A survey of bacterial colonisation of historic limestone buildings: Lincoln Cathedral and St. Peter at Gowts, United Kingdom.

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ABSTRACT: Built heritage may be at risk from the effects of biofilms (a microbial community encapsulated in a matrix of sugars, protein and DNA). Some microbes in biofilms damage stone surfaces and cause staining. Although biofilm research has been carried out in Mediterranean regions, few studies cover temperate Northern Europe climates, or the UK. This research concentrates on bacterial colonization of limestone, a building material that is highly vulnerable to many agents of deterioration. We recorded environmental conditions on damaged and undamaged stone at two Lincoln sites, and sampled surfaces for bacteria. A correlation between low surface pH and damage was observed. Some bacteria cultured were able to acidify their environment, which may have a significant contribution to loss of limestone through acidic decay mechanisms. As well as increasing our knowledge in a currently under-researched area of environmental microbiology, this study provides valuable information for the conservation of historic buildings.

1 INTRODUCTION

In England alone there are currently 374,081 listed building entries. These are buildings which have been identified as nationally or internationally important, and worthy of preservation (English Heritage, 2013). A search of the listed buildings database shows that almost 36,000 of these listings relate to heritage sites composed partially or entirely of limestone. With such a substantial amount of built heritage, it is important that the mechanisms of its deterioration are understood, in order to inform conservation treatments and decision making.

It has long been acknowledged that the deterioration seen on stone surfaces results from chemical, physical and biological processes. Biological processes of decay range from plants to bird droppings, but can also be due to the colonisation of the stone surface by microorganisms, such as bacteria, fungi and algae, in the form of biofilms.

A biofilm is a community of microorganisms which excrete protective materials, usually proteins and sugar polymers, to produce an extracellular polysaccharide matrix (EPS) (Lewis, 2001). The EPS protects the microorganisms from desiccation and physical damage while providing a greater surface area for them to occupy. It is gradually becoming recognised that many bacterial species will spend at least part of their life cycle in a biofilm. Recent studies have identified biofilms containing bacteria and fungi from many sources of historically relevant stonework including Mediterranean statuary (Miller *et al*, 2012; Dakal and Cameotra, 2012).

Bacterial colonisation has also been studied on a range of historic artifacts, from leather to wallpaintings. Species of interest that have been implicated in deterioration include *Bacillus sporothermodurans, Bacillus pumilus, Arthrobacter agilis, Micrococcus luteus* and many others (Heyrman and Swings, 2001; Dakal and Cameotra, 2012; May *et al*, 2000; Allsopp *et al*, 2004).

There is evidence to suggest that although some biofilms may have a detrimental effect on stone, in some circumstances biofilms may be protective of stonework (Doehne and Price, 2010). It is thought that growth of biofilms on surfaces can produce discoloration, chemical deterioration and promote physical deterioration. Discoloration can be caused by excreted bacterial by-products staining or chemically changing surfaces, as well as the natural pigmentations of the bacteria or other microorganisms (Urzi, 1992; Urzi, 1993). Bacterial metabolism often results in acidic by-products which can result in chemical damage, as can the scavenging of minerals essential for bacterial growth and excretion of salts and EPS. The damage caused by these mechanisms can seed physical deterioration processes by producing the initial disruption of the stone surface. In addition, the EPS may affect the pores within the stonework through a physical process, as the biofilm swells and shrinks within the pore network which may cause microfractures (Dornieden *et al*, 2000). The alteration of moisture circulation through the pores of the stone may also impact on stability of the stone itself (Warscheid and Braams, 2000). Furthermore, evidence suggests that biofilms may encourage the build-up of pollutants from the atmosphere on the stonework, thus accelerating the deterioration rates Young, 1996; Mansch and Bock, 1998). However with such mixed evidence, concentrating on the Mediterranean climate, further research is needed to understand the colonisation of bacterial species on stone, and the potential effects on deterioration.

To protect our built cultural heritage from potentially damaging biofilms, standard conservation treatments use chemicals to kill bacteria, and cleaning to remove bacterial growth. These methods are effective for only a short time, as regrowth and recolonisation is rapid. As conservation is based around ethics of minimal intervention, the ideal treatments would remove potentially damaging or unsightly species from the biofilm, while leaving the stonework patina intact. By further understanding the bacterial colonisation of stone, this will aid conservation treatment decisions and development of cleaning or control methodologies.

A significant number of historic properties in the county of Lincolnshire, UK are built from Lincoln limestone, a locally available stone which is still actively quarried for use on local buildings, with 96% of the buildings in the British Geological Survey report of historic properties in Lincolnshire (2013) being built at least partially from Lincoln Limestone. Although it is predominantly present in the Lincoln area, it has been used in properties from Winsdsor Castle, London to York Railway Station (British Geological Survey, 2013). Limestone is a sedimentary rock composed largely of different crystalline forms of calcium carbonate, and as such is soluble in the presence of weakly acidic solutions. As one of the potential mechanisms of bacterial deterioration of stone is the production of acids, colonization by acid producing bacteria would be likely to have a detrimental effect on this stone.

This study focuses on identifying bacterial colonisation in an oceanic climate, and considering their potential effects on the stonework, specifically buildings made from limestone in an urban environment, i.e. a settlement with greater than 10,000 population (Defra, 2013). The oceanic climate differs from the Mediterranean climate by having no dry season, the Mediterranean climate can see up to 6 months without significant precipitation. The average high summer temperatures in an Oceanic climate are also lower than those seen in a Mediterranean environment (Peel *et al*, 2007), 15°C in the Lincolnshire region of the United Kingdom (Met Office, 2000). In collaboration with Lincoln Cathedral and the Diocese of Lincoln we carried out sampling of bacterial biofilms present on Lincoln Cathedral, a more exposed site close to the edge of the Lincoln Cliff at 72.8m above sea level, and Saint Peter at Gowts in Lincoln, a site in the valley below the cliff at 20.4m above sea level. Both buildings were made of Lincoln limestone, and are shown in figure 1. Information obtained in this study will aid our understanding of bacterial colonisation on historic built heritage. Environmental measurements were also taken, to compare the differing conditions at the sites. This will enable us to investigate whether the environment had a role in the bacterial colonisation of stonework, and whether an environmental marker could be found which would enable areas of concern to be located quickly through a simple testing process.

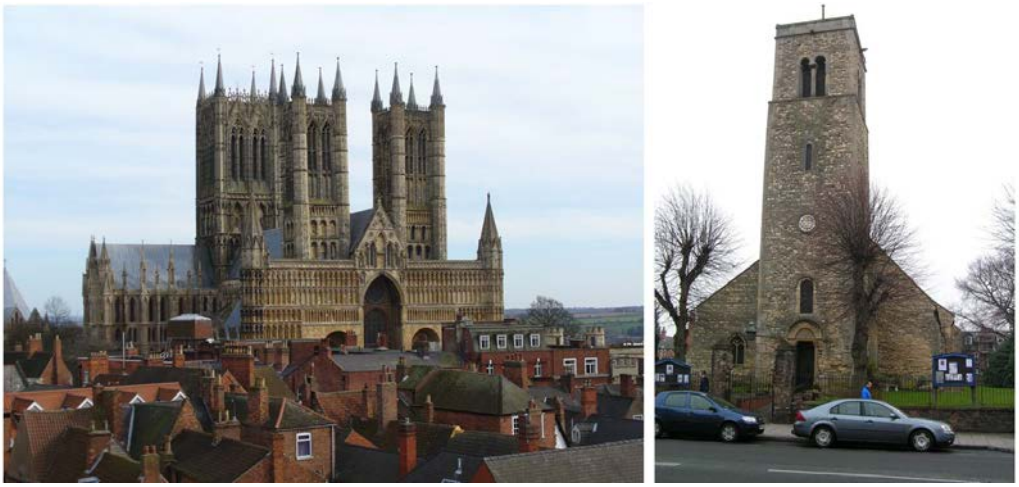

Figure 1: Lincoln Cathedral, left, and Saint Peter at Gowts, High Street Lincoln, right.

2 METHODS

Five external sites were sampled from Lincoln Cathedral, with four external sites sampled from St Peter at Gowts, High Street, Lincoln, UK. Where possible, sites were chosen where an undamaged stone was adjacent to a damaged stone, with both being sampled in order to achieve a direct comparison of bacterial colonisation. Damaged stones were defined as having greater than ten percent of the surface showing a minimum deterioration of 2mm depth. At two sites at the Cathedral samples of bacteria were taken only from undamaged stone, these being an area of the Cathedral with extensive copper staining, and a recently built addition to the Cathedral (completed 2008). At St Peter at Gowts, a sample was taken from a flat gravestone within the path, which was observed to have a highly mucoidal surface. Each site was photographed and all areas of damage recorded during the sampling.

2.1 Sampling - Sellotape and swabs

Sampling of the stonework was performed in duplicate. To obtain a sample of biofilm for microscopy work a 4 cm long piece of double sided sellotape was pressed firmly against the stonework. A glass slide was then placed against the side without the sample on it. To obtain samples for culturing the bacteria, a sterile swab was dipped in M9 salts and wiped over a 1cm square region of the stonework next to the sellotape sampling area. At all stages of sampling nitrile gloves were worn to prevent contamination of the samples with skin fauna.

2.2 *Environmental data recording*

Relative humidity and light (lux and UV) measurements were taken at each recording site using an Elsec 765 Environmental Monitor (Littlemore Scientific).

Moisture readings of the surface of each stone sampled were obtained using a protimeter (GE Protimeter Mini BLD2000). Surface pH readings were taken using narrow range pH paper (Whatman, pH 4-6 and 6-8) moistened with distilled water.

2.3 *Staining of sampled biofilms*

Biofilms were stained with FilmTracer™ FM® 1-43 Green Biofilm Cell Stain and FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain with the only deviation from the manufacturers protocol being a further two fold dilution of the stain in distilled water. This change was based on the experimental observation that at higher concentrations of the FM 1-43 stain it was difficult to see the Ruby Biofilm matrix staining. Unstained samples were also observed to identify any autofluoresence in the sellotape, stone or biofilms.

Observation of the stained biofilms was performed with a Nikon ECLIPSE E800 model fluorescent microscope as per the manufacturers protocol.

2.4 *Bacterial identification*

Bacterial species were initially isolated from the samples based on physical characteristics and identification was initiated using traditional microbiological tests. All species were also identified by the genomic sequence of their 16S rRNA gene, a region of genomic DNA commonly used for bacterial identification. This was achieved by amplifying a region of the 16S rRNA gene through the polymerase chain reaction (PCR) to ensure sufficient material of a high enough purity was available for the sequencing.

2.5 *Traditional methods of bacterial identification*

Micro-organisms were isolated from the swabs by adding 1ml of M9 salts to the swab holder and vortexing at full speed for 5 minutes. The resulting suspension was then plated out onto Nutrient Agar (Oxoid CM0003B), and grown for 96 hours at 25ºC. Plates were then inspected and where possible bacterial colonies were selected and re-streaked onto Nutrient Agar based on variations in colour and morphology, they were then grown for 96 hours at 25ºC.

On plates where the growth was confluent, samples were taken and re-streaked onto Nutrient Agar and grown for 96 hours at 25ºC. This process was repeated until single colonies could be isolated.

Following the isolation of single species on the plates they were then left for a further 96 hours at 25ºC to confirm that there were no slower growing organisms whose presence was obfuscated by the selected species. In cases where these were seen the two species were separated and re-streaked, again being grown for the appropriate period at 25ºC.

Initial identification of the isolated bacteria was by gram staining using standard protocols, at the same time the species were tested using Catalase and Oxidase tests. Based on the data from these tests, bacteria where it was considered that rapid identification could be performed based on sugar metabolism, were tested for metabolism of glucose, lactose, xylose and maltose using an Oxidative-Fermentative test.

2.6 *Identification of micro-organisms – DNA extraction protocol*

The bacterial genomic DNA was extracted using an in house protocol which is suitable for both gram positive and gram negative bacteria. Cells were grown as a static planktonic culture for 96 hours at 25ºC in 4ml of Nutrient Broth (Oxoid CM0001B). 1ml of the broth was taken and centrifuged at full speed in a microcentrifuge for 2 minutes to pellet the cells. The supernatant was disposed of and the pellet resuspended in 100µl of TE (Sigma, T9285-100ML) buffer with 2µg of a 1mg/ml stock Lysozyme (Sigma, 62971-10G-F) added and incubated, shaken, for 30 minutes at 37ºC. 50µl of a 10% SDS stock was then added and the sample was incubated at 60ºC for 30 minutes. The tube was then microcentrifuged at full speed for 5 minutes and the supernatant transferred to a fresh tube. A further 100µl of TE buffer was added to the supernatant to increase the volume for ease of handling, and then a standard phenol chloroform isoamyl alcohol extraction was performed with a final chloroform step to ensure the elimination of any phenol from the sample. DNA was precipitated with ice cold 70% Ethanol and then resolubilised in 30µl of ultrapure water. Where it was not possible to use the extracted DNA in a PCR reaction straight away, samples were stored at -20ºC.

2.7 *Identification of micro-organisms – 16S rRNA sequencing with primer sequences*

Regions of the 16S rRNA gene were then PCR amplified from the extracted genomic DNA for each isolated species. The amplification was performed twice, once with the commonly used Universal primers amplifying a 1498bp region between nucleotides 27 and 1525 and once with in-house primers which amplify a 322bp region between nucleotides 764 and 1084. The PCR machine was run with an initial denaturing step of 95 ºC for 10 minutes followed by thirty four cycles of 95 ºC for 30 seconds, 47.9 ºC for 30 seconds and 72 ºC for 1 minute 30 seconds. The run ended with a final extension step of 72ºC for 10 minutes. Amplification was performed using OneTaq® 2x Master Mix (New England Biolabs, M0482) The in-house primers were used alongside the Universal primers to improve accuracy. PCR products from both reactions were sent to Beckman Coulter Genomics for sequencing. Sequencing data was reviewed using the FinchTV software package provided by Geospiza and then used to identify the individual species by BLAST searching the sequence on the NCBI website[1].

Primer name	Primer sequence
16S-rRNA_F27	AGAGTTTGATCMGGC
16S-rRNA_R1525	AAGGAGGTGWTCCARCC
MS_BACT-16S_For	GGATTAGATACCCTGGTAGTCC
MS_BACT-16S_Rev	TCGTTGCGGGACTTAACCCAAC

Table 1: Primers used for PCR and sequencing of the 16S rRNA genes from bacterial studies. 16S-rRNA-F27 and 16S-rRNA-R1525 are universal primers commonly used for this purpose. MS_BACT-16S_For and MS_BACT-16S_Rev are highly robust in house primers used for the same purpose by the University of Lincoln. All primers were produced by Sigma.

3 RESULTS

In order to investigate the presence of biofilms on the stone of the two buildings, sites were chosen for sampling based on the following criteria:

- Evidence of biological patina on the stonework, i.e. discolouration, mucoidal appearance to the surface.
- Damaged stone surfaces next to undamaged (this was not possible at every sample site).
- Location; where possible samples were taken at ordinal points (namely north, south, east and west sides).

3.1 *Determining the presence or absence of biofilms*

Almost all locations at the two sites tested positive for the presence of intact biofilm based on sellotape sampling, as illustrated in figure 2. While some autofluoresence of the limestone was observed it did not interfere with the visualisation of the biofilms through the fluorescent stains. The only site where we were unable to acquire biofilm using the sellotape method was site 4 at Lincoln Cathedral, under the main window, which had been chosen because of extensive copper staining to the stonework from the main window. As known biofilm forming bacteria, including *Bacillus cereus var mycoides* were isolated from this site it is possible that the copper staining interfered with sample acquisition. Alternatively, it may be that the copper inhibited full biofilm formation, as it is known to inhibit growth of a number of other bacteria, including some of the Bacillus species (Chang and Tien, 1969).

Figure 2: Sellotape sample from undamaged stone at site 2 of Lincoln Cathedral showing intact biofilm as large red areas, orange clusters within the areas show a high concentration of bacteria, green clusters are algal species

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3.2 *pH and moisture measurements of damaged and undamaged stone*

Readings of dampness obtained with a Protimeter across the sites varied from 12% to 55% wood moisture equivalent (WME). WME is a standard reading of the equivalent moisture level of wood when in equilibrium with the stone. However the level of 55% was found only at the copper saturated site, and will be excluded from further discussions as no biofilm was detected in that area. The remainder of the dampness readings were in the 12-30% range.

The average dampness reading from undamaged stone was 18.3%, whereas the average reading from damaged stone was 22%, which is not a statistically significant variation ($p=0.2$, Student's t-test).

Each site was also checked for acidity levels, which ranged from 6.5 to 5. It is interesting to note that the least acidic stone, at pH 6.5, was the most recently quarried stone, with that area of the Cathedral having been completed in 2008.

Damaged stone was found to have an average pH of 5.36, whereas undamaged stone had an average pH of 5.92. Using a Student's t-test to compare the pH readings, there is a statistically significant difference ($p<0.05$) between the pH of damaged and undamaged limestone.

3.3 *Bacterial Identification*

A total of 42 separate species were identified from the two sites, 16 of which were only associated with damaged stone, 12 were associated with damaged and undamaged stone and 14 were only associated with undamaged stone (Table 2).

Only on damaged	Only on undamaged	Found on both
Acinetobacter calcoaceticus	*Acinetobacter venetianus*	*Arthrobacter sp*
Arthrobacter agilis	*Advenella kashmirensis*	*Bacillus cereus*
Bacillus licheniformis	*Arthrobacter phenanthrenivorans*	*Bacillus cereus var mycoides*
Bacillus muralis	*Chryseobacterium sp*	*Bacillus simplex*
Bacillus pumilus	*Frigoribacterium sp.*	*Bacillus sp.*
Bacillus sporothermodurans	*Paenibacillus sp.*	*Micrococcus halobius*
Bacillus subtilis	*Paenibacillus sp. 1105*	*Micrococcus luteus*
Brevibacillus brevis	*Penicillium chrysogenum*	*Micrococcus roseus*
Curtobacterium flaccumfaciens	*Pseudomonas fluorescens*	*Pseudomonas sp.*
Isoptericola variabilis	*Psychrobacter sp.*	*Pseudomonas stutzeri*
Microbacterium sp.	*Solibacillus silvestris*	*Staphylococcus xylosus*
Paenibacillus pabuli	*Stenotrophomonas maltophilia*	*Sporosarcina saromensis*
Pseudomonas putida	*Stenotrophomonas rhizophila*	
Pseudomonas sp. HZ06	*Streptococcus gp B*	
Acinetobacter sp.		
Streptomyces sp.		

Table 2: Bacterial species identified during the study. 16 species were associated solely with damaged stone surfaces, 14 with undamaged stone and a further 12 species were associated with both damaged and undamaged stone.

On average 4.37 species were isolated from each of the undamaged stones sampled, and 5.86 species from each of the damaged stones, suggesting a trend towards greater biodiversity on damaged stone.

With the species that were associated with both damaged and undamaged stone it is of interest that *Micrococcus luteus* was isolated at twice as many damaged stone sampling points as undamaged.

Of the other species identified, *Bacillus simplex*, *Micrococcus roseus* and *Staphylococcus xylosus* were more prevalent on damaged stone than undamaged. Conversely, *Bacillus cereus*, *Micrococcus halobius* and *Bacillus cereus var mycoides* were more commonly isolated from undamaged stone. However further data will be needed to determine if there is any preference for damaged or undamaged stone by the species, or whether this observed differentiation has arisen by chance.

Bacillus licheniformis was isolated at three of the seven damaged sites compared to the other species identified which were unique to the sites where they were isolated. Preliminary results from additional sampling in the South West of the UK also shows isolation of *B. licheniformis* exclusively from damaged stone, increasing the potential significance of this observation.

Previous studies have identified bacterial species in the Order of Actinomycetes as being associated with damage to stonework so the species identified were also analysed by Order. For the damaged stone 15 out of 39 were identified as the Order Actimomycetes, compared to 10 out of 33 for undamaged stone. Again further data will be required before we could comment on the statistical significance of this observation.

4 CONCLUSIONS

Although there was no clear link between stone moisture content and damage, we found a clear and significant correlation between pH of stonework and deterioration. Damaged stone was generally more acidic, with a pH of 5.5 or less. In general, undamaged stone had a pH of around 6. As the relatively new, undamaged stone had a pH of 6.5, it suggests that the stone becomes more acidic over time, as deterioration processes occur. While there are a number of potential causes for increasing acidity on the stone surface, including the effects of pollution, colonisation by bacteria which produce acids may contribute to this. When examining the data from adjacent stones, it is clear that the damaged stone of the pair had a more acidic surface than the directly adjacent undamaged stone. It is likely that airbourne pollutants would affect stonework equally when they are in close proximity, implying that local, surface effects are making a contribution to this acidity.

The increased acidity levels suggests that taking pH measurements of stone may be a marker for deterioration, with the possibility of a change in acidity suggesting a need for conservation treatment before damage is visible to the eye. This is an area which would benefit from further investigation in order to fully explore this possibility, especially as species such as *B. pumilis*, *P. putida* and *M. roseus*, which were detected on stonework in this study, have been shown experimentally to acidify their environment under the correct conditions.

Of the species unique to damaged stone, only the *B. licheniformis* and unidentifiable *Acinetobacter* species were found on more than one location. *B. licheniformis* has been previously associated with biodeterioration of mural paintings in the Servilia Tomb (Necropolis of Carmona, Seville, Spain) by Heyrman and Swings (2001) and frescos in Assisi (Radaelli *et al*, 2004). The bacterial species on both damaged and undamaged stones, named in column three of table 2, were found at multiple sampling sites at both Lincoln Cathedral and Saint Peter at Gowts.

When taken to the level of Order rather than Species for bacterial identification the dominant Orders of bacteria were Bacillus and Actinomycetes, this ties in well with previous studies (May *et al*, 2000) where the isolates of Acintomycetes on damaged versus undamaged stone were at least 24 fold higher.

Within the scope of this study we have been unable to confirm whether *B. lichenoformis* is responsible for damage to stone, or whether the environment created by other associated species is more conducive to its growth. However while further study is needed, the data from this study and the further research previously mentioned shows a high correlation between *B. lichenoformis* and damaged stone. This makes it, at the very least, a strong marker for damage. Depending on when *B. licheniformis* colonises damaged stone, detection of this species may also provide an ‘early warning system’.

5 ENDNOTES

1. http://blast.ncbi.nlm.nih.gov/Blast.cgi

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8 SUPPLIERS

Whatman pH paper, Lysozyme and TE buffer are available from Sigma-Aldrich Company Ltd. Dorset, England

OneTaq 2x PCR Master Mix is available from New England Biolabs, Hitchin, England.

Elsec 765 from Littlemore Scientific Gutchpool Farm, Gillingham, Dorset SP8 5QP

GE Protimeter from Mini Survey Express Services, 218 - 220 Brownhill Road, London, SE6 1AT.