsis*, also present in Seville and Granada samples. This cyanobacterium can survive extreme cold, heat and arid conditions and it may be the single autotrophic organism most tolerant to environmental extremes (Graham and Wilcox, 2000). The identification of *Chroococcidiopsis* indicates that endolithic growth may occur in the analysed monuments since its presence has been reported in studies concerning endolithic growth in rocks (Friedmann, 1982; Banerjee et al., 2000). The nitrogen-fixing cyanobacterium *Pleurocapsa* was detected in all analysed photosynthetic biofilms. Tomaselli et al. (2000) reported that the most widespread cyanobacteria occurring in photosynthetic communities dwelling on stone monuments were *Chroococcidiopsis*, *Pleurocapsa*, *Leptolyngbya* and *Plectonema*. Three nitrogen-fixing cyanobacteria, *Cylindrospermopsis*, *Nostoc* and *Pleurocapsa*, were found in our work. The presence on buildings of nitrogen-fixing cyanobacteria has been considered as possibly relevant for the establishment and development of other organisms, such as heterotrophic bacteria (Grant, 1982).

Samples from the Cathedral of Seville presented the largest proportion of microorganisms belonging to Bacteria. Proteobacteria was the most representative phylum detected in these samples. Their presence have been previously reported and comprises common members of the bacterial community considering stone materials (Gurtner et al., 2000; Piñar et al., 2002; Akatova et al., 2007; Cappitelli et al., 2007). Stone monuments can represent extreme habitats, favouring the growth of a variety of specialized microorganisms. For instance, the detection of *Marinobacter*, belonging to Gammaproteobacteria, in samples from Cathedral of Seville could be an indicative of salt efflorescences since these organisms are capable of extremophilic lifestyles and are moderately halophilic and mesophilic (Gauthier et al., 1992). *Methylobacterium* is a facultative methylotroph, mostly found in soils and plants, which has the ability to grow by reducing carbon from methyl groups or short chain aliphatic compounds. Aliphatic hydrocarbons are examples of those sorts of organic compounds in building stones located in urban environments, which have been detected in samples from Seville Cathedral by Saiz-Jimenez (1993). The presence of this bacterium was reported in Lisbon and Seville samples, and also in samples derived from the wall paintings of Catherine Chapel in the Castle of Herberstein (Gurtner et al., 2000).

Since the bacteria detected in the analysed photosynthetic biofilms are generally heterotrophic microorganisms, their growth must be dependent on the presence of organic matter. In fact, photoautotrophs, as major primary producers, are considered pioneer inhabitants of stone materials and heterotrophic microorganisms are known to inhabit these microbial communities (Ortega-Calvo et al., 1993; Urzì and Krumbein, 1994). Organic matter provided by extracellular polymeric substances release by the phototrophs is the main source of organic carbon for the heterotrophic microorganisms. Furthermore, the phototrophic microorganisms, when dead, become a nutrient source for heterotrophic bacteria. Instead, stone monuments located in urban environments act as repositories of organic and inorganic pollutants which accumulate on their surfaces. Aliphatic hydrocarbons are examples of organic compounds found on building stones

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located in urban environments, which were detected in samples from Seville Cathedral by Saiz-Jimenez (1993).

Our results showed that the studied photosynthetic biofilms can develop as a small ecosystem with many different functional groups of organisms. There is increasing evidence that there are specific bacterial taxa associated with photosynthetic microorganisms. Interactions of algae and bacteria range from symbiotic, via commensal, to parasitic interactions. Most studies on bacterial–algal interactions have focused in phytoplankton (González et al., 2000; Pinhassi et al., 2005). Therefore, experiments with microbial communities composed by different groups of organisms are essential for microbial ecology studies. In this respect, the culturing experiments with phototrophic biofilms performed in this work aimed to exhibit a high resemblance with the natural phototrophic communities. However, our results demonstrated that phototrophic biofilms cultured in the laboratory do not easily generate stable and diverse microbial communities. We have monitored five different biofilm cultures and only one of them reached stable conditions with an acceptable natural-looking diversity. According to Roeselers et al. (2006), the end of exponential growth does not necessarily mean that a stable climax community has established. The biofilms may be still in a transient state, developing slowly towards a final convergence. The cases probably following this common evolution were Lisbon and Seville biofilm cultures, which inocula showed the highest microbial diversity and the greatest variability during the incubation time, likely due to shortage or absence of special nutrient requirements. In the case of Seville biofilm, the culture variability indicates that a rather unstable community was cultivated or the biofilm culture was still in a transient state and a stable community has not established. The reason for this instability is probably due to the presence of bacteria degrading recalcitrant compounds (e.g. polycyclic aromatic hydrocarbons) (Saiz-Jimenez, 1995; Ortega-Calvo and Saiz-Jimenez, 1997). Obviously these bacteria were unable to grow under laboratory conditions, where no recalcitrant compounds were added.

In contrast, the cultured community obtained from Coimbra showed a stable and diverse community after 75 days of incubation. This community included algae, cyanobacteria and bacteria which were also the major components of the natural green biofilm causing deterioration. This is a requirement for comparative monitoring and evaluation of biodeterioration studies under laboratory conditions. Biodeterioration studies, monitoring and control, require the development of standard procedures to quantify and approach these phenomena under reproducible conditions. One of the essentials of these procedures is the use of an acceptable microbial community able to simulate natural conditions of colonisation and in order to satisfy this requirement it is necessary to obtain diverse microbial communities composed by a stable set of components. In this study we have been able to obtain a stable microbial community reflecting natural components and maintaining its diversity along the time.

Further work should focus the study of the biodeterioration processes and the interaction between microorganisms and the mineral substrate through laboratory-based stone colonisation experiments. Artificial biofilms should be obtained by inoculating the stone substrates with the multiple-species phototrophic community culture obtained in this work.

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