'Biodeterioration of limestone: role of bacterial biofilms and possible intervention strategies'

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PhD Thesis

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To my wife, Lynda Skipper.

Without whom none of this would have been possible.

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This dissertation is the result of my own work and includes nothing that is the outcome of work done in collaboration except where it is specifically indicated in the text.

Abstract

Limestone built heritage is at risk from the effects of biofilms, a microbial community encapsulated in a matrix of sugars, protein and extracellular DNA. Although biofilm research has been carried out in Mediterranean regions, few studies cover temperate Northern Europe climates, or the UK. This study concentrates on bacterial colonisation of Lincoln limestone, a highly vulnerable building material, and identifies the species, their role in biodeterioration and the efficacy of biocides against them.

As part of this study the core species which comprise the bacterial component of the limestone microbiome have been characterised for the first time; this has allowed the identification of non-core species which are significantly associated with damaged and undamaged surfaces.

Four mechanisms of biodeterioration have been identified, one previously unidentified, and isolated species have been characterised as to whether they are biodeteriorative and the mechanisms of biodeterioration that they employ. Two species, *Curtobacterium flaccumfaciens* and *Solibacillus silvestris*, have been characterised as producing biofilm matrix which actively causes biomechanical damage to the oolitic limestone structure as opposed to the passive enhancement of physical weathering which has been previously associated with biofilm matrix. Species capable of biodeterioration have also been shown to be present on both damaged and undamaged surfaces, something which has not been previously investigated.

Environmental sampling, species identification and characterisation of species for biodeterioration have all combined to identify markers of biodeterioration, ie both physical markers and biomarkers. Specifically, a surface pH of 5.5 or lower and the presence of *B. licheniformis* is indicative of biodeterioration with a proportionally higher level of *M. luteus* when comparing damaged and undamaged stone.

Finally this study brings the literature on conservation methods up to date by testing biocides which are in current usage, as many biocides in the literature are discontinued. This study is also the first in the field to show their efficacy against biofilm encapsulated bacteria and their propensity for chemically disrupting the biofilm matrix.

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Professional development from this study

Research outputs from this study.

Peer reviewed publications

Skipper, P.J.A., Williams, D.R. and Dixon, R. (Manuscript in Draft, expected submission to Frontiers in Microbiology), Characterisation of the microbiome of damaged limestone in an oceanic climate.

Skipper, L.K., Skipper, P.J.A. and Dixon, R. (Manuscript submitted to Journal of the Institute of Conservation), The effect of biocides on salt contaminated and naive limestone.

Skipper, P.J.A. & Skipper, L.K., 2014. A survey of bacterial colonisation of historic limestone buildings: Lincoln Cathedral and St. Peter-at-Gowts, United Kingdom. In R. Amoêda, S. Lira, & C. Pinheiro, eds. *Rehab 2014 – Proceedings of the International Conference on Preservation, Maintenance and Rehabilitation of Historic Buildings and Structures.* Barcelos, PORTUGAL: Green Lines Institute, pp. 1003–1012.

Skipper, P.J.A., Schulze, H. Williams, D.R. and Dixon, R., 2016. Biodeterioration of Limestone Built Heritage: A Multidisciplinary Challenge. In J. Hughes & T. Howind, eds. *Science and Art: A Future for Stone. Proceedings of the 13th International Congress on the Deterioration and Conservation of Stone - Volume I*. Paisley: University of the West of Scotland, pp. 139–146.

Conference presentations

Skipper, P.J.A., Schulze, H. Williams, D.R. and Dixon, R., 2016. Biodeterioration of Limestone Built Heritage: A Multidisciplinary Challenge. 2016, 13th International Congress on the Deterioration and Conservation of Stone - Volume I. Paisley

Skipper, P.J.A., Gillett, A. and Dixon, R., 2013, Clinically relevant polyculture biofilm produces protective environment for the development of resistance, Third European Congress on Microbial biofilms (Eurobiofilms 2013), 2013, Ghent.

Training and Development

Conferences / meetings attended

13th International Congress on the Deterioration and Conservation of Stone, 2016, Paisley
University of Lincoln Postgraduate Symposium, 2015, Lincoln
Third European Congress on Microbial biofilms, 2013, Ghent
IBBS Antimicrobial Strategies for Biofilm Control, Winchester, 2012

Workshops

To date I have designed and taught two master classes for the Conservation Department, School of History and Heritage, University of Lincoln:

Microscopy for Conservators, 2015
Bacterial sampling and identification for Conservators, 2016

I have also attended in house poster presentation skills workshops and training sessions on Scanning Electron Microscopy and Fluorescent microscopy.

I attended a week long training workshop in 2012 on biofilm growth, characterisation and antimicrobial testing at Health Protection Agency, Porton Down, Salisbury, UK.

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 showed 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 composed of limestone, addressing its preservation is vital for ensuring that this built heritage remains accessible for future generations. It is therefore 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 (Doehne & Price, 2010). Biological processes of decay range from the impact of 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 protective matrix.

Current research into bacterial biodeterioration of stone does not provide a consistent picture of the species or processes involved, and is dominated by studies based around the Mediterranean or Southern Europe, e.g. Clara Urzi's research group whose studies centre around Rome, Italy (Urzi *et al.*, 1992, 1993; Young, 1996b; Urzì *et al.*, 2008; De Leo *et al.*, 2012). The current literature therefore lacks applicability in Northern Europe due to the differences in climate. This study aims to address these issues and provide a solid baseline for future research to build upon.

1.1 Aims

The overall aim of this research is for the first time to detail a 'typical' bacterial microbiome, the microorganisms which inhabit a particular environment, for limestone as a heritage material, using traditional and molecular characterisation to describe the prokaryotic microbiome in an oceanic climate. The role of bacteria in the biodeterioration of stone is under represented in the literature when compared to other microorganisms such as fungi and algae, potentially due to the fact that identification of these microorganisms can be carried out with a light microscope unlike bacteria. It is important therefore to provide a thorough definition of the role of bacteria in the biodeterioration of built heritage. A vital aspect of this will be to identify which of the specific bacteria in the microbiome are responsible for damage to stonework. Most of the literature

makes the assumption that the presence of a species on a damaged surface is responsible for specific damage. This study seeks to establish whether this is indeed the case.

In relation to this, the efficacy of commonly used biocides in heritage conservation will also be investigated. Many biocides used by conservators to clean stone are manufactured to eliminate lichens, fungi and algae; the manufacturers make no claims that they are effective against bacteria. Testing of biocides for potential efficacy for cleaning surfaces of bacterial biofilms will include checking for breakthrough of the individual species, growth of the species into the treated area, as this will provide evidence for which species will drive recolonisation of the cleaned surface.

The homogeneity of limestone chemistry and environmental flexibility of bacterial species means that aspects of these results will be applicable to all but extreme environments. A key aim is to determine whether the environment has a role in the bacterial colonisation of stonework, and to what extent an environmental marker can be found which would enable areas of concern to be located quickly through a simple testing process such as culture and ID or 16S rRNA sequencing.

The specific aims and objectives of the study are as follow:

1. To characterise the limestone lithic microbiome by traditional as well as molecular methods.

   Both traditional and molecular methods will be undertaken in order to address the fact that over 90% of known bacterial species are unculturable in the laboratory (Stewart, 2012). Current research tends not to look at the microbiome as a whole, but focuses on laboratory grown isolates from damaged stone, assuming that if it is present on damaged stone then it must be responsible for the damage. It is also rare to find studies looking at the effects of pollutants on the microbiome, in order to address this we have tested for isolates in a rural location in addition to the urban locations which will provide the opportunity to identify differences which could be due to pollutants. Sampling will be carried out with the specific aim of understanding the differences in the microbiome between damaged and undamaged stone, as well as rural and urban environments. Current studies also assume that the species isolated are biofilm producers as they have been isolated from a stone surface, testing the surface for biofilm or the isolates for biofilm production is rarely seen.

   Hypothesis 1: The limestone microbiome will have discernible differences in identified species between damaged and undamaged surfaces.

Null hypothesis: There will be no significant difference in the species isolated from the damaged and undamaged surfaces.

Hypothesis 2: The limestone microbiome will have discernible differences in identified species between rural and urban locations.

Null hypothesis: There will be no significant difference in the species isolated from rural or urban environments.

Hypothesis 3: Biofilm production will be recognised in > 90% of the species which can be cultured under laboratory conditions.

Null hypothesis: Biofilm production will not be observed in the majority of species.

2. To characterise the physical and geochemical properties of Lincoln limestone in order to characterise its potential bioreceptivity.

   Hypothesis 4: Lincoln limestone provides a surface which encourages biological colonisation.

   Null hypothesis: Lincoln limestone provides a surface which is neutral or inimical to biological colonisation.

3. To identify and characterise species which are involved in bacterial mediated damage to limestone.

   Many species of bacteria are capable of producing acids under normal growth conditions which damage limestones. Isolated species will be characterised as damaging to limestone if they test positive for calcium carbonate dissolution or show evidence under controlled conditions of micropitting, etching or physical damage when observed with the Scanning Electron Microscope (SEM). With any form of acid production being seen as a key indicator of biocorrosive capacity in the literature, the ability to grow well at lower pHs is likely to be phenotypic of isolates from damaged surfaces. The assumption which the current literature is based upon, that only species from damaged surfaces cause damage, will also be tested.

   Hypothesis 5: Species isolated from the limestone microbiome will demonstrate the capability to cause biocorrosion or physical damage to the stone or stone matrix.

Null hypothesis: Species isolated from the limestone microbiome will not alter the stone in a deleterious fashion.

Hypothesis 6: Species isolated from the limestone microbiome that demonstrate low optimum growth pH will be associated with damaged surfaces.

Null hypothesis: Optimum growth pH will not be significantly associated with the surface the species is isolated from.

Hypothesis 7: Species isolated from the limestone microbiome which cause damage will belong to a subpopulation of the damaged microbiome.

Null hypothesis: Species which cause damage will not be solely associated with damaged stone.

4. To test biocides commonly used in conservation processes for efficacy against isolated species, to identify effectiveness against species identified as damaging and to provide guidance to conservators. Biocide manufacturers make no distinction in their claims as to whether their product efficacy testing is against planktonic or biofilm encapsulated bacteria. As has been seen with clinical antimicrobials, biofilm encapsulation often results in reduced efficacy (Parsek & Singh, 2003; Sepandj *et al.*, 2004). Manufacturers of biocides also claim that their products clean the surfaces without providing any information on degree to which they remove microbial biofilm.

   Hypothesis 8: Biofilm production will result in reduced efficacy of biocides.

   Null hypothesis: Efficacy of the biocides will not be effected by the presence of biofilm.

   Hypothesis 9: Chemical cleaning by biocides will remove biofilm matrix from the test surface.

   Null hypothesis: Biocides will be ineffective for chemical removal of biofilm matrix.

1.2 Limestone

Historically limestone, and it's metamorphic relative marble, have been used as a building material on a global scale for both aesthetic and practical reasons, with examples ranging from the pyramids of Giza, Nummulite limestone with a Tura limestone casing (Park & Shin, 2009), the Diocletian's Palace in Split built from Brač limestone (Marasović *et al.*, 1976), to the Gothic Cathedrals of Europe (Dhami *et al.*, 2014). As well as being a source of building stone it is also a

valuable resource of crushed rock aggregates suitable for construction fill and sub-base road materials (Harrison *et al.*, 2005). As a building stone in antiquity limestone could be easily worked even with flint tools, with evidence of carving and quarrying as a building stone as early as the Naqada I culture in pre-dynastic Egypt, 3900-3500 B.C. (Waelkens *et al.*, 1992) and with Belas Knap, a chambered long barrow in Gloucestershire, UK, being of a similar vintage and using limestone lintels (English Heritage, 2013). Worked limestone decorative carvings have been found and dated as early as 32,000 B.C., specifically the Willendorf Venus, Germany (Sandars, 1995).

1.2.1 Lincolnshire limestones

As a building stone Lincoln limestone is one of the most famous building stones in England (Pevsner *et al.*, 2002). 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. 96% of the buildings in the 2013 British Geological Survey report of historic properties in Lincolnshire (British-Geological-Survey, 2013) are built at least partially from Lincoln limestone. Although it is predominantly present in the Lincoln area, it has been used in properties from Windsor Castle (London) to York Railway Station (British-Geological-Survey, 2013). While Lincoln limestone, which for the purposes of this study refers only to limestone quarried within a 5 mile radius of Lincoln, makes for a locally convenient material to test, the vulnerability of all limestones to biocorrosion is due to their composition of calcite and aragonite and therefore aspects of the data accumulated in this study will be relevant globally.

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 colonisation mechanisms related to bacterial deterioration of stone is the production of acids, colonisation by acid producing bacteria would be likely to have a detrimental effect on this stone.

The limestones of Lincolnshire are calcereous inferior oolites from the middle Jurassic period (Harrison *et al.*, 2005) and is described as follows.

“Limestone, typically calcilutites, and peloidal wackestones and packstones in the lower part (Lower Lincolnshire limestone) and high energy ooidal and shell fragmental grainstones in the upper part (Upper Lincolnshire limestone). Commonly includes sandy limestone in basal part and may contain substantial units of mudstone particularly from the Lincoln area northwards.” (Griffiths *et al.*, 2006)

With the composition varying throughout the deposit, not all of the 80 ft. deep deposit of limestone around Lincoln make good building stone, Pevsner *et al*. (2002) cite only 3 or 4 feet of the deposit in the Dean and Chapter Quarry owned by Lincoln Cathedral as being suitable for construction.

The most recent publication on the chemical composition of Lincoln limestone is Barber, 1974. Barber's methodology involves dissolving the limestone in acetic acid, hydrogen peroxide and analysis by X-ray diffraction on the insoluble components. Curiously Barber identifies the major component as strontium with measurements between 301-376 ppm depending on whether the limestone is impure or pure! Vanadium comes next with measurements between 57.8 and 17.5 with calcium oxide, due to acid dissolution, coming in at 45.3 to 53.64. Other studies into limestones correctly identify calcium carbonate as the major component (Miller *et al.*, 2009; Park & Shin, 2009) so it is unusual that Barber does not. In fact nowhere in the paper does it discuss these unexpected proportions. The trace elements identified in the paper, magnesium, manganese and iron, together with the calcium carbonate, are all key elements required for bacterial growth and are the key components along with ammonium chloride and potassium phosphate of M9 Minimal Media (MM), a standard bacterial and fungal growth media (Rédei, 2008). The presence of these trace elements therefore may provide a good foundation for bacterial growth on the stone surface.

A few isolates from a separate study by Mary Webster, from Oxwich head limestone and Herefordshire tufa, were also included in this study for identification and characterisation. Oxwich head limestone is a member of the Pembroke limestone group and consists of thick bedded packstones making for a more dense substrate than Lincoln limestone (British Geological Society, 2016). It is also considered a very high purity limestone with trace elements much lower than those described for Lincoln limestone (Mitchell, 2011). The Herefordshire tufa has been generated by the action of underground springs on the Bishop's Frome limestone bed resulting in the dissolution and redeposition of the calcium carbonate resulting in variations in the trace chemical composition depending on the region it was quarried from.

Physical and chemical deterioration processes of limestone have been better characterised than the biological processes. Hydromechanical processes including spalling, granular disintegration and fragmentation are well studied as part of the freeze thaw cycle, where damage is not only caused by the volume expansion of the ice, but also by the migration of unfrozen water in the stone which causes an increase in pressure in the pores (Walbert *et al.*, 2016). As well as the freeze thaw cycle, changes in the atmospheric relative humidity (RH), and rain associated wetting

and drying cycles, effects the swelling of expandable clay particles (smectite) within the stone matrix leading to mechanical damage of the stone (Cherblanc *et al.*, 2016; Wangler *et al.*, 2016).

Water absorption sufficient to cause weakening of the mechanical properties of limestones does not require heavy rain or flooding, an RH of 97% or greater is sufficient (Cherblanc *et al.*, 2016), something which is not uncommon in an oceanic climate. Figure 1 shows the fluctuations in RH in Lincolnshire over a 4 year period, with measurements over the autumn and winter months peaking over 97%. The profile for the county as a whole matches that for the closest weather station to Lincoln, RAF Waddington, where the majority of the buildings sampled in this survey are located.

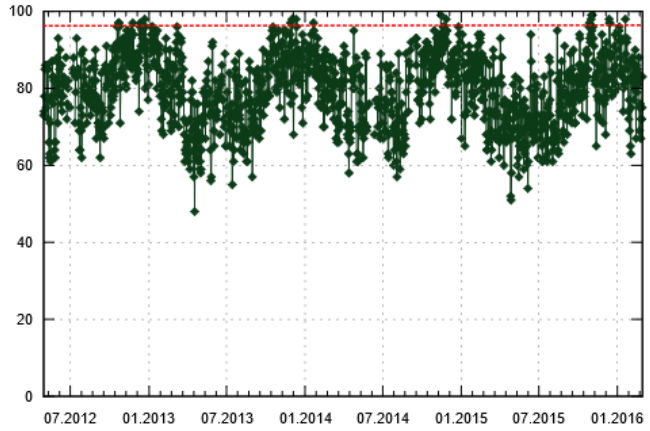


Figure 1: Relative humidity for Lincolnshire, for the period from April 2012 to February 2016 with RH of 97% and higher indicated by the red dashed line. Chart produced using weatheronline.co.uk. Between October and February the RH regularly peaks over 97% RH with occasional peaks between March and June.

1.2.2 The bioreceptivity of limestone

The absorbance of water into the limestone matrix can be modified by the presence of biofilms coating and penetrating the surface. Equally the ability of the limestone matrix to retain water will directly effect its potential for supporting microbial growth. The nature of the biofilm is dependent on the composition of the microbiome which in turn is directly affected by the primary bioreceptivity of the limestone matrix.

Bioreceptivity was originally defined in 1995 as “the aptitude of a material (or any other inanimate object) to be colonised by one or several groups of living organisms without necessarily undergoing any biodeterioration.” (Guillitte, 1995). It is worth noting the choice of the word colonised, this is to exclude transiently present organisms. The core concept of bioreceptivity is to

encompass the conditions provided by the material to support an ongoing biome. Guillette defined three phases of bioreceptivity; primary bioreceptivity is the inate ability of the unmodified material to support colonisation, bioreceptivity of damaged or colonised stone encompases the secondary phase and stone which has undergone conservation treatment the tertiary. He then went on to define methodologies for laboratory studies of bioreceptivity for microorganisms which are still used today, such as determining the porosity, surface roughness, mineralogical composition as well as observing colonisation rates (Guillitte & Dreesen, 1995).

Primary bioreceptivity of limestones has mainly been studied using Portuguese and Spanish limestones (Miller *et al.*, 2012). In these studies physical characteristics of the limestone were compared with the increase in biomass from an initial defined inoculum over 90 days. Analysis looking for correlations between physical characteristics and biomass increase demonstrated that high surface roughness and capillary coefficient were more significant for determining the stones bioreceptivity than open porosity and permeability (Miller *et al.*, 2010). These characteristics of the limestone regulate surface adhesion through reduction in shear forces, increase in surface area, and result in increased availability of water and nutrients (Baer & Snethlage, 1997; Morton *et al.*, 1998). As such, limestones with a high surface roughness and capillary coefficient have a higher capacity for retaining water, this enhances their viability for initial colonisation by air spora microorganisms.

The availability of minerals was also analysed for the Spanish and Portuguese limestones with similar trace elements being present to Lincoln limestone, within the limitations of the one paper published which were discussed earlier.

Abrasion pH, the pH resulting from physical and chemical weathering of a stone surface, was also measured and gave an average pH of 8.556, which is slightly higher than that of pure calcium carbonate (Summerfield, 2014).

Capillary coefficient measures the speed at which water is taken up through the open pore network of the stone, the higher the measurement the greater the capacity for the stone to take up and retain water (Delgado, 2016). The limestones studied showed a range of capillary coefficients, measured in $g.m^{-2}.s^{-0.5}$, Escúzar stone (268.79±77.54), San Cristobal stone (199.30±31.05), Lecce stone (128.76±3.04), Ançã limestone (57.25±1.17), and Lioz limestone (0.20±0.06) (Miller *et al.*, 2010). While there was high variation between the capillary coefficients there was lower variation observed for the surface roughness measurements which ranged from 8.16 to 1.59µm.

With no studies to date involving Lincoln limestone there is still a degree of information available through accessible quarry data sheets. Capillary coefficient, measured according to BSEN 772-11 : Determination of Water Absorption Coefficient by Capillarity Perpendicular to Bedding, is available for Lincoln limestone (Ashall & Webb, 2013) giving the capillary coefficient as 171.48±19.9 $g.m^{-2}.s^{-0.5}$ for Lincoln stone. When comparing the results of the Spanish and Portuguese limestones, Escúzar stone and San Cristobal stone were shown to have the highest bioreceptivity, the Lincoln stone capillary coefficient is in the same range as these two which suggests that it will show a reasonable level of bioreceptivity, however details on the surface roughness and a reliable analysis of the chemical composition are not available in the literature.

1.3 Limestone microbiome and its role in biodeterioration

There are three principal approaches to the colonisation of stone surfaces by microrganisms, direct colonisation as exploited by some fungi and algae, the formation of symbiotic structures, for example lichens, and encapsulation of microorganisms within a secreted, protective, film known as a biofilm (Pinna, 2014).

The primary role of biofilm secretions are to provide a protective, stable environment for the microbial population (Figure 2). A biofilm is a community of microorganisms which excrete protective materials, usually proteins, extra cellular DNA 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 most bacterial species will spend at least part of their life cycle in a biofilm (Lewis, 2001).

As with any ecosystem the organisms present have the potential to engineer their environment both directly and indirectly to adapt it to their needs (Wright *et al.*, 2006); which can result in physical, chemical and aesthetic modification of the stone surface.

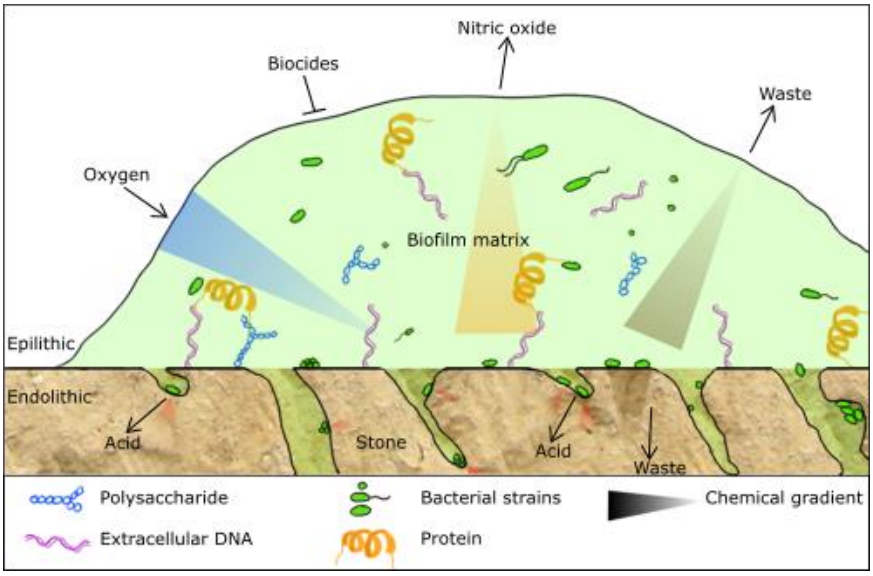


Figure 2: Biofilm structure on a stone surface, the biofilm matrix consists of polysaccharides, extracellular DNA and proteins making up a structural matrix which the bacteria inhabit. Chemical gradients occur in the matrix with secretion of waste products such as nitric oxide and acids into the atmosphere and the stone surface. The matrix also provides protection against biocides. Diffusion of the waste products such as organic acids into the limestone results in weakening of the structure allowing increased penetration of the biofilm matrix into the stone. These mechanisms both result in the deterioration of the stone surface. Diagram by author and not to scale.

The order of the methods of colonisation previously mentioned also follows the progression of colonisation observed on new or freshly cleaned surfaces. Initial colonisation is predominantly by phototrophs, for example algae, cyanobacteria and lichens, this results in an accumulation of organic matter and debris on the surface which supports the colonisation by fungi and bacteria with the species which do not require the protection of a biofilm generally being the secondary colonists (Li *et al.*, 2016).

The role of lichens, fungi, algae and cyanobacteria within the microbiome are well covered in the literature, with general consensus on their roles in colonisation of the surface as well as considerations such as aesthetic impact, bioprotection and biodeterioration having been reached. The role of bacteria and bacterial biofilms is less well researched, lacking the full characterisation of the microbiome and the effects of the species which is found in other areas of microbiology. This skew in the research is something which has been acknowledged since the 1930s, as can be seen in the quote below, but remains unaddressed.

“Living organisms also contribute to the decay of stone and similar materials and, although their action is, generally, of somewhat less importance than certain of the other deleterious agencies

which have been considered, their study presents numerous features of interest. The effect of certain organisms, such as bacteria, is still a matter of controversy, but the effect of others, such as the growth of ivy, is generally considered to be detrimental." (Schaffer, 1932)

1.3.1 The bacterial microbiome

Analysis of the bacterial component of limestone and other calcium carbonate rock microbiomes is understandably a niche area in academia; the majority of papers look at a subpopulation of the microbiome from a biodeterioration perspective (Dakal & Cameotra, 2012), and a few publications looking at the subject to enhance understanding of the role of microorganisms in the biogeological deposition of rock (Banks *et al.*, 2010). Analysis of the microbiome is usually limited to looking at damaged stone surfaces for biodeterioration and undamaged stone surfaces for biogeological studies and the scope of the studies are constrained by the climate of the site studied (Table 1).

There is currently minimal research into biodeterioration based around lithic microbiomes. The recent review by Dakal & Cameotra (2012) covering algal, fungal and bacterial biodeterioration only found 30 studies to review, by broadening the scope of this review to include murals a further 9 papers were found. Since the 2012 review a further 4 papers have been published on the topic (De Leo *et al.*, 2012; Ortega-Morales *et al.*, 2013; Diaz-Herraiz *et al.*, 2014; Wu *et al.*, 2015), with a further 4 on the related topic of biodeterioration of murals. The 47 studies mainly focus on damaged surfaces, and none make direct comparisons between isolates from damaged and undamaged stone.

The majority (85%) of these studies are geographically limited to central and southern Europe. Of the remaining 15%, only 8% look at northern Europe. While 11% of the total studies look at an oceanic environment as found in the UK, these sites are classified as Köppen and Geiger Cfb (Peel *et al.*, 2007) see Appendix B for classification details, all but one of these studies are based inland in central and southern Europe where the average temperature and rainfall push them into the same Cfb classification.

Of the 47 studies discussed, 27 were looking at deterioration of stones composed primarily of calcium carbonate (Table 1). Given its status as a building material and how easily it can be damaged, it is of little surprise that limestone predominates in the current studies of biodeterioration, with sandstones and granites making up the majority of the other studies (Dakal & Cameotra, 2012).

The depth to which the microbiome is investigated also varies between studies, while the majority of the studies attempted to identify the isolates to species level, many studies only looked as far as the level of genus or class (Videla *et al.*, 2000; Mitchell & Gu, 2001; Berdoulay & Salvado, 2009; Diaz-Herraiz *et al.*, 2014) something which is made more acceptable as it is used in metagenomics studies where it is described as an Operational Taxonomic Unit (OTU) (Schloss & Westcott, 2011). Not every study in the literature addresses which species or class of organism is present in the sampled population, several studies look at a very broad picture stating that they have found 'bacteria' on the surface, and therefore the bacteria must be causing the damage (Papida *et al.*, 2000; Wu *et al.*, 2015; Sasso *et al.*, 2016)

The studies are regularly based on a small number of samples from a highly discrete area, for example Diaz-Herrais *et al*, 2014 in Nature – Scientific Reports which looks at a random section of the bacterial population from 3 points of damage on 1 mural in a tomb in southern Italy.

Reviewing these papers does identify some core species, *Bacillus cereus, Bacillus mycoides, Bacillus licheniformis, Bacillus pumilis, Bacillus subtilis, Micrococcus luteus, Pseudomonas putida,* and *Stentrophomonas maltophilia* were all found in 3 or more papers. Unfortunately each of these species is said to cause damage by the papers looking at damaged surfaces and, typically for this field, said to not cause damage in the papers which were looking at undamaged surfaces (Flores *et al.*, 1997; Videla *et al.*, 2000; Radaelli *et al.*, 2004; Saarela *et al.*, 2004; Nuhoglu *et al.*, 2006).

All the studies to date have focused on damaged or undamaged stone (Table 1) and therefore lack a comparative approach, which would allow the breakdown of the microbial community into those associated solely with damaged stone, those associated with undamaged stone and the species which overlap the two, in other words establishing clearly which subset of the bacterial population are associated with damage to the stone.

Only 9 of the 27 papers (Table 1) tested their isolates to see whether they had biodeteriorative potential, 7 of these had not identified the isolates to species level and went on to make sweeping statements about particular genera being the cause of the damage.

To give an example, there are currently at least 316 recognised *Bacillus* species (Parte, 2013), for a publication to state that an isolate of *Bacillus* genus, or worse of the Bacillaceae family, caused biodeterioration leaves open the question of which of the 316 species, or thousands of members of the family, was the cause.

Paper	Damaged	Undamaged	Köppen-Geiger classification	Continent
Banks *et al*, 2010	X		Dfa	North America
Berdoulay *et al*, 2009		X	Cfb	Central Southern Europe
Berner *et al*, 1997	X		Dfc	Central Southern Europe
Cacchio *et al*, 2003	X		Csb	Central Southern Europe
Cappitelli *et al*, 2007	X		Cfa	Central Southern Europe
Cuzman *et al*, 2010	X		Csa	Central Southern Europe
De Leo *et al*, 2012	X		Csa	Central Southern Europe
di Bonaventura, 1999	X		Cfb	Central Southern Europe
Diaz-Haerraiz *et al*, 2014	X		Cfb	Central Southern Europe
Dupont *et al*, 2007		X	Cfb	Central Southern Europe
Flores *et al*, 1997	X		Csa	Central Southern Europe
Laiz *et al*, 2009	X		Csb	Central Southern Europe
Mansch and Bock, 1998	X		Dfb	Central Southern Europe
Mitchell & Gu, 2001	X		Dfb	North America
Nuhoglu *et al*, 2006	X		Dfb	Western Asia
Ortega-Morales *et al*, 2013	X		Am	South America
Papida *et al*, 2000	X		Cfb	Northern Europe
Peraza Zurita *et al*, 2005	X		Csa	Central Southern Europe
Pinar *et al*, 2009	X		Dfb	Central Southern Europe
Radaelli *et al*, 2004	X		Cfa	Central Southern Europe
Saarela *et al*, 2004		X	Csa	Central Southern Europe
Sakr *et al*, 2013	X		BWh	North Africa
Sarro *et al*, 2006	X		Csa	Central Southern Europe
Urzi *et al*, 2008	X		Csa	Central Southern Europe
Videla *et al*, 2000	X		Aw	South America
Wu *et al*, 2015		X	Cfa	China
Zammit *et al*, 2011	X		Csa	Central Southern Europe

Table 1 : The literature to date looking at biodeterioration by microorganisms on limestone surfaces, whether the sampling was from damaged stone, undamaged stone or a comparison of both and the climate and general geographic location of the sampling. Only 5 of the papers look at oceanic climates (Cfb) and of these 4 are inland continental sites. Köppen-Geiger classifications can be found in Appendix B.

Publications of this nature while moving towards a characterisation of the microbiome are still contributing to the controversy noted by Schaffer in 1932.

Equally limiting the ability to draw comparisons with other studies in the literature is Papida *et al.* (2000) who performed testing for biodeteriorative potential without any sort of identification of the species.

Only one paper identified species in detail on a damaged surface and went on to directly test whether the identified species were involved in the biodeterioration (Sakr *et al.*, 2013). In this study they isolated and identified 8 different *Streptomyces* species from pigmented salt efflorescence discovered on limestone surfaces and murals in an Egyptian tomb. The pigmentation was shown experimentally to be produced by *Streptomyces canaries*, but only under growth conditions matching the salt levels found on the surface it was isolated from. SEM imaging of samples of damaged limestone taken from the tombs showed *Streptomyces* mycelia penetrating the stone surfaces. Finally correlations between the occurrence of the salting and the presence of the *Streptomyces* species were demonstrated, although they were unable to tell whether the isolates were causing the salting.

Of the 18 papers which did not test their isolates, 11 identified isolates to species level, 6 to genus or family level and one (Zammit *et al.*, 2011) made no attempt to identify the microorganisms in the biofilm but chose to make educated guesses about what would be present from the colour of the patina, based on a literature search. All 18 worked on the assumption that because they had isolated organisms from a damaged surface then the isolates were causing the damage, without providing any supporting evidence for this.

The current literature on the limestone microbiome (Table 1) identifies a total of 205 isolates of which only 106 were identified to species level. One hundred and fifty four of these isolates were considered to cause biodeterioration based purely on their site of isolation. Seventeen of these had also been isolated from undamaged stone and identified as not causing damage. As only 4 of the 27 studies were looking at undamaged stone it is extremely likely that more of the species associated with damage in the literature will also turn out to be commensal to the limestone microbiome rather than specific to damaged sites.

It is clear that the literature to date, while adding to our understanding of the limestone microbiome, is fragmented and relies on assumptions which are not supported with experimental evidence.

The present study addresses the failings of the studies referred to in this review, providing for the first time a thorough analysis of the bacterial component of the limestone microbiome which will clarify the conflicts found in the current literature and contributes, through a multidisciplinary approach, to the knowledge and understanding of the field. This is important as the variable, and sometimes contradictory, information which makes up the current understanding of the limestone microbiome effects does not just effect our understanding of biodeterioration, but it is

also the foundation upon which decisions regarding the protection of historic buildings and monuments, against biodeterioration by microorganisms, are based.

1.3.2 Interpreting the bacterial microbiome

When selecting the methodologies for the analysis of the limestone microbiome in this study the identification of the bacterial species present required the greatest consideration. The other methodologies required, characterisation of the stone surface for bioreceptivity, sampling of the stone surface, characterisation of the microorganisms and their role in biodeterioration were mostly approached using standard, published methods with additional tests being put in place to support these.

The current literature, as previously discussed, has approached the identification of species using a range of methods, some of which did not identify to the species level. For this study the decision was made that where possible all isolates should be identified to species level, but two approaches were available, direct isolation and culture of organisms or metagenomic analysis. It is worth noting that whatever method is chosen the results are susceptible to the possibility of contamination both from the researcher's personal microbiota as well as the laboratory environment in which the analysis occurs, with rates as high as 2.02% contamination being observed in some laboratories (Morrell & Wasilauskas, 1992).

Classical microbiological methods of sampling, culturing and then identifying microorganisms through morphology of the colonies and cells, growth characteristics such as cell clustering or growing in chains, together with biochemical tests for the ability to metabolise chemicals, such as nitrates and carbohydrates, provides a robust method of identification with the results from the tests being checked against Bergey's Manual (Rainey *et al.*, 2015). While this approach has the weight of history behind it and uses methodologies that Pasteur originally formulated (Pasteur, 1878), it runs into the problem of so called unculturable bacteria. These are species where there is insufficient information about their biology and so culture media which will support their growth has not yet been developed (Stewart, 2012). Evidence for this was first uncovered in the mid 1980s when it was shown that the number of cells visible under the microscope exceeded the number of colonies which were grown on media, sometimes by several orders of magnitude (Staley & Konopka, 1985).

Culture isolation of microorganisms therefore needs either experimentally specified limits, for example isolating only copiotrophs, species which are capable of growing on a wide range of

nutrients, or cyanobacteria, or requires the researcher to provide as wide a range of growth media as possible to ensure that as many growth environments as possible are provided. In previous studies (Table 1) where isolation was undertaken, the researchers chose to look at a limited sample of the population. They extracted it from the total population using the appropriate growth media. Twenty one of the papers took this approach, of the others, the approach taken by Zammit et al has already been discussed, one chose to identify species by direct microscopic observation (Peraza Zurita *et al.*, 2005) and the remaining 4 took a metagenomics approach hence looking at the total population (Berdoulay & Salvado, 2009; Ortega-Morales *et al.*, 2013; Diaz-Herraiz *et al.*, 2014; Wu *et al.*, 2015)

Of the studies which isolated microorganisms, 4 of the 21 used a culture medium which was inappropriate for the aims of the study. Banks *et al* having initially cultured species on a non-selective agar then used tryptone soya agar (TSA), selective for heterotrophs, to try and "purify the cultures" as it "often allows separation of mixed cultures" (Banks *et al.*, 2010). TSA was also used in two further studies where they were not intending to limit the selection to heterotrophs with no explanation about why they had chosen to use it (Sarró *et al.*, 2006; Urzì *et al.*, 2008). The final study used nutrient agar from the start, also selecting heterotrophs, when they intended to look at a total population of bacteria causing biodeterioration (Videla & Characklis, 1992). The use of traditional techniques to identify microorganisms was more common in studies looking at fungi and algae, where microscopy is often sufficient to identify the species, than those looking at bacteria. In the majority of cases the genomic DNA was extracted and isolates were characterised according to their 16S rRNA gene.

Following the advent of DNA analysis of species, the 11 bacterial phyla described by Woese in 1987 has grown to at least 85, the majority of which have no cultured representatives (Stewart, 2012). Sequencing the 16S rRNA gene is one of the more recent developments in species identification. With over 8168 bacterial species identified (Janda & Abbott, 2007) out of the $1x10^7$ estimated to exist (Schloss & Handelsman, 2004), 16S rRNA gene sequencing provides a straightforward approach to identifying bacterial species. The concept is based on the fact that the 16S rRNA gene is highly conserved and so collects mutations at a very slow rate, approximately the same rate as speciation (Ochman *et al.*, 1999). With direct sampling isolation and sequencing of the 16S rRNA gene, the variable regions can give accurate species level identification (Janda & Abbott, 2007). When carrying out direct sampling, the sequencing is carried out using Sanger sequencing with an average of 700bp good quality read, although up to 1kb is possible (Sanger *et al.*, 1977). As the average length of the 16S rRNA gene is 1522bp this

means coverage of half to two thirds of the gene is possible allowing the inclusion of several of the variable regions increasing the accuracy of the identification (Chakravorty *et al.*, 2007).

16S rRNA sequencing is not a perfect method as many species contain more than one copy of the 16S rRNA gene. Only 15% of the genomes surveyed in Větrovský & Baldrian (2013) had a single 16S rRNA gene per genome, the highest number of copies found was 15 with the average being 4.2 copies per genome. 95.4% of genomes tested had a mean 16S rRNA similarity of over 97%, and the probability of dissimilarity within the genome increases with copy number (Větrovský & Baldrian, 2013). While many of the species with multiple copies of the 16S rRNA gene will have copies which all identify it as the same species, variation between these copies can be as high as 45 base pair substitutions in the gene before it is below the accepted 97% similarity for species identification. These differences are sufficient to cause problems with Sanger sequencing, especially if they include deletions or insertions in non-functional copies of the gene (Chakravorty *et al.*, 2007).

To overcome the issues associated with traditional isolation and identification, either by physical and biochemical testing or 16S rRNA sequencing, total population identification using PCR amplified fragments of the 16S rRNA gene, metagenomics, is now considered the most effective approach. This approach extracts the total genomic DNA from every microorganism in the sampled population, without isolating them from each other, and extracts a 300 base pair region of the 16S gene by PCR. A next generation sequencer is then used to sequence the heterogeneous mixture of 16S gene fragments and the results analysed to give a more complete picture of the population. These results are the endpoint for the method as there are no isolates available for further testing.

Metagenomic analysis, like any system, suffers from limitations. As previously mentioned species can carry more than one copy of the 16S rRNA gene which, if it is homogeneous or heterogeneous but identifying the same species, will artificially amplify the presence of the species. If the species carries copies of the gene which they have captured from other species, this will result in false positives. This also limits the potential for the 16S rRNA gene to be used to accurately quantify the microbiome, while the data on the percentage of species which contain multiple copies (Větrovský & Baldrian, 2013) could be used to provide an estimate of the number of bacteria present on a surface; methods such as applying the exponential of Shannon's diversity index to the sampling data will give an estimation of the total population size (Jost, 2007; Jørgensen *et al.*, 2010).

Extraction methods will also limit the data set as if the cells are not sufficiently lysed they will not contribute to the pooled genomic DNA (Gilbert *et al.*, 2014), or if the sampling environment contains PCR inhibitors which are inadequately removed amplification of some species may be prevented (Schrader *et al.*, 2012). The nature of most next generation sequencers used for 16S rRNA metagenomics sequencing provides the next limitation, with only 454 pyrosequencing producing read lengths larger than 300bp. The sequencers also have a higher error rate than Sanger sequencing, up to 1.5% as opposed to 0.01% (Sanger *et al.*, 1977; Goodwin *et al.*, 2016). A maximum read length of 300bp leads to the compromise of only being able to look at one variable region with in the 16S rRNA gene. Most studies use the Earth Microbiome Project primers which cover variable region 4, bases 515 to 806 (Walters *et al.*, 2015). While considered ideal for the broad scope of the Earth Microbiome Project (Gilbert *et al.*, 2014) other studies have found this region to be less suitable due to a higher degree of sequence conservation when compared to other hypervariable regions (Chakravorty *et al.*, 2007). In fact the primers have recently been redesigned to include variable region 5 as well (Walters *et al.*, 2015) to improve identification, unfortunately this pushes them past the maximum read length for many systems which has resulted in poor uptake of the modified primers by service providers who are not using the more expensive, out dated, 454 pyrosequencers (Walters *et al.*, 2015; Goodwin *et al.*, 2016).

As an example based on the statistics provided by Větrovský & Baldrian 2013, within a population of 1000 extracted genomes at least 46 of the amplified 16S rRNA genes are likely to be from multiple copies that have <97% identity with the host organism. Based on the averages that would reduce the population by 11 species, with multiple copies. The size of the population is further reduced by the bacterial species which carry more than one copy at >97%. Of the remaining population, 15% would have a single copy of the 16S rRNA gene (143) and the rest of the population would be averaging 4.2 copies per genome meaning that instead of 811 there would be 194 genomes giving a total of 337 genomes identified.

The example above is less of a concern when taking the sequence data down to species level. The genes from species which carry multiple copies of the 16S rRNA gene which have >97% identity with each other would be identified as that species, albeit different strains. This would collapse the data set giving the result closer to the true population size, possibly a slight underestimate if multiple strains were in fact present. When the analysis is to OTU level, usually class, then the data set becomes artificially inflated as every strain identified becomes an individual count, whether it is a genuine single species or one of the multiple copies of the 16S rRNA gene that are found in some genomes. The strength in OTU analysis at the class level is based upon the

weakness in the identification of species by 16S rRNA, specifically that the databases are incomplete. The 8168 bacterial species identified in 2007 is a fraction of the total number of bacterial species present on Earth, the estimated minimum species richness is 35,498 with the actual figure expected to be between 10^7 and 10^9 species (Schloss & Handelsman, 2004). Based on the minimum species richness only 23% of the bacterial species can be identified by 16S rRNA sequencing, a number which drops as low as 0.0008% should the species richness actually be 10^9. By analysing the sequences to the taxonomic class level it is possible to obtain information from the whole data set rather than just the proportion of the population where the species have been identified.

With this in mind the decision was made to carry out both types of testing, metagenomics to better characterise the whole microbiome and direct culturing and isolation of a subpopulation of the microbiome to better understand the role of those species in biodeterioration.

1.3.3 Biodeterioration

The study of biodeterioration is the elucidation of the physical and chemical processes of decay, where the causative factor is of a biological, rather than environmental origin (Dakal & Cameotra, 2012). Biological processes of decay in the environment ranges from the macroscopic, for example large mammals, plants and bird droppings, to the microscopic which mainly consists of the colonisation of the stone surface by microorganisms, such as bacteria, fungi and algae.

One of the more obvious effects of biodeterioration is the obfuscation of the worked surface; the growth of ivy and other creepers, and coatings of guano are notable examples. The patina produced by the growth of microorganisms is another example which can change the character of masonry completely (Warscheid & Braams, 2000). Maintaining a balance between the aesthetic of an aged building, and removing biological growth which is contributing to the deterioration of the structure, is a challenge for conservators (Doehne & Price, 2010).

In the case of microorganisms, patina (Figure 3) has been shown, in many cases, to result in physical and chemical damage as well as aesthetic change. Both lichens and the biofilm encapsulated organisms contribute to the aesthetic changes to the surface through pigmentation and physical structure. The asthetic and physical effects of lichens in particular are well covered in the literature with the general conclusion being that conservation efforts should only be made where the lichen is causing physical or chemical damage to the stone substrate (Pinna, 2014). The patina produced by the growth of biofilms on surfaces produces discoloration, chemical deterioration and promotes physical deterioration.

Figure 3: Lincoln Cathedral between the Women's Chapel and the Song School. Stonework in the foreground shows biological patina (dark stains) and damage, stonework in the background has been recently cleaned. On the left hand wall biological growth and spalling of the stone work are evident, especially where overhanging stonework provides a protective environment; this is especially clear at the bottom of the wall where there is a clear gradient between patinated stone and clean stone moving down from the overhang. On the right hand of the image, the Song School, water flow down the wall due to insufficient guttering, and shelter of the courtyard, has led to a damper environment than that on the left, patina is clearly evident working from the left near the door across towards the gutter downpipe where a combination of improved guttering and lead salts, which can be seen as a slight greying of the stone behind the downpipe itself, have led to a reduction in biological growth.

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 *et al.*, 1992, 1993). This study will not be considering whether the aesthetic alteration of the surface by microbial growth should be considered biodeterioration, as it is more of an artistic interpretation

than a scientific one. There are also ethical considerations involved as the presence of rare microorganisms which make up the patina may skew the consideration towards their conservation rather than that of the structure (Doehne & Price, 2010).

Biofilm encapsulated microorganisms such as fungi, algae and bacteria are less well studied, with the study of bacteria being the most limited to date. Recent studies into biodeterioration of the built environment have, when detection was part of the study, identified the presence of biofilms containing bacteria, algae and fungi from many sources of historically relevant stonework including Mediterranean statuary (Dakal & Cameotra, 2012; Miller *et al.*, 2012; Dhami *et al.*, 2014).

Where the patina is due to a biofilm, the surface film will trap and concentrate airborne particles and pollutants (Flores *et al.*, 1997). Should the film block the pores in the stone substrate the accumulation results in the development of unsightly and damaging crust formations (Ortega-Calvo *et al.*, 1995). In the UK the key pollutants measured are nitrogen and sulphur compounds, ammonia, ammonium, nitrogen dioxide, nitrates, sulfur dioxide and sulfate, with the key differences in Lincolnshire being a high concentration of nitrates in urban areas with rural areas having a high concentration of ammonia (Defra, 2016). Only one of the studies in the current literature has considered the effects of different pollutants on biodeterioration of limestone (Mitchell & Gu, 2001). In the paper they gave no clear definition of how they defined low pollution and the two sampling sites studied were both in urban areas close to major roads which would suggest that while the levels of pollutants may have varied, the type of pollutant would not.

Physical erosion is promoted when secretions of the microorganisms penetrate the surface. Narrowing of the pores due to the presence of biofilm increases capillary water uptake, which enhances differential stress and therefore weathering is observed due to expansion and contraction of the secretions. There is also an increase of the depth beneath the surface of the stone where salt precipitation can occur (Smith *et al.*, 2005) and weakening of the structure internally due to the microbial excretion of inorganic and organic acids. It is also suggested that salting prompts changes in the microbiome towards a population consisting of halotolerant and chemolithotropes, which in turn accelerates the promotion of salting (Papida *et al.*, 2000).

The damage caused by these mechanisms seeds 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 can 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 & Braams, 2000) through the swelling and contraction of clay particles which compose part of the stone matrix (Herrera & Videla, 2009; Cherblanc *et al.*, 2016). Furthermore, evidence suggests that biofilms may encourage the build-up of pollutants from the atmosphere on and in the stonework, thus accelerating the deterioration rates (Young, 1996a; Mansch & Bock, 1998).

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. This acts in addition to the effects of the matrix on physical weathering and salting with the production of volatile compounds, especially organic and inorganic acids, resulting in specific chemical attack of the stone matrix, biocorrosion (Warscheid & Braams, 2000). The majority of organic acids produced by bacteria are byproducts of metabolism, mainly carbohydrates, and as such their production is dependant upon their environment (Audrain *et al.*, 2015). Sulfur containing compounds released by bacteria can also be linked to biocorrosion of the limestone matrix accelerating the production of gypsum from calcium carbonate (Papida *et al.*, 2000).

Bacterial volatile compounds also include nitrogen containing compounds which are primarily basic in nature such as trimethylamine oxide, an electron acceptor, and ammonia, used to promote biofilm production as part of the stress response (Audrain *et al.*, 2015). While these alkalizing agents will act to inhibit biocorrosion their production by species is generally ignored in the biodeterioration literature which ignores the fact that some species may be neutralising the acids that they produce before they can cause damage. The high diversity of bacterial volatile compounds and the level to which this environmental niche has been studied means that the distribution of acid and alkali production in the bacterial component of the microbiome is difficult to determine (Schulz & Dickschat, 2007), especially as the majority of papers in the field have only identified organic acid genesis through its presence in biochemical characterisation of the type strain.

In addition to the organic acids, sulfates and alkalizing agents, bacteria also release terpenes, ketones and alcohols. These products are unlikely to cause biochemical weathering of the limestone matrix and are involved in signalling and competition within the biofilm (Audrain *et al.*, 2015).

The organic acids most commonly ascribed to biocorrosion, oxalic, oxaloacetic, citric, gluconic, glyoxalic and fumaric (Nuhoglu *et al.*, 2006), are all secondary metabolites, usually waste products and as such are excreted into the environment (Prakash, 2012). Other than calcium oxalate the

salts produced by these acids are water soluble and so their role in biocorrosion is clear. Calcium oxalate deposits have the potential to create a protective film on the stone surface and indeed the production of these deposits using ammonium oxalate is used in conservation. The low pH of oxalic acid has been shown to damage the surface and results in the formation of friable separate small crystals rather than a contiguous film with protective properties (Cezar, 1998; Doehne & Price, 2010).

With over 80 years of research since Schaffer's 1932 report on weathering there is still controversy over the role of microorganisms in the processes of stone decay (Doehne & Price, 2010). The controversy in question is not whether the bacteria are having an effect on the chemical and physical properties of the stone matrix, but whether these effects are detrimental. For example, calcium oxalate films, the product of lichen, fungal and bacterial excretion of oxalic acid, have been shown to produce a protective effect when an intact oxalate film is produced.

Di Bonaventura *et al.* (1999) and Saikia (2008) identify *Bacillus licheniformis* as a producer of oxalic acid and suggest that it's presence on a calcium carbonate based stone would be protective, in contrast Nuhoglu *et al*. (2006) identify *B. licheniformis* as damaging due to the production of biocorrosive acids and include oxalic acid as biocorrosive which is more in line with the research discussed earlier. Reviewing 32 papers covering the biodeterioration of limestones, sandstones and granites resulted in 14 bacterial species out of the 118 identified being considered as both damaging and undamaging, with direct contradictions between studies.

With contradictory information being published it is not surprising that there is controversy over the effects of the microorganisms.

1.4 Conservation cleaning of limestone

The challenges associated with the conservation of limestone are mainly centred on cleaning and consolidation of damaged stone. Conservation treatments of historic monuments are governed by a code of ethics first defined in the Venice Charter of 1964 (Gazzola *et al.*, 1964). This document defines not only the appropriate approaches to conserving monuments, which includes statuary and buildings, such as not changing the layout, decoration and setting of the monument but defines a historic monument as any which has gained significance with the passing of time.

“Article 1.

The concept of a historic monument embraces not only the single architectural work but also the urban or rural setting in which is found the evidence of a particular civilization, a significant development or a historic event. This applies not only to great works of art but also to more modest works of the past which have acquired cultural significance with the passing of time.” (Gazzola *et al.*, 1964)

Before the articles of the charter approach the impact of the actions on the monuments themselves, they address the need for conservators to work with scientific researchers, a collaborative approach which was uncommon at the time, to ensure that the work carried out is based on a thorough understanding of the material. Article two therefore makes a significant statement in a time when interdisciplinary study was far less common than it has become since the 1990s (Jacobs & Frickel, 2009).

“**Article 2.**
The conservation and restoration of monuments must have recourse to all the sciences and techniques which can contribute to the study and safeguarding of the architectural heritage.” (Gazzola *et al.*, 1964)

Unfortunately Article 2 is not always adhered to, a special session was held in the most recent stone conference, the 2016 13th International Congress on the Deterioration and Conservation of Stone, to try to overcome this issue regarding the use and efficacy of stone consolidants. Results from a survey of conservators, architects and researchers had exposed serious problems in the ability to access research findings and best practice as well as a lack of tools for conservators to use to determine the efficacy of the treatments (Gerdwilker *et al.*, 2016).

In any conservation program of consolidation or repair cleaning plays an important role, and where microorganisms are involved there is a requirement for the cleaning method to eliminate the microorganisms. This also protects the built cultural heritage from unsightly or potentially damaging biofilms. Standard conservation treatments use chemicals to clean the surface and kill microorganisms, and non-chemical or physical cleaning methods to remove bacterial growth. Of the non-chemical cleaning methods, only exposure to biocidal UV wavelengths is a totally non-physical method, even then, UV exposure may damage pigments on a painted surface. The other main methods used, steam cleaning, pressure washing and laser cleaning, all rely on surface

ablation to remove the microorganisms and their associated biofilm matrices (Doehne & Price, 2010). These methods are effective for only a short time, as regrowth and recolonisation is rapid (Warscheid & Braams, 2000; Dakal & Cameotra, 2012). 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 and causing little or no damage to the stone (Doehne & Price, 2010).

1.4.1 Biocides

While treatment with biocides is currently widely accepted in the profession, concerns are entering the conservation field regarding their use. Many species are now showing resistance to recommended concentrations of biocide, or demonstrate the ability to rapidly recolonise treated areas once the biocide has been diluted by environmental conditions and wicking through the stone matrix. Acidic biocides can cause direct damage to calcium carbonate based surfaces and alkaline ones can cause iron staining on some stone types (Allsopp *et al.*, 2004). There are also concerns that some biocides are depositing harmful salts, which accelerate physical and chemical weathering (Eklund, 2013).

There are 22 main biocides quoted as being tested on stone surfaces in the conservation literature. In order to understand the level of knowledge relating to the use of commercial biocides in the removal of bacteria the author reviewed product notes and MSDS's for these biocides. When reviewing the manufacturer's literature for the biocides, only two biocides stated that they were effective against bacteria, the rest claim efficacy against some combination of lichens, fungi and algae, with one only stating microorganisms (Table 2). With conservators frequently accepting visual analysis of the surface as a comparison method for biocide efficacy (Long & Young, 2016), it is not surprising that there is a lack of hard data on the efficacy of commercial biocides which are compatible with limestone and effective against bacteria.

The methodologies used when cleaning with biocides are still under active investigation. A recent study into two biocides on a building in Sydney, Australia, which had been damaged by pressure washing, chose to eliminate physical cleaning and rely purely on the chemical activity, comparing a biocide impregnated cellulose poultice with direct application with a brush (Long & Young, 2016). Poulticing was shown to be as visually effective as pressure washing, without the associated abrasion of the surface.

Long and Young also made certain that the trials of the biocides they carried out were performed on an area of the building where any chemical incompatibilities with the components of the stone would not affect the exterior appearance of the building.

The other main concern when using biocides, other than material compatibility, is the fact that the surface being treated is host to a mixed community of microorganisms (Dakal & Cameotra, 2012), with the attendant risk that one or more of the species present may show some resistance to the biocide. Resistant species within the biofilm have been shown to have the potential to provide protection to non-resistant species, and that selective pressure could lead to a reduction in sensitivity to the biocides used or even promote full resistance (Sterflinger & Piñar, 2013; Sasso *et al.*, 2016). Treatments of fungal contamination from 1963 onwards in the Lascaux Caves using antibiotics, formol, and biocides such as benzalkonium chloride and isothiazolinone amongst others resulted in the development of resistant fungal and bacterial species, rather than the desired cleaning effect (Saiz-Jimenez *et al.*, 2012).

Biocide	Study	Lichens	Mould	Algae	Bacteria	Microorganisms	Moss	Discontinued/banned	Available in UK
Proxymousse	Moreau *et al*, 2008	X	X	X					
New Des 50	Blazquez *et al*, 2000				X				
Bio Estel						X			
Mergal S97			X	X				X	
Preventol R80		X	X	X					
Preventol A6				X				X	
Preventol A9D			X					X	
Metatin N5810/101			X	X				X	
ALGOPHASE	Ascaso *et al,* 2002	X	X	X	X				
ACTICIDE® LV706	Camara *et al*, 2011	X	X	X				X	
ACTICIDE® CF			X	X				X	
ACTICIDE® IOG			X					X	
Wet and Forget	Long & Young, 2016	X	X	X			X		X
Boracol 100RH		X	X	X					
Captan	Fernando, 2008		X					X	
Mancozeb			X					X	
Folicur			X					Ban due 2017	
Paraquat		X	X	X				X	
Mergal S 89			X	X					
Microtech	Biocide tested in this study	X	X	X					X
Algo biocide	Biocide tested in this study			X		X			X
Soluguard	Biocide tested in this study		X	X			X		X

Table 2: Biocides used in stone conservation broken down by the microorganisms which they effect, their current status as a commercial product, and their availability in the UK. The majority of biocides in the literature are not currently available in the UK and 10/23 are banned or due to be banned. Soluguard, tested in this study, is based on the same quaternary ammonium compound as Preventol R80.

1.4.2 UVC light and cold plasma

Cleaning surfaces by exposure to UVC light, UV light between 100-280nm as opposed to UVA, 315-400nm or UVB 280-315nm, is considered an effective approach against microbial growth (Meulemans, 1987). This is most commonly obtained using low pressure UV mercury vapour lamps which emit a single monochromatic wavelength peaking at 253nm. UVC doses between 10 and 300 J m^{-2} have been shown to effectively kill 99.9% of viable planktonic bacteria by dimerisation of thiamine molecules in the DNA preventing reproduction of the organism (Bak *et al.*, 2009; Lakretz *et al.*, 2011). Chemical oxidation of pollutants absorbed from the atmosphere in the biofilm generates free radicals which damage the biofilm matrix resulting in its breakdown (Lakretz *et al.*, 2011).

There is a lack of literature or guidance for conservators or researchers regarding decontamination and cleaning using UVC light, in particular regarding light sources, methodology and treatment times (Stanley *et al.*, 2016). This is an effective technique but currently has one accessible publication on its use as a tool for conservation (Stanley *et al.*, 2016). This paper does discuss the required variables and directs conservators to product guidelines produced by Phillips (Philips Electronics N.V., 2006). The study, which focuses on fungal contamination, took pre and post treatment samples and identified the species tested and the efficacy of the treatment against each, something which was not available in the literature up until this point. The only other publication on UVC treatment is only accessible to those who have a print copy of the 10th Congress of the International Committee for the Conservation of Mosaics (Stewart *et al.*, 2008).

The scarcity of literature and guidance in this case is due to a lack of successful application of the technique by conservators due to the wrong UV wavelengths being used, and even a desire to keep effective methods secret to ensure better business. Failure and the desire for secrecy has stopped conservation professionals from publishing in this area (Personal communication, September 2016, Naomi Luxford, co-author of Stanley *et al*. 2016)

An alternative to UV sterilisation which is being assessed by stone conservators is cold plasma sterilisation (Aibéo, 2012). Plasma sterilisation is effective because of the production of UV light and chemically reactive radicals at relatively low temperatures (<= 50 °C) greatly reducing the chances of damage to stone surfaces (Moisan *et al.*, 2002; Aibéo, 2012). Cleaning is carried out by passing the plasma torch over the surface, removing contaminants such as oil, dust, oxides and organic materials. While Aibéo (2012) did not test against biofouling of stone surfaces, there is

evidence in medical biofilm literature which suggests that cold plasma would be highly effective against the biofilm matrix (Ziuzina *et al.*, 2015).

1.4.3 Laser cleaning

Laser cleaning of stone surfaces is typically carried out using an Nd:YAG (neodymium-doped yttrium aluminium garnet) based laser system emitting pulses at 1064nm. The system has a long history of testing and publication starting in the late 1980s (Cooper, 1998; Siano *et al.*, 2012).

Laser cleaning is most commonly applied to the removal of pollution crusts, especially black gypsum encrustations formed on limestones and marbles (Grammatikakis *et al.*, 2015). One of the advantages of laser cleaning is that unlike biocides, UVC and pressure washing, the treatment can be applied in stages allowing a gradual removal of the surface contaminants. This not only allows for highly controlled cleaning but also addresses a conservation issue, that of minimal intervention. In the case of the Porta Nigra in Trier, Germany, cleaning the Roman gate back to the base grey sandstone would have altered the appearance which resulted in the name, 'Black Gate'. Application of laser treatment allowed the removal of sufficient of the damaging gypsum and pollution crusts to stabilise the condition of the stonework, without excessively changing the visual appearance of the monument (Auras *et al.*, 2016). Additional applications in the removal of graffiti and biofilms has been investigated, but requires the use of higher energy wavelengths which can result in damage or modification of the stone surfaces, especially in the case of softer stones such as limestone (Siano *et al.*, 2012).

1.4.4 Presure washing and steam cleaning

Pressure washing is a problematic cleaning method. Contractors generally approach the issue with industrial pressure washers operating at around 2,500 psi, at this pressure the removal of the stone surface as well as the surface contamination is highly likely. Safe pressures for limestones are generally considered to be below 200 psi. This low pressure misting of the surfaces is often successful due to surface contaminants being bound up in gypsum which is water soluble (Normandin & Slaton, 2015). The work on biocides and poulticing referred to earlier (Long & Young, 2016), was as a direct result of damage by contractors using high pressure washing systems on a stone matrix which was unsuitable. Any form of cleaning which relies on soaking the surface with water also runs the risk of increasing salting, freeze thaw damage, and hydric expansion of smectite in the stone matrix (Normandin & Slaton, 2015; Cherblanc *et al.*, 2016).

Steam cleaning is a development of low pressure washing where the water is heated under pressure prior to release. Temperatures range from 90 to 180°C at pressures up to 102 psi, the release from the nozzle of the cleaner results in the water boiling as it hits atmospheric pressure

and generating the steam (Odgers, 2013). The systems are promoted for removing generally flexible materials such as biofilms, and in many cases are effective (Odgers, 2013). Problems arise when the presence of gypsum crusts, bioprecipitated calcium coatings on the surface of the biofilms, and the biofilm blocking steam penetration into the stone pores (Di Bonaventura *et al.*, 1999; Cacchio *et al.*, 2003; Banks *et al.*, 2010; Dhami *et al.*, 2014), results in the use of the steam cleaners degenerating into a method more appropriate to pressure washers and as a result damaging the surface (Personal communication, Clara Willett, Historic England, September 2016).

1.4.5 Selecting the appropriate method

While additional systems such as grit blasting, detergents, and latex or chemical poultices are also used it is to a lesser degree (Doehne & Price, 2010; Normandin & Slaton, 2015), and therefore will not be discussed further.

More physical methods are by their nature unlikely to result in the development of resistance, but may be problematic for other reasons. In the study at Newport Roman Villa the conservators chose to box in the area requiring UVC treatment for health and safety reasons, this caused localised heating of the mosaic resulting in rapid salting after the treatment was completed (Stewart *et al.*, 2008).

Laser cleaning imparts sufficient energy to the stone matrix to cause chemical changes and the formation of iron rich nano-particles, which can cause yellowing to limestones when compared to other treatments such as biocide application or steam cleaning (Godet *et al.*, 2016)

Post cleaning inhibition of microbial recolonisation by coating surfaces with resins and waxes, while once considered an acceptable approach (Doehne & Price, 2010), is now frowned upon due to deleterious effects on the stone and because studies have shown the resins and waxes to be easily metabolised by the very organisms they were looking to inhibit (May, 2003; Cappitelli *et al.*, 2007; Dhami *et al.*, 2014).

Therefore while there are several cleaning methods available for the conservation of stone, the issues associated with them, the cost of purchasing specialised equipment and its ongoing maintenance, and lack of guidance in their use means that conservators often fall back on the familiar use of biocides despite the concerns related earlier.

2 Materials and Methods

2.1 Sampling

Initial sampling was carried out at two locations in Lincoln, with Dr. Lynda Skipper assisting by recording the data and taking photographs of the sample sites, further sampling for the metagenomics work was carried out solely by the author at 3 urban locations, including the two original locations, and 1 rural location. In addition to these 4 locations, 3 rural locations in Herefordshire were sampled by Mary Webster for her MA in conservation for isolation of organisms, the isolates found were incorporated into this study.

Where possible, sites were chosen where an undamaged stone was adjacent to a damaged stone with the same orientation and exposure, both being sampled in order to achieve a direct comparison of bacterial colonisation. Surfaces were considered physically damaged if spalling or fragmentation to a depth > 10mm from the surface had occurred (Cherblanc *et al.*, 2016)

Each site was photographed and the exact location noted as well all areas of damage recorded during the sampling on a standardised sampling sheet, see Figure 4 for an example sheet.

2.1.1 Sellotape and swab method to sample surfaces

Sampling of the stonework was performed in duplicate. To obtain a sample of biofilm for microscopy work, adhesive tape sampling was carried out (Urzì & Leo, 2001; Cutler *et al.*, 2012).

To obtain samples for culturing the bacteria or extracting the total genomic DNA from the microbiome for metagenomics studies, a 1cm^2 area was swabbed with sterile M9 salts (M6030, Sigma). Fresh M9 salts were used for each sample to prevent cross contamination between sampling sites. At all stages of sampling nitrile gloves were worn to prevent contamination of the samples with skin microbiota.

2.1.2 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.

Information regarding details of the sampling location which could have an effect on the biome such as guttering, vegetation proximity and water flow were recorded along with the direction the stone face (aspect), height from ground level and location on the wall, also shown in Figure 4. The latter data, along with photographs of the sampling site, were taken to allow accurate resampling at a future date if necessary. Finally a sketch of the sampling site was produced.

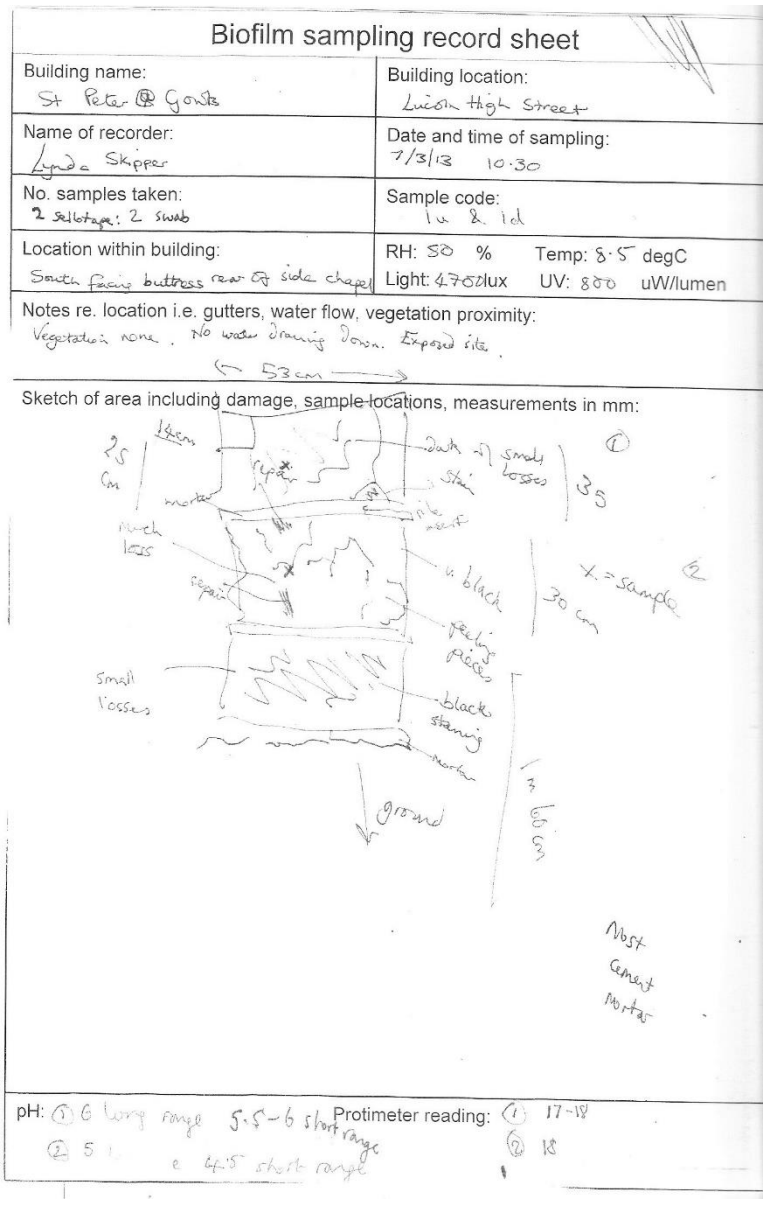


Figure 4: Sample sheet from site 1 at Saint Peter-at-Gowts showing recording information to allow for accurate resampling if necessary. Environmental considerations such as aspect, light and UV levels, temperature were recorded to eliminate sampling bias for identifying the core microbiome. In addition to the sketch of the area sampled photographs were taken and the sampling point marked on a map of the building.

2.1.3 Staining of sampled biofilms

Biofilms were stained with FilmTracer™ FM® 1-43 Green Biofilm Cell Stain and FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (Invitrogen) 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 using the TRITC filter (570-620nm barrier filter) as per the manufacturer's protocol.

2.1.4 Characterisation of Lincoln limestone

Samples of quarried limestone which had been finished to an equivalent surface to those used for repairs on the cathedral were sent to Alice Gillett at the University of Chester for surface profile analysis, samples were analysed using CCI profilometer (Micromeasure2, STIL, France).

To determine the chemical composition of the limestone 7x 1 cm^{-3} blocks of Lincoln limestone, provided newly cut by the Lincoln Cathedral Quarry, were mounted on stubs with carbon tabs and earthed to the stub with silver conductive paint (G3790, Agar Scientific). The samples were imaged on an FEI 2017/11 Quanta Inspect SEM using FEI's xT microscope control software (version 4.1.0.1910). One hundred and twenty six spectra were read across the 7 samples using EDX to determine the molecular composition. EDX was carried out with the Oxford Instruments INCAx-act attached to the FEI 2017/11 Quanta Inspect and controlled through Oxford Instruments INCA software (version 4.11).

2.2 Characterisation of microorganisms

2.2.1 Culturing and isolation of bacteria

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 25°C in normal atmosphere. 25°C was selected experimentally as the temperature at which the majority of species isolated from the initial sampling showed optimal growth under laboratory conditions. Plates were then inspected and single colonies were isolated using standard culture techniques. Following the isolation of single

colonies the plates were incubated for a further 96 hours at 25°C to confirm that there were no slower growing organisms present.

2.2.2 Identification of isolated species

Initial identification of the isolated bacteria was by gram staining using standard protocols, and the colonies were tested using Catalase and Oxidase tests, using Kovacs Oxidase reagent for the oxidase test. Based on the data from these tests, where it was considered that rapid identification could be performed on the bacteria using traditional methods, biochemical tests based on sugar metabolism were performed. These were tests for metabolism of glucose, lactose, xylose and maltose using an Oxidative-Fermentative test (Hugh & Leifson, 1953) as well as gelatin dissolution (Thirst, 1957).

Bacterial genomic DNA was extracted using an in house protocol which is suitable for both gram positive and gram negative bacteria. Briefly, cultured cells were pelleted by centrifugation (17,000 x g in a Hereus Pico 17 microcentriguge). 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 then incubated, shaken, for 30 minutes at 37°C. 50 µl of a 10% SDS stock was then added and the sample was incubated at 60°C for 30 minutes. Insoluble material was pelleted by centrifugation and the supernatant transferred to a fresh tube. 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 immediately, samples were stored at -20°C.

2.2.3 Identification of micro-organisms – 16S rRNA sequencing with primer sequences.

Regions of the 16S rRNA gene were PCR amplified (Table 3a) 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 (Maciel *et al.*, 2009) and once with highly efficient in-house primers, designed by Michael Shaw, which amplify a 322bp region between nucleotides 764 and 1084 (Table 3b).

Where poor sequence was returned species were checked for multiple 16S rRNA genes using 2% agarose gels on the PCR product using PS_16S_F555, designed by the author based on analysis of the 16S rRNA gene, and MS_BACT-16S_Rev as primers (Table 3b). This primer pair covers variable

regions 4-8 giving sufficient coverage for accurate identification of species, while producing a product of 769bp, which allows sequence overlap of the whole product when sequenced in both directions for additional robustness. The PCR product was then TOPO cloned into a pCR4 as per the manufacturer's protocol (K457502, Invitrogen). Plasmids were isolated using a QIAGEN Mini-Prep plasmid extraction kit (27104, QIAGEN) and the 16S rRNA fragment was then sequenced using M13 forward and reverse primers.

Analysis of the species identified at the sites was carried out using Geneious version R9 (http://www.geneious.com, M. Kearse et al. 2012) for phylogenetics and the metagenomeSeq package for R (Joseph N Paulson *et al.*, 2013) for statistical significance.

Step	Temperature (°C)	Time	
Initial denaturation	95	10 minutes	
Denaturation	95	30 seconds	34 cycles
Annealing	47.9	30 seconds	
Elongation	72	1 minute 30 seconds	
Final elongation	72	10 minutes	
Hold	4	until analysis	

Table 3a: Cycling instructions for the amplification of 16s rRNA fragments.

Primer name	Primer sequence
16S-rRNA_F27	AGAGTTTGATCMGGC
16S-rRNA_R1525	AAGGAGGTGWTCCARCC
MS_BACT-16S_For	GGATTAGATACCCTGGTAGTCC
MS_BACT-16S_Rev	TCGTTGCGGGACTTAACCCAAC
PS_16S_F555	CTCCTACGGGAGGCAGCAGT

Table 3b: 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 designed by, and used with permission of, Michael Shaw, molecular biology technician at the University of Lincoln. All primers were produced by Sigma. PS_16S_F555 was designed by the author.

2.2.4 Planktonic growth curves

Starter cultures were grown as a static planktonic culture, bacterial colonies being inoculated into a growth media to produce a culture of single cells floating planktonically in the media as opposed to encapsulated in a biofilm, for 96 hours at 25°C in 4ml of nutrient broth. 3 dilutions of each

isolate were made to a final OD 580nm (Fisher Scientific model 45 colorimeter) of 0.01. Aliquots of 100μl of each dilution for each species were then placed into a 100 well Bioscreen C plate and incubated at 25°C for 72 hours. Readings were taken every 30 minutes at 600nm with the samples being shaken for 10 seconds prior to and after reading. All growth curves were produced from the mean of the three repeats.

The Bioscreen C is a modified plate reader system designed to automate routine planktonic microbiology methodologies (*Bioscreen C MBR*, 2017). In order to obtain highly accurate temperature control it uses a custom micro-plate format of 100 wells arranged in a honeycomb format. Plates are placed in a moving carriage cassette system which is heated from the base through a recirculating heat transfer fluid to maintain the desired temperature and has an electronically heated lid which allows the plate lid to be run at a slightly higher temperature to avoid condensation which would interfere in the readings.

The system provides a temperature controlled incubator, reader and shaker and is equipped with 8 filters, 7 which read between 405 nm and 600 nm and one wide band filter. For increased sensitivity the system uses a nephelometer (light scatter turbidity meter) for measurements as opposed to direct absorbance as measured by a standard spectrophotometer plate reader (Underwood & Doermann, 1947).

With computer control the system can be set up to run tests extending from a single data point up to one year with the only limitation being the life of the bulb. Temperature control is accurate to 0.1 degree centigrade.

2.2.5 pH profiling

Optimal pH for growth was ascertained by culturing the species in buffered media. A final concentration of 0.2 mM Phosphate/Citrate buffer (Sigma citric acid monohydrate, C7129, and sodium phosphate dibasic, S3264) was used to produce buffered nutrient broth at pH 4.6, 5, 5.6, 6, 6.6, 7 and 7.6. OD 580nm 0.01 cultures were produced as per the planktonic growth curves using the buffered media and an unbuffered control. Growth curves were then measured over 72 hours at 25°C reading every 30 minutes on a BMG Fluostar Optima at 620nm. All growth curves were produced from the mean of three repeats.

2.2.6 Characterisation of biofilm formation

OD 580nm 0.01 cultures for each species were produced as per the planktonic growth curves. Aliquots of 100μl of each dilution for each species was placed into a 96 well plate and a Nunc

Immuno TSP 96 peg lid (Fischer Scientific, 445497) was applied. The plates were sealed with parafilm (Sigma P7793) and incubated at 25°C for 72 hours to allow all of the species sufficient time to produce a mature biofilm (Ceri *et al.*, 1999).

The mature biofilm was quantified by staining with crystal violet for 2 minutes followed by 2 ten minute washes in an excess of deionized 18MΩ water. Crystal violet which was bound to the biofilm matrix was eluted in 100% ethanol and the eluate read on a BMG Fluostar Optima at 570nm. All results were produced from the mean of three repeats.

2.3 Metagenomics

Total genomic DNA was extracted from swabbed samples of stonework using the phenol chloroform protocol described in 2.2.2. The extracted DNA was given a final wash in 50µl of 10mM Tris (Sigma T6791), 5mM EDTA (Sigma E6758) and 5mM EGTA (Sigma E0396) then the ethanol precipitation step was repeated to remove any remaining calcium ions which could interfere with the PCR amplification of the 16S genes. Samples were sent to Nottingham Trent Universities Genomics service to be run on an Illumina MiSeq next generation sequencing system using the Earth Microbiome Project primers (Table 4). These amplify between 515F/806R and give > 80% identity of bacterial and archaeal species. Raw data from the sequencing, that is the direct, unprocessed, output from the sequencer, was returned and all analysis was carried out by the author using Geneious version R9 (http://www.geneious.com, Kearse et al. 2012) and the metagenomeSeq package for R (Joseph N Paulson *et al.*, 2013).

515f PCR Primer Sequence – Forward primer

Field description (space-delimited):

1. 5′ Illumina adapter
2. Golay barcode
3. Forward primer pad
4. Forward primer linker
5. Forward primer (515f)

AATGATACGGCGACCACCGAGATCTACAC XXXXXXXXXXXX TATGGTAATT GT

GTG**Y**CAGCMGCCGCGGTAA

806r PCR primer sequence – Reverse primer

Field description (space-delimited):

1. Reverse complement of 3′ Illumina adapter
2. Reverse primer pad
3. Reverse primer linker
4. Reverse primer (806r)

CAAGCAGAAGACGGCATACGAGAT AGTCAGTCAG CC GGACTAC**N**VGGGTWTCTAAT

Table 4: Earth Microbiome Project primers description for Illumina next generation sequencing, taken from Caporaso et al. (2012).

2.4 Biocide testing

Four biocides were tested in this study, Microtech by Wykamol, Algo bioinhibitor by Tensid UK, Soluguard Multi-Surface Biocide by Safeguard Europe Ltd and finally Industrial methylated spirits (IMS). These biocides were selected based on the results of a questionnaire submitted to UK based stone conservators. Thirty five stone conservators were directly surveyed as well as the survey being released on the Conservation Distribution list, which has a subscription of several thousand international conservators, to ensure that the biocides tested for this part of the research were relevant to current practice.

The questions were as follows:

Q1: What brand of biocide do you use?
Q2: What concentration do you use the biocide at?
Q3: How regularly do you use the biocide?
Q4: What material(s) do you use the biocide on?
Q5: If possible can you tell me why you selected that biocide (i.e. recommendation, sales pitch etc.)?
Q6: Would you like to be updated on the results of this research? (Please leave email address in the box below)

The 3 commercial biocides had maximum and minimum recommended dilutions within similar ranges so a range from 1/24 to 1/150 was tested as this covered the ranges for all 3 biocides. IMS was tested from 100% to 40% for spot plates and biofilm resistance, and from 80% to 0.5% for planktonic growth with the change in range being due to experimental constraints. All tests were run with appropriate positive and negative controls.

2.4.1 Agar spot test

Biocide testing was performed as per Hernández *et al* (2005) with the following modifications. Strains were cultured in nutrient broth and agar and plated onto nutrient agar plates in a 50% nutrient top agar. 10 μl aliquots of biocide concentrations spanning the manufacturer's recommended dose range were spotted onto the plates and then incubated at 25°C for 48 hours. Inhibition was scored as positive if the zone was wider than 10mm diameter. The plates were then incubated for a further 48 hours at 25°C to test for breakthrough. Breakthrough was scored

as positive if visible colonies had formed in the cleared area. All results were calculated from the mean of three repeats.

2.4.2 Microdilution

To measure the minimum inhibitory concentration of the biocides (MIC) in planktonic culture, MICs were performed using the standard microdilution method for clinical organisms (EUCAST, 2000) with the following modifications. Nutrient broth was used instead of Mueller-Hinton broth and cultures were incubated for 48 hours at 25°C and shaken at 180 rpm in an orbital shaking incubator (labnet 211DS) instead of 35°C for 24 hours throughout, to allow for the growth conditions required for these species. All biocides were tested across the manufacturers recommended range. All results were produced from the mean of three repeats.

2.4.3 Biofilm Calgary Peg method

The "Calgary Peg method" is a development of the characterisation of biofilm growth protocol in 2.2.6. Following the growth of the mature biofilm the peg lid is transferred to plates containing 100μl biocide at concentrations spanning the manufacturers recommended range and then incubated for 24 hours at 25°C. The peg lids are then transferred to nutrient broth as a recovery media for a further 24 hours at 25°C. Following the 24 hour incubation any growth in the recovery media was measured at 620nm, any remaining biofilm matrix on the peg lids was stained and the crystal violet was eluted and the eluate measured as per the biofilm growth protocol in section 2.2.6. All results were produced from the mean of three repeats.

2.4.4 Testing for potential enhancement of salt weathering by biocide

Salt weathering testing was carried out using freshly cut 1 cm^{-3} limestone cubes which were immersed in distilled water for 7 days, in addition to limestone cubes which had been saturated in a 4.66M sodium chloride solution for 7 days prior to drying in a Genlab Mino Economy Oven at 40°C for 42 hours. Sodium chloride was selected for testing as it is one of the most common salts detected in weathering (Rodriguez-Navarro & Doehne, 1999).

10μl of biocide at the manufacturers maximum recommended concentration was spotted onto the centre of each block and allowed to dry at room temperature for 48 hours. Blocks were photographed for analysis using a Nikon Coolpix L840 digital camera at 16mp resolution. The chemical composition of the surfaces was analysed using SEM-EDX. The above work was carried out by Doctor Lynda Skipper, the anaylsis described below was carried out by the author.

Surface roughness of the SEM images was analysed using the Roughness Calculation plugin (Chinga & Dougherty, 2002) for ImageJ (Schneider *et al.*, 2012). Photographic analysis of colour change was carried out using the GNU Image Manipulation Program RGB histogram tool (Kimball & Mattis, 2016), taking the mean colour readings of red, green and blue components of the image over a 12269 pixel area of the each block, following the method from Concha-Lozano *et al.* (2013).

2.5 Testing for active dissolution of limestone

2.5.1 Effects of bacterial metabolism on the dissolution of calcium carbonate

Dissolution of calcium carbonate by bacterial metabolic by-products was tested on a solid agar medium as per Di Bonaventura et al. (1999), with the following modifications. Calcium carbonate in the enriched medium was reduced from 50g/L to 20g/L which resulted in a more homogenous dispersion of calcium carbonate through the medium. The additional supplementation of calcium carbonate for the carbonate solubilisation test was shown to be unnecessary. Glucose in the enriched medium was reduced from 67g/L to 1g/L, in line with more commonly used growth media, after it was shown to inhibit growth of many species at the original concentration. All results were produced from the mean of three repeats.

2.5.2 pH modification of environment through growth curve.

Modification of environmental pH was measured using nutrient broth supplemented with 0.0008% Bromocresol purple (Sigma B5880). Bromocresol purple is a non-toxic, pH sensitive dye with a colour range shifting from 590 nm (purple) to 427 nm (yellow) across a 6.8-5.2 pH range used in both selective and non-selective microbiological growth media. In order to confirm that the Bromocresol purple was not altering bacterial growth rates control reactions were run without Bromocresol purple, initial (0.008 at OD 590nm) and final OD for the runs with and without Bromocresol purple were compared to each other. A random sample of species was also plated out for colony counts to confirm that both Bromocresol purple supplemented media and unsupplemented media had reached the same density of cell growth.
Measurement was carried out using a Labsystems Bioscreen C at 492nm, 25°C, for 72 hours with minimal shaking before readings.
Bromocresol purple growth curves compared with the control growth curve indicated that acidification caused suppression of the signal, whereas alkalisation caused an increase in the signal. All growth curves were produced from the mean of three repeats.

2.5.3 SEM analysis of monoculture biofilms

Isolates were cultured in a liquid form of the growth media described in Di Bonaventura et al. (1999), without the addition of calcium carbonate to the media. Instead a 1 cm^3 sterile limestone cube was partially immersed in the media to provide a source of calcium carbonate and a surface for the bacteria to form a biofilm matrix on. The culture was grown statically at 25°C for a 3 month period with growth media being refreshed under sterile conditions every fortnight.

Samples were prepared for viewing on the SEM by coating in 0.1M sodium cacodylate (Sigma C0250), fixing in 3% glutaraldehyde (Sigma G7776), washed in deionized water then dehydrated by working through a series of 50, 70 and 100% methanol (Sigma 322415) then a final step of 100% acetone (Sigma 439126). The stone blocks were then air dried, attached to SEM stubs and splutter coated using a gold palladium electrode to reduce damage to the biofilms and earthed with silver conductive paint to prevent charge build up. As with the characterisation of the limestone imaging was carried out with the FEI 2017/11 Quanta Inspect SEM.

2.6 Analysis of data

2.6.1 Analysis of metagenomic data

Analysis of the data provided was carried out using Geneious version R9. For each of the data sets received the following process was followed for analysis. In order to ensure that only high quality reads were used the raw reads were trimmed to ensure that the primers had been removed and to trim any regions of sequence data which had more than a 5% chance of error per base based on the quality data within the FASTQ file.

The raw data was provided as interlaced paired reads, in other words both the sequence and complement strands were sequenced and these were identified by the addition of a /1 or /2 on the end of the file name. Paired reads were identified and merged to provide consensus sequences for each read. Where reads were of too low a quality they remained unmerged and stored in a separate list.

The merged paired reads were then assembled using a customised de-novo assembly with high stringency allowing 2% maximum gap per read, maximum gap size of 1bp, maximum mismatches per read of 2% and maximum ambiguity of 4. The assembler was also set up to save any ambiguities as N in order to facilitate BLAST (NCBI Resource Coordinators, 2013) searching.

BLAST searching was carried out against the NCBI nr database obtaining the best hit for each sequence allowing a maximum E-value, the chance of achieving this result randomly, of $1x10^{-1}$.

On completion of the BLAST search duplicate results were removed before downloading the full sequences from the NCBI server. Any sequence results which were ambiguous, for example the organism being identified as 'Bacteria' or 'Unclassified' were filtered out and stored in a separate directory as were any hits which were identified as 'Eukaryotes'.

The assembly, classification and filtering process was then carried out on the unmerged reads.

Once the data had been assembled and classified for the merged and unmerged reads a final stringency filter was applied removing any organisms which had been identified at < 97% identity to ensure that all identifications were at species level (Větrovský & Baldrian, 2013).

This provided two data sets for each of the sample sites, sequences with identity above 97% for species level analysis and the total population of characterised data for analysis at the OTU level.

2.6.2 Statistical analysis of data

Analysis of data was carried out using R, version 3.3.1, or where packages were not available for R using Microsoft Excel 2013.

Shannon's index of diversity was selected as a simple measure to test whether the diversity found in the sampling of the surfaces was representative of the populations. By quantifying the uncertainty or information content in the sample, based on the number of isolates of each species found in a sample, the diversity of the total population can be calculated (Shannon, 1948). Shannon's index of diversity and Shannon's equitability were both calculated using Excel using the equations provided in the original paper (Shannon, 1948)

$$H_s = -\sum_{i=1}^{S} p_i \ln p_i$$

Equation 1: Shannon's index of diversity (Hs), the proportion of species (i) relative to the total number of species (pi) is calculated, and then multiplied by the natural logarithm of this proportion (ln pi). The resulting produce is summed across the total number of species in the community (S) and multiplied by -1

$$E_H = H_s/(H_s/\ ln\, S)$$

Equation 2: Shannon's equitability is calculated by dividing the result of Shannon's index of diversity (Hs) by the natural logarithm of the total number of species in the community (S). The resulting product is divided by the result of Shannon's index of diversity (Hs)

Microbiome analysis was carried out on the species identified from the metagenomic and directly sampled studies. This was achieved using the metagenomeSeq package in R (J.N. Paulson *et al.*, 2013), which was selected as it is designed to handle smaller data sets and is specifically targeted at analysis of differences between microbial communities. The plotMRheatmap function of metagenomeSeq was used to generate a structural overview heatmap to provide visualisation of correlations and clustering. Log normal permutation testing (Equation 3) was applied to the data to determine whether there were species, or OTUs, significantly present in one sample when compared to the other.

$$z = \left(\frac{1}{n}\sum_{i=1}^{n} ln(x_i)\right) \pm 2\left(\sqrt{\frac{\sum_{i=1}^{n} ln(x_i)^2 - \frac{1}{n}\left[\sum_{i=1}^{n} ln(x_i)\right]^2}{n-1}}\right)$$

Equation 3: Log normal permutation test where z is a quartile from the standard normal distribution, n is sample size and x is the sample mean

Discovery odds ratio testing, implemented using Fisher's exact test (Equation 4), was used to determine whether some species, or OTUs, were present at significantly higher levels in one sample than another.

$$p = \frac{\binom{a+b}{a}\binom{c+d}{c}}{\binom{n}{a+c}}$$

Equation 4: Fisher's exact test where a is column 1 row 1 of the data set, b is column 2 row 1, c is column 1 row 2, d is column 2 row 2 and n is the sum of the columns and rows.

Finally presence-absence testing and unique feature searches were carried out to determine whether species, or OTUs, were found solely in the sample of interest.

Normality testing, to determine whether the distribution of the data was normal and therefore applicable to the population as a whole, was carried out using Lilliefors normality test as implemented by the nortest package in R (Gross & Ligges, 2015). This was selected as being suitable for the range of the sample size.

Sample mean	$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$
Standard deviation	$s = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$
Normalised sample values	$Z_i = \frac{x_i - \bar{x}}{s}$
Lilliefors test statistic	$T = sup[F^*(x) - S(x)]$

Equation 5: Lilliefors test statistic calculated from the sample mean, the standard deviation and the normalised sample value for each sample. n is the size of the sample, x is the values of the sample, F(x) is the cumulative distribution function of a normal distribution with mean zero and standard deviation one and S(x) is the empirical distribution of the values of Z_i calculated using the normalised sample values equation.*

Skewness testing was carried out in Excel 2013 using the SKEW function which implements the Fisher-Pearsons test (Equation 6) in order to determine whether the distribution of the data was skewed towards either end with the null hypothesis being normal symmetrical distribution. Significance of this was carried out by calculating the test statistic (Equation 7), where the test statistic is > 2 or < -2 the skewness is considered significant, p-value = 0.05 (Wright & Herrington, 2011).

$$G_1 = \frac{\sum_{i=1}^{N}(Y_i - \overline{Y})^3/N}{s^3}$$

Equation 6: Fisher Pearsons test, G_1 is the measure of skew, Y is the data set, $\bar{Y}$ is the mean, s is the standard deviation, and N is size of the sample being tested.

$$Z_{g1} = G_1/SES \text{ where } SES = \sqrt{\frac{6n(n-1)}{(n-2)(n+1)(n+3}}$$

Equation 7: Test statistic for skewness and Standard Error of Skewness (SES) where G_1 is the Fisher-Pearson skewness test result and n is the size of the original sample.

Non-paired Student's t-tests (Equation 8) were carried out using the embedded function in R (R Core Team, 2016) having first checked that the data showed the correct homoscedasticity using Fishers F-test (Equation 9), also in R.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{S^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

$$S^2 = \frac{\sum_{i=1}^{n_1}(x_i - \bar{x}_1)^2 + \sum_{j=1}^{n_2}\left(x_j - \bar{x}_2\right)^2}{n_1 + n_2 - 2}$$

Equation 8: Non-paired Student's t-test where $\bar{x}_1$ and $\bar{x}_2$ are sample means, s^2 is the pooled sample variance, n_1 and n_2 are the sample sizes and t is a Student t quartile with n1+n2-2 degrees of freedom.

$$F = \frac{\sum_{i=1}^{K} n_i(\bar{Y}_i - \bar{Y})^2/(K-1)}{\sum_{i=1}^{K}\sum_{j=1}^{n_i}\left(Y_{ij} - \bar{Y}_i\right)^2/(N-K)}$$

Equation 9: Fishers F test where $\bar{Y}_i$ denotes the sample mean in the i-th group, n_i is the number of observations in the i-th group, $\bar{Y}$ denotes the overall mean of the data and K denotes the number of groups, Y_{ij} is the jth observation in the ith out of K groups and N is the overall sample size.

Testing for correlation between data sets was carried out using Pearsons correlation coefficient testing was carried via the embedded function in R (Equation 10).

$$r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[n(\sum x^2) - (\sum x)^2][n(\sum y^2) - (\sum y)^2]}}$$

Equation 10: Pearsons correlation coefficient where n is the sample size x is data set 1 and y is data set 2.

One way anova testing (Equation 11) was implemented using the native functions implemented in R.

$$F = \frac{\frac{\sum_{j=1}^{p} n_j\left(\bar{x}_j - \bar{x}\right)^2}{p-1}}{\frac{\sum_{j=1}^{p}\sum_{i=1}^{n_i}\left(x_{ij} - \bar{x}_j\right)^2}{n-p}}$$

Equation 11: One way anova where p is the number of groups, n is the total entries in a group, $\bar{x}$ is the mean of the group and x is the grand mean of all the groups.

3 Sampling of the limestone microbiome

3.1 Introduction

In order to greater understand the limestone microbiome as a whole, as well as the specific variation resulting from rural or urban environments and damaged or undamaged surfaces, 4 locations were selected for sampling. The 4 churches suitable for the study were identified by the Diocese of Lincoln's conservation officer (see Figure 5 for locations). To avoid confusion each church is referred to as a location, and sampling points on the church would be sites (therefore one location could have several sites sampled). One church was designated as a rural location as it was over half a mile away from the nearest A road (Lincolnshire has no M roads) which ensured that localised pollution would not skew comparisons between this church and the urban microbiomes, the distance was based on the ambient air quality mapping provision by Defra (Defra, 2016). The aim of this is to ensure that the core microbiome detected was not purely urban and to enable an initial comparison of the bacteria in polluted vs. non polluted areas. Environmental measurements were taken at each site to eliminate the possibility that the microbiome present was due to factors such as aspect, light etc.

Permission was obtained from the Vicars of the churches before sampling was carried out. In addition, permission was granted by the Masters Committee of The Cathedral Church of the Blessed Virgin Mary of Lincoln (Lincoln Cathedral) to allow sampling to take place around the site.

Where isolation of microorganisms was intended, multiple samples were taken at selected sites in order to ensure that the species cultured were representative of the microbiome across the building. Isolates were cultured on media which would select for copiotrophs as these represent the faster growing, more adaptable members of the population. Cultured isolates were then tested for physical and biochemical properties and the species confirmed using 16S rRNA sequencing.

The initial sampling for the direct isolation of cultured species for identification from Lincoln Cathedral (Figure 6A) and Saint Peters-at-Gowts (Figure 6B) was carried out as an internally funded collaboration with Dr. Lynda Skipper of the University of Lincoln's School of History and Heritage, with Dr. Skipper defining the broader project scope and assisting with the recording of the sampling, sampling site details can be found in Table 5. The experimental design, laboratory work, species identification and analysis was carried out by the author.

Sampling for metagenomic analysis was carried out at Lincoln Cathedral, Saint Peter-at-Gowts, Lincoln, Saint Botolph-by-Bargate, Lincoln (Figure 6C), and Saint Nicholas, Saint Andrew and the Blessed Virgin Mary's Church, Burton Pedwardine (Figure 6D). Sampling for the metagenomics study was carried out solely by the author, sampling site details can be found in Table 5. Pure DNA samples were sent to Dr. McNally of Nottingham Trent University who returned the raw data, in nucleotide quality scored FASTQ format, with the raw data being processed solely by the author to identify the components of the microbiome to species level.

In addition to the sites sampled for this study, rural sampling and environmental measurements were carried out by Mary Webster as part of her Masters project (Webster, 2016) at Weobley Castle (Figure 6E) and Oxwich Castle (Figure 6F) on the Gower Peninsula in Wales and Saint Michael and All Angels church (Figure 6G) at Moccas in Herefordshire with the permission of the land owners; the isolates from these buildings were integrated into this study. Mary carried out the initial sampling and isolation of the colonies for her study, sampling site details can be found in Table 5. Identification and characterisation of the species isolated was carried out by the author.

Both the cultured isolates and species identified in the metagenomics study were then analysed to determine whether there were significant differences between the microbiomes found on damaged and undamaged stone, basic analysis was also carried out to identify differences between the urban and rural samples.

In addition to sampling the microbiome the key characteristics for bioreceptivity of the surface were measured, capillary co-efficient and surface roughness, characterised by the Ra and Rz measurements as per Miller et al. (2009). Data regarding the capillary co-efficient was available from quarry data sheets for Lincoln limestone. The surface measurements were kindly carried out by Alice Gillet of the University of Chester, as a surface profilometer was not available in house, with the interpretation of the results being carried out by the author of this study. The only study of the chemical profile of Lincoln limestone (Barber, 1974) was contains significant inaccuracies, therefore in addition to the physical characterisation of the stone, the chemical profile of the limestone was analysed using SEM-EDX.

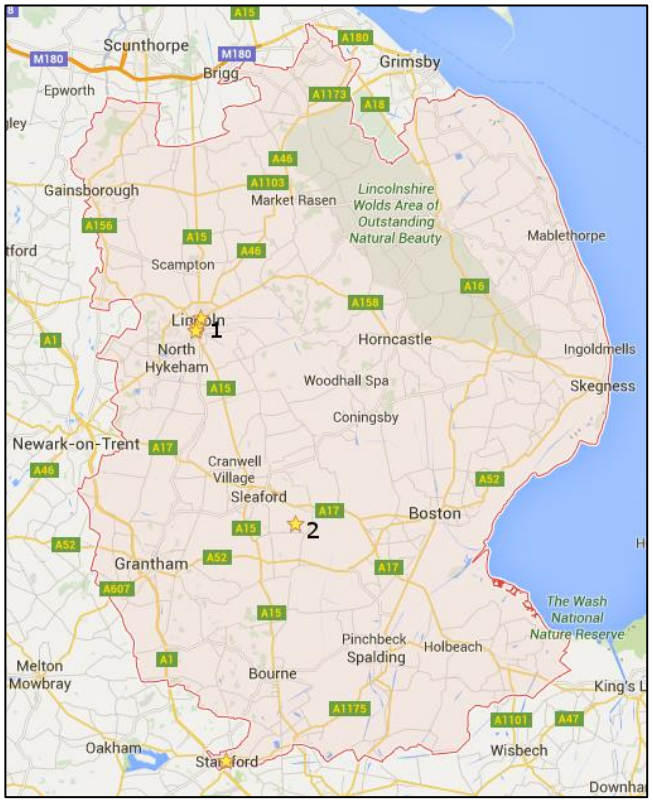


Figure 5: Lincolnshire sampling locations 1) Lincoln (from north to south): The Cathedral Church of the Blessed Virgin Mary of Lincoln, Saint Peters-at-Gowt, and St. Botolphs-by-Bargate. 2) Burton Pedwardine: Saint Nicholas, Saint Andrew and the Blessed Virgin Mary's Church. Map © OpenStreetMap contributors.

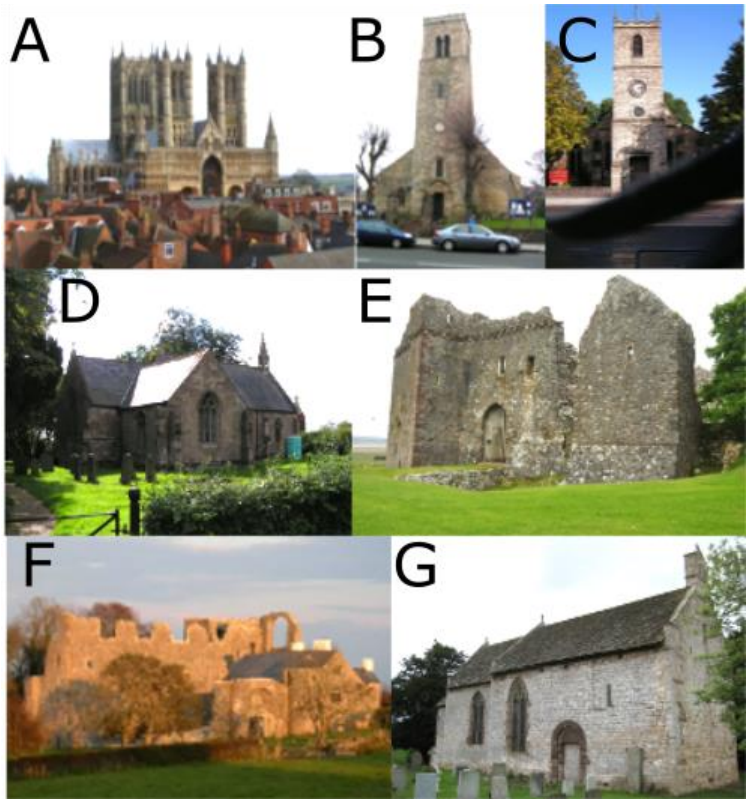


Figure 6: Locations sampled, A, Lincoln Cathedral, B, Saint Peter-at-Gowts, C, Saint Botolph-by-Bargate, D, Saint Nicholas, Saint Andrew and the Blessed Virgin Mary's Church, Burton Pedwardine, E, Weobley Castle, F, Oxwich Castle, G, St. Michaels at Moccas. Photographs of Oxwich Castle, Weobley Castle and St. Michaels at Moccas are copyright to Chris Henley, 'Nilfanion' of Wikimedia Commons and Philip Halling and are licenced for resuse under Creative Commons Licence, all other images are the authors own.

Location	Site	Position	Aspect	Origin (Quarry)	Date built (A.D.)	Samples taken		Method of analysis	
						Damaged	Undamaged	Isolation of organisms	16S rRNA Metagenomics
Lincoln Cathedral	1	SE Transept Roof	SE	Lincoln	1235	X	X	X	
	2	NW Transept	NE	Lincoln	1235	X	X	X	X
	TB	New toilet block	SE	Lincoln	2008		X	X	
	3A	Flemming Chapel	SW	Ancaster	1431	X		X	
	3L	Flemming Chapel	SW	Lincoln	1431	X		X	
	4	East End	NE	Lincoln	1280		X	X	
St. Peters-at-Gowts (Lincoln)	1	Chantry Chapel	SSW	Lincoln	1347	X	X	X	X
	2	St. Andrews Chapel	NNE	Lincoln	>1800	X	X	X	
	3	Gravestone	n/a	Lincoln	1832		X	X	
	4	Tower	WNW	Lincoln	>1000	X	X	X	
St. Botolphs-by-Bargate (Lincoln)	1	South Chapel	N	Lincoln	1721 (reused stone from >1100)	X	X		X
St. Nicholas, St. Andrew and the Blessed Virgin Mary's Church (Burton Pedwardine)	1	NW chancel	WSW	Lincoln	>1300	X	X		X
Oxwich Castle, Gower	1	SE wall	NW	Oxwich head	>1500	X	X	X	
	2	NW wall	SE	Oxwich head	>1500	X	X	X	
	3	NW wall	SE	Oxwich head	>1500	X	X	X	
Weobley Castle, Gower	1	West wall	E	Oxwich head	>1000	X	X	X	
	2	South wall	N	Oxwich head	>1000	X	X	X	
	3	NNE wall	SSE	Oxwich head	>1000	X	X	X	
Saint Michael and All Angels Church (Moccas)	1	North wall	S	Herefordshire Tuffa	>1100	X		X	
	2	East end	W	Herefordshire Tuffa	>1100	X		X	
	3	SE chancel	NW	Herefordshire Tuffa	>1101		X	X	

Table 5: Sampling locations detailing the site, aspect at which the sample was taken, the age and origin of the stone, the surface sampled and the use of the isolates in the study. X denotes, where appropriate, whether samples were taken from damaged or undamaged stone and the final use of the samples.

3.2 Environmental sampling results

Sampling was carried out on exterior stone, selecting (where possible) paired sites where an undamaged stone was directly adjacent to a stone which showed evidence of physical damage (loss of surface >10mm depth). Environmental measurements of relative humidity, surface wetness, lux, UV, temperature and surface pH at the sampling sites (Table 6) were recorded. Environmental data for Moccas, Weobley and Oxwich are not included as sampling occurred in wet weather which would have prevented accurate readings for relative humidity, surface wetness and surface pH (Webster, 2016), data from sampling sheets from Mary's dissertation is available in Appendix A: Data from M. Webster Thesis.

Analysis of the environmental data gave significant (p-value < 0.05) correlations between the data which was not related to the stone surface, for example correlations were found between relative humidity and temperature, relative humidity and lux, relative humidity and UV, and so on. Significant correlations were not found between these and the surface wetness or surface pH for the sampled stone. This supports the initial assumption that having sampling sites adjacent to each other would reduce the likelihood of the damage being caused by external environmental factors.

Surface wetness on damaged surfaces gave a mean of 21.11, and a mean of 21 for undamaged. A student's t-test showed that there was no significant (p-value = 0.98) difference between damaged and undamaged stone. A significant (p-value < 0.01) positive correlation, 0.7653, between surface wetness of damaged stone surfaces and pH of damaged stone surfaces was shown.

Analysis of pH gave a mean of 5.25 for damaged stone surfaces and a mean of 5.94 for undamaged stone which was a significant difference between the two (p-value = 0.001). Variation in temperature, relative humidity and light, both lux and UV, was related to the times the sample was taken.

All sampling sites tested proved positive with sellotape sampling and staining for biofilm (Figure 7) with the exception of site 4 at Lincoln Cathedral which was below the main east window and showed heavy copper staining. Presence of algae was also identified in the sellotape sampling at all sites.

						Protimeter (WME)		Surface pH	
Location	Site	Relative Humidity (%)	Temperature (°C)	Light (lx)	UV (μW/lumen)	Damaged	Undamaged	Damaged	Undamaged
Lincoln Cathedral	1	83.9	11.9	2600	1000	18	12	5.5	6
	2	83.4	12.2	500	800	18	15	5.5	6
	TB	82.1	12.1	1000	1000	-	14	-	6.5
	3A	80.4	13.2	3000	980	25	-	5.5	-
	3L	80.4	13.2	3000	980	19	-	5.5	-
	4	76	14	13800	914	-	55		5
Saint Peter-at-Gowts, Lincoln	1	80	8.5	4700	800	18	18	4.5	6
	2	77	8.9	6740	980	26	30	5	6
	3	76.6	8.5	7800	1000	-	25	-	5.5
	4	73.2	9.4	2721	927	26	19	5.5	6
Burton Pedwardine	1	72.8	17.1	9324	1273	24	12	5	6
Saint Botolphs-by-Bargate, Lincoln	1	50.6	26.4	58834	4	16	10	5.5	6

Table 6: Environmental and surface measurements taken at each site. Environmental measurements were relative humidity, temperature, light and UV. Surface measurements were protimeter, a qualitative measurement, and surface pH. No correlation was found between the environmental measurements and surface measurements indicating that the differences between the damaged and undamaged surfaces were not being influenced by the environment.

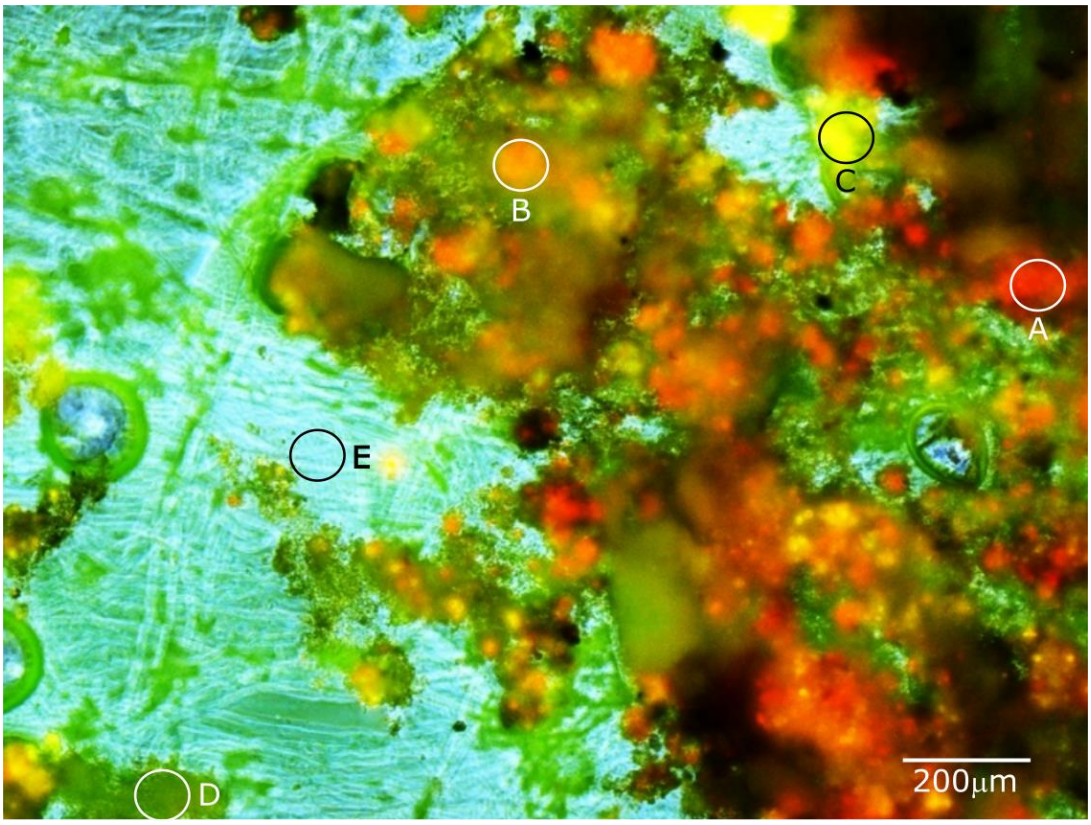


Figure 7: Sellotape sample from undamaged stone at site 2 of Lincoln Cathedral. Intact biofilm binds the FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain which can be seen the red areas in the image (A). Bacteria bind the FilmTracer™ FM® 1-43 Green Biofilm Cell Stain, when high concentrations are present within the biofilm this results in a yellow to orange fluorescence (B) in combination with the Ruby Biofilm Matrix stain. Bright yellow-green areas (C) are high concentrations of bacterial cells which are either unencapsulated in biofilm matrix or minimally encapsulated. The dull green clusters (D) are algal species, for example the green area at the bottom left of the image. The blue background (E) is due to the autofluoresence of the sellotape used to mount the biofilm.

3.3 Physical characterisation of bioreceptivity

Surface roughness profiling was carried out (Courtesy of Alice Gillet of the University of Chester) on 4 samples of ashlar surfaced Lincoln limestone, provided freshly cut from the Lincoln Cathedral Quarry. Ashlar is the most common surface finish for limestone buildings (Pevsner *et al.*, 2002) and showed a low surface roughness when compared to other limestones (Miller *et al.*, 2009). The measured Rz value, 1.63±0.44 µm, was approximately 5-6 times higher than Ra, 8.43±1.45 µm (Table 7).

	Amplitude parameters		Spacing parameters	
	Ra (μm)	Rz (μm)	Rsm (mm)	Rdq (degrees)
Sample 1	1.79	8.68	0.27	3.65
Sample 2	1.97	9.91	0.275	3.38
Sample 3	0.989	6.44	0.194	2.36
Sample 4	1.79	8.69	0.275	3.36
Average	1.63	8.43	0.25	3.19
Standard Deviation	0.44	1.45	0.04	0.57

Table 7: Summary of Surface Roughness profiling results with averages and standard deviations of the 4 samples. Ra is the average of all absolute distances between peaks and valleys across the surface. Rz is the average maximum peak to valley of five consecutive sampling lengths. Rsm is the mean width between peaks and Rdq the slope in degrees of the peaks. From these measurements we can see that the surface consists of steep peaks and valleys, 3.19° slopes between the peak and the valley, which are well spaced out. The surface roughness profile is within the range considered suitable for bacterial adhesion and colonisation.

Determination of water absorption coefficient by capillarity perpendicular to bedding, was found to be available for Lincoln limestone from quarry data sheets (Ashall & Webb, 2013b, 2013a) giving the capillary coefficient as 171.48±19.9 $g.m^{-2}.s^{-0.5}$ for Lincoln stone. This measurement was based on an average of 6 tests with the error being calculated from the data sheet by the author.

Analysis of the chemical composition of Lincoln limestone was carried out using SEM-EDX. Table 8 shows the results from the spectral readings with the proportions of atoms in the spectra being expressed as a percentage.

The most common combination was carbon, oxygen and calcium, followed by carbon, oxygen, silicon and calcium as would be expected from a limestone material. 126 spectra were read from 7 samples giving an average overall composition of 26.1% carbon, 27.7% calcium, 28.3% oxygen, 9.3% silicon, 2% phosphorous, 1.8% sulphur, 1.4% aluminium, 1.1% sodium, 0.9% magnesium, 0.7% potassium, 0.2% bromine, 0.2% iron and 0.2% tantalum (Figure 8).

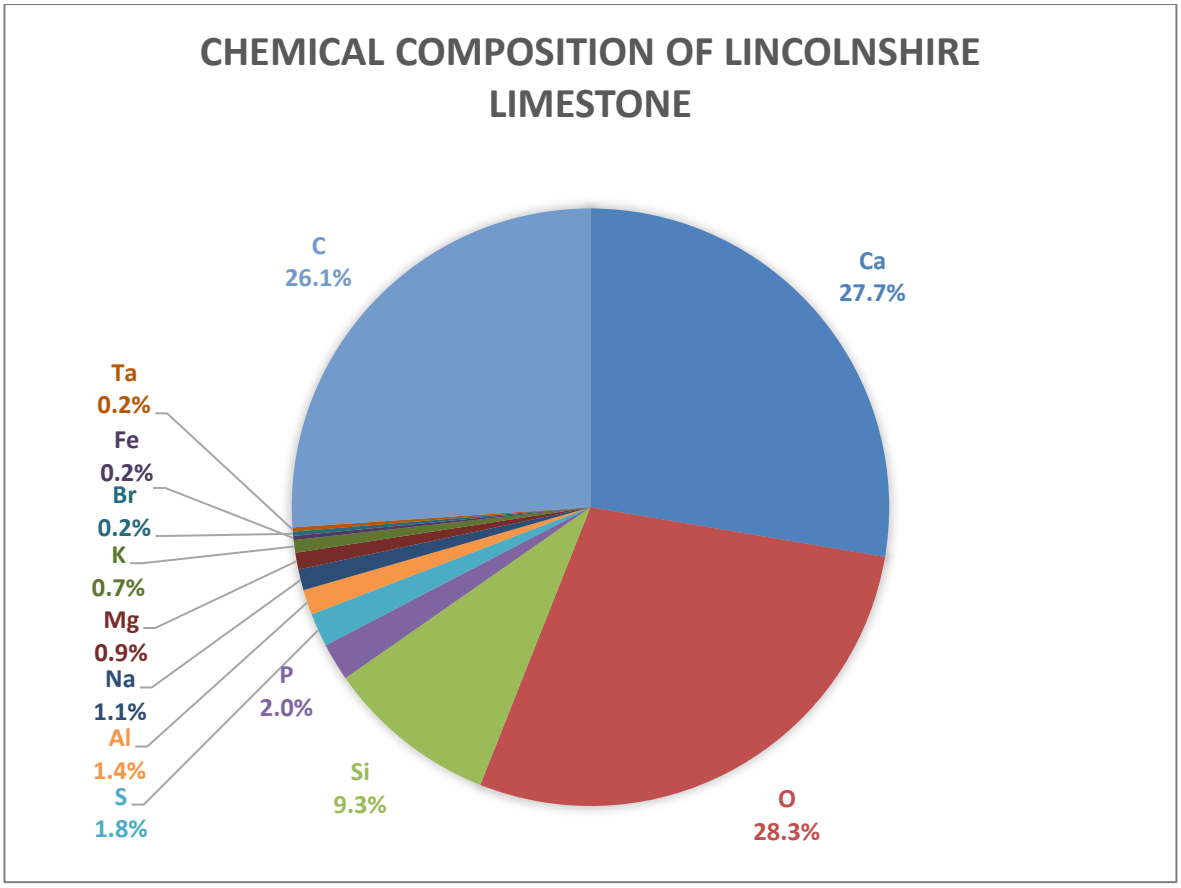


Figure 8: SEM-EDX analysis of Lincoln limestone showing the percentage composition of the detected atomic components. The main composition, as expected, is calcium carbonate and the majority of trace elements are consistent with the biological origins of the limestone deposits.

Spectra showing this profile	C	O	Na	Mg	Al	Si	P	S	K	Ca	Fe	Br	Ta
67	52.74	26.67								20.58			
30	56.21	30.24				0.76				12.8			
6	50.06	34.71					1.92			13.32			
3	53.05	29.81						0.89		16.25			
2	56.43	27.12	1.01		6.06	2.82				11.96			
2		58.75	6.46	2.39	3.37	24.19				4.85			
2		58.04						5.29		36.67			
2	82.05	17.95											
2		39.21								60.79			
1	47.79	35.53		0.76			3.31	0.79		11.8			
1	54.39	29.15				0.69	1.08	1.29		13.4			
1	54.82	26.45					2.41	0.67		15.65			
1	55	25.34								16.8			2.86
1	55.65	23.99				0.83				8.31	11.22		
1		53.81			11.39	26.54			4.64	3.62			
1		56.82	5.73	1.98		24.21				4.85		6.1	
1		56.69			8.3	24.02			10.99				
1						13.79			86.21				
1		38.95				45.22				15.83			

Table 8 : EDX analysis of Lincoln limestone showing the elements identified, percentage atoms per grouping, and the number of times that they occurred in the 126 spectra measured (first column). Combining the data from these measurements allowed the chemical profile of Lincoln limestone to be

3.4 Identification of cultured isolates

An initial 193 copiotrophic bacterial colonies were isolated from Lincoln Cathedral, Saint Peter-at-Gowts, St. Michael and All Saints Church, Oxwich Castle and Weobley Castle.

Isolates were characterised for morphology, gram stain, oxidase activity, the presence of cytochrome c oxidase, and catalase activity as well as the ability to utilise glucose, sucrose, lactose, xylose and maltose and the ability to hydrolyse gelatin (Table 9). Finally identification of all isolates to the species level by 16S rRNA sequencing was carried out.

When compared to the species identified as belonging to the limestone microbiome in the literature the level of gram negative species was much higher. This is potentially due to the higher water retention of Lincoln limestone compared to the characterised limestones as well as the climatic differences, producing an environment more amenable to gram negative growth.

Sixty four distinct species and 6 strain variants of *B. licheniformis* (2), *B. muralis* (3), *B. pumilis* (2), *B. safensis* (2) and *B. subtilis* (2) were identified and profiled for physical and biochemical properties (Table 9). Of these 19 were shown to have multiple 16S rRNA genes. Seventeen of the 19 isolates were shown to have copies of the 16S rRNA gene which while heterogeneous were still within the 97% similarity margin for species identification, therefore all of the genes sequenced provided the same identity for the isolate.

The remaining two isolates *Enterococcus hirae* and *Bacillus muralis* strain WE2D1c had heterogeneous 16S rRNA genes which matched different species. *E. hirae* had two copies and *B. muralis* had 5 copies, 3 of which identified the species as *B. muralis.*

The additional copy in *E. hirae* matched *Enterococcus faecium* with 100% identity. Characterisation was carried out on blood agar to look for β haemolytic activity, as none was shown this confirmed the identification as *E. hirae.*

The additional copies in *B. muralis* identified as *Bacillus simplex* (100% identity) and *Citrobacter freundii* (99% identity). *C. freundii* is gram negative therefore a gram stain was sufficient to eliminate it as a possibility. *B. muralis* is oxidase positive and *B. simplex* oxidase negative so an oxidase test was able to confirm that the isolate was in fact *B. muralis*.

Sample code	Species	Gram stain	Morphology	Spore former	Oxidase	Catalase	Glucose	Sucrose	Lactose	Xylose	Maltose	Gelatin
PAG2U1	*Acinetobacter baylyi*	-	rod	-	-	+	+	-	-	-	-	-
PAG4D10b	*Acinetobacter calcoaceticus*	-	rod	-	-	+	+	-	-	-	-	-
MO34	*Acinetobacter johnsonii**	-	rod	-	-	+	-	-	-	-	-	-
MO2D2a	*Acinetobacter lwoffii**	-	rod	-	-	+	variable	-	-	-	-	-
LCA2U4a	*Advenella kashmirensis*	-	coccus	-	+	+	+	-	-	-	-	-
LCA1U3	*Bacillus subtilis*	+	rod	+	+	+	-	-	-	-	-	-
LCA3L3	*Arthrobacter agilis*	+	coccus	-	+	-	-	-	-	-	-	-
WE1D5a	*Arthrobacter protophormiae**	+	coccus	-	+	-	-	-	-	-	-	-
LCA4U3	*Arthrobacter phenanthrenivorans*	+	coccus	-	+	-	+	-	-	-	-	-
PAG4D11a	*Bacillus aerophilus*	+	rod	+	+	+	+	-	-	-	-	-
LCA4U4	*Bacillus cecembensis*	+	rod	+	+	+	-	-	-	-	-	-
LCA3A1	*Bacillus cereus*	+	coccus	+	+	+	-	-	-	-	-	+
PAG3U1	*Bacillus mycoides*	+	rod	+	-	+	-	-	-	-	-	-
WE3D1a	*Bacillus foraminis**	+	rod	+	+	+	+	+	+	+	+	-
PAG2D1	*Bacillus infantis*	+	rod	+	-	+	-	-	-	-	-	-
PAG2D4	*Bacillus licheniformis*	+	rod	+	-	+	-	-	-	-	-	-
LCA1D6a	*Bacillus muralis*	+	rod	+	+	+	-	-	-	-	-	-
OX2D8	*Bacillus muralis**	+	rod	+	+	+	-	-	-	+	-	-
WE2D1c	*Bacillus muralis**	+	rod	+	+	+	-	-	-	+	-	-
MO1D1a	*Bacillus niacini**	+	rod	+	variable	+	+	+	variable	+	+	n/a
MO36c	*Bacillus psychrosaccharolyticus**	+	rod	+	n/a	+	n/a	n/a	n/a	n/a	n/a	n/a
OX1D5a	*Bacillus pumilis**	+	rod	+	-	+	n/a	n/a	n/a	n/a	n/a	n/a
OX1D5b	*Bacillus pumilis**	+	rod	+	-	+	n/a	n/a	n/a	n/a	n/a	n/a
PAG4D2	*Bacillus pumilis*	+	rod	+	+	+	-	-	-	-	-	-
LCATB3	*Bacillus safensis*	+	rod	+	+	+	-	-	-	-	-	+
OX3U10	*Bacillus safensis**	+	rod	+	+	+	-	-	-	-	-	+
OX3U5	*Bacillus simplex**	+	rod	+	-	+	+	+	-	-	-	-

Sample code	Species	Gram stain	Morphology	Spore former	Oxidase	Catalase	Glucose	Sucrose	Lactose	Xylose	Maltose	Gelatin
WE1U1a	*Bacillus* sp. *BC11**	+	rod	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
OX1U3	*Bacillus* sp. *PVS08**	+	rod	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
LCA3A3a	*Bacillus sporothermodurans*	+	rod	+	+	+	-	-	-	-	-	-
LCA1D9	*Bacillus subtilis*	+	rod	+	+	+	-	-	-	-	-	-
OX2U1	*Bacillus thuringiensis**	+	rod	+	-	+	+	variable	-	-	+	-
PAG2D2	*Brevibacillus brevis*	+	rod	+	+	-	-	-	-	-	-	-
WE2U6	*Microbacterium ginsengisoli**	+	rod	-	+	-		+	-	-	+	-
LCA3L7	*Curtobacterium flaccumfaciens*	+	rod	-	+	+	-	-	-	-	-	-
WE1D2b	*Enterococcus hirae**	+	coccus	-	-	-	+	+	+	+	-	-
WE1U3b	*Escherichia coli**	-	rod	-	-	+	-	-	-	-	-	-
WE1U1c	*Exiguobacterium sibiricum**	+	rod	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
LCA1D8b	*Isoptericola variabilis*	+	rod		-	+	-	-	-	-	-	-
WE1D4	*Lysinibacillus fusiformis**	+	rod	+	+	+	-	-	-	-	-	-
LCA3A6	*Lysinibacillus parviboronicapiens*	+	rod	+	+	+	-	-	-	-	-	-
PAG4D12a	*Microbacteriaceae* sp. *PAG4D*	+	rod	n/a	+	+	+	-	-	-	-	+
WE1D2c1	*Microbacterium pseudoresistens**	+	rod	-	-	+	n/a	n/a	n/a	n/a	n/a	n/a
LCA3L5	*Microbacterium schleiferi*	+	rod	-	+	+	-	-	-	-	-	-
PAG4D11c	*Microbacterium thalassium*	+	rod	-	-	+	-	+	-	-	-	-
LCA1D3	*Micrococcus halobius*	+	coccus	-	+	-	-	-	-	-	-	-
PAG4D12c	*Micrococcus luteus*	+	coccus	-	-	+	-	-	-	-	-	-
PAG2U2	*Micrococcus roseus*	+	coccus	-	+	+	+	+	-	-	-	-
WE2D3a1b	*Paenibacillus lactis**	-	rod	+	+	+	+	+	+	-	+	-
MO1D2b	*Paenibacillus lautus**	+	rod	+	+	-	+	+	+	+	+	-
LCA3L1	*Paenibacillus pabuli*	-	rod	+	+	+	-	-	-	-	-	-
LCA4U6	*Paenibacillus polymyxa*	+	coccus	+	+	-	-	-	-	-	-	-
LCA4U5	*Paenibacillus* sp.*1105*	+	coccus + filaments	+	+	-	-	-	-	-	-	-
PAG4D1	*Pseudomonas brassicacearum*	-	rod	-	+	+	-	-	-	-	-	+

Sample code	Species	Gram stain	Morphology	Spore former	Oxidase	Catalase	Glucose	Sucrose	Lactose	Xylose	Maltose	Gelatin
PAG3U3b	*Pseudomonas brenneri*	-	rod	-	+	+	-	-	-	-	-	+
PAG3U4b	*Pseudomonas fluorescens*	-	rod	-	+	+	+	-	-	-	-	+
PAG4D11b	*Pseudomonas putida*	-	rod	-	+	+	+	-	-	-	-	+
PAG4D7a	*Pseudomonas* sp. *HZ06*	-	rod	-	+	+	+	-	-	-	-	-
PAG2U4	*Pseudomonas stutzeri*	-	rod	-	+	+	+	-	-	-	-	-
MO32a	*Psychrobacillus psychrodurans**	+	rod	+	+	+	-	-	-	-	-	-
PAG3U5b	*Psychrobacter faecalis*	-	coccus	-	+	+	-	-	-	-	-	-
LCATB4	*Solibacillus silvestris*	+	rod	+	-	+	-	-	-	-	-	-
WE3U2b	*Sphingobacterium anhuiense**	-	rod	-	+	+	+	+	+	-	+	-
WE3U2b1b	*Sphingobacterium faecium**	-	rod	-	+	+	+	+	-	-	+	-
LCATB2	*Spongiibacter* sp. *IMCC21906*	-	rod	-	+	+	-	-	-	-	-	+
PAG3U3a	*Sporosarcina saromensis*	+	rod	+	+	+	-	-	-	-	-	-
PAG4U6	*Staphylococcus xylosus*	+	coccus	-	-	+	+	-	-	+	-	-
LCA2U3	*Stentrophomonas maltophilia*	-	rod	-	+	+	+	+	+	-	+	+
LCA2U5c	*Stentrophomonas rhizophila*	-	rod	-	+	+	+	-	-	+	+	
LCA3A5	*Streptomyces microflavus*	+	coccus + hyphae	+	+	+	-	+	-	-	-	-

*Table 9: Morphological and biochemical test results for bacterial isolates from all sites. * = data included from Bergey's manual of determinative bacteriology for completeness. Where data was not available n/a has been used. Isolate codes are three letters for the location, number for the site sampled, D or U for damaged or undamaged surface and an alphanumeric identifier for the isolate. LCA is Lincoln Cathedral, PAG is Saint Peter-at-Gowts, BOL is Saint Botolphs-by–Bargate, BUP is St. Nicholas, St. Andrew and the Blessed Virgin Mary's Church, Burton Pedwardine, OX is Oxwich Castle, WE is Weobley Castle and MO is St. Michael and All Angels Church, Moccas.*

Sampling codes, column one Table 9, identified species according to the sites that they were isolated from and allowed tracking of whether they were isolated from damaged stone, undamaged stone or both. Specimens were considered identified to species level if there was a match of > 97%, with the majority of species identified showing a match of > 99%. Where more than one species was identified the sampling code was assigned as a strain name.

The data was tested to identify any significant correlations between the physical and biochemical characteristics of the species isolated and the surface, damaged or undamaged, that they were isolated from. When comparing cell wall structure, gram positive or negative, to isolation surface, a highly significant, p-value = $8x10^{-4}$, positive correlation, 0.454, was demonstrated between gram positive bacteria and damaged surfaces.

Sporulation was also significantly, p-value = 0.004, positively correlated, 0.342, with damaged surfaces, which ties in the correlation between gram positive species and damaged surfaces. Morphology, rod vs cocci, showed a significant, p-value = 0.04, negative correlation, -0.243, with undamaged surfaces showing that there was a significant correlation between undamaged surfaces and bacteria with a coccus morphology.

No other significant correlations were found between physical and biochemical characteristics and the isolation surface.

In one case identification to the species level was not possible. *Microbacteriaceae* sp. *PAG4D* was confirmed to family level with the closest matches being 93%, as 97% is considered the cut off for species level identification (Větrovský & Baldrian, 2013) this means that the isolate is most likely a new species, if not genus.

Phylogenies were calculated based on the closest relatives with *Microbacteriaceae* sp. *PAG4D* sitting between *Mycetocola* species and *Clavibacter* species with a phylogenetic distance based on substitutions per base of 0.044 and 0.046 respectively (Figure 9).

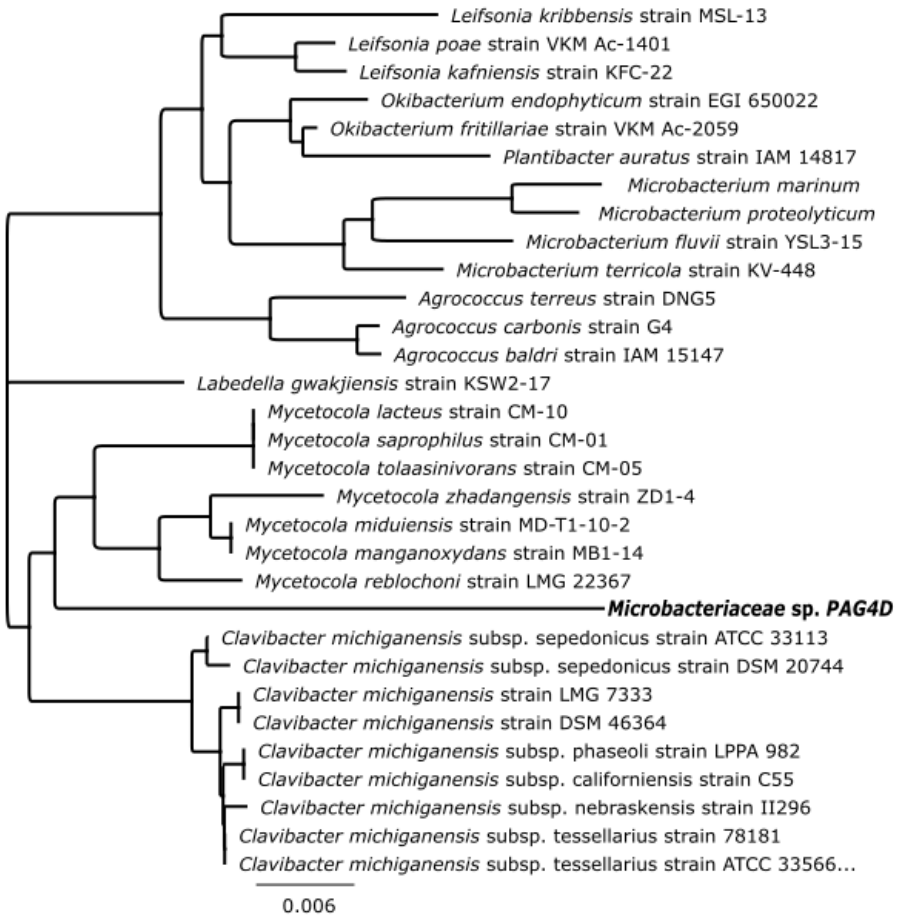


Figure 9: Microbacteriaceae sp. PAG4D with the closest relatives based on substitutions per site. Distance between PAG4D and closest relative Mycetocola miduiensis is 0.044, distance to closest Clavibacter michiganensis subsp. Sepedonicus strain ATCC 33113 is 0.046. Distance between the Mycetocola and Clavibacter species is 0.023.

With direct culturing an average of 3.4 species were isolated from undamaged surfaces, and 5 species from damaged surfaces.

Applying Shannon's index of diversity to the sampling data provides a measure of how successful the sampling has been in representing the population sampled. Where Shannon's equitability, the evenness of the distribution of species across the sample, is higher than 0.8 this indicates that the isolates obtained from the surface were evenly distributed across the population, in other words the sampling showed no bias. All sites sampled (Table 10) showed, where data was available, no major bias in sampling. Shannon's index of diversity for each of the individual samples showed a poor to moderate ecosystem (Table 10), something which would be expected due to the selective culturing techniques used.

When applying Shannon's index of diversity to the combination of the two populations, the species abundance (H_s) for the population from damaged surfaces was 3.38 with an evenness of

distribution (E) of 0.88, the H_s for the population from the undamaged surfaces was 3.51 with an E of 0.98, measurements for both populations indicate a good level of diversity and are therefore considered to represent healthy ecosystems (Jørgensen *et al.*, 2010).

Conversion from Shannon's index of diversity to an estimate of number of equally common species in the population can be obtained by calculating the exponential of Shannon Entropy (Jost, 2007) and allows a direct comparison of the two populations. Shannon's Entropy resulted in the estimated population of the damaged surfaces consisting of 28 species compared to the 35 of the undamaged population.

Sample site	H_S	E	Status of ecosystem	Sample site	H_S	E	Status of ecosystem
LCA1D	1.96	1.01	Poor	MO1D	0.68	0.98	Bad
LCA1U	n/a			MO1U	0.68	0.98	Bad
LCA2D	1.8	1	Poor	MO2D	1.4	1.01	Poor
LCA2U	1.96	1.01	Poor	MO5D	0.68	0.98	Bad
LCA3AD	2.16	1.04	Moderate	OX1D	1.11	1.01	Poor
LCA3LD	1.6	0.99	Poor	OX1U	n/a		
LCATBU	1.6	0.99	Poor	OX2D	0.68	0.98	Bad
LCA4U	1.8	1	Poor	OX2U	0.68	0.98	Bad
PAG1D	0.68	0.98	Bad	OX3D	n/a		
PAG1U	1.11	1.01	Poor	OX3U	0.68	0.98	Bad
PAG2D	1.4	1.01	Poor	WE1D	1.6	0.99	Poor
PAG2U	1.8	1	Poor	WE1U	1.4	1.01	Poor
PAG3U	1.8	1	Poor	WE2D	1.4	1.01	Poor
PAG4D	2.06	0.98	Moderate	WE2U	n/a		
PAG4U	1.11	1.01	Poor	WE3D	1.11	1.01	Poor
				WE3U	1.11	1.01	Poor
				Combined Damaged	3.38	0.88	Good
				Combined Undamaged	3.51	0.98	Good

Table 10 : Shannon's diversity index (Hs) and Shannon's equitability (E) measured for each sampling site based on isolated copiotrophs as well as the sites combined as damaged and undamaged. Status of ecosystem gives an interpretation of the diversity index results, the lower the diversity index the poorer the ecosystem. Shannon's equitability measure shows the evenness of sampling with 0.8 or higher demonstrating an even selection of species across the population. Where results are not available this is due to species diversity being too low to calculate the index. Sampling codes are three letters for the location, number for the site and D or U for damaged or undamaged surface. LCA is Lincoln Cathedral, PAG is Saint Peter-at-Gowts, BOL is Saint Botolphs-by–Bargate, BUP is St. Nicholas, St. Andrew and the Blessed Virgin Mary's Church, Burton Pedwardine, OX is Oxwich Castle, WE is Weobley Castle and MO is St. Michael and All Angels Church, Moccas.

3.5 Identification of species from metagenomic data

Metagenomic sampling resulted in the identification of 1048 species which could be assessed based on the number of sample sites they were isolated from, the surface they were sampled from and whether the sample was rural or urban (Appendix C: Species identified in metagenomic analysis); 59 of these were found in > 75% of the sites sampled forming a core microbiome; these included *A. lwoffii, M. luteus, A. agilis* and *P. fluorescens*, all of which were isolated from the stone surfaces during sampling. This made up 6% of the total population. The majority of species identified, 73% of the population, were present in < 25% of the sites sampled indicating a strong geographical component to the microbiome.

Shannon's index of diversity, species abundance, Shannon's equity, evenness of species distribution, and Shannon's exponential, estimated total population, were calculated for each sample and showed that a healthy, undisturbed, population had been sampled (Table 11).

As expected Shannon's exponential came out higher than the total number of species in each sample (Table 11). The undamaged samples from Saint Botolphs-by–Bargate (BOL-1U) and Burton Pedwardine (BUP-1U) had exceptionally high percentages for identification of the total estimated population at 95.33% and 97.8% respectively, the rest of the samples ranged from 10.95% identity, Burton Pedwardine damaged (BUP-1D), up to 44.48% identity, Lincoln Cathedral (LCA-2U), which closer to the range expected as the sequence databases, and the microbial census (Schloss *et al.*, 2016), are currently incomplete.

When comparing the directly cultured isolates to the species identified in the metagenomic survey, 33 species were identified in both studies. Seventeen remained unchanged from the direct sampling to the metagenomic sampling. Eight of the species which had only been found at damaged sites in direct sampling were found at both sites in metagenomic sampling. These were *Acinetobacter calcoaceticus, Acinetobacter lwoffii, Arthrobacter agilis, Bacillus pumilis, Curtobacterium flaccumfaciens, Escherichia coli, Exiguobacterium sibiricum* and *Pseudomonas putida.*

Seven of the species which were only found at undamaged sites in direct sampling were found at both in metagenomic sampling. These were *Acinetobacter baylyi, Paenibacillus* sp.*1105, Pseudomonas fluorescens, Pseudomonas stutzeri, Sphingobacterium faecium, Stentrophomonas maltophilia* and *Stentrophomonas rhizophila.* This results in the final analysis for surface isolation of the cultured species (Table 12).

	Total contigs	Total isolates	Isolates at >97%	Shannon's Diversity Index	Shannon's Equitability	Status of Ecosystem	Shannon's Exponential	% identified
LCA2D	6864	700	376	7.21	1	Good	1352.63	27.80
LCA2U	7044	608	302	6.52	1	Good	678.92	44.48
PAG1D	2661	565	287	7.23	1	Good	1374.65	20.88
PAG1U	6110	694	375	7.21	1	Good	1347.27	27.83
BOL1D	11441	2154	803	7.99	1	Good	2947.90	27.24
BOL1U	5111	503	178	5.23	1	Good	186.71	95.33
BUP1D	3961	634	313	7.96	1	Good	2859.29	10.95
BUP1U	6672	740	299	5.72	1	Good	305.74	97.80

Table 11: Metagenomics counts and analysis of Shannon's index of diversity, equitability and exponential results together with the percentage of species identified out of the total population estimated by Shannon's exponential. As all the Shannon's diversity index results are above 3 they represent a good ecosystem status. Shannon's Equitability of 0.8 or above demonstrates even sampling across the species in the total population. Shannon's Exponential gives an estimate of the total population size allowing the number of species identified to be estimated based on this and the number of isolates characterised at >97% identity. Sampling codes are three letters for the location, number for the site and D or U for damaged or undamaged surface. LCA is Lincoln Cathedral, PAG is Saint Peter-at-Gowts, BOL is Saint Botolphs-by–Bargate and BUP is St. Nicholas, St. Andrew and the Blessed Virgin Mary's Church, Burton Pedwardine.

Damaged	Both	Undamaged
Acinetobacter johnsonii	*Acinetobacter baylyi*	*Advenella kashmirensis*
Arthrobacter protophormiae	*Acinetobacter calcoaceticus*	*Arthrobacter phenanthrenivorans*
Bacillus aerophilus	*Acinetobacter lwoffii*	*Bacillus cecembensis*
Bacillus foraminis	*Arthrobacter agilis*	*Bacillus* sp. *BC11*
Bacillus infantis	*Bacillus cereus*	*Bacillus* sp. *PVS08*
Bacillus licheniformis strain *LCA3A7*	*Bacillus mycoides*	*Bacillus thuringiensis*
Bacillus licheniformis strain *PAG2D*	*Bacillus pumilis* strain *OX1D5a*	*Microbacterium ginsengisoli*
Bacillus muralis strain *LCA1D6a*	*Bacillus pumilis* strain *PAG4D2*	*Paenibacillus polymyxa*
Bacillus muralis strain *OX2D8*	*Bacillus safensis* strain *LCATB3*	*Pseudomonas brenneri*
Bacillus muralis strain *WE2D1c*	*Bacillus safensis* strain *OX3U10*	*Psychrobacter faecalis*
Bacillus niacin	*Bacillus simplex*	*Solibacillus silvestris*
Bacillus psychrosaccharolyticus	*Bacillus subtilis* strain *LCA1D9*	*Sphingobacterium anhuiense*
Bacillus sporothermodurans	*Bacillus subtilis* strain *LCA1U3*	*Spongiibacter* sp. *IMCC21906*
Brevibacillus brevis	*Curtobacterium flaccumfaciens*	
Isoptericola variabilis	*Enterococcus hirae*	
Lysinibacillus fusiformis	*Escherichia coli*	
Lysinibacillus parviboronicapiens	*Exiguobacterium sibiricum*	
Microbacteriaceae sp. *PAG4D*	*Micrococcus halobius*	
Microbacterium pseudoresistens	*Micrococcus luteus*	
Microbacterium schleiferi	*Micrococcus roseus*	
Microbacterium thalassium	*Paenibacillus* sp.*1105*	
Paenibacillus lactis	*Pseudomonas fluorescens*	
Paenibacillus lautus	*Pseudomonas putida*	
Paenibacillus pabuli	*Pseudomonas stutzeri*	
Pseudomonas brassicacearum	*Sphingobacterium faecium*	
Pseudomonas sp. *HZ06*	*Sporosarcina saromensis*	
Psychrobacillus psychrodurans	*Staphylococcus xylosus*	
Streptomyces microflavus	*Stentrophomonas maltophilia*	
	Stentrophomonas rhizophila	

Table 12: Species identified through direct sampling analysed according to the environment they are isolated from adjusted in accordance with the sampling data from the metagenomics analysis. The additional data from the metagenomic sampling demonstrates that the majority of species isolated from undamaged surfaces were not specific to that environment. The damaged surfaces having double the number of specific species isolated when compared to the undamaged surface.

3.6 Analysis of the microbiome at the Operation Taxonomic Unit (OTU) level

3.6.1 Analysis of cultured isolates

To allow direct comparison with studies in the literature the data was analysed at the family level in the taxonomy, in addition to genus or species level which will be discussed in the next section, the breakdown of this analysis is shown in Table 13.

It is immediately apparent from Table 13 that the sampling at Oxwich Castle does not show the same level of diversity when compared to the other sites, all the species being of the Bacillaceae family. Due to the low diversity the sample data from the Oxwich sites could not be normalised for analysis at the family level, therefore to determine whether there was a significant difference between the isolates from damaged and undamaged stone a Student's t-test was carried out and demonstrated a significant difference between the isolates (p-value < 0.01).

The identified families were analysed based on the surface, damaged or undamaged, that they were isolated from. A heat map, Figure 10 , was derived from this data grouping the families by the significance of their presence vs the surface that they were extracted from. Sampling sites produced two main groups in the tree produced from heat map which corresponded with the surface sampled. All damaged sites grouped together, with the exception of Moccas which grouped with the undamaged sites due to the presence of Promicronosporaceae family members and Saint Peter-at-Gowts undamaged sites which grouped with the damaged sites due to the presence of Moraxellaceae and Micrococcaceae family members. The tree grouping the damaged and undamaged sites also demonstrates subdivision into rural and urban locations with the exception of the Lincoln Cathedral undamaged sites.

While the majority of sites sampled grouped according to damaged and undamaged when plotted as a heat map, Figure 10, none of the families were found to be solely present on either damaged or undamaged stone, or to show a significant difference between rural or urban sampling locations.

Discovery odds testing showed a statistically significant (p-value < 0.05) difference between damaged and undamaged stone for members of the Bacillaceae family with a greater probability of them being isolated from damaged stone. Xanthomonadaceae family members were shown to be significantly (p-value < 0.02) more likely to be isolated from undamaged stone. When comparing the rural and urban isolates there was a significant (p-value < 0.01) difference for Bacillaceae and Micrococcaceae families, with a greater probability of isolating them from urban sites. The Promicromonosporaceae family were shown to be significantly (p-value < 0.01) more likely to be isolated from rural sites.

	Sample site									
Family	LCA-D	LCA-U	PAG-D	PAG-U	OX-D	OX-U	WE-D	WE-U	MO-D	MO-U
Alcaligenaceae		1								
Bacillaceae	9	7	10	3	11	5	16	1	11	6
Bacillales incertae sedis								1		
Enterobacteriaceae								1		
Enterococcaceae							4			
Flavobacteriaceae		1								
Microbacteriaceae	2		2	1			4	2		
Micrococcaceae	12	7	15	8			1			
Moraxellaceae			1	2					2	2
Paenibacillaceae	1	2					1		1	
Phyllobacteriaceae										1
Planococcaceae	2			1						
Promicromonosporaceae	1							1	6	4
Pseudomonadaceae			3	3				1		
Sphingobacteriaceae								3		
Spongiibacteraceae		1								
Staphylococcaceae	2			1						
Streptomycetaceae	2		2	1						
Xanthomonadaceae		3						1		
Total isolates	31	22	33	20	11	5	26	11	20	13

Table 13: Isolates obtained at each sampling site by family, sample site codes ending in D are from damaged stone, sample site codes ending in U are from undamaged stone. LCA is Lincoln Cathedral, PAG St. Peter-at-Gowts, OX Oxwich Castle, WE Weobley Castle, and MO All Saints Church, Moccas. Oxwich Castle, unlike the other sampling sites, demonstrates no diversity in the families present on the sampled surfaces. This is potentially due to recent conservation work at the site.

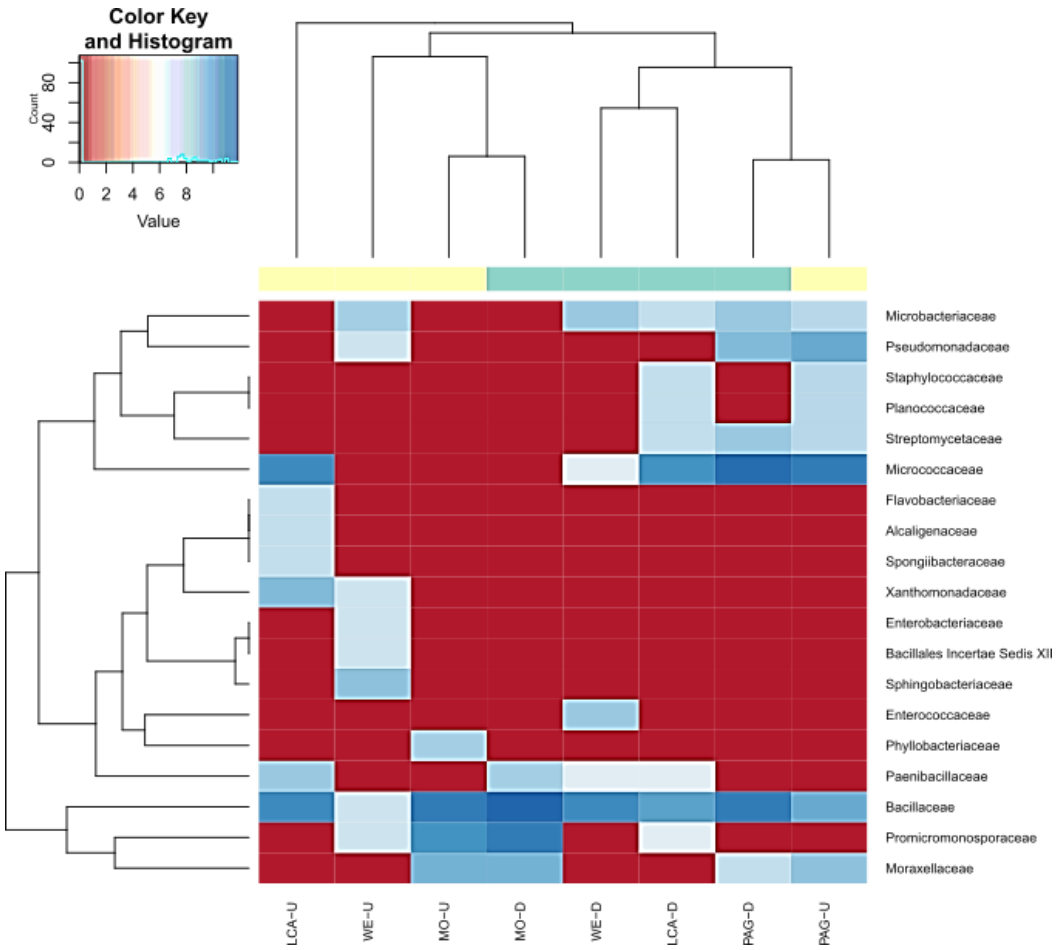


Figure 10: Analysis of the isolates at a family level shows significant (p-value = 0.05) clustering according to whether the surfaces are: damaged –blue; or undamaged; yellow. Presence of the families at each location is indicated using the colour range in the key, for example Micrococcaceae shows a high presence (12 isolates, dark blue) in the St. Peter-at-Gowt damaged sample, a medium presence (6 isolates, white) in the Weobley Castle damaged sample, but is absent in the Weobley Castle undamaged sample (0 isolates, red)

3.6.2 Analysis of metagenomic isolates

With the metagenomic data the characterisation of the total population was carried out at the class level of the taxonomy (Rousk *et al.*, 2010; De Leo *et al.*, 2012; Wu *et al.*, 2015) as this requires a lower percentage identity species level and allows the use of the full data set. Charts were automatically generated for the full data set of each sample filtered to the class level of taxonomy, Figure 11. The data produced was compiled for further analysis in metagenomeSeq.

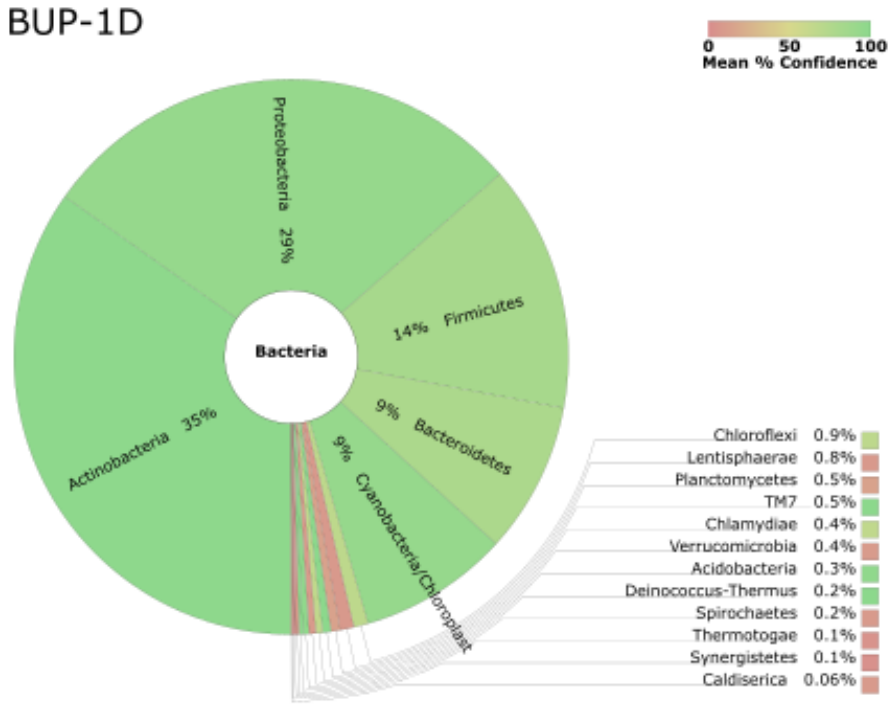
Analysis was carried out based on sampling location and the surface, damaged or undamaged, sampled. A heat map and tree, Figure 12, was produced to determine whether the metagenomics data produced significant clusters at the OTU level. Other than the Lincoln Cathedral samples,

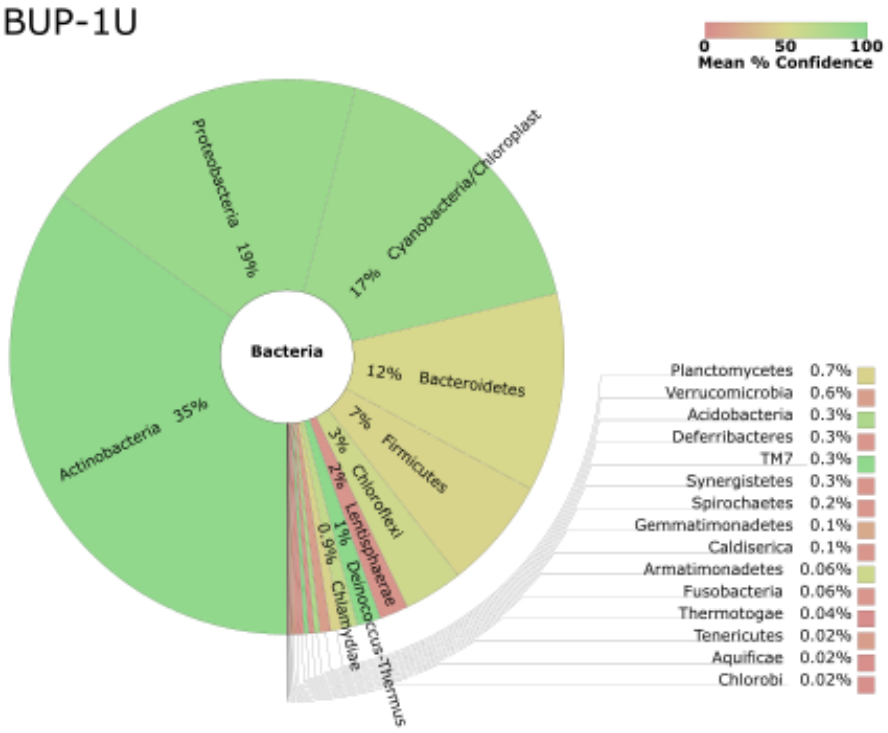
damaged and undamaged samples grouped together in a significant (p-value = 0.05) fashion. No significant clustering was observed for rural and urban sites in the tree, however deeper analysis demonstrated significant differences.

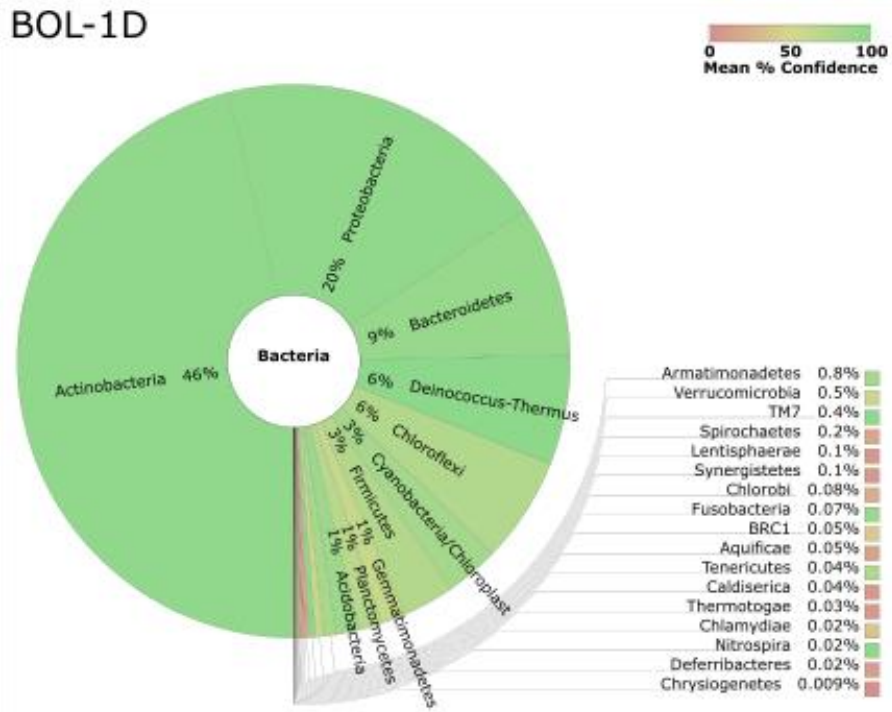
Log normal permutation testing did not identify any significant differences in the presence of the classes between the damaged and undamaged surfaces, therefore all classes identified were present to some extent or other on each surface. When comparing rural and urban, Deinococcus-Thermus was shown to be highly associated with urban locations with the coefficient of interest p-value < 0.05. While sampling numbers of Nitrospira and Elusimicrobia were too low to be classed as significantly associated with the urban environment, both classes of bacteria were only isolated from it within this data set. In the classes associated most strongly with rural locations, TM7 and Chlamydiae were shown to be highly associated with the coefficient of interest, p-value < 0.05. No classes of bacteria were shown to be solely isolated from the rural environment in this data set.

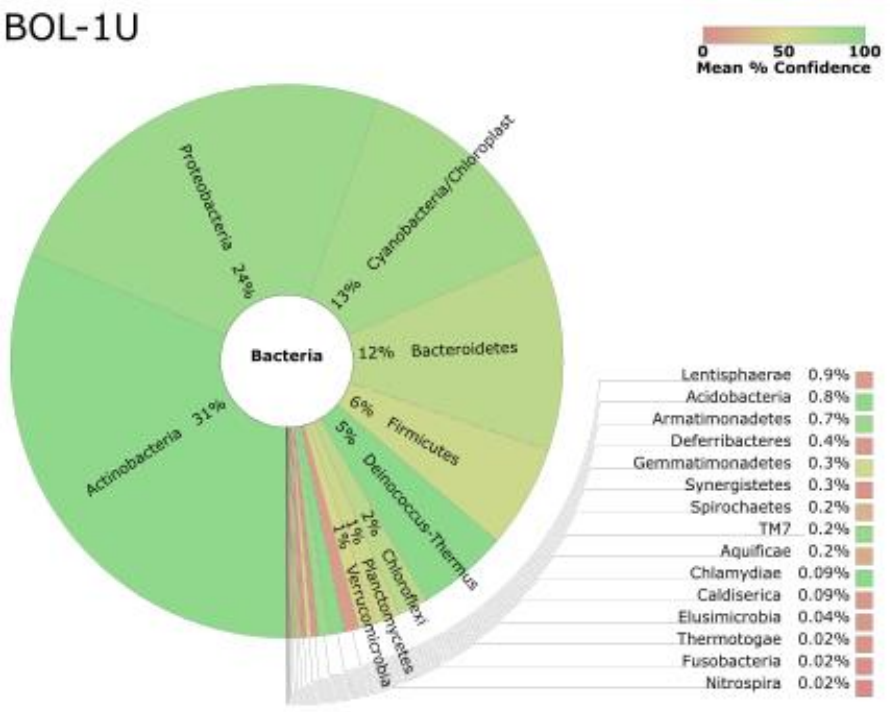
To determine whether there were significant differences in the levels at which the classes were present on the two surfaces, discovery odds were also calculated for each class of bacteria based on surface, damaged or undamaged. Bacterial classes that were significantly (p-value < 0.05) more likely to be discovered on damaged surfaces were Actinobacteria, Proteobacteria, Firmicutes, Acidobacteria, TM7 and Thermotogae. Bacterial classes that were significantly (p-value < 0.05) more likely to be isolated from undamaged surfaces were Bacteroidetes, Cyanobacteria, Planctomyces, Armatimonadetes, Verrucomicrobia, Lentisphaerae, Sunergistetes, Caldiserica, Chlamydiae and Elusimicrobia. Of the classes associated most strongly with undamaged stone Cyanobacteria and Lentisphaerae were shown to be highly associated with the coefficient of interest, undamaged stone, with p-value < 0.05. Bacterial class Elusimicrobia was found to be solely isolated from undamaged stone surfaces.

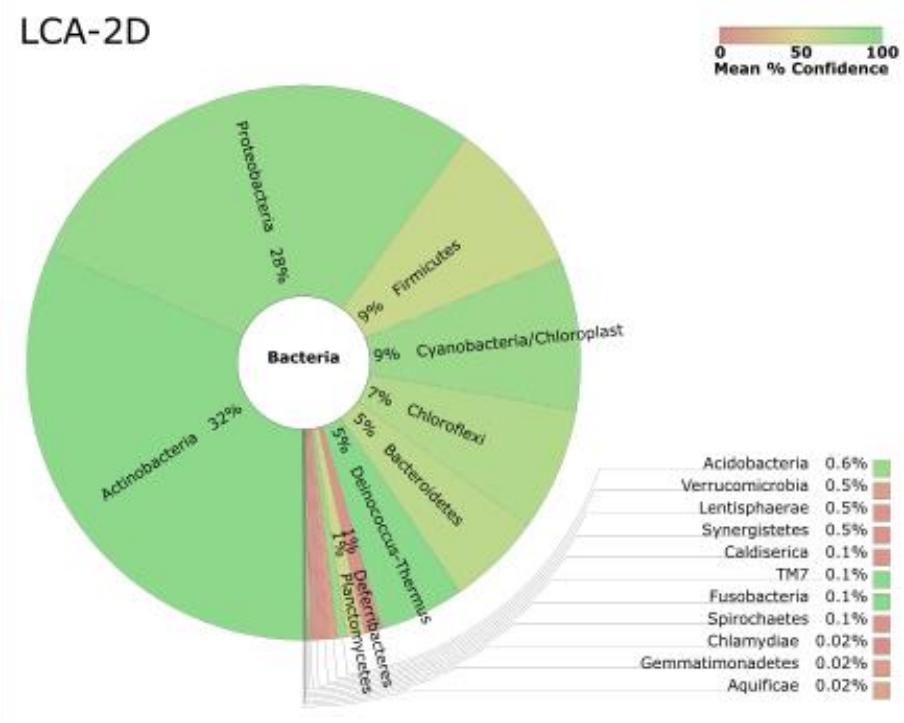
Discovery odds were also calculated for each class of bacteria identified based on sampling location, rural or urban. Bacterial classes which were significantly (p-value < 0.05) associated with urban sample locations were Actinobacteria, Deinococcus-Thermus, Chloroflexi, Gemmatimonadetes, Planctomyces, Acidobacteria, Armatimonadetes, Verrucomicrobia, Aquificae and Deferribacteres. Bacteroidetes, Cyanobacteria, Firmicutes, TM7, Lentisphaerae, Thermotogae and Chlamydiae were the bacterial classes which were significantly (p-value < 0.05) associated with rural sample locations.

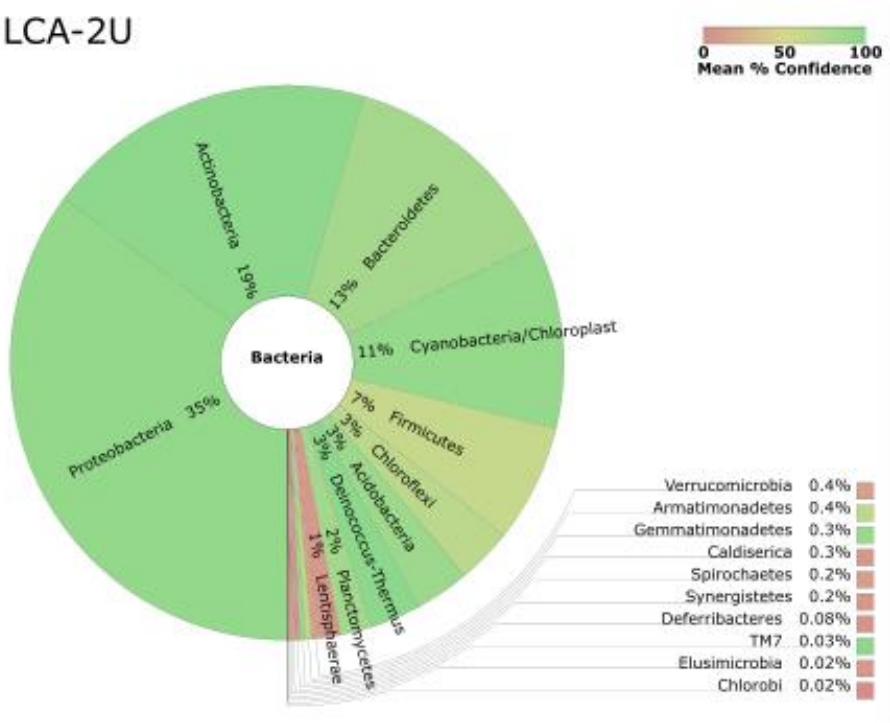

BUP-1D
0 50 100
Mean % Confidence
Bacteria
Proteobacteria 29%
14% Firmicutes
9% Bacteroidetes
9% Cyanobacteria/Chloroplast
Actinobacteria 35%
Chloroflexi 0.9%
Lentisphaerae 0.8%
Planctomycetes 0.5%
TM7 0.5%
Chlamydiae 0.4%
Verrucomicrobia 0.4%
Acidobacteria 0.3%
Deinococcus-Thermus 0.2%
Spirochaetes 0.2%
Thermotogae 0.1%
Synergistetes 0.1%
Caldiserica 0.06%


BUP-1U
0 50 100
Mean % Confidence
Bacteria
Proteobacteria 19%
17% Cyanobacteria/Chloroplast
12% Bacteroidetes
7% Firmicutes
3% Chloroflexi
2% Lentisphaerae
1% Deinococcus-Thermus
0.9% Chlamydiae
Actinobacteria 35%
Planctomycetes 0.7%
Verrucomicrobia 0.6%
Acidobacteria 0.3%
Deferribacteres 0.3%
TM7 0.3%
Synergistetes 0.3%
Spirochaetes 0.2%
Gemmatimonadetes 0.1%
Caldiserica 0.1%
Armatimonadetes 0.06%
Fusobacteria 0.06%
Thermotogae 0.04%
Tenericutes 0.02%
Aquificae 0.02%
Chlorobi 0.02%


BOL-1D
0 50 100
Mean % Confidence
Bacteria
Actinobacteria 46%
Proteobacteria 20%
Bacteroidetes 9%
Deinococcus-Thermus 6%
Chloroflexi 6%
Cyanobacteria/Chloroplast 3%
Firmicutes 3%
Gemmatimonadetes 1%
Planctomycetes 1%
Acidobacteria 1%
Armatimonadetes 0.8%
Verrucomicrobia 0.5%
TM7 0.4%
Spirochaetes 0.2%
Lentisphaerae 0.1%
Synergistetes 0.1%
Chlorobi 0.08%
Fusobacteria 0.07%
BRC1 0.05%
Aquificae 0.05%
Tenericutes 0.04%
Caldiserica 0.04%
Thermotogae 0.03%
Chlamydiae 0.02%
Nitrospira 0.02%
Deferribacteres 0.02%
Chrysiogenetes 0.009%


BOL-1U
0 50 100
Mean % Confidence
Bacteria
Actinobacteria 31%
Proteobacteria 24%
Cyanobacteria/Chloroplast 13%
Bacteroidetes 12%
Firmicutes 6%
Deinococcus-Thermus 5%
Chloroflexi 2%
Planctomycetes 1%
Verrucomicrobia 1%
Lentisphaerae 0.9%
Acidobacteria 0.8%
Armatimonadetes 0.7%
Deferribacteres 0.4%
Gemmatimonadetes 0.3%
Synergistetes 0.3%
Spirochaetes 0.2%
TM7 0.2%
Aquificae 0.2%
Chlamydiae 0.09%
Caldiserica 0.09%
Elusimicrobia 0.04%
Thermotogae 0.02%
Fusobacteria 0.02%
Nitrospira 0.02%


LCA-2D
0 50 100
Mean % Confidence
Bacteria
Proteobacteria 28%
Firmicutes 9%
Cyanobacteria/Chloroplast 9%
Chloroflexi 7%
Bacteroidetes 5%
Deinococcus-Thermus 5%
Deferribacteres 1%
Planctomycetes 1%
Actinobacteria 32%
Acidobacteria 0.6%
Verrucomicrobia 0.5%
Lentisphaerae 0.5%
Synergistetes 0.5%
Caldiserica 0.1%
TM7 0.1%
Fusobacteria 0.1%
Spirochaetes 0.1%
Chlamydiae 0.02%
Gemmatimonadetes 0.02%
Aquificae 0.02%


LCA-2U
0 50 100
Mean % Confidence
Bacteria
Actinobacteria 19%
Bacteroidetes 13%
Cyanobacteria/Chloroplast 11%
Firmicutes 7%
Chloroflexi 3%
Acidobacteria 3%
Deinococcus-Thermus 3%
Planctomycetes 2%
Lentisphaerae 1%
Proteobacteria 35%
Verrucomicrobia 0.4%
Armatimonadetes 0.4%
Gemmatimonadetes 0.3%
Caldiserica 0.3%
Spirochaetes 0.2%
Synergistetes 0.2%
Deferribacteres 0.08%
TM7 0.03%
Elusimicrobia 0.02%
Chlorobi 0.02%

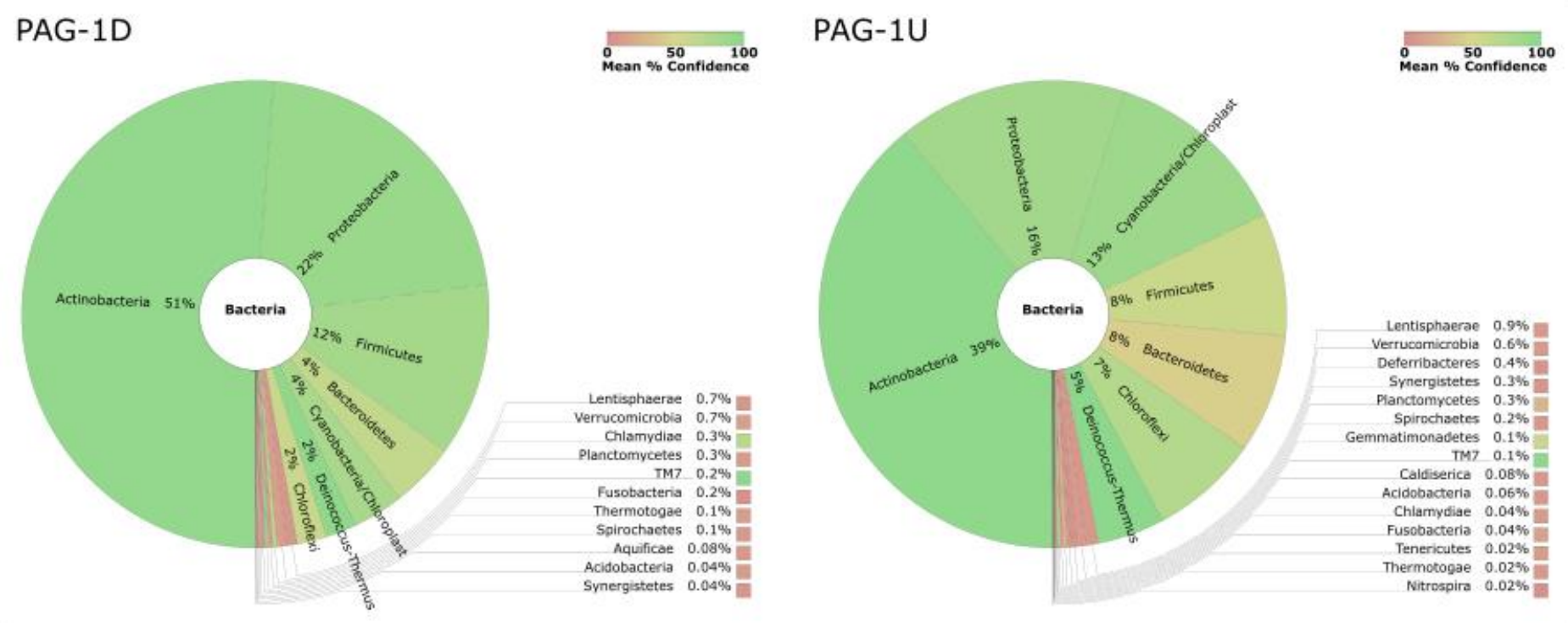


Figure 11: 16S Biodiversity charts from Geneious R9 for each of the sample sites. Analysis of the data showed a significant association between Actinobacteria, amongst others, with damaged surfaces and Cyanobacteria, amongst others, with undamaged surface. Deinococcus-Thermus demonstrated a significant association with urban sites with TM7 and Chlamydiae being significantly associated with rural surfaces.

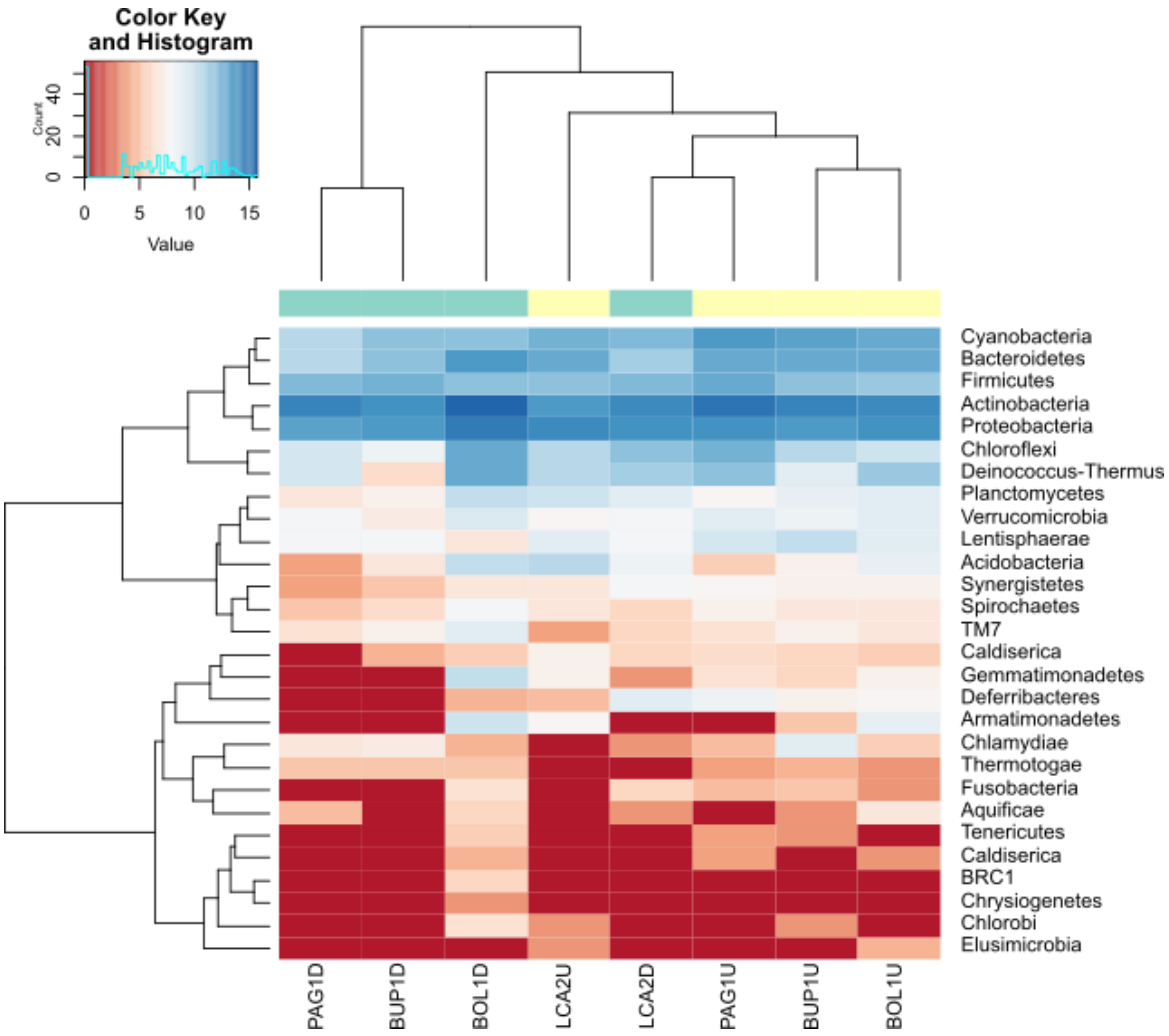


Figure 12: Heat map and tree of metagenomic data analysed at the OTU level. Analysis of the isolates at this level shows significant (p-value = 0.05) clustering according to whether the surfaces are: damaged – blue; or undamaged – yellow, with the exception of Lincoln Cathedral where the presence of Deferribacteres results in the clustering of the damaged sample with undamaged and the higher level of Acidobacteria grouping the undamaged site in with damaged sites. Presence of the families at each location is indicated using the colour range in the key, for example Deinococcus-Thermus shows a high presence (12 isolates, dark blue) in the St. Botolph-by Bargate damaged sample (BOL1D), a medium presence (6 isolates, white) in the St. Peter-at-Gowts undamaged sample (PAG1U), and a lower presence at Burton Pedwardine damaged undamaged sample (BUP1U, 2 isolates, pink)

3.7 Analysis of the microbiome at the Species level

3.7.1 Analysis of cultured isolates

The 70 isolates identified by direct culturing from the surfaces were analysed to determine whether there were significant associations with the surface, damaged or undamaged, that they were isolated from. A heat map, Figure 13, was derived from this data grouping species by the

significance of their presence vs the surface that they were extracted from. This showed significant (p-value = 0.05) clustering of samples based on the surface which they were isolated from. Clustering was also observed between rural and urban sites with the sole exception of the damaged samples from St. Peter-at-Gowts which clustered with the rural sites due to the presence of *Bacillus pumilis.* Significant differences, p-value > 0.05, were identified via log normal permutation testing between the two surfaces with *Bacillus licheniformis* and *Micrococcus halobius* both being identified as contributing to these differences. *Bacillus mycoides* and *Micrococcus halobius* were confirmed as being significantly associated with an urban environment, as were *Acinetobacter baylyi, Acinetobacter calcoaceticus, Bacillus aerophilus, Bacillus infantis, Bacillus weihenstephanensis, Brevibacillus brevis, Microbacterium thalassium, Micrococcus luteus, Pseudomonas brassicacearum, Pseudomonas brenneri, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas* sp*. HZ06, Pseudomonas stutzeri, Psychrobacter faecalis, Staphylococcus xylosus* and *Streptomyces microflavus*.

Analysis of the discovery odds for species isolated from damaged surfaces vs. undamaged surfaces showed *Bacillus licheniformis* (p-value < 0.001) and *Micrococcus halobius* (p-value < 0.05) as being significantly associated with damaged stone. *Bacillus muralis*, *Bacillus safensis* and *Micrococcus luteus* all showed a high but non-significant association with damaged stone (p-value < 0.2). Based on other studies of ecosystems further analysis of these three species was considered appropriate (Chmielewski & Rotzer, 2001). The sampling data for *Bacillus muralis* showed it had only been isolated from damaged sites, just an insufficient number for a higher significance to be assigned. *Bacillus safensis* and *Micrococcus luteus* sampling data showed both had been isolated from damaged and undamaged stone. The sampling data was run through a Student's t-test with no significant difference for *Bacillus safensis* as the means came out identical. A significantly higher presence on damaged stone was demonstrated when the comparison was made between the damaged and undamaged populations for *Micrococcus luteus* (p-value < 0.05). None of the species isolated showed a significant or close association with undamaged stone.

The analysis of discovery odds for rural vs. urban showed *Micrococcus luteus* as being significantly associated with urban environments (p-value < 0.001) with *Sporosarcina saromensis, Micrococcus halobius, Bacillus simplex, Bacillus safensis* and *Bacillus mycoides* showing strong associations with urban environments (p-value < 0.2). Presence absence testing for damaged vs undamaged and rural vs urban confirmed the previous results.

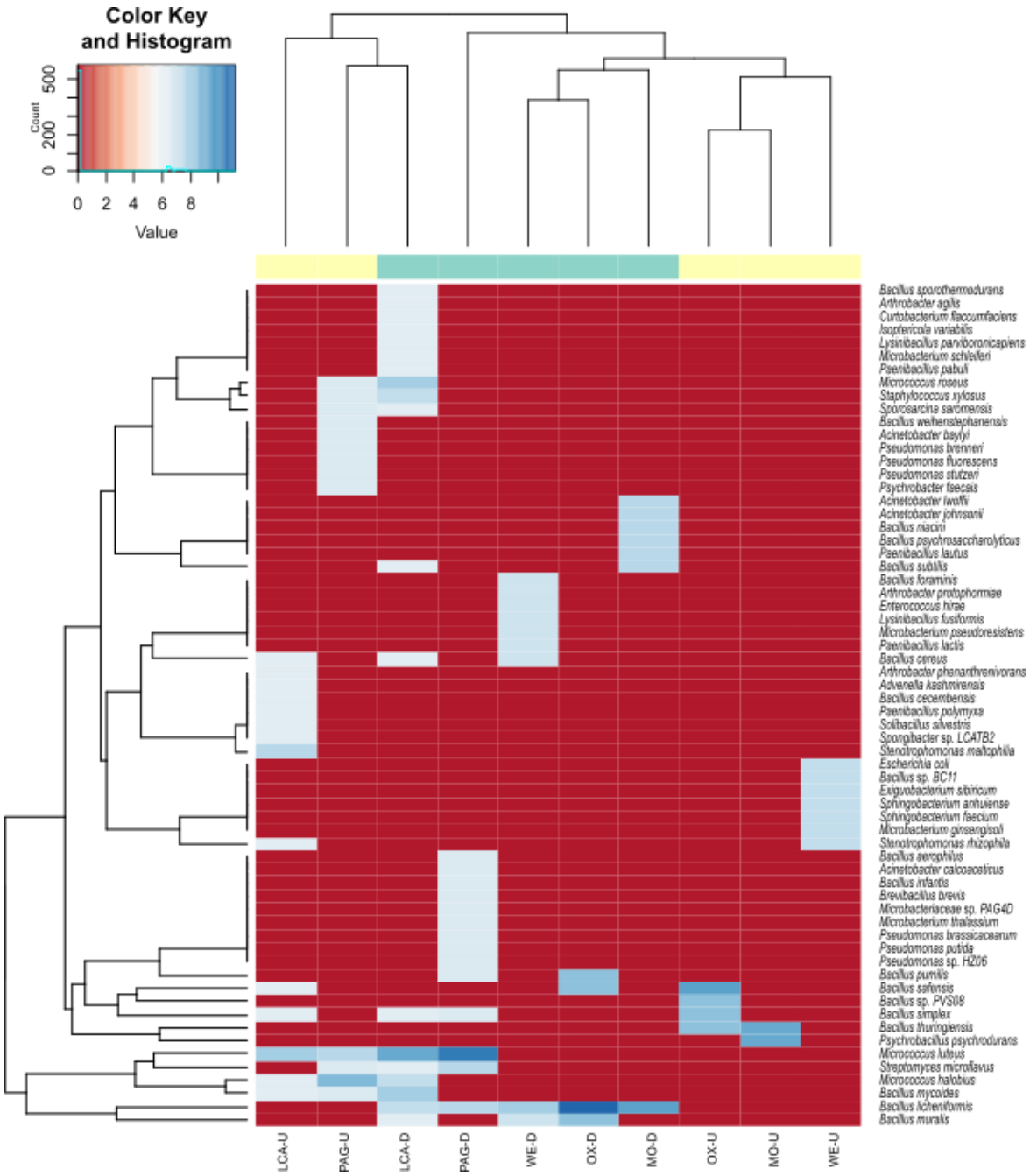


Figure 13: Identified species show clustering based on whether they were isolated from rural or urban locations and damaged or undamaged stone with the exception of Saint Peter-at-Gowts which clusters with rural samples instead of urban. Samples from damaged sites are indicated with a turquoise box at the end of the upper tree, undamaged with a yellow box. Red blocks in the heat map indicate that the species was not found at that sampling location, blue indicates positive identification at that location with the intensity of colour being indicative of the number of isolates found for example Bacillus licheniformis shows a high presence (12 isolates, dark blue) in the Oxwich Castle damaged sample (OX-D) when compared to the undamaged samples for all sites (0 isolates, red)

3.7.2 Analysis of metagenomic isolates

The species identified from the metagenomic data were analysed to determine whether any were significantly associated with the surface, damaged or undamaged, that they were isolated from. A heat map and tree, were derived from this data grouping species by the significance of their presence vs the surface that they were extracted from, the tree is shown in Figure 14. Significant (p-value = 0.05) clustering was observed based on the surface the sample was from. No significant clustering was observed with aspect, light level or UV level and further analysis showed no correlations between species sampled and these measurements. No significant clustering was observed with rural and urban locations although further analysis identified species significantly associated with both.

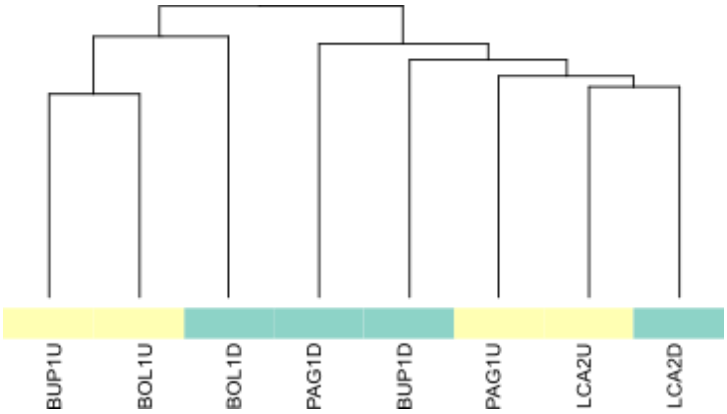


Figure 14: Tree generated from species characterised by 97% or greater match to NCBI database sequences for Saint Peter-at-Gowts (PAG1U & 1D), Lincoln Cathedral (LCA2U &2D), Saint Botolphs-by-Bargate (BOL1U & 1D) and Burton Pedwardine (BUP1U & 1D).

A Log Normal Permutation test was carried out to determine whether there were significant differences between the species present on the two surfaces. The results identified species which were significantly different (p-value = 0.05) between damaged or undamaged surfaces. *Kineococcus bacterium, Shigella coli, Gemella morbillorum, Brevundimonas* sp.*0312MAR21U9, Peptoniphilus* sp.*EL1, Bifidobacterium longum, Haemophilus influenza, Gordonibacter* sp.*S475, Rhodococcus* sp.*320, Friedmanniella sagamiharensis* and *Bacillus licheniformis* all showed a significant association with damaged stone. *Pedobacter panaciterrae, Kocuria rhizophila, Blastococcus ginsenosidimutans, Gemmata* sp.*Br1-2, Balneimonas flocculans, Hyphomicrobium* sp.*ColF, Bacillus pumilis, Kingella oralis, Phenylobacterium aquaticus, Sphingomonas* sp.*DUSK, Rothia mucilaginosa, Calothrix* sp.*PCC, Solirubrobacter ginsenosidimutans* and *Pseudonocardia seranimata* all showed a significant association with undamaged stone and *Kocuria kristinae* showed a significant association with both.

Of the species which were significantly associated (p-value = 0.05) with urban or rural sites, *Actinomyces* sp.*ICM47* was the only species to show a significant association with urban sites being present at all sampling locations except Saint Botolph-by-Bargate's undamaged site. *Kocuria rhizophila* showed a significant association with both urban and rural sites. *Micrococcus endophyticus, Staphylococcus auricularis* and *Hymenobacter yonginensis* showed a significant association with rural sites being present on both damaged and undamaged stone but only at the rural sampling site. The further 125 species, Appendix D: Species solely found in the rural environment, were identified as being significantly associated with the rural site.

Bacterial species which were significantly (p-value < 0.05) associated with damaged surfaces when testing with discovery odds were *Afipia* sp*. 42S5, Bacillus licheniformis,* and *Rhodococcus* sp.*320*. *Corynebacterium glucuronolyticum, Modestobacter* sp.*I12A-02988* were identified as being significantly (p-value < 0.05) associated with undamaged surfaces.

Species which were only found in the damaged stone microbiome and present in over 50% of the sites sampled were *Rhodococcus* sp.*320, Bacillus licheniformis, Paenibacillus* sp.*1105, Pseudomonas argentinensis, Haemophilus influenza, Gordonibacter* sp.*S475, Pantoea septica,* and *Friedmanniella sagamiharensis*. Only 4 species, *Corynebacterium glucuronolyticum, Streptococcus parasanguinis, Solirubrobacter ginsenosidimutans*, and *Streptococcus constellatus* were solely associated with undamaged stone and present in over 50% of the sites sampled. When presence absence testing was applied to the data set to determine whether any species were solely found on a particular surface, *Bacillus licheniformis* and *Rhodococcus* sp.*320* were identified as being present solely on damaged surfaces in all locations, no species were identified as being solely specific to undamaged surfaces in all locations.

Bacterial species which were significantly (p-value < 0.05) associated with the urban environment were *Streptococcus* sp.*3192A, Nocardioides* sp.*9_67, Actinomyces* sp.*ICM47* and *Micrococcus luteus*.

Lactobacillus gasseri, Flavobacterium sp.*JSC-P2-223-10, Clostridium perfringens, Micrococcus endophyticus, Leifsonia* sp.*215, Staphylococcus auricularis* and *Hymenobacter yonginensis* were identified as being significantly (p-value < 0.05) associated with the rural environment.

Micrococcus endophyticus, Staphylococcus auricularis and *Hymenobacter yonginensis* were all shown to be present solely on surfaces in the rural environment in all locations. A further 59 species were identified as being significantly associated with the urban environment and present in >50% of urban sites sampled (Table *14*), of these *Actinomyces* sp.*ICM47, Anaerococcus*

octavius, Aurantimonas sp.*HC-3* and *Nocardioides* sp.*9_67* were found in over 80% of urban sites sampled (62.5% of the total sites) and can therefore be considered to represent an urban core microbiome.

Acidiphilium sp.*N29*	*Mesorhizobium* sp.*ADC-19B*
Actinomyces sp.*ICM47 **	*Methylocapsa palsarum*
Actinoplanes lichenis	*Microbacterium trichothecenolyticum*
Advenella mimigardefordensis	*Modestobacter* sp.*I12A-02988*
Amaricoccus sp.*YIM125*	*Moraxella nonliquefaciens*
*Anaerococcus octavius **	*Nocardioides* sp.*9_67 **
Arthrobacter arilaitensis	*Paenibacillus* sp.*1105*
Aurantimonas glaciistagni	*Pantoea agglomerans*
Aurantimonas sp.*4M3-2*	*Pantoea septica*
Aurantimonas sp.*HC-3 **	*Pedobacter* sp.*2P1H2*
Blastococcus endophyticus	*Pedobacter suwonensis*
Bosea sp.*CC11C2*	*Phyllobacterium* sp.*JCM*
Campylobacter ureolyticus	*Plantibacter* sp.*H53*
Chryseobacterium joostei	*Porphyromonas bennonis*
Corynebacterium glucuronolyticum	*Prevotella salivae*
Corynebacterium tuscaniense	*Prevotella timonensis*
Derxia sp.*CB*	*Pseudonocardia zijingensis*
Enterobacter cloacae	*Psychrobacter immobilis*
Friedmanniella sp.*Pao16*	*Psychrobacter* sp.*44(2016)*
Humicoccus bacterium	*Ralstonia pickettii*
Hymenobacter algoricola	*Ralstonia proteobacterium*
Hymenobacter perfusus	*Rathayibacter* sp.*JSM*
Hymenobacter soli	*Rothia* sp.*B18*
Kineococcus lusitanus	*Salinibacterium soli*
Kitasatospora sp.*1C-32*	*Serratia bacterium*
Lachnospiracea incertae sedis Dorea	*Sorangium cellulosum*
Lactobacillus iners	*Sphingobacterium* sp.*FM2*
Leifsonia sp.*2EM3*	*Sphingomonas cucumeris*
Mesorhizobium mediterraneum	*Sporosarcina luteola*
	Streptococcus gastrococcus

Table 14: Species identified solely in urban samples and present in >50% of sites sampled. *=species which form an urban core microbiome.

3.7.3 Analysis of species common to both metagenomics and direct isolation

Thirty three species characterised within the metagenomics data set were identified as being common with the direct sampling. Metagenomics sampling counts for these species were merged with the counts for the direct sampling for analysis. Sample sites MO1D, OX1U, WE2U and WE3D could not be included in the data set as they only contained one or none of the 33 species identified and therefore could not be normalised to support analysis of the sparse dataset.

A heat map and phylogenetic tree, Figure 15, was derived from this data grouping species by the significance of their presence vs the surface that they were extracted from. Clustering was observed with damaged and undamaged samples significantly (p-value = 0.05) grouping together and the metagenomics samples clustering due to the high presence *of Arthrobacter agilis, Acinetobacter lwoffii, Pseudomonas fluorescens, Escherichia coli* and *Micrococcus luteus* in the metagenomics samples. Removing these 5 species from the dataset results in the metagenomics data set and sampled data set merging and still showing significant (p-value = 0.05) clustering based on damaged and undamaged sampling (data not shown). Clustering was also observed for rural and urban sites at a significant (p-value =0.05). Geographical differences in the microbiome also becomes evident in with the central cluster consisting mainly of the sites sampled in Wales and Herefordshire.

Log Normal Permutation testing was carried out to determine whether there were significant differences between species present on the two surfaces. *Bacillus aerophilus, Bacillus licheniformis, Paenibacillus* sp.*1105* and *Psychrobacillus psychrodurans* were identified as being significantly (p-value = 0.05) present on damaged stone, no species were identified as being significantly associated with undamaged stone with this test. *Micrococcus luteus* and *Paenibacillus* sp.*1105* were identified as being significantly associated (p-value = 0.05) with the urban environment. *Bacillus licheniformis* was identified to be significantly present (p-value = 0.05) in both rural and urban environments, on damaged surfaces, and *Bacillus safensis* was identified as significantly present (p-value = 0.05) in rural environments.

With discovery odds testing *Bacillus licheniformis* was identified as being significantly (p-value < 0.01) associated with damaged stone. Again *M. luteus* showed a p-value of 0.06 suggesting that further analysis was required. Repeating the students t-test for the isolation of *M. luteus* using the combined metagenomic and direct sampling counts again gave a significant (p-value = 0.05) association between *M. luteus* and damaged stone. *Bacillus licheniformis* was identified as being significantly (p-value < 0.05) present on damaged surfaces in both rural and urban environments. *M. luteus* was also significantly (p-value < 0.02) associated with the urban environment.

Testing for species solely isolated from either the damaged and undamaged environment identified *Bacillus aerophilus, Bacillus licheniformis* and *Psychrobacillus psychrodurans* as being only found in the damaged environment. *Arthrobacter phenanthrenivorans* and *Solibacillus silvestris* were identified as being solely associated with the undamaged environment. Presence absence testing confirmed the significance of *Bacillus licheniformis* (p-value < 0.01) being associated with damaged stone, the other species identified were not present in the data set in high enough numbers to confirm whether this was significant or not. Testing for species which were only isolated from either the rural or urban environments identified *Enterococcus hirae* as being solely isolated from the rural environment. *Micrococcus halobius, Paenibacillus* sp*. 1105, Bacillus subtilis, Staphylococcus xylosus, Acinetobacter baylyi, Bacillus aerophilus* and *Sporosarcina saromensis* were all solely associated with the urban environment.

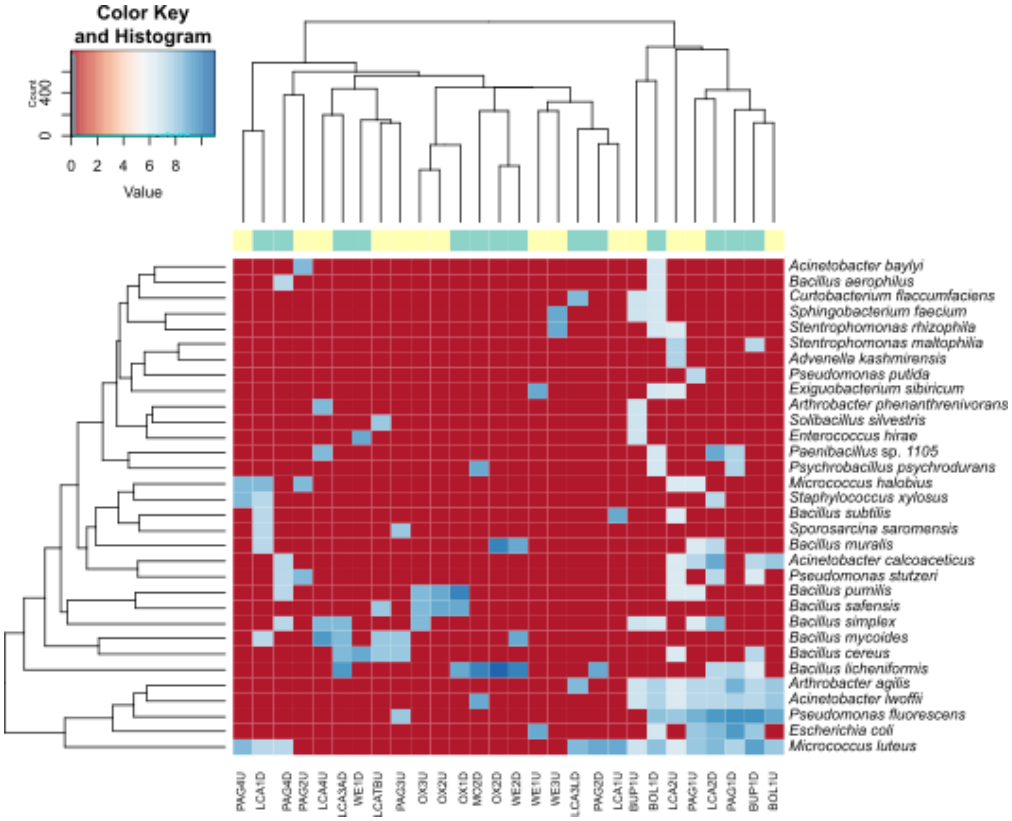


Figure 15: Heatmap and phylogenetic tree produced from the counts of species common to both direct sampling and metagenomic analysis. Clustering of damaged and undamaged sites is still present within the tree as are geological differences between the microbiomes with the sites at Oxwich Castle, Weobley Castle and Moccas making up the majority of the central branch. Presence of the species at each location is indicated using the colour range in the key, for example <u>Micrococcus luteus</u> shows a high presence (12 isolates, dark blue) in the Lincoln Cathedral site 1 undamaged sample (LCA1U), a lower presence (7 isolates, pale blue) in the Burton Pedwardine site 1 undamaged sample (BUP1U), but is absent in all of the Weobley Castle samples (WE, 0 isolates, red)

3.8 Discussion

3.8.1 Environmental measurements

The environmental measurements and site recording taken during sampling were primarily to ensure that the sampling sites had no major differences between them, for example overhanging foliage resulting in soiling of the surface by honeydew which will also provide a nutrient source for the microorganisms already present (Hallmann *et al.*, 2011). This was confirmed by the lack of correlation between the measurements taken from the stone surfaces, stone moisture content and surface pH, and those taken from the surrounding environment, relative humidity, temperature, lux and UV. The most directly comparable measurements at each site were stone moisture content and surface pH. In addition to this there was no significant link between the species isolated and the aspect or light and UV levels of each site. As a result the aspect, exposure and environment of each site should have minimum impact on the core microbiome detected.

Although there was no significant link between stone moisture content and surface damage a significant difference between surface pH of damaged and undamaged stonework was found. The surface of damaged stone was found to be more acidic, with a pH of 5.5 or less (mean pH 5.25). Undamaged stone surfaces had a pH range between 5 and 6.5 (mean pH 5.94). As the recently cut, undamaged stone sampled from the Cathedral's new toilet block had a pH of 6.5, it suggests that the surface of the biofilm coated stone becomes more acidic over time as biodeterioration processes occur. While in a newer structure, the toilet block was 5 years old at the time of sampling, the higher surface pH could be due to the leaching of hydroxide ions from fresh cement causing an elevation of pH (Wei *et al.*, 2013) sufficient time had passed to eliminate this as a cause (Setunge *et al.*, 2009).

The effects of purely physical or chemical weathering on limestone pH are well studied, with an abrasion pH of 8.0 in the literature for calcite, with other carbonates ranging between pH 6.9 and 9.2 (Summerfield, 2014; Lottermoser, 2016). While there are a number of potential causes for increasing acidity on the stone surface, including the effects of pollution, colonisation by acid producing bacteria will contribute to this. When examining the data from adjacent stones, < 1 metre between sampling points, 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 airborne pollutants would affect stonework equally when they are in close proximity, implying that local, surface effects are making a contribution to this acidity.

A significant positive correlation was also found between surface wetness and pH on damaged surfaces. The mechanism which is causing the low pH is not physical weathering as the dissolution

of calcium carbonate through the action of dilute carbonic acid present in rainwater results in the neutralisation of the acid and an alkaline abrasion pH of 8.0 (Lottermoser, 2016) which suggests that the correlation is not causative. The innate requirement of bacteria for water provides one reasonable link for the non-causative correlation seen between water retention of the stone substrate and the pH on damaged stone, especially as the close proximity between sampling sites makes it highly unlikely to be due to atmospheric pollution or other environmental conditions.

The increased acidity levels suggests that pH measurements of stone could be used as a novel marker for deterioration, with the change in acidity suggesting a need for conservation treatment before damage is visible to the eye.

3.8.2 Limestone as a habitable environment

The complete analysis of the bioreceptivity of Lincoln limestone is a unique occurrence for a British stone. One other stone, Mansfield dolomite (Papida *et al.*, 2000), has been partially analysed, although this analysis did not include the chemical composition or surface roughness which were considered to be a vital component of the assessment of bioreceptivity in the European studies of limestones (Miller *et al.*, 2009, 2012).

Sellotape sampling to confirm the presence of biofilm was positive at all sites, except for site 4 at Lincoln Cathedral which was beneath the main East End window where deposited copper salts on the surface were observed. The species isolated from this site, including *Bacillus mycoides*, are known biofilm formers so it is possible that the copper deposits interfered with sampling as it has been shown in the literature that the presence of copper ions, as well as being biocidal, inhibits biofilm formation in Bacillus species (Chang & Tien, 1969; Keevil, 2004). An alternative explanation is the presence of *Paenibacillus polymyxa* at this site which produces biosurfactant lipopeptides which have been shown to be effective against biofilms produced by *Bacillus subtilis, Micrococcus luteus, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus bovis* and could therefore have been contributing to the lack of detectable biofilm (Sadekuzzaman *et al.*, 2015). Other than site 4 at Lincoln Cathedral, the clear and easily detectable presence of biofilm at each sampling location confirms that the species growing on these surfaces are part of a biofilm community, whether they are strong biofilm formers or not. As the presence of biofilm is indicative of colonisation (Garrett *et al.*, 2008), the absence of easily detected biofilm at site 4 of Lincoln Cathedral means that there is a greater probability that the isolates recovered were transient rather than species which had colonised the surface.

In order to provide comparative data for the bioreceptivity of Lincoln limestone, the chemical composition and surface roughness were measured (Table 15). Having comparative data for

bioreceptivity of specific stone types, combined with sampling data will allow future researchers and conservators to make predictions about which biodeteriorative species have a high probability of being present on damaged surfaces. The presence of aluminium oxide relates to the presence of clay minerals in the limestone and puts Lincoln limestone as having a higher clay content than the stones tested in the other studies, 1.4% compared to a maximum of 0.79%. Calcium carbonate composition is approximately the same. Magnesium, potassium and sodium are all higher than the stones in Millers study, with iron and silicon being in the same range. While Millers study detected manganese which was not detected in Lincoln limestone, Barbers study in 1974 showed that there were only very low traces of manganese in Lincoln limestone, so it is likely that the SEM-EDX was not a sufficiently sensitive technique to pick up the traces of manganese present. Lincoln limestone also showed the presence of phosphorous, sulphur, bromine and tantalum which were not detected in the Spanish and Portuguese limestones. While Barbers 1974 study did not detect these, trace levels of tantalum are regularly found in limestones (Salminen, 2016), and limestone is one of the most common phosphate bearing stones (Prothero & Schwab, 2004). Bromine has been shown to leach from Lincoln limestone (Edmunds, 1996), and the sulphur present was probably absorbed from pollutants in the atmosphere which reacted with the calcium carbonate to form gypsum nanoparticles.

It is interesting to note, although unsurprising given the biological origin of limestone deposits (Emery *et al.*, 1988), that the trace minerals are sufficient to support bacterial growth. The chemical composition of limestone is a very close match to the recipe for M9 minimal media which is a common growth media for bacteria. Sodium phosphate, potassium phosphate, sodium chloride, ammonium chloride, magnesium sulfate and calcium carbonate together with a carbon source make up the M9 minimal media, all that Lincoln limestone is missing is the nitrogen and carbon sources but these are available from the environment. Comparison with the limestones analysed by Miller et al. (2009) showed that they had similar trace elements.

A surface roughness of below 30nm is sufficient for bacterial adherence (Yoda *et al.*, 2014) so while the surface in both Miller *et al* and this research does not reach the millimetre levels of roughness observed in some studies (Korkanç & Savran, 2015), it is more than sufficient to promote surface attachment. The surface roughness parameters quoted in Miller et al were Ra and Rz, when compared using these measurements Lincoln limestone showed a surface roughness almost identical to Ançã limestone, Ra 1.63±0.44µm for Lincoln compared to 1.61±0.40µm for Ançã with Rz being 8.43±1.45µm and 8.52±1.9µm respectively (Table 15).

Lincoln limestone has a capillary coefficient of 171.48±19.9 $g.m^{-2}.s^{-0.5}$ compared to 57.25±1.17 $g.m^{-2}.s^{-0.5}$ for Ançã limestone. As Miller showed that physical bioreceptivity is a combination of Ra, Rz and Capillary coefficient, with the higher the roughness and the capillary coefficient the better the bioreceptivity, this would place Lincoln limestone as having a higher bioreceptivity than Ançã stone due to the better water retention. Lincoln limestones capillary coefficient was closest to San Cristobal stone, actually a calcareous sandstone, the stone which showed the second highest bioreceptivity in their study (Table 15).

	Na (% weight)	Mg (% weight)	Al (% weight)	Si (% weight)	K (% weight)	Fe (% weight)	Mn (% weight)	Capillary coefficient ($g.m^{-2}.s^{-0.5}$)	Ra (μm)
Lincoln	1.1	0.9	1.4	9.3	0.7	0.2	no data	171.48+-19.9	1.63+-0.44
Ançã	0.15	0.40	0.41	3.12	0.13	0.12	0.00	57.25+-1.17	1.61+-0.4
Lioz	0.15	0.25	0.12	2.15	0.22	0.11	0.00	0.20+-0.06	1.59+-0.42
San Cristobal	0.12	0.10	0.20	38.45	0.05	0.41	0.01	199.30+-13.05	8.16+-2.16
Escúzar	0.29	0.32	0.25	1.19	0.08	0.16	0.02	268.79+-77.54	5.98+-1.28
Lecce	0.29	0.45	0.79	0.33	0.13	0.28	0.00	128.76+-3.04	6.14+-0.63

Table 15: Comparison of Lincoln limestone trace elements and physical characteristics with those found in Miller et al. (2009). The chemical composition of Lincoln limestone is similar to all the samples in Millers study, except San Cristobal which should really be considered a calcareous sandstone because of the high levels of silicon present. Similarly the surface roughness and capillary coefficients are within the ranges which Millers study showed to be positive for bioreceptivity.

Water retention is obviously essential information to determine the bioreceptivity of the stone, but will also, as future studies emerge, provide a guide for which species are likely to have colonised the surface. Species which have no adaptations to a xerophytic environment will be less likely to colonise surfaces with a low capillary coefficient or wide pore network. In this study Lincoln limestone has been shown to have high water retention properties. The high water retention is reflected in the relatively high level of gram negative species identified when compared to the bacterial members of the microbiome published to date, as gram negative species have a lower resistance to dehydration due to the structure of their cell walls (Mille *et al.*, 2005).

It is difficult to state whether the higher presence of gram negative species is a significant environmental finding, due to the lack of studies which include the undamaged environment. This

study showed a strong positive correlation between gram positive species and damaged stone surfaces, which would be expected as they have been shown to have a wide range of acid resistance systems when compared to gram negative species (Cotter & Hill, 2003). If the correlation between gram positives and damaged surfaces is true for all limestones then the absence of gram negative species in the literature may be due to the failure to test the whole microbiome.

Based on the geochemical composition, the comparison of Lincoln limestone with the limestones in Millers study, the detection of biofilm on almost all surfaces, and isolation from Lincoln limestone of bacteria and other microorganisms, we conclude that there is sufficient data to reject the null hypothesis for hypothesis 4, and confirm that Lincoln limestone provides a surface which encourages biological colonisation. The unidentified algal species detected microscopically while staining for the presence of biofilms will enhance the bioreceptivity of the surface for bacteria by fixing atmospheric carbon and nitrogen (St. Clair & Seaward, 2004), the two elements needed that are not easily accessed from the geochemical composition of the limestone.

Analysis of the bioreceptivity will also provide a basis for comparison of the microbiomes found on other stone surfaces. As the field expands it is likely that correlations will be found between chemical composition, availability of water and the species present on the surface. Previous studies on bioreceptivity have been mainly laboratory based, looking at the physical characteristics of the stone and then using model organisms to determine bioreceptivity through the number of organisms that the surface can support using a range of different techniques. There is variation in the techniques used to characterise the stone surface with only half of the studies in Millers 2012 review of bioreceptivity having looked at surface roughness. Where studies have looked at pH they have solely looked at abrasion pH, a measure of the pH under physical or chemical weathering. While valuable for this study as it gives a baseline pH to work from, all of the studies in the review failed to analyse whether the pH was effected by the presence of microorganisms.

3.8.3 The limestone bacterial microbiome

3.8.3.1 Cultured isolates

Of the 64 species identified from the isolates, 24 were identified as part of the limestone microbiome recognised in the current literature. When comparing the isolates at the genus level, which allows the inclusion of papers which did not provide identification to species level, 19 of the 25 genus identified are found in the literature. *Advenella, Enterococcus, Lysinibacillus,*

Psychrobacillus, Psychrobacter and *Spongiibacter* are the genera which were not identified, of these only *Enterococcus* and *Lysinibacillus* were isolated from damaged stone. With the sparsity of studies in the literature looking at undamaged stone it is not surprising to have identified 4 genera associated solely with undamaged limestone.

The absence of any family other than Bacillaceae at Oxwich Castle could be explained by the fact that a program of conservation at Oxwich Castle was completed in September 2012 (Cadw, 2012). While full details of the program are currently unavailable it is likely that cleaning and/or biocide treatment would have been carried out during this period, which would explain the lower diversity on the stones which were sampled 9 months later. While the Oxwich Castle isolates do little to enhance understanding of the microbiome as a whole they allowed the identification of several species which are capable of biocorrosion.

Close to half of the species (10/24) identified both in this study and the literature call into question the current assignment of the species as damaging. This is unsurprising as the failure of the previous studies to look as the species specific to the undamaged portion of the population means that they have only been working with half the data set compared to this study. *Bacillus cereus, thuringensis,* and *simplex, Micrococcus halobius, luteus* and *roseus, Paenibacillus polymyxa, Pseudomonas fluorescens, Pseudomonas stutzeri* and *Stentrophomonas maltophilia* are all in conflict with the literature for the surfaces they were isolated from. In this study *Bacillus cereus, Bacillus simplex*, and *Micrococcus halobius, luteus* and *roseus,* were isolated from both damaged and undamaged surfaces with the rest of the species isolated from undamaged surfaces. In the literature, they were isolated from damaged surfaces and classified as damaging due to this. As they have been isolated from undamaged surfaces in this study it would be fallacious to claim that they are damaging without further investigation. It is worth noting that *Micrococcus luteus* is significantly associated with urban damaged surfaces and therefore may provide an easy biomarker for biodeteriorative processes.

Correlation testing identified a a significant, p-value < 0.01, positive correlation, 0.45, between damaged surfaces and gram positive bacteria, a significant, p-value < 0.05, negative correlation, -0.243, between undamaged surfaces and the presence of rod morphology and a significant, p-value <0.01, positive correlation, 0.34, between damaged surfaces and sporulators.

With the majority of sporulators being gram positive bacteria the fact that gram positive bacteria and sporulators both correlate to damaged surfaces is unsurprising. As the damaged environment has been shown to have a significantly lower surface pH overall when compared to the

undamaged environment the significant correlation between gram positives and the damaged surfaces is most likely due to the fact that gram positive bacteria have a high number of mechanisms to overcome the challenges posed by acidic environments (Cotter & Hill, 2003).

While the undamaged environment is less extreme in surface pH than the damaged environment it also has a lower surface area (Korkanç & Savran, 2015) and is more exposed. This will result in more frequent desiccation of the biofilm, something cocci shaped bacteria with their lower surface area are better suited to tolerate (Hirai, 1991), hence the significant correlation.

The rougher surface area of the damaged surface and the correlation between damaged surfaces and gram positive species helps to explain the higher number of isolates recovered when compared to undamaged surfaces. A more diverse population will have arisen through a combination of less frequent desiccation of the biofilm combined with a higher proportion of species which can survive the desiccation through sporulation.

The isolate classified as *Microbacteriaceae* sp. *PAG4D* is potentially a new species and may even represent a new genus. The distance between the two closest species, *Mycetocola miduiensis* and *Clavibacter michiganensis* subsp. *Sepedonicus* strain *ATCC 33113* species is 0.023, the distance between PAG4D and these species is 0.044 and 0.046, respectively a distance sufficient to imply that it is of a different, currently unidentified genus (Chakravorty *et al.*, 2007; Větrovský & Baldrian, 2013).

A blast search of the 16S rRNA sequence for *Microbacteriaceae* sp. *PAG4D* against the assembled metagenomics data did produce partial matches to contigs. All contigs found were shown to be the close relatives previously identified (Figure 9). The partial matches which were found were due to the shorter sequence length used in metagenomics, 250 base pairs, than that used for sampling id, 740 base pairs. When the contigs identified by a local BLAST search were searched against the ncbi database the matches mentioned above were always more closely related, 99% similarity or greater, to the species which were identified as close relatives in Figure 9, than to *Microbacteriaceae* sp. *PAG4D*. This further supports the assumption that *Microbacteriaceae* sp. *PAG4D* is a previously unidentified species.

The Poor and Moderate diversity on Shannon's index for isolated species from each sample site is understandable as the isolation of copiotrophs only retrieves a subsection bacterial component of the microbiome, metagenomics data showed healthier populations as it revealed all of the identifiable bacterial species present in the sample, subject to the previously discussed limitations of 16S rRNA metagenomics sequencing. The results of Shannon's equitability measure are in this

study more important than the index for isolates species as they demonstrate whether the sampling is biased. In all cases the sampling showed an even selection of species from across the bacterial component of the population.

Having taken samples from damaged and undamaged stone allows, for the first time, a comparative approach to be taken, which clarifies the conflicts in the association of species with surfaces found in the current body of literature. It is important to recognise that the presence or absence of a bacterial species on a damaged surface does not provide evidence as to whether it is damaging or not, and for the species which are found on both surfaces further analysis is necessary to determine whether they are predominantly associated with one surface or the other. The addition of the metagenomic data set to the direct isolation allowed greater accuracy in profiling the isolates with regard to the surface that they were sampled from.

3.8.3.2 Metagenomics

Analysis of the species identified using metagenomics showed that there was a common core microbiome that was found in the majority of the sites sampled, across the limestone surfaces in both rural and urban locations. These species made up only 5.6% of the total species identified in the study. When comparisons were made between the rural site and the urban sites a further 4 species were identified which are components of the core microbiome in urban environments. As only one rural site was sampled there is insufficient data to identify the species which are specific to the core microbiome in rural environments although these are likely to be represented in the 125 species which were solely isolated from that environment.

The identification of the key species which are common to the limestone microbiome will help clarify the role of the species represented in the current literature, for example *Acinetobacter lwoffii* has been identified as being isolated from damaged surfaces (De Leo *et al.*, 2012), as has *Arthrobacter agilis* (Heyrman & Swings, 2001; De Leo *et al.*, 2012). The discovery of these species in their studies is not surprising as this study has shown that they are common to the limestone microbiome and were in fact found on every surface sampled for metagenomic analysis. The elucidation of the core species common to the limestone microbiome is therefore of key importance to future studies as well as the retrospective analysis of the existing literature; to support this the full listing of species complete with the surface they were isolated from and whether they were found in rural or urban environments has been included as Appendix C.

As well as the species which are common to the limestone microbiome as a whole, this study has identified species which are specific to the microbiome found on damaged stone, as well as those

which have the capacity for damage but are found universally. The species which have biodeteriorative potential which are also found on undamaged stone provide an opportunity for wider research to answer the question of why they are not actively causing damage.

A total of 1048 species were identified in the metagenomic sampling across the 4 locations. When comparing the species identified through direct sampling with the metagenomics results, directly sampled species were found in the metagenomics data to a level which meets the expectations of the literature for selective sampling (Table 16). The directly isolated species which were also found in the metagenomic sampling were not just confined to the core microbiome. Species which had a lower chance of being found on a surface were also isolated, confirming that the distribution of isolates from the direct sampling was representative of the microbiome as a whole, as was demonstrated by the normality testing. While some conflicts were found between the literature and the association with damaged and undamaged surfaces of the species identified in this study, these were mostly due other studies only isolating species from damaged surfaces.

Location	Site	Surface	%
Lincoln Cathedral	2	U	1.72
	2	D	2.1
Saint Peter-at-Gowts	1	U	2.19
	1	D	1.34
Total population	-	-	3.14

Table 16: Comparison of total species identified in metagenomics sampling to total species identified in direct culturing expressed as a percentage of the total for Lincoln Cathedral, Saint Peter-at-Gowts and the total population for both sampling methods. Based on the literature direct culturing should be between 0.1 – 1% of the total population. U refers to undamaged surface, D to damaged.

The 20 species which were isolated though direct sampling and culture which were not found as part of the metagenomics analysis had all been isolated from sites which were not included in the metagenomic sampling. These species are likely to be regionally specific for those isolated in Wales and the Southwest of England, or due to microclimate when isolated in Lincolnshire, as clustering of the locations outside of Lincolnshire has been observed in the analysis (Figure 15).

Analysis of the sampled populations at the species level gave all 8 samples as coming from healthy, well distributed ecosystems using Shannon's index of diversity. The sampling was also shown to be unbiased using Shannon's equitability test. The percentage of species identified was for the most part between 20-40%, which is consistent with the current state of the microbial census (Schloss *et al.*, 2016).

Of the species solely isolated from, or predominantly associated, with damaged stone, *B. aerophilus, B. licheniformis, P.* sp. *1105* and *P. psychrodurans* have all been shown to have a significant association with damaged stone. Of these 4 only *B. licheniformis* has been shown experimentally to cause biocorrosion (Chapter 5). *M. luteus* has been shown to have a weaker but potentially significant (p-value = 0.06) association with damaged stone in the urban environment, although the significance gave a p-value = 0.05 with a direct students t-test on the sampling data, and has also been proven experimentally to cause biocorrosion (Chapter 5). *B. licheniformis* has been previously associated with biodeterioration of mural paintings in the Servilia Tomb (Necropolis of Carmona, Seville, Spain) (Heyrman & Swings, 2001) and frescos in Assisi (Radaelli *et al.*, 2004) as well as limestone surfaces in Erzurum (Nuhoglu *et al.*, 2006). It should be noted that in these studies only areas of damage were sampled and no testing of the isolate for biocorrosive activity was performed, so the claim was presumptive, whereas in this project a statistically significant association with damaged surfaces and biocorrosive capability in some strains has been proven.

Other species such as *Rhodococcus* sp*. 320, Paenibacillus* sp*. 1105, Pseudomonas argentinensis* and *Friedmanniella sagamiharensis* showed a significant association with damaged surfaces, the significance of *Paenibacillus* sp*. 1105* having been demonstrated by the additional data provided from the metagenomic sampling when compared to the direct sampling alone. The additional metagenomic data also highlighted the significant association of *P. psychrodurans* with damaged surfaces.

While the isolates associated with undamaged surfaces from the direct sampling did not produce any species which were significantly associated with that surface, the richer metagenomic data set identified 4 species. This analysis provides evidence for rejecting the null hypothesis for hypothesis 1. For each sample location there is a significant difference between populations on damaged and undamaged stone, confirming that there is a discernible difference in identified species between damaged and undamaged stone.

There were no families of bacteria solely associated with damaged and undamaged stone surfaces at the highest level of metagenomic analysis. Given the number of species which can be encompassed by a family this is not surprising, differences are most likely to be encountered at a genus or species level.

The discovery odds ratio data was interesting as it showed a greater chance of discovering members of the Bacillaceae family on damaged stone which corresponds with the species and genus reported

in the current literature. The greater probability of discovering Xanthomonadaceae family members on undamaged stone conflicts with the current literature, this is most likely due to the fact that the majority of the studies have only looked at damaged stone, and therefore did not have the data available to make a comparison between damaged and undamaged surfaces. This is supported by the positive correlation between gram positive bacteria and damaged surfaces discussed earlier

Analysis of the metagenomic data at the OTU level produced significant differences between the classes of bacteria found on damaged and undamaged stone. As well as the classes identified in damaged surfaces in the literature (De Leo *et al.*, 2012; Kusumi *et al.*, 2013; Diaz-Herraiz *et al.*, 2014), Acidobacteria, Actinobacteria, Firmicutes and Proteobacteria, TM7 and Thermotogae were identified as significantly associated with damaged surfaces. The presence of Actinobacteria on damaged stone is also supported by the direct sampling, where the 3 families of Actinobacteria isolated, Microbacteriaceae, Micrococcaceae and Promicromonosporaceae, were all associated with damaged stone.

Cyanobacteria were identified as being more likely to be associated with rural environments and undamaged stone, a completely different association to that in the tropics where the majority of the published work on Cyanobacteria on limestone has taken place (Kusumi *et al.*, 2013; Bartoli *et al.*, 2014). This association is only apparent at the OTU level due to the low levels of terrestrial Cyanobacteria 16S rRNA genes present in the ncbi nucleotide database. A search of the ncbi database yielded 169 species, 81% of which were isolated from an aquatic environment (Guiry & Guiry, 2017). Of the 32 terrestrial species 22 were associated with soil, 7 with stone surfaces and 3 were lichen phycobionts. Based on the estimated bacterial population given by Shannon's exponential and the percentage of Cyanobacteria in the population from the metagenomic data it becomes clear that the majority of the species on the limestone surface cannot be identified at the species level (Table 17). Only three Cyanobacteria were identified to the species level from the metagenomic data, *Hassallia* sp.-*C76, Calothris* sp*. PC7507* and *Machrochaete lichenoides*, a phycobiont of lichen *Placynthium nigrum*.

While Cyanobacteria have been shown to be significantly associated with undamaged stone in this study the level at which they have been identified does not eliminate some species from having a role in biodeterioration; they also play a large role in the aesthetic alteration of surfaces due to their pigmentation.

	Shannon's Exponential	% Cyanobacteria	Estimated number of cyanobacteria species
LCA2D	1352.63	9	122
LCA2U	678.92	11	75
PAG1D	1374.65	4	55
PAG1U	1347.27	7	94
BOL1D	2947.9	3	88
BOL1U	186.71	13	24
BUP1D	2859.29	9	257
BUP1U	305.74	17	52

Table 17: Estimation of the number of cyanobacteria present on each surface sampled for the metagenomics survey. Only 32 16S rRNA genes from terrestrial species of Cyanobacteria are present in the ncbi nucleotide database which explains the low level of Cyanobacteria (3) identified to the species level in this study.

Bacteroidetes and Planctomycetes were identified as being associated with damaged stone in Diaz-Herraiz *et al.* (2014). Again it is difficult to determine whether this is in conflict with this study as they did not sample undamaged surfaces for comparison so it is not possible to state if their findings were significant or not.

Analysis of the metagenomic data at the OTU level also provides evidence for the rejection of the null hypothesis for hypotheses one, specifically showing discernible differences between the identified species, in this case at the class level, between damaged and undamaged surfaces. It is worth noting that as many of the species which are components of the OTU data are currently unidentified or unculturable this analysis would not be possible without metagenomic sequencing.

With the results of the analysis of the classes present on damaged and undamaged limestone surfaces at the OTU levels as well as the analysis at the species level, we can confidently reject the null hypothesis for hypothesis one, and state that there are discernible differences between the identified species making up the microbiome on damaged and undamaged surfaces.

The limited analysis of the microbiome according to whether the sampling was from a rural or urban environment showed that the core species, those found in over 75% of the sample sites, were not affected by the different environments. For urban microbiomes the core species are expanded by the presence of urban specific species, and it is likely that species specific to the rural environment will be identified in the future which will expand the core species in a similar fashion. The differences observed at all levels, OTU analysis and species level analysis, identify significant differences between the urban and rural environments for the rest of the population.

As previously discussed only Mitchell and Gu (2001) have made an attempt at looking at the effects of pollutants on the limestone microbiome. Their definition of non-polluted was still within an urban environment and less than half a mile of a major road which would explain why the results that they obtained could not be analysed to give significant differences between polluted and non-polluted microbiomes.

Twenty one out of the 27 studies on limestone biodeterioration sampled in areas which would be considered urban, or polluted, based on the definition of this study. This is as all of the rural studies in the literature have been carried out less than half a mile away from a main road. Only 3 of the studies sampled from rural locations, with the final 3 sampling from both rural and urban sites. None of the 3 which isolated samples from rural and urban sites (Berner *et al.*, 1997; De Leo *et al.*, 2012; Diaz-Herraiz *et al.*, 2014) had deliberately selected the sample sites based on their environment and so did not analyse their data accordingly. It is interesting to note that the bacterial classes which were found to be associated with damaged surfaces in the Diaz-Herraiz et al. (2014) study match those which are significantly associated with urban damaged environments in this study. While their study was not considering the effects of urban pollution on the microbiome the Etruscan tomb they were sampling was close to a major road which would explain the distribution of classes identified in the study.

In general there was consensus between the classes of bacteria associated with rural and urban environments in the literature, and those from the analysis performed in this study. The sparsity of comparative studies and sampling from environments with low levels of pollution, combined with the significant differences between the microbiomes in this study, helps to demonstrate the need for a wider program of basic investigation in this field.

The results obtained from analysing the microbiomes sampled from the 1 rural and 3 urban environments serve to highlight differences at both the OTU and species levels between the two environments. Other than the isolates from the damaged stone at Saint Peters at Gowts where the presence of *Bacillus pumilis* appears to have made it cluster with the rural isolate over urban isolates, but still at a significant distance from them, there is a discernible difference between the species isolated between rural and urban locations. This was further supported by *Micrococcus luteus* amongst other species showing a significant association with urban sampling locations.

Several species were also identified as significantly associated with the rural environment in the analysis of the metagenomic data, these included *Micrococcus endophyticus, Staphylococcus auricularis* and *Streptococcus constellatus*. Unfortunately none of the species which were

identified as being solely associated with the rural environment in the metagenomic sampling had been recovered in the direct sampling and culturing, so while this study can raise the importance of the difference between the microbiomes between the two environments it is not presently possible to characterise the species which are associated with damaged surfaces in the rural environment for biodeteriorative potential. Equally the geographic clustering observed in the Herefordshire and Welsh samples identifies potential areas for future research.

While sufficient data are not available to confirm this, it is likely that the differences between rural and urban isolates are due to atmospheric pollutants as seen in other studies (Mitchell & Gu, 2001). Analysis of atmospheric pollutants between Lincoln and Burton Pedwardine show the major differences to be based on nitrogen chemistry, urban Lincoln has high levels of nitrogen dioxide from vehicle emissions with high levels of agricultural emission of ammonia being the main cause of pollution around Burton Pedwardine (Defra, 2016). This ties in with the species shown to be significantly associated with the rural sample site as both *Staphylococcus auricularis* and *Streptococcus constellatus* have been shown to be associated with environments containing higher levels of ammonia in other studies (Ismail *et al.*, 2016). Significant clustering of rural and urban sampling locations was also observed in the combined data set for the species common to direct sampling and metagenomic analysis (Figure 15) further supporting the rejection of the null hypothesis for hypothesis two.

The urban versus rural comparison of the metagenomic data at the OTU level gave classes of species specifically associated with each, and in each case these are comprised of classes which are specifically associated with damaged or undamaged surfaces. Acidobacteria and Actinobacteria were both significantly associated with damaged surfaces and urban environment whereas Firmicutes, Thermotogae and TM7 were significantly associated with damaged surfaces and rural environments. Armatimonadetes, Planctomyces and Verrucomicrobia were significantly associated with both undamaged surfaces and urban environments. Bacteroidetes, Chlamydiae, Cyanobacteria and Lentisphaerae were significantly associated with undamaged surfaces and rural environments.

The differences between the rural and urban environments do not prevent analysis at the class level from clustering the damaged and undamaged surfaces together, with the noted exception of Lincoln Cathedral where the presence of Deferribacteres class species changing the grouping. This is of concern when considering that the current understanding of the limestone microbiome with regards to biodeterioration is almost entirely based on studies which, according to this studies definition, are classified as urban environments. While this study has only taken an initial look at

the differences between these environments, it is clear that the absence of studies encompassing unpolluted areas in the literature means that a large proportion of the microbiome is not being sampled and its role in biodeterioration is therefore unknown.

4 Characterisation of isolated bacteria

4.1 Introduction

Characterisation of basic capabilities of the isolated bacterium is essential for determining their role in the limestone microbiome. The analysis of growth and biofilm formation characteristics carried out in this chapter builds on the physical and biochemical characterisation in the last chapter creating a firm foundation for testing the isolates for their biodeteriorative potential and understanding the efficacy of biocides against them.

Despite the evidence for strain to strain variability in the general microbiology literature (Croes *et al.*, 2009; Kostaki *et al.*, 2012; Sant'Ana *et al.*, 2012), in this field there is a general assumption that because one strain in the species behaves in a certain way that all of them will show the same characteristics. With this in mind, and with the environmental data from the sampling showing a significant difference in pH between damaged and undamaged stone, analysis of the characteristics of the isolated species is a necessity.

In this chapter the isolated species are characterised for growth rate, biofilm formation and optimal growth pH. Growth rate and biofilm formation play a key role in colonisation of the surfaces (Garrett *et al.*, 2008) and access to this information helps to characterise the roles of the various species within the microbiome, for example faster growing species have the potential to dominate the microbiome depending on the development phase of the biofilm, and their location within the biofilm, which effects whether the bacterial cells are acting as R or K strategists (Davies *et al.*, 1998; Spormann, 2008). In addition to the growth characteristics the isolates were also tested for the metabolism of Industrial methylated spirits (IMS) as a sole carbon source, a metabolic function which was effecting the results of testing of IMS as a biocide.

Species which are not strong biofilm formers will rely on other species to maintain the matrix. The higher levels of biofilm produced by strong biofilm formers have the potential to more rapidly enhance physical weathering than the lower levels produced by weak biofilm formers, as low level production would take longer to reach quantities where the biofilm was enhancing physical stresses .

With optimal growth pH there is the potential that some species are producing acids or alkalising agents to modify their environment to gain a selective advantage by optimising the pH for their particular growth requirements. By looking at these 3 basic characteristics we can start to see the roles of the species within their environment.

4.2 Planktonic growth curves

Growth rate was analysed, with the time to mid log phase on the growth curve selected to be used as a comparative measurement, this was carried out by taking the mean of the growth curve and identifying the appropriate time point (Figure 16). The time taken to reach mid log growth phase varied between the strains isolated for each species and ranged from 12 hours for *Bacillus senegalensis* to 42.5 hours for *Psychrobacillus psychrodurans*.

As the isolates recovered from direct sampling will only be a subsection of the total copiotrophs within the microbiome it is possible that the results from the sample would not be representative of the total population. Normality testing provides a way to test whether the distribution of growth rates, or any characteristic of the organism; if the results show a normal distribution, it means that conclusions drawn from them would be applicable to the environmental population and to other studies (Ghasemi & Zahediasl, 2012). Therefore, normality was tested using Lilliefors normality test in R, as this is suitable for the sample size being tested (Ghasemi & Zahediasl, 2012).

The normality testing of the growth rates from the total isolates from the limestone microbiome gave a positive, significant, result for the total population (p-value < 0.01) demonstrating that these isolates show a distribution of growth rates which would be found in the total population of copiotrophs in the limestone microbiome.

To ensure that the growth rates measured were representative of the variations in the sampled populations found on damaged and undamaged surfaces, normality testing was carried out on two subsections of the total isolates. The total isolates found on damaged stone, that is the damaged population combined with the population found on both damaged and undamaged stone, and the total population of isolates found on undamaged stone, undamaged and both in (Figure 16), were tested. The normality testing on the total isolates from damaged stone gave a positive, significant, result (p-value < 0.01) demonstrating that the distribution of growth rates was within the range that would be found in the total population of copiotrophs on damaged limestone surfaces. The normality testing for the total isolates found on undamaged stone also gave a positive, significant, result (p-value < 0.01).

This confirms that the range of growth rates observed in the isolates from this study is an accurate representation of the growth rates in the total population of copiotrophs found in the limestone microbiome, and that the conclusions drawn from the analysis of these results can be applied to the whole population as well as the sampled isolates.

Inter-strain variability in growth rate can be observed in the species where multiple strains were recovered. The two *B. subtilis* strains showed the lowest variability with the fastest growing strain being 1.07 times faster than the slower growing strain, the *B. licheniformis* strains showed a difference between growth rates of 1.53 times, *B. pumilis* a difference of 1.58 times and *B. safensis* a difference of 2 times between growth rates. *B. muralis* was the only species where more than two strains were able to be tested, when compared to the slowest growing strain, *OX2D8, LCA1D6a* was 1.28 times faster and *WE2D1c* was 3.37 times faster.

When comparing the growth rates observed between populations isolated from damaged and undamaged stone, as well as those found on both, damaged stone was more likely to host species which took over 24 hours to reach mid log growth phase; 25% of the isolates, when compared to 17.6% for undamaged stone, and 10.3% compared to isolates that were found on both surfaces. The subsection of the population which took 12-24 hours to reach mid log growth phase was 41.6% for damaged and undamaged and 65% for those isolated from both surfaces. The isolates from damaged surfaces which showed the fastest growth rate made up 33% of the damaged population. The proportion of isolates from undamaged stone which showed the fastest growth rate made up 41.2% of that population. The isolates which were found on both damaged and undamaged surfaces had the lowest proportion of fast growers in the population with only 24.1%.

As previous studies have not looked at isolates from undamaged surfaces, no comparisons have been made between the distribution of growth rates for species between the damaged and undamaged populations. In order to determine whether there was a significant difference in the distribution of growth rates between the isolates from damaged and undamaged limestone surfaces, the data sets for each were tested to see whether it was skewed towards faster growth, slower growth or whether the distribution fitted a standard distribution.

The skewness measure for growth rate in the total damaged population, including isolates which were found on both damaged and undamaged stone, gave G_1 (skew) = 0.88 showing a slight skew in the data towards slower growing species, p-value = 0.05. There was no significant skew in the growth rate data for the total undamaged population.

To determine whether the isolates which were found on both surfaces were responsible for the damaged population demonstrating a skew towards slower growing species the data set was split in 3, damaged, undamaged and both, and retested.

The skewness measure for growth rate in the population isolated solely from undamaged stone remained insignificant. Isolates found on both surface also showed no significant skew. Isolates

solely found on damaged surfaces gave a statistically significant $G_1 = 0.967$, p-value = 0.05, showing an even higher skew towards slower growing species when the isolates found on both surfaces were removed from the data set. With the lower pH found on damaged stone, slower growth may well be an adaption to the more extreme environment (Bell, 2012).

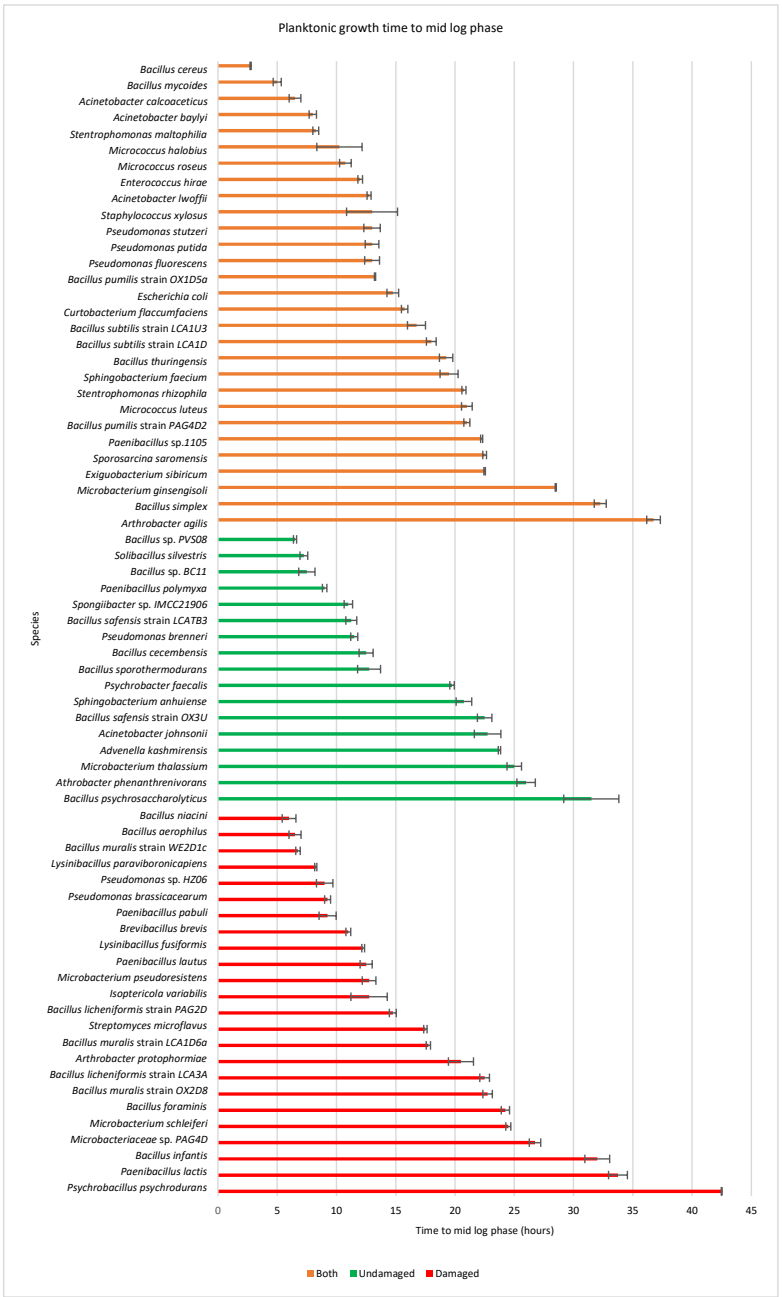


Figure 16: Time taken for species to reach mid log phase in their growth curve in hours. Data shown is the average of n=3. Slower growing species such as P. psychrodurans, P. lactis and B. infantis are more common in the damaged population (red), whereas the isolates from the undamaged population (green) and both surfaces (orange) showed a normal distribution of growth rates.

4.3 Biofilm formation

Biofilm formation was measured by growing the individual isolates using the "Calgary peg" plate system with a ratio of biofilm formation: negative control being used to allow direct comparison between plates. Biofilm formation ratios ranged from 36.49 for *P. psychrodurans* down to 1.02 for *S. saromensis* which was considered not to produce biofilm under the test conditions, the biofilm OD reading being less than 1 standard deviation from the control. All other isolates which gave a low ratio had OD readings which were distinctly separate from the controls by two standard deviations (n=3) and were therefore producing measurable amounts of biofilm.

Biofilm formation by isolated species was compared by grouping the species based on whether they had been isolated from damaged stone, undamaged stone or both (Figure 17).

The normality testing of the biofilm formation of the total isolates gave a positive, significant, result (p-value = 4.41×10^{-11}), showing that these isolates are representative of the range of biofilm formation capability in the total population of copiotrophs within the limestone microbiome. Breaking the population down into the total damaged and total undamaged populations was carried out for comparative analysis as per the analysis of the planktonic growth rates. The total damaged population gave positive, significant, results (p-value = 1.23×10^{-9}) and the distribution seen is therefore representative of the range seen in the population of copiotrophs found on damaged limestone. Similarly the total undamaged population also gave a positive, significant, result (p-value = 2.52×10^{-6}). This demonstrates that the rates observed in the isolates for the two sub populations are representative of the biofilm formation capacity for their respective surfaces in the total population and that the analysis of these results is applicable to the copiotrophs found on limestone.

Species which produced a ratio below 1.36 for biofilm formation, cut off marked on Figure 17, were considered as poor or non-biofilm forming. The cut-off point was calculated by taking the average of the lower quartile of the complete data set plus two times the standard deviation as per Croes et al. 2009.

Based on the cut off defined by Croes et al. 2009, 17.64% of species which were found undamaged stone were poor or non-biofilm formers. The isolates which were found on damaged surfaces, and those isolated from both damaged and undamaged stone, showed a percentage of poor or non-biofilm formers in the population which was much closer, 29.2% on damaged stone and 32% on both damaged and undamaged stone.

The presence of biofilm has been shown to enhance physical weathering of the limestone surface (Warscheid & Braams, 2000). As previous studies have not looked at isolates from undamaged surfaces, no comparisons have been made between the capacity for species to form biofilm between the damaged and undamaged populations. In order to determine whether there was a significant difference in biofilm formation between the isolates from the two surfaces, the distribution of rates of biofilm formation was tested for skew.

The skewness measure for biofilm formation in the total damaged population, including isolates from both damaged and undamaged stone, gave $G_1 = 2.59$ showing a skew in the data towards high biofilm formation, p-value = 0.05.

All isolates from undamaged surfaces, including isolates found on both surfaces, were tested for skewness giving $G_1 = 1.37$ showing a lesser skew towards high biofilm formation than the damaged population, p-value = 0.05.

In order to determine whether the isolates which were found on both surfaces were responsible for the undamaged population demonstrating a skew towards high biofilm formers the data set was split in 3, damaged, undamaged and both, and retested.

The population isolated solely from undamaged stone showed no significant variation from a normal distribution. The isolates from both surfaces gave a skew of $G_1 = 1.55$, p-value = 0.05, demonstrating a skew towards high biofilm formers.

The highest skew shown in biofilm formation was found in the isolates solely from damaged stone, $G_1 = 3.29$, p-value = 0.05. The skew in this data set was influenced by the extremely strong biofilm production of *P. psychrodurans*, removing this result from the data set dropped the figure to $G_1 = 1.3$, p-value = 0.05, which was still a significant skew towards high biofilm formers.

This study has therefore shown for the first time that the copiotroph population found as part of the limestone microbiome on damaged surfaces is significantly skewed towards high biofilm formers unlike the population found solely on undamaged stone.

To determine whether the differences in percentage of low biofilm formers between the 3 groups, damaged, undamaged and both, was significant, a comparison of the 3 populations using a one way ANOVA for independent samples was carried out, giving a p-value > 0.05 showing that the higher percentage of poor biofilm formers in the population found on undamaged stone surfaces was not significant.

Inter-strain variability in biofilm formation can be observed in the species where multiple strains were recovered. Unlike the results from the planktonic growth rate *B. pumilis* and *B. subtilis* showed the lowest variation between biofilm formation capacities with a difference of 1.2 between strains. *B. safensis* showed a difference of 1.5 between strains followed by *B. licheniformis* with a difference of 2.45. Finally *B. muralis* had the highest variation between strains, interestingly though in this case strain *OX2D8* was the outlier with a biofilm formation capacity approximately 5 times higher than the other two strains (6.46) when compared to *WE2D1c* (1.25) and *LCA1D6a* (1.3)

In order to better understand the basis of the differences between the microbiomes found on damaged and undamaged surfaces, and to determine whether the higher proportion of slower growing species found on damaged stone was linked to the larger proportion of high biofilm forming species on damaged stone, correlation testing was carried out. Biofilm formation and planktonic growth rate demonstrated a positive, significant correlation between the two data sets, correlation = 0.885, p-value = 2.2×10^{-16}.

Plotting growth rate against biofilm formation (Figure 18) demonstrates the correlation between the two, as well as providing a graphical representation of the skew that the isolates from the damaged surface and both surfaces show towards slow growth as well as towards high biofilm formation. Isolates from the undamaged surface demonstrate a linear trend between growth rate and biofilm formation as would be expected from with a normal distribution, the isolates from the damaged and both surfaces demonstrate a logarithmic trend representing the skew in the data.

To enhance the understanding of biofilm production by the species isolated, further correlation testing was carried out between biofilm formation levels and the physical and biochemical characteristics tested for in the last chapter. Of these only catalase production showed a significant positive correlation, cor = 0.23, p-value = 0.05. This is unlikely to be a causative correlation, as catalase secretion into the biofilm environment has been shown to enhance protection against hydrogen peroxide (Frederick *et al.*, 2001).

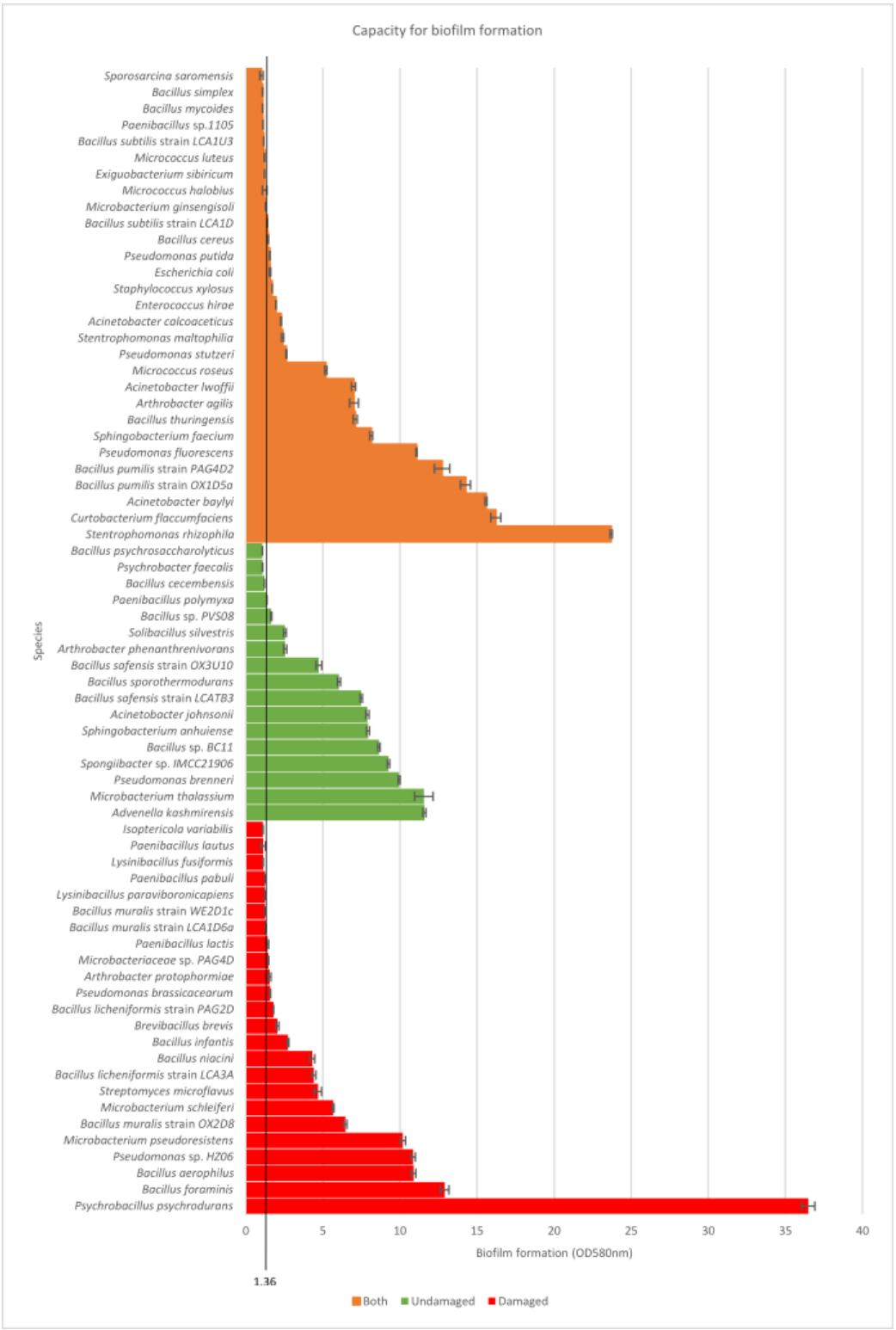


Figure 17: Biofilm formation capacity as a ratio of the OD580nm readings of the biofilm coated peg and the negative control peg. Data shown is average of n=3. Species below 1.36 are poor or non-biofilm formers. A positive skew towards high biofilm formers is present in the isolates from damaged surfaces (red) as well as those isolates from both surfaces (orange). Isolates from the undamaged surface (green) demonstrate a normal distribution in their ability to form biofilms.

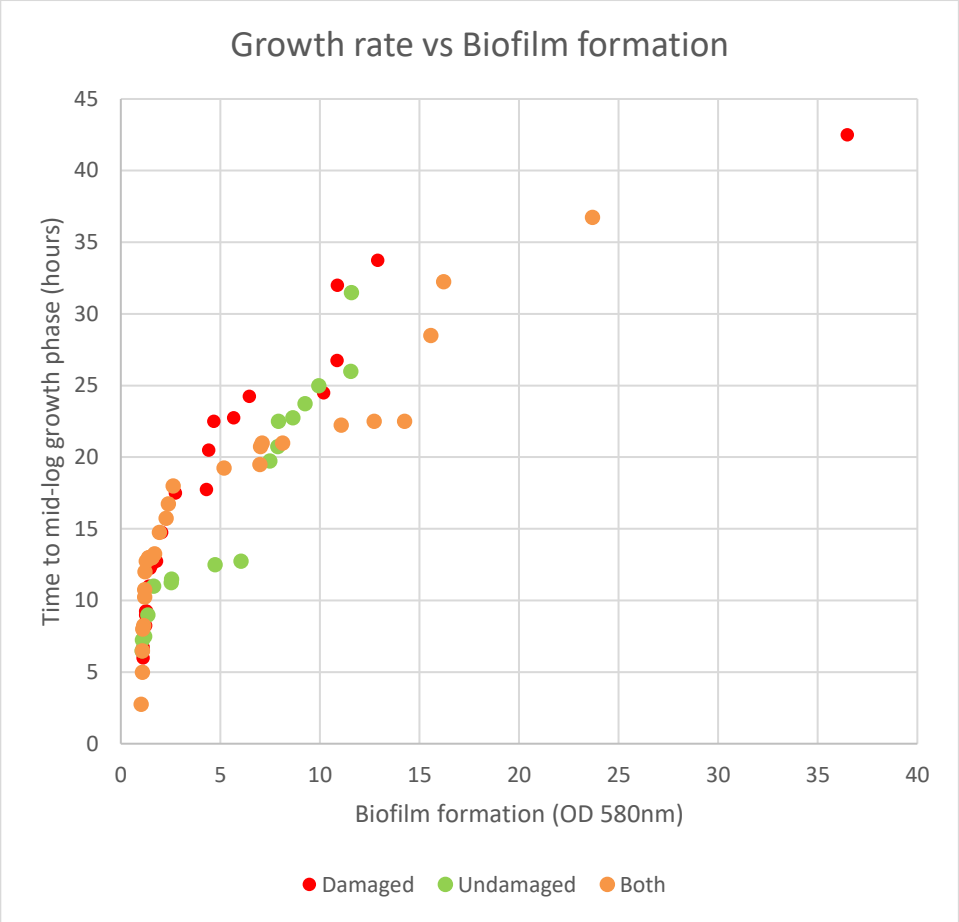


Figure 18: Growth rate to mid-log phase for the isolates plotted against biofilm formation. A low correlation was found between the two, R = 0.241652, with slower growing species demonstrably producing higher levels of biofilm than faster growing species. The skew towards slower growing species and high biofilm formation for isolates from damaged surfaces (red) and those found on both (green) becomes more apparent here, the plot for isolates from undamaged surfaces (green) shows a different distribution to damaged and both with the time to mid-log growth phase against biofilm formation demonstrating a more linear trend when compared to the curves produced by the others.

4.4 Optimal growth pH

Optimal growth pH was identified by calculating the time taken to reach mid log growth phase. The pH at which the isolate reached mid log growth phase first was identified as the optimal growth pH for that isolate. *Pseudomonas putida* and *Pseudomonas stutzeri* provide good examples of the differences between species with *P. putida* showing optimal growth between pH 6.6 and 7.6, whereas in a buffered environment *P. stutzeri* will barely grow at these pH's but shows optimal growth between pH 5 and 6 (Figure 19).

Optimal growth pH varied across the range tested in each grouping, damaged, undamaged and both (Table 18). *B. foraminis, L. parviboronicapiens* and *P. pabuli* would not grow in the presence of citrate phosphate buffer and so data points are not available for these 3 species.

The data sets showed normal distribution (p-value = 6.25×10^{-14}), the lowest optimal pH for species which grew across a wide range was used for this test. Distribution of optimal growth pH within the sampled isolates is therefore applicable to the copiotroph population in the limestone microbiome as are the results from further analysis.

Species which show a low optimal growth pH have the potential to acidify their environment in order to optimise their growth conditions and gain a competitive advantage against species which require a more neutral pH for optimal growth. In order to understand the distribution of optimal growth pH the data set was tested for skew to determine whether there was a significant skew towards species which had a low optimal growth pH in the isolates found on damaged stone (Hypothesis 6).

The total damaged population was skewed, $G_1 = -1.342$, p-value = 0.05, towards higher optimal growth pH. Analysis of the subpopulation found solely on damaged surfaces was also skewed towards higher optimal growth pH, $G_1 = -2.3$, p-value = 0.05.

Similarly the total undamaged population demonstrated a skew, G1 = -1, p-value = 0.05, towards higher optimal growth pH. The subpopulation found solely on undamaged surfaces was also skewed towards higher optimal growth pH, $G_1 = -1.33$, p-value = 0.05. Analysis of the subpopulation found on both damaged and undamaged stone showed no significant skew.

The majority of species which are solely found on damaged or undamaged stone have been shown to have a higher optimal growth pH, 6.8 - 7.6 (Table 18) and therefore those found on damaged stone are unlikely to be actively acidifying the environment.

No significant correlation between optimal growth pH and growth rate (p-value = 0.8416) or optimal growth pH and biofilm formation (p-value = 0.2916) was found. No significant correlations were found when comparing the optimal growth pH to physical and biochemical characteristics either. There was a significant, p-value <0.02, negative correlation between optimum pH and isolates found on both damaged and undamaged stone, these isolates were more likely to have an acidic optimum pH.

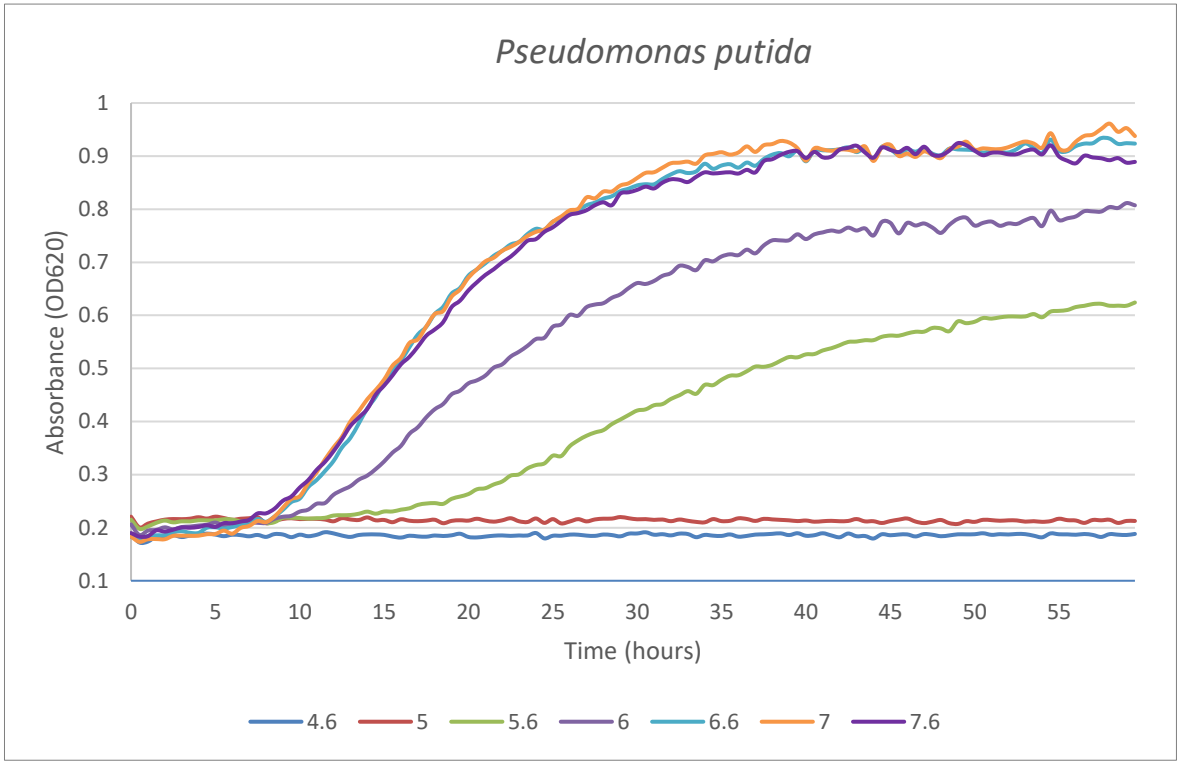
The species which show a broad range of optimal growth pH have the potential to modify the environment in order to obtain a selective advantage over other organisms. The majority of

isolates with this capability were in the population isolated from both surfaces, 34.5%, and those from undamaged surfaces, 30.7%. Only 3.6% of the isolates solely found on damaged surfaces had a broad range of optimal growth pH.

Four of the 30 isolates from the population isolated from both damaged and undamaged surfaces grew at a pH of 5.6 or below (Table 18), the range observed for damaged stone surfaces. *P. stutzeri* showed optimal growth between pH 5 and 6.6 as did *A. baylyi*. *Stentrophomonas rhizophila* showed a broad range for optimal growth between pH 5.6 and 7.6. *Micrococcus halobius* demonstrated the broadest range growing optimally at all pH's tested.

In the undamaged population, only 1 of the 17 grew at a pH of 5.6 or below, this was *Paenibacillus polymyxa* which showed an optimal growth range of between pH 5-6.

In the population found on damaged stone *Psychrobacillus psychrodurans* grew optimally across the whole range tested and demonstrated the ability to grow optimally in the range of surface pH's found on damaged stone. *Bacillus muralis* strain OX2D8 showed pH 5 as its optimal growth condition, also within the surface pH range for damaged stone.



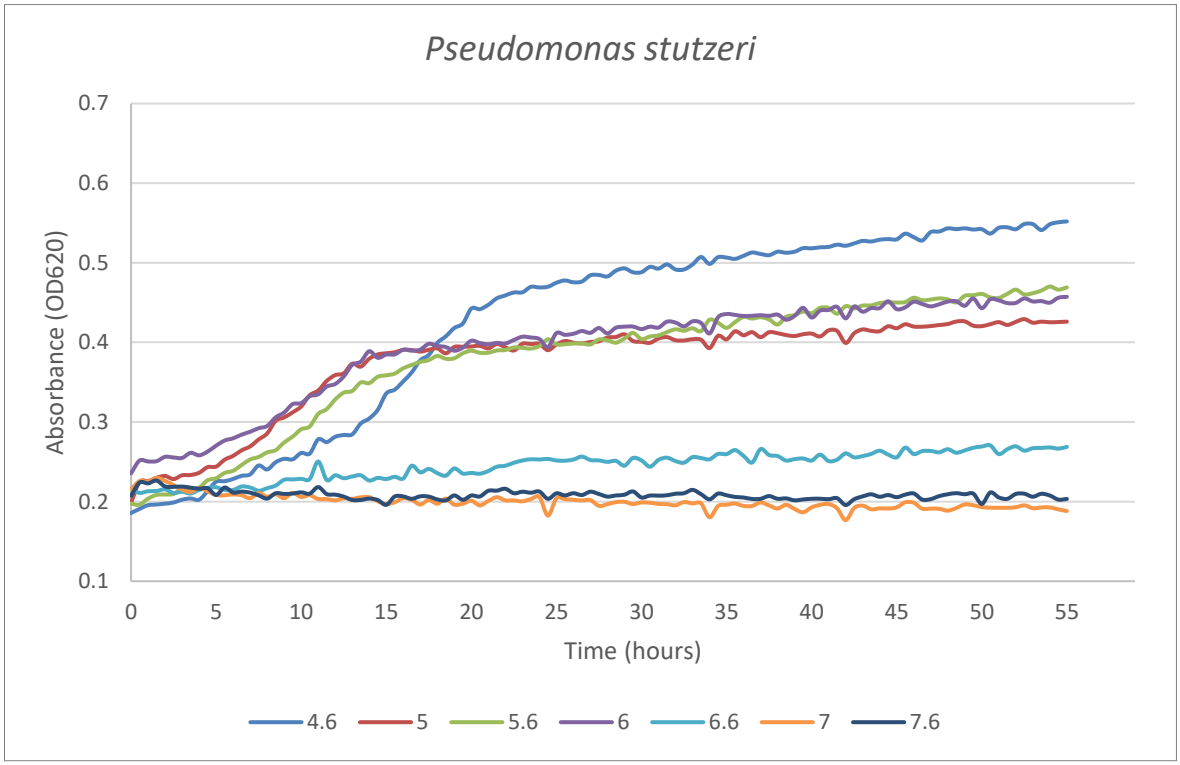


Figure 19: Optimal pH growth curves for Pseudomonas putida and Pseudomonas stutzeri, which demonstrates growth over a range of pH's including the typical growth lags seen at suboptimal pH. The optimum pH for growth is identified as the pH at which the growth curve reaches mid log growth phase the fastest. Where several growth curves reach mid log growth phase at the same time this is considered the optimal range. Data shown is average of n=3

Damaged	pH	Undamaged	pH	Both	pH
Psychrobacillus psychrodurans	4.6-7.6	*Paenibacillus polymyxa*	5-6	*Micrococcus halobius*	4.6-7.6
Bacillus muralis strain *OX2D8*	5	*Microbacterium ginsengisoli*	6-7.6	*Pseudomonas stutzeri*	5-6.6
Streptomyces microflavus	6.3	*Bacillus* sp. *BC11*	6.6-7.6	*Acinetobacter baylyi*	5-6.6
Bacillus niacin	6.6	*Bacillus thuringensis*	6.6-7.6	*Stentrophomonas rhizophila*	5.6-7.6
Pseudomonas brassicacearum	6.8	*Pseudomonas brenneri*	6.8	*Bacillus safensis* strain *OX3U10*	6-7.6
Acinetobacter johnsonii	7	*Sphingobacterium anhuiense*	6.8	*Paenibacillus* sp.*1105*	6
Lysinibacillus fusiformis	7	*Advenella kashmirensis*	7	*Pseudomonas fluorescens*	6-7.6
Microbacterium thalassium	7	*Bacillus cecembensis*	7	*Sporosarcina saromensis*	6
Bacillus muralis strain *LCA1D6a*	7.3	*Bacillus* sp. *PVS08*	7	*Sphingobacterium faecium*	6-7.6
Bacillus muralis strain *WE2D1c*	7.3	*Arthrobacter phenanthrenivorans*	7.6	*Arthrobacter agilis*	6.6
Microbacterium schleiferi	7.3	*Psychrobacter faecalis*	7.6	*Enterococcus hirae*	6.6-7.6
Arthrobacter protophormiae	7.6	*Solibacillus silvestris*	7.6	*Escherichia coli*	6.6-7.6
Bacillus aerophilus	7.6	*Spongiibacter* sp. *IMCC21906*	7.6	*Pseudomonas putida*	6.6-7
Bacillus infantis	7.6			*Stentrophomonas maltophilia*	6.6-7.6
Bacillus licheniformis strain *LCA3A7*	7.6			*Acinetobacter calcoaceticus*	7
Bacillus licheniformis strain *PAG2D*	7.6			*Micrococcus luteus*	7
Bacillus psychrosaccharolyticus	7.6			*Staphylococcus xylosus*	7
Bacillus sporothermodurans	7.6			*Acinetobacter lwoffii*	7.3
Brevibacillus brevis	7.6			*Bacillus cereus*	7.3
Isoptericola variabilis	7.6			*Exiguobacterium sibiricum*	7.3
Microbacteriaceae sp. *PAG4D*	7.6			*Bacillus mycoides*	7.6
Microbacterium pseudoresistens	7.6			*Bacillus pumilis* strain *OX1D5a*	7.6
Paenibacillus lactis	7.6			*Bacillus pumilis* strain *PAG4D2*	7.6
Paenibacillus lautus	7.6			*Bacillus safensis* strain *LCATB3*	7.6
Pseudomonas sp. *HZ06*	7.6			*Bacillus simplex*	7.6
Bacillus foraminis	NA			*Bacillus subtilis* strain *LCA1D9*	7.6
Lysinibacillus parviboronicapiens	NA			*Bacillus subtilis* strain *LCA1U3*	7.6
Paenibacillus pabuli	NA			*Curtobacterium flaccumfaciens*	7.6
				Micrococcus roseus	7.6

Table 18: Optimal growth pH for species based on time taken to reach mid-log phase of growth in nutrient broth buffered with phosphate citrate buffer. Optimal growth pH datum is not available for the species with NA in the pH column as they would not grow in the presence of phosphate citrate buffer. Species shaded blue grew optimally across a range of two or more pH units, as can be seen this ability was most common in species which were isolated from both damaged and undamaged surfaces.

4.5 Metabolism of Industrial methylated spirits as a sole carbon source

In order to investigate the ability of the species to metabolise IMS, ⅓ of the isolates were tested for the ability to metabolise IMS as a sole carbon source and a bioinformatics search performed for alcohol dehydrogenase genes for each isolate. All species were later tested at sub-lethal levels of IMS, in parallel with the biocide testing to determine whether they could tolerate or metabolise IMS in the presence of a different carbon source.

Of the 64 species in this study 15 showed no evidence of alcohol dehydrogenase genes in the NCBI database; none of these had had their genomes sequenced and the majority were solely represented by 16S rRNA gene fragments, so the lack of alcohol dehydrogenase genes in the database is understandable. Of the 50 isolates where alcohol dehydrogenase genes were identified, the lowest number of alcohol dehydrogenase genes identified was one, *A. baylyi,* with most species having several members of the alcohol dehydrogenase family of genes each serving different a metabolic function.

24 of the 64 species were tested for the capability to utilise IMS as a carbon source for metabolism, selection was based on habituation in the presence of IMS, resistance to IMS when grown in a biofilm or resistance of the biofilm matrix to cleaning by IMS. *Pseudomonas putida* and *Pseudomonas fluorescens* were included as controls which had shown none of these characteristics.

Figure 20 shows typical growth curves for species which were unable to metabolise IMS, *Curtobacterium flaccumfaciens*, and those which were able to metabolise IMS, *P. brenneri*. In both cases growth is better supported by nutrient broth as this is formulated to provide optimal growth conditions. In all cases where the isolate was able to utilise IMS as a carbon source the growth curve was slower than nutrient broth or M9 salts with glucose and failed to reach the same density of growth.

15 of the species tested showed the ability to utilise 2% IMS as a sole carbon source (Table 19). Interestingly only 12 species were capable of utilizing 0.1% IMS. Two species, *Bacillus* sp*. PVS08* and *Psychrobacillus psychrodurans* showed no growth in M9 minimal media with either glucose or IMS as a carbon source.

Tolerance of IMS in the presence of a different carbon source was demonstrated for all but 5 of the isolates (Table 20), namely *B. niacini, C. flaccumfaciens, P.* sp*. 1105, P. polymyxa* and *M.*

halobius. On average species showed tolerance to 4.5% IMS with the median being 4%. The lowest level of tolerance observed was growth in the presence of 2% IMS.

Strain to strain variation was observed in all cases except *B. subtilis*. For *B. safensis, B. licheniformis* and *B. pumilis* one strain showed tolerance at 4% and the other at 8%. With *B. muralis* where 3 strains were tested both, *LCA1D6a* and *OX2D8* showed tolerance at 2% with *WE2D1c* showing tolerance at 4%.

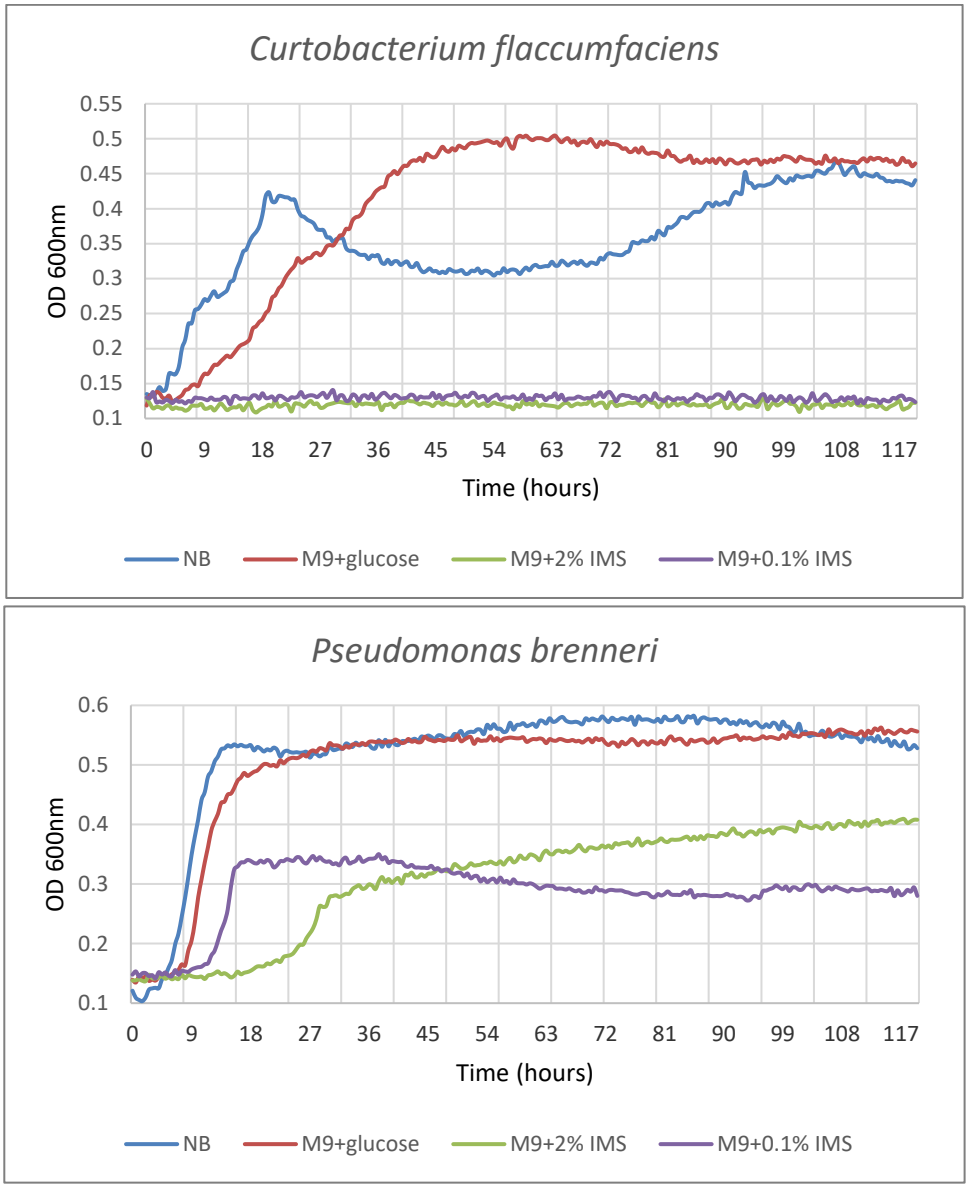


Figure 20: Growth curves for Pseudomonas brenneri and Curtobacterium flaccumfaciens demonstrating growth, or lack thereof, when utilising IMS as a sole carbon substrate. Data shown is average of n=3.

Species	Number of alcohol dehydrogenase genes on NCBI	Genome present on NCBI	metabolism of 2% IMS	metabolism of 0.1% IMS
Acinetobacter johnsonii	8	yes	Yes	yes
Arthrobacter agilis	0	no	Yes	yes
Arthrobacter phenanthrenivorans	45	yes	Yes	yes
Bacillus muralis strain *WE2D1c*	18	yes	Yes	yes
Bacillus pumilis	8	yes	Yes	yes
Bacillus safensis	5	yes	Yes	no
Bacillus safensis strain *LCATB3*	6	yes	Yes	no
Bacillus sp. *BC11*	0	no	Yes	no
Bacillus sp. *PVS08*	0	no	No	no
Bacillus thuringensis	0	no	yes	no
Brevibacillus brevis	4	yes	yes	yes
Curtobacterium flaccumfaciens	11	yes	No	no
Escherichia coli	72	yes	yes	yes
Microbacterium ginsengisoli	7	no	No	no
Microbacterium schleiferi	0	no	No	no
Pseudomonas brenneri	5	yes	yes	yes
Pseudomonas fluorescens	97	yes	yes	yes
Pseudomonas putida	107	yes	No	yes
Psychrobacillus psychrodurans	0	no	No	no
Solibacillus silvestris	9	yes	yes	yes
Sphingobacterium anhuiense	0	no	No	no
Sphingobacterium faecium	0	no	No	no
Spongiibacter sp. *IMCC21906*	0	no	No	yes
Streptomyces microflavus	0	no	yes	no

Table 19: Species tested for the ability to metabolise IMS as a sole carbon source showing the compiled data from bioinformatics search and IMS metabolism testing. Half of the species where there was no evidence of alcohol dehydrogenase genes in the bioinformatics search demonstrated the ability to grow using IMS as a sole carbon source, for example Streptomyces microflavus. With the exception of Curtobacterium flaccumfaciens and Microbacterium ginsengisoli, all of the species which were identified in the bioinformatics search as having alcohol dehydrogenase genes demonstrated the ability to grow using IMS as a sole carbon source under experimental conditions.

Damaged	IMS tolerance (%)	Undamaged	IMS tolerance (%)	Both	IMS tolerance (%)
Acinetobacter johnsonii	8	*Advenella kashmirensis*	8	*Acinetobacter baylyi*	2
Arthrobacter protophormiae	8	*Arthrobacter phenanthrenivorans*	4	*Acinetobacter calcoaceticus*	8
Bacillus aerophilus	4	*Bacillus cecembensis*	8	*Acinetobacter lwoffii*	8
Bacillus foraminis	2	*Bacillus* sp. *BC11*	8	*Arthrobacter agilis*	4
Bacillus infantis	8	*Bacillus* sp. *PVS08*	4	*Bacillus cereus*	16
Bacillus licheniformis strain *LCA3A7*	4	*Bacillus thuringiensis*	4	*Bacillus mycoides*	16
Bacillus licheniformis strain *PAG2D*	8	*Microbacterium ginsengisoli*	4	*Bacillus pumilis* strain *OX1D5a*	8
Bacillus muralis strain *LCA1D6a*	2	*Paenibacillus polymyxa*	0	*Bacillus pumilis* strain *PAG4D2*	4
Bacillus muralis strain *OX2D8*	2	*Pseudomonas brenneri*	2	*Bacillus safensis* strain *LCATB3*	4
Bacillus muralis strain *WE2D1c*	4	*Psychrobacter faecalis*	8	*Bacillus safensis* strain *OX3U10*	8
Bacillus niacin	0	*Solibacillus silvestris*	4	*Bacillus simplex*	2
Bacillus psychrosaccharolyticus	64	*Sphingobacterium anhuiense*	4	*Curtobacterium flaccumfaciens*	0
Bacillus sporothermodurans	8	*Spongiibacter* sp. *IMCC21906*	8	*Escherichia coli*	2
Bacillus subtilis strain *LCA1D9*	4			*Exiguobacterium sibiricum*	4
Bacillus subtilis strain *LCA1U3*	4			*Micrococcus halobius*	0
Brevibacillus brevis	4			*Micrococcus luteus*	4
Enterococcus hirae	4			*Micrococcus roseus*	32
Isoptericola variabilis	2			*Paenibacillus* sp.*1105*	0
Lysinibacillus fusiformis	4			*Pseudomonas fluorescens*	4
Lysinibacillus parviboronicapiens	2			*Pseudomonas putida*	2
Microbacteriaceae sp. *PAG4D*	4			*Pseudomonas stutzeri*	4
Microbacterium pseudoresistens	8			*Sphingobacterium faecium*	2
Microbacterium schleiferi	2			*Sporosarcina saromensis*	8
Microbacterium thalassium	4			*Staphylococcus xylosus*	4
Paenibacillus lactis	4			*Stentrophomonas maltophilia*	4
Paenibacillus lautus	4			*Stentrophomonas rhizophila*	2
Paenibacillus pabuli	4			*Streptomyces microflavus*	8
Pseudomonas brassicacearum	2				
Pseudomonas sp. *HZ06*	2				
Psychrobacillus psychrodurans	4				

Table 20: Percentage of IMS at which isolates are capable of growth.

4.6 Discussion

Analysis of the growth rate and biofilm formation ratios for the isolated species further supported the isolates as being representative of the environmental copiotroph population as both data sets showed normal distribution.

The different growth rates observed between strains was within the range seen in the literature for other species (Sant'Ana *et al.*, 2012) except for *B. muralis* strain *WE2D1c*. It is interesting to note that *B. muralis* strain *WE2D1c* was also the strain which showed evidence of having taken up DNA from other species, specifically the 16S rRNA genes from *B. simplex* and *C. freundii*. It is likely that additional genes were also integrated into the genome of this strain, potentially giving it a selective advantage as far as growth rate is concerned.

Variation in growth rate between populations has also been observed. The damaged stone environment, because of the lower average pH, can be considered a more extreme environment when compared to the undamaged stone environment where the average pH is closer to the bacterial physiological norm. Slower growing species are often found in more extreme environments (Bell, 2012) so the difference observed between the growth rates of damaged and undamaged surfaces is not unexpected.

Biofilm formation showed normal variation between the species, a subsection of the population were weak biofilm formers with *Sporosarcina saromensis* demonstrating no significantly measureable biofilm formation. 18 species were classified as showing weak biofilm formation under laboratory test conditions, although the test conditions do provide an environment optimised for growth which in some cases could result in a suppression of biofilm formation (Hsueh *et al.*, 2006; Zhang *et al.*, 2014). With only *S. saromensis* identified as being a non-biofilm forming species, the null hypothesis for hypothesis 3, that biofilm production will not be observed in the majority of species, can be rejected. While the weak biofilm formers are less likely to be contributing to enhanced physical weathering, species such as *P. psychrodurans, B. foraminis, S. rhizophila, C. flaccumfaciens, A. baylyi* and *B. pumilis*, all of which are strong biofilm formers, are likely to be contributing to the physical weathering of the stone surface through the production of biofilm matrix.

While it is likely that biofilm formers are the early bacterial colonisers, none of the current studies in the literature identified the species colonising the surfaces, just that they were present (Saiz-Jimenez, 1984; Viles & Gorbushina, 2003). This study provides a foundation for the future analysis of early colonisers by having characterised the species isolated for biofilm forming capability.

The link in the literature between enhanced physical and chemical weathering and biofilm production is supported by the skew measured in the damaged population towards high biofilm formers, and the skew measured towards high biofilm formation for those isolates found on both, when no significant skew is observed in the population isolated solely from undamaged surfaces.

The low correlation between biofilm formation and growth rate means that growth rate is one factor of many in biofilm formation rate but a significant component of it. When looking at the growth rates between the populations, 50% of isolates from each population showed a growth rate to mid log phase of 12-24 hours under laboratory conditions, with 26-36% of the population growing faster than this. With 70% upwards of the population showing a fast to medium growth rate, maintenance of the protective biofilm matrix is assured as is a healthy microbial environment.

While most physical and biochemical properties showed no significant correlations with biofilm formation, catalase production showed a low, 0.23, but significant (p-value = 0.05) positive correlation with biofilm formation. As discussed earlier the secretion of catalase into the biofilm matrix plays a part in protecting the cells from environmental hydrogen peroxide and is therefore not a causative correlation.

In close to half the species the optimal growth pH was not in the pH range found at the surface of damaged and undamaged stone, the fact that the species are growing in a biofilm matrix in their natural environment provides a probable explanation for this discrepancy as pH can vary significantly within the biofilm environment (Alan *et al.*, 1999; Babauta *et al.*, 2012). Another explanation for the observed discrepancy is that the metabolic pathways active in the bacterial cell effect the homeostasis of the cytoplasm and therefore the ability of the cell to survive in different pH's (Booth, 1985), thus the standardised testing of the wide range of bacterial species found in this study could have pushed many of the species into using metabolic pathways which effected their tolerance of low environmental pH.

With damaged surfaces while the population as a whole was not creating the more acidic environment found when measuring the pH of the stone surface, potential candidates for engineering the pH of the damaged stone were identified. Based upon the optimal growth pH testing data these were *P. psychrodurans,* pH 4.6-7.6, and *B. muralis* strain *OX2D8*, pH 5.

In the population isolated solely from undamaged stone the majority of the species show optimal growth ranges above pH 6. The sole exception to this is *Paenibacillus polymyxa* which has an optimum growth range between pH 5 and pH 6. *Paenibacillus polymyxa* was isolated from LCA-

4U, the copper staining under the Cathedral's main window, which had a pH of 5, the lowest found for any of the undamaged surfaces, and due to the copper staining could be considered atypical when compared to the other undamaged sampling sites.

The isolates from both damaged and undamaged stone had the highest number of species with wide optimum growth ranges, 10 out of the 29 isolates. Four of the 10 species which had wide optimal growth ranges were able to grow optimally in the pH range found on damaged stone surfaces in the initial sampling. *P. stutzeri* and *M. halobius* demonstrated optimal growth ranges which went from pH 4.6 up, *A. baylyi* and *S. rhizophila* demonstrated optimal growth ranges which went from pH 5.6 up. There is the potential for these species, having been introduced to a stable, undamaged, environment, to actively acidify the environment and thus create a situation in which they have a competitive advantage. This is supported by the significant negative correlation demonstrated between optimum growth pH and the species isolated from both damaged and undamaged surfaces.

When considering the problems with the effects of the chemical buffer on the growth media, and the growth media potentially initiating metabolic pathways that alter tolerance for low pH environments, an alternative method would have been running these experiments in a chemostat. Growing the isolates in a chemostat would control of the environment pH without using a buffer. This method was rejected as running all 70 isolates at 7 different pH's with 3 repeats allowing for 3 days per growth curve would have taken just over 12 years.

The ability to metabolise IMS was tested in order to address issues with biocide testing in the spot plate format. Fifty of the 64 species isolated had positive identification of alcohol dehydrogenase genes in a bioinformatics search, the remaining 15 were only present in the database as 16S rRNA genes. Of the 24 species tested for the ability to metabolise IMS all of the species, with the exception of *Curtobacterium flaccumfaciens* and *Microbacterium ginsengisoli*, which had scored positively in the bioinformatics search were able to utilise IMS as a sole carbon source. Of the remaining ten species, which showed no evidence of alcohol dehydrogenase genes in the bioinformatics search, 4 were able to utilise IMS as a sole carbon source. Based on these results 86% of the species isolated should be able to metabolise IMS as a sole carbon source, all but 5 of the 64 species (92%) showed the ability to tolerate IMS in the presence of an alternative carbon source which supports the bioinformatics and sole carbon source test results.

5 Bacterial biocorrosion of limestone

5.1 Introduction

As was discussed in the introduction only 2 of the 27 papers which make up the current literature on the biodeterioration of limestone tested species to determine whether they had the potential to cause biocorrosion. While a further 7 studies carried out testing for biodeteriorative potential they failed to identify the isolates to a species level, and therefore made sweeping statements about the genus or family to which the isolates belonged.

The mechanisms of deterioration most commonly investigated are the acceleration of physical weathering by the biofilm matrix, and weakening of the stone matrix through biocorrosion (Doehne & Price, 2010). As all species which are biofilm formers will have the potential to accelerate the effects of physical weathering to a degree (Warscheid & Braams, 2000), and the majority of the species in this study have been shown to produce biofilms, this chapter therefore characterises the species for biocorrosive properties. In the literature to date two approaches have been taken, testing for dissolution of calcium carbonate in an agar based medium (Di Bonaventura *et al.*, 1999), and direct culturing on limestone blocks followed by direct observation using scanning electron microscopy (Herrera & Videla, 2009).

Researchers in this field have a tendency to rely on the literature as to whether bacterial species produce organic acids, or not. They therefore do not take into account the possibility of strain to strain variation, or that the acid production pathways tested for in the biochemical characterisation of the species may not be active under the metabolic constraints imposed by the limestone environment.

In this study both of the commonly used tests have been carried out as well as a third, assessing the isolates modification of the pH of growth media as a function of the growth cycle. Identifying the modification of the environment as an aspect of the growth cycle can be used to rapidly characterise the isolates for organic acid production, as well as looking at differences in the acidification or alkalisation of the environment between different sampling environments.

5.2 pH modification of the environment.

The 70 isolates were tested for pH modification of their environment, as this will show whether they have the potential to cause chemical damage to limestone through corrosion of the stone

substrate. Figure 21 shows typical results for species which acidified, alkalised, had no effect or did both. Acidification and alkalisation were characterised for each species by calculating the area under the growth curve as described in Figure 21, from the actual data, under which these conditions occurred. Measurements which were below two times the standard error of the negative control were eliminated from the growth curve analysis. Acidification and alkalisation were then characterised as strong or weak with the cut off as the mean of the lower quartile plus two times the standard deviation of the lower quartile (Croes *et al.*, 2009).

Of the 70 isolates tested only 8 demonstrated no modification to the pH of the environment over the growth curve. Seventeen isolates acidified the environment at some stage of the growth curve, and 32 isolates alkalised their environment. The remaining 13 isolates acidified and alkalised their environment at different stages of the growth cycle.

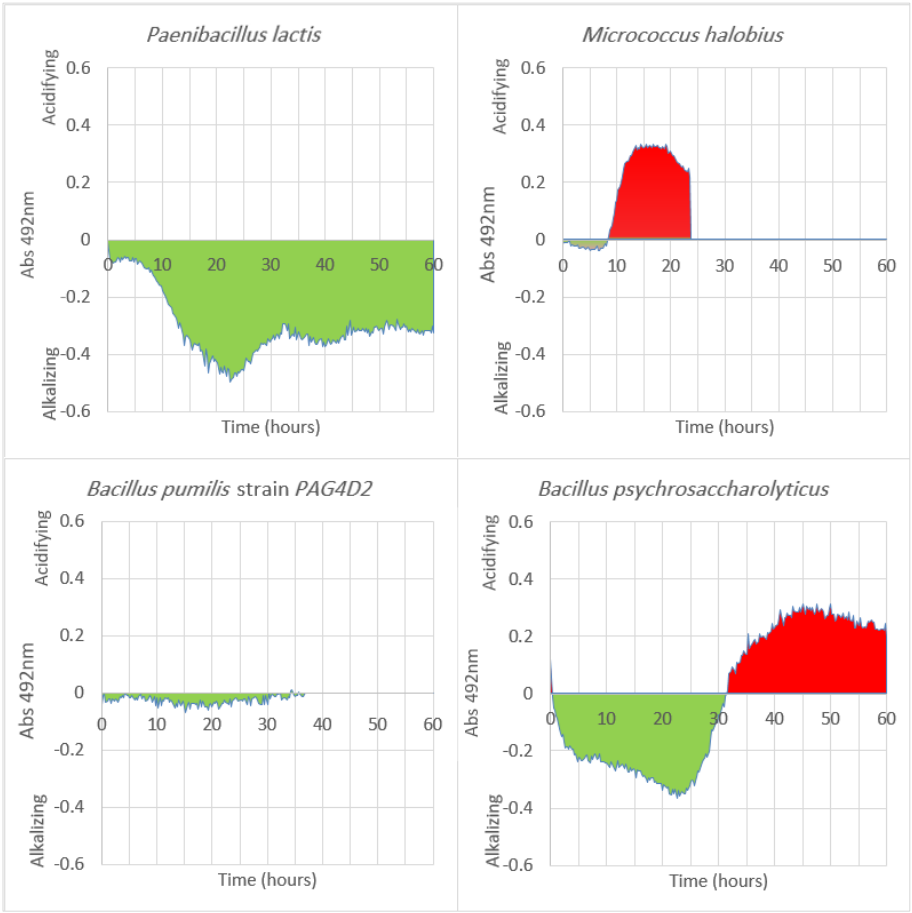


Figure 21: Examples of acidification and alkalisation growth curves from the Bromocresol Purple media; these are calculated as the difference in absorbance when compared to the control culture resulting in a negative signal for alkalization (green area) and a positive signal for acidification (red area). The area of acidification or alkalization under the curve is then calculated to allow comparisons to be made between species. Data shown is the average of n=3.

Inter-strain variability was observed in *B. licheniformis, B. muralis and B. safensis* (Table 21). *Bacillus licheniformis* strain *PAG2D* showed weak acid production, whereas strain *LCA3A7* showed a strong alkalisation of the media. *Bacillus muralis* strains also showed variation, *LCA1D6a* was a strong alkaliser*, OX2D8* acidified more than alkalised and *WE2D1c* was weak but equal in acidification and alkalisation. Both *Bacillus safensis* strains were alkalisers, but *LCATB3* (10) was very weak compared with *OX3U10* (209.3).

In order to better characterise the distribution of acidifiers and alkalisers in the population the data set was subdivided into isolates solely found on damaged surfaces, those solely found on undamaged surfaces and those found on both. As the populations were different sizes the analysis is expressed as percentages, Figure 22.

In the 3 populations there was very little difference in the percentage of the population which acidified the environment, 21.4% for isolates from damaged surfaces, 23.1% for isolated from undamaged surfaces, and 27.6% for the isolates found on both.

There was no difference between isolates which only alkalised the environment between the isolates from damaged and undamaged surfaces, both were 46%. The population found on both surfaces which alkalised the environment was slightly lower at 44%.

The difference between populations becomes more distinct when the weak acidifiers or alkalisers are excluded from the analysis. The percentage of isolates from damaged surfaces and the isolates from both surfaces which acidify their environment remains at the same percentage. The percentage of isolates from undamaged surfaces drops from 23.1% to 7.8%.

The isolates which were identified as being solely alkalisers drop in all 3 populations by similar amounts, 46.4% to 39.3% for isolates from damaged surfaces, 46.2% to 38.5% for isolates from undamaged surfaces and 44.9% to 37.9% for the isolates found on both surfaces.

A similar change in profile to that demonstrated for alkalisers is seen for those species which both acidify and alkalise. For isolates from damaged surfaces the percentage of the population changes from 25% to 14.3%. Isolates from undamaged surfaces change from 15.4% to 7.7%, and those found on both surfaces drop from 13.8% to 6.9% for both.

The isolates which are considered to be neither acidifying nor alkalising the environment rises from 7.1% to 25% for the species solely isolated from damaged surfaces. For undamaged surfaces the percentage of isolates which are not acidifying or alkalising increases from 15.4% to 46.2%.

Finally the isolates which are found on both damaged and undamaged surfaces increases from 13.8% to 27.6%.

When comparing the acidification or alkalisation of media to the physical and biochemical characteristics which were tested, there was a significant (p-value = 0.04) positive correlation, cor = 0.25, between spore formation and alkalisation, with 26 of the 36 spore forming bacteria alkalising the environment and 45 of the total 70 isolates being alkalisers. None of the other physical or biochemical characteristics gave a significant correlation and neither did the surface the isolates were retrieved from.

Therefore the isolates from the damaged surfaces and those isolated from both damaged and undamaged surfaces had a higher percentage of strong acidifiers in the population than those isolated from undamaged surfaces.

For isolates which only caused strong acidification of the environment during the growth curve, those found solely on damaged surfaces were present in significantly, p-value = 0.05 Chi-squared test, higher numbers than those found solely on undamaged surfaces. Isolates which only caused strong acidification of the environment which were found on both damaged and undamaged surfaces were also significantly, p-value = 0.01, higher than those found solely on undamaged surfaces. There was no significant difference between alkalisation for the 3 populations.

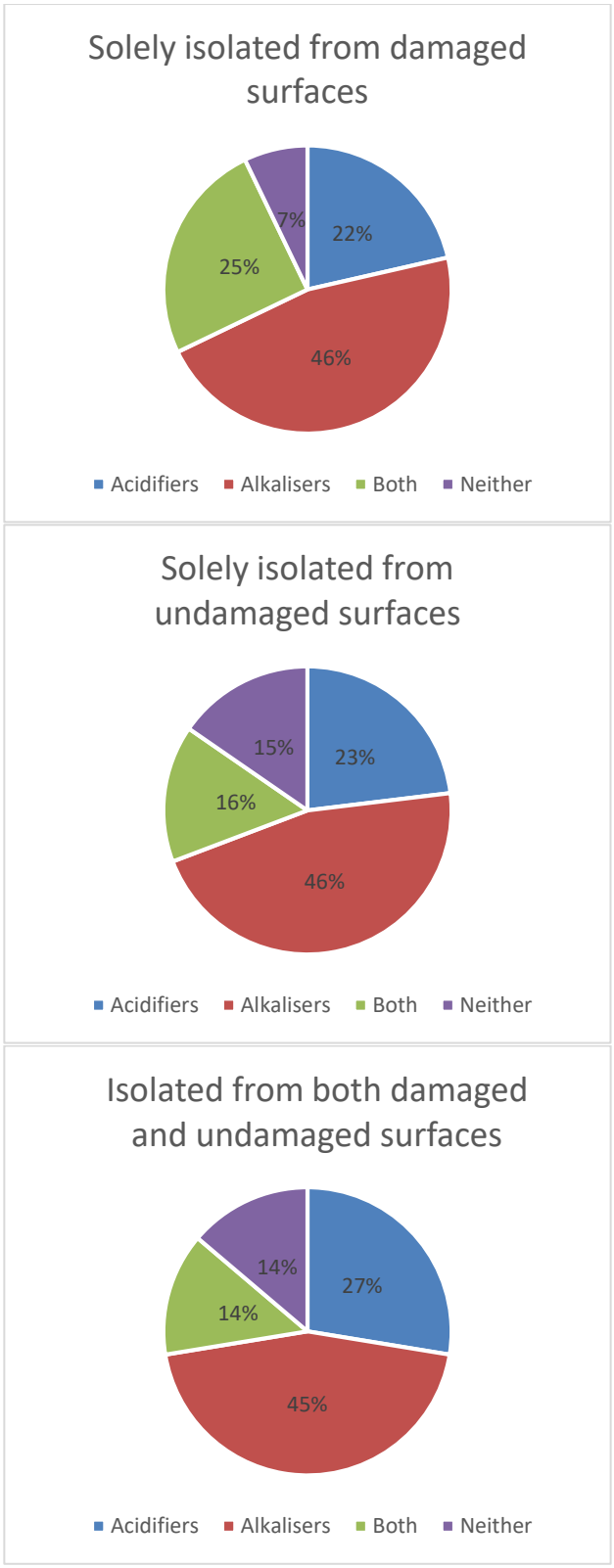


Figure 22: Distribution of isolates which are acidifiers (blue), alkalisers (red) and those which do both (green) or neither (purple), across the three populations of the microbiome.

Damaged	Area of acidity	Area of alkalinity	Undamaged	Area of acidity	Area of alkalinity	Both	Area of acidity	Area of alkalinity
Acinetobacter johnsonii	112.2		*Advenella kashmirensis*		166.6	*Acinetobacter baylyi*		677.8
Arthrobacter protophormiae		340.2	*Arthrobacter phenanthrenivorans*	924.8	87.5	*Acinetobacter calcoaceticus*	191	
Bacillus aerophilus	705.4	10.4	*Bacillus cecembensis*	3.24		*Acinetobacter lwoffii*		224.7
Bacillus foraminis		564.9	*Bacillus* sp. *BC11*		136.6	*Arthrobacter agilis*	84.716	
Bacillus infantis	203.1	492.8	*Bacillus* sp. *PVS08*		10.6	*Bacillus cereus*	93.15	
Bacillus licheniformis strain *LCA3A7*		121.6	*Bacillus thuringiensis*		240.4	*Bacillus mycoides*		
Bacillus licheniformis strain *PAG2D*	16		*Microbacterium ginsengisoli*		112.1	*Bacillus pumilis* strain *OX1D5a*		
Bacillus muralis strain *LCA1D6a*		157.3	*Paenibacillus polymyxa*	8.78		*Bacillus pumilis* strain *PAG4D2*		
Bacillus muralis strain *OX2D8*	95.5	59.1	*Pseudomonas brenneri*		167	*Bacillus safensis* strain *LCATB3*		10
Bacillus muralis strain *WE2D1c*	20.9	21.4	*Psychrobacter faecalis*			*Bacillus safensis* strain *OX3U10*		209.3
Bacillus niacini			*Solibacillus silvestris*	24.1		*Bacillus simplex*	167	134.4
Bacillus psychrosaccharolyticus	391.7	419.5	*Sphingobacterium anhuiense*			*Bacillus subtilis* strain *LCA1D9*		1448.2
Bacillus sporothermodurans		38.4	*Spongiibacter* sp. *IMCC21906*	279.3	1.2	*Bacillus subtilis* strain *LCA1U3*		1095.7
Brevibacillus brevis	5.26					*Curtobacterium flaccumfaciens*		155.9
Isoptericola variabilis		131.8				*Enterococcus hirae*		1.43
Lysinibacillus fusiformis	186.6					*Escherichia coli*	1035.3	15.4
Lysinibacillus parviboronicapiens	211.3	23				*Exiguobacterium sibiricum*	241	
Microbacteriaceae sp. *PAG4D*		36.23				*Micrococcus halobius*	22.9	
Microbacterium pseudoresistens	389.74					*Micrococcus luteus*	92.3	
Microbacterium schleiferi	48.4	82.5				*Micrococcus roseus*		443.6
Microbacterium thalassium		77.6				*Paenibacillus* sp.*1105*	93	
Paenibacillus lactis	170.2					*Pseudomonas fluorescens*		196.7
Paenibacillus lautus		1085.3				*Pseudomonas putida*	943.2	33.9
Paenibacillus pabuli		298.2				*Pseudomonas stutzeri*		
Pseudomonas brassicacearum		59.3				*Sphingobacterium faecium*		116.5
Pseudomonas sp. *HZ06*		89.2				*Sporosarcina saromensis*		99.16
Psychrobacillus psychrodurans						*Staphylococcus xylosus*	2.46	
Streptomyces microflavus		245.8				*Stentrophomonas maltophilia*	320.2	66.23
						Stentrophomonas rhizophila		279.5

Table 21: Area of acidification or alkalisation for all isolates, divided into damaged, undamaged and both categories based on the surfaces the species were isolated from. Isolates with no results did not modify the pH of the growth media. The area of acidity or alkalinity was calculated from the growth curves produced in the presence of Bromocresol purple as described earlier and shown in Figure 21.

5.3 Bacterial dissolution of calcium carbonate

Bacterial dissolution of calcium carbonate was observed as an endpoint experiment using solid growth media which is made opaque by the presence of insoluble calcium carbonate. Dissolution results in first the colony becoming more translucent and then a zone of translucency around the colony. Figure 23 shows the zone of clearance around *M. roseus*, which took 15 days to develop.

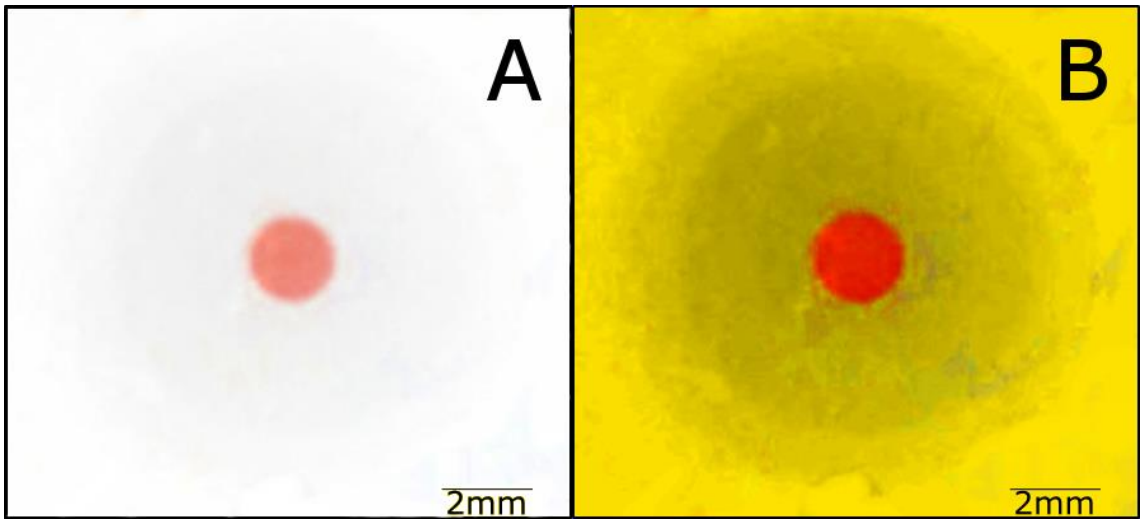


Figure 23: Dissolution of calcium carbonate by Micrococcus roseus, image A, is duplicated on the right (image B) and has been overlaid on a yellow background and the contrast adjusted using GIMP version 2.8 for clarity. Colony diameter is 2.15mm, zone of dissolution radius is 9.97mm.

Fourteen species have been identified as actively dissolving calcium carbonate in this test, *Bacillus sporothermodurans* from the damaged stone population, *Pseudomonas brenneri, Solibacillus silvestris,* and *Spongiibacter* sp. *IMCC21806* from the undamaged population and *Acinetobacter calcoaceticus, Arthrobacter agilis, Bacillus cereus, Bacillus safensis* strain *LCATB3, Micrococcus luteus, Micrococcus roseus, Pseudomonas putida, Pseudomonas stutzeri, Staphylococcus xylosus* and *Stentrophomonas maltophilia* from the isolates found on both surfaces.

As shown in Table 22, *P. putida* showed the strongest dissolution with a clear 10mm diameter halo around the colonies within 7 days. The remaining 12 species took 3 weeks to develop identifiable zones of dissolution, ranging from 6-12mm diameter around the colonies with *Micrococcus luteus* being the weakest showing translucency under the colony with 1mm diameter visible clearance around the colonies.

In order to determine whether the distribution of species with the capacity to dissolve calcium carbonate was applicable to the copiotroph subpopulation of the microbiome, the time taken and clearance zones were tested for normality using Lilliefors normality test in R but the distribution

was not normal, p-value for time = 0.64, p-value for clearance zone = 0.38, and therefore these results have to be accepted as specific to the species and not representative of the population.

Correlation testing of physical and biochemical characteristics against calcium carbonate dissolution showed a positive correlation, cor = 0.36, p-value=0.002, to gelatin hydrolysis. None of the other characteristics showed any correlation.

Surface isolated from	Species	Time taken (days)	Clearance zone (mm)
Damaged	*Bacillus sporothermodurans*	10	8
Undamaged	*Pseudomonas brenneri*	21	4
	Solibacillus silvestris	18	4
	Spongiibacter sp. *IMCC21906*	14	7
Both	*Acinetobacter calcoaceticus*	11	12
	Arthrobacter agilis	11	12
	Bacillus cereus	14	3
	Bacillus safensis strain *LCATB3*	14	7
	Micrococcus luteus	22	1
	Micrococcus roseus	15	10
	Pseudomonas putida	7	10
	Pseudomonas stutzeri	16	4
	Staphylococcus xylosus	16	8
	Stentrophomonas maltophilia	18	6

Table 22: Time taken (days) and zone of clearance (mm) for the dissolution of calcium carbonate by species which tested positive. Zone of clearance is radius of clearance beyond the edge of the colony.

5.4 SEM analysis of monoculture biofilms on limestone

By testing for biocorrosion through direct observation in a different growth environment the potential for growth media to alter metabolic pathways and suppress acid production can be partially overcome. As analysis of biocorrosion using SEM imaging is highly time consuming and can be subjective, only species from the damaged population along with those which had tested positive for calcium carbonate dissolution from the other two populations, as controls, were selected for testing.

SEM imaging was carried out with species being scored as positive for biofilm formation, micro pitting and etching of the surface based on direct comparison with known examples from the literature (Flores *et al.*, 1997; Videla *et al.*, 2000; Ascaso *et al.*, 2002; Viles & Gorbushina, 2003; Gorbushina *et al.*, 2004; Nuhoglu *et al.*, 2006; Herrera & Videla, 2009; Sgobbi *et al.*, 2010; Ortega-Morales *et al.*, 2013).

Of the 28 isolates found solely on damaged stone, 9 showed clear evidence of biocorrosion or other biodeteriorative processes when cultured on limestone blocks and observed under the SEM. The 10 controls all tested positive for biocorrosion.

All species inoculated onto the limestone blocks showed evidence of biofilm formation on the surfaces, see Figure 24 for examples.

Micro pitting, where individual cells have dissolved the surface they were sitting on, was visible at a bacterial scale with some surfaces showing evidence of bacterial etching of the surface, etched troughs in what should otherwise be smooth crystalline surfaces. An example of this is highlighted in the *A. agilis* SEM image, Figure 24 image A, with a black circle surrounding the pit, false coloured red, which is the exact dimension of the surrounding cells. This section of the picture has been enlarged for clarity, Figure 24 image B.

Evidence of bacterial etching of the surface, areas false coloured in red, were identified on nailhead spar crystals of calcium carbonate which have completely smooth faces when uncorroded. These are the crystals with hexagonal cross sections seen in the SEM image of *B. cereus*, Figure 24 image C.

B. sporothermodurans was one of the species which showed evidence of calcification of the biofilm matrix, false coloured blue, Figure 24 image D. Calcification was identified from structures which matched those shown in the literature as calcified biofilm, or biofilm structures which matched the literature but were also encrusted. A particularly good example of this is the calcified biofilm string in the top centre of the *B. sporothermodurans* image. Levels of calcification varied between species, for *S. maltophilia* and *S. silvestris* only calcified biofilm was observed where for the other species calcification appeared to be present only in mature biofilms.

Finally both *C. flaccumfaciens* (Figure 24 image E) and *S. silvestris* showed evidence of physical damage with the biofilm matrix growing through the limestone oolite and lifting them from the surfaces. Both of these species produce a biofilm sheet, false coloured violet in the SEM image, which was not observed in other species, as well as the globular biofilm matrix which is found elsewhere. The small oolite which have been removed from the surface by the *C. flaccumfaciens* biofilm have been false coloured red to distinguish them from the biofilm sheet. This mode of physical damage is not one which has been previously identified.

With the calcium carbonate media tests and the SEM observations combined there are a total of 19 species which demonstrated a measureable capacity for biodeterioration, 18 of these were due to biocorrosion.

As biofilm formation is directly linked in the literature to biodeterioration, analysis was carried out to determine whether biocorrosive potential showed any links with biofilm formation. When comparing the species with biodeteriorative potential to the total population for biofilm formation the size of the data set was insufficient to get statistically significant results. Normality testing demonstrated a trend towards normal distribution, Lilliefors normality test p-value > 0.2, with the distribution of species across the total population being even for isolates from the damaged environment, the undamaged environment and those isolated from both. This trend suggests that biofilm formation rate is not directly connected to biocorrosive potential although further work would be needed to confirm this.

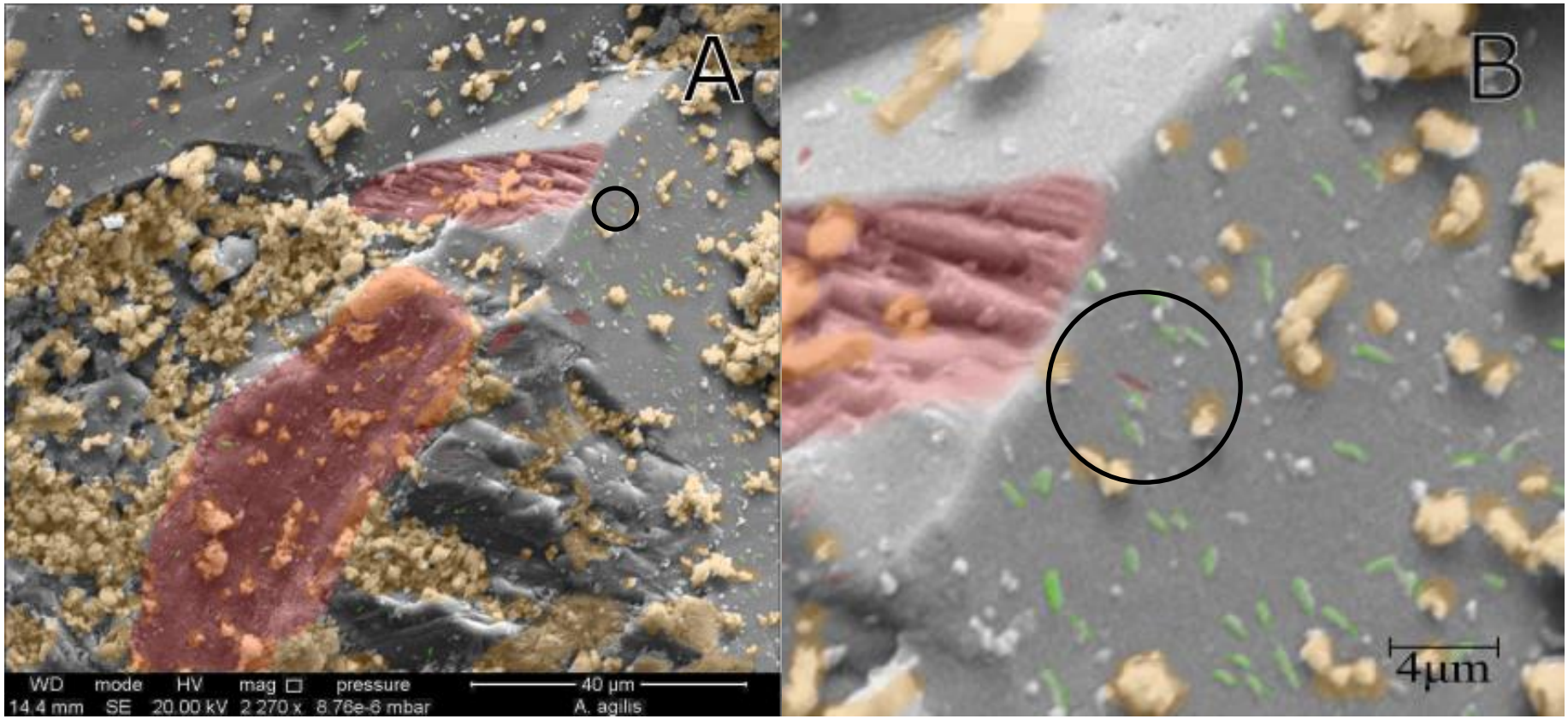


Figure 24: SEM images showing biofilm presence and areas of pitting and etching. Biofilm is shaded orange unless calcified when it is blue or a biofilm sheet when it is violet. Bacterial cells are shaded green when not encapsulated in biofilm matrix. Areas of damage are shaded red.

Images A and B: Black ring on Arthrobacter agilis picture highlights a very good example of bacterial pitting. Image B shows an enlarged copy of the area within the ring, the bacterial pit is highlighted in red and is the same dimensions as the surrounding cells, highlighted in green.

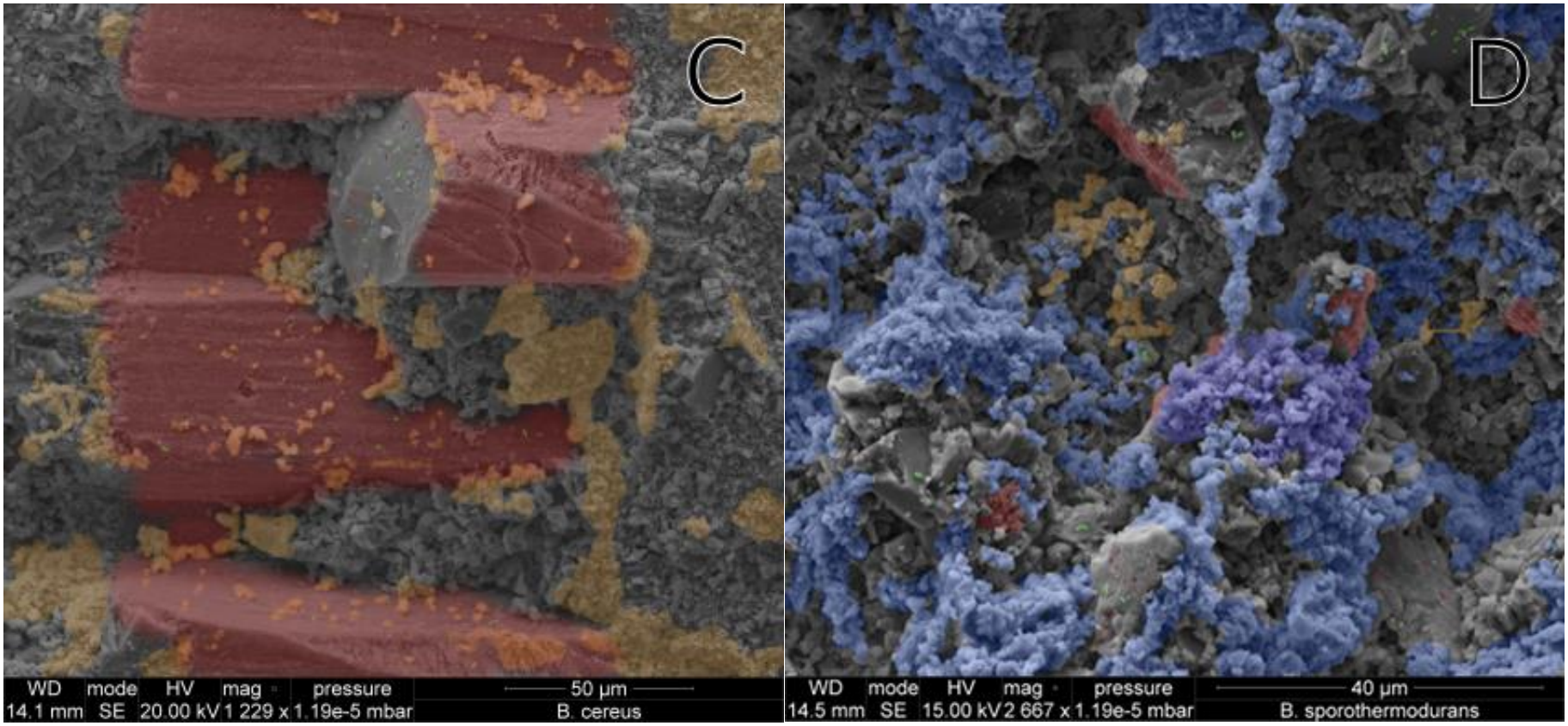


Figure 24 image C: SEM image of Bacillus cereus showing high levels of etching on nailspar crystals, red, the crystal surface is naturally smooth making it easy to identify the pitting (holes in the surface) and etching (long grooves in the surface). Biofilm is shaded orange. Bacterial cells are shaded green when not encapsulated in biofilm matrix.

Figure 24 image D: Bacillus sporothermodurans demonstrated high levels of calcification of the biofilm matrix, blue. Uncalcified biofilm is shaded orange with pitting and etching shaded red. Bacterial cells are shaded green when not encapsulated in biofilm matrix.

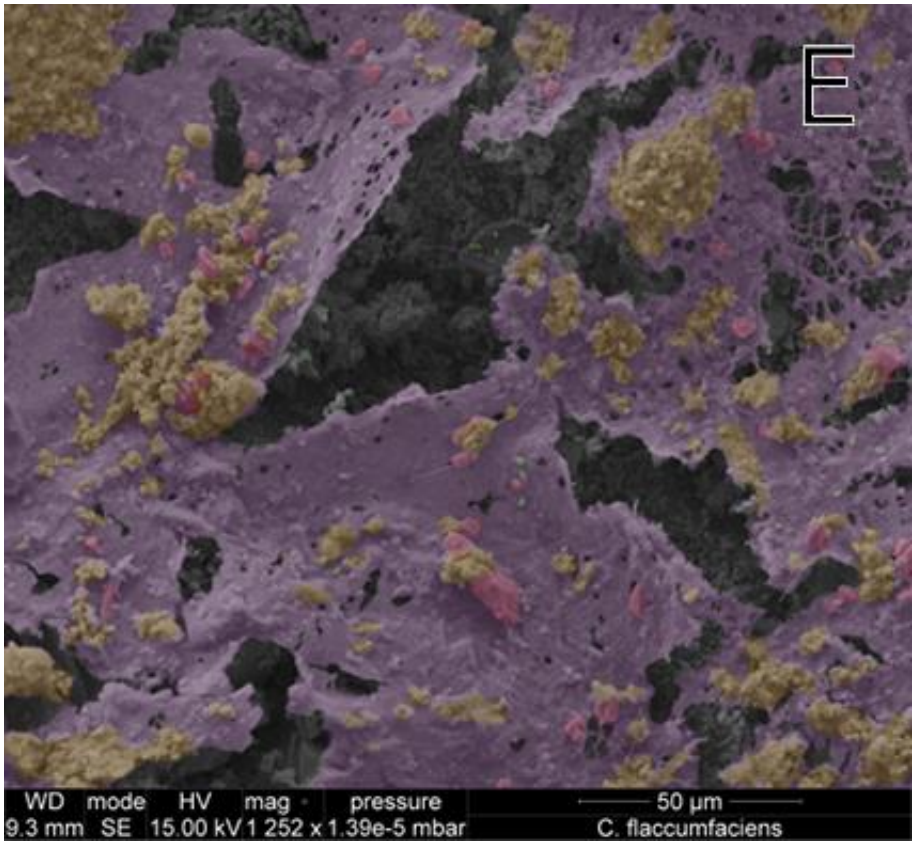


Figure 24 image E: <u>Curtobacterium flaccumfaciens</u> produced two forms of biofilm matrix, sheets and clusters. The sheets showed evidence of physical damage caused by the biofilm matrix, shaded purple, growing through the limestone oolitic matrix and lifting the smaller oolites, shaded red, from the surface. Where the biofilm formed in clusters it is shaded orange, bacterial cells are shaded green when not encapsulated in biofilm matrix.

5.5 Discussion

Considering the claims made in the literature regarding individual species potential for biodeterioration, especially biocorrosion, it seems surprising that so few studies took the opportunity to test their isolates. As a result, the understanding of species roles in biocorrosion in the majority of the literature is based on a peer reviewed version of Chinese whispers, where references are made to papers, which cite another paper, which cites Bergey's manual, which states that certain organic acids are produced under specific test conditions. The obvious problem with this literature based methodology is that it assumes that any species which shows capacity for acid production is directly associated with biocorrosion. This is an assumption which was not supported by the analysis of acidification and biocorrosion in this study with species such as *A. phenanthrenivorans, I. variabilis* and *E. sibiricum* which are all capable of strongly acidifying their environments showing no evidence of dissolution of calcium carbonate in growth media.

In order to help address the assumptions in the literature that organic acid production under test conditions of type strains means that the species cause biocorrosion all isolates were tested to see how they modified the pH of their environment. Out of the 70 isolates tested in this study 28 were shown to acidify their environment, only 13 of these showed any evidence of biocorrosion. In fact 6 of the species, *B. sporothermodurans, P. lautus, P. brenneri, B. safensis* strain *LCATB3, P. putida,* and *S. xylosus,* when analysed only demonstrated alkalisation of their environment while testing positive for biocorrosion in both the calcium carbonate growth media and under the SEM. When comparing the species based on the surface that they were isolated from there were significantly higher numbers of species which were strong acid producers isolated from damaged surfaces, and both damaged and undamaged, than those which were solely isolated from undamaged surfaces.

A comparison of the 4 species with a broad range of optimal growth pH identified in the last chapter with the pH modification of the environment results shows that *Micrococcus halobius, Pseudomonas stutzeri* and *Stentrophomonas rhizophila* all strongly acidify their environments under these test conditions, supporting their identification as species which may actively modify the pH of their environment for competitive advantage. *Acinetobacter baylyi* on the other hand was shown to be a strong alkaliser under the specific test conditions. This obviously does not eliminate the possibility that the *A. baylyi* would acidify the environment under different growth conditions or in the presence of competing species.

Strain to strain variation was also observed in the pH modification of the media. *Bacillus licheniformis* strain *PAG2D* showed weak acid production and was observed to cause pitting under the SEM, but not in the calcium carbonate test, whereas strain *LCA3A7* showed a strong alkalisation of the media and did not show evidence of biocorrosion in either test. With *Bacillus muralis* strain *OX2D8*, the strain which showed the strongest evidence of acidification, showing evidence of biocorrosion when observed using the SEM. Both *Bacillus safensis* strains were alkalisers, but the weaker of the two, *LCATB3*, dissolved calcium carbonate in the plate assay and showed evidence of calcification and etching under the SEM.

The analysis of the pH modification of the environment by species raises questions regarding the mechanisms by which biocorrosion takes place. Of the species which dissolved calcium carbonate in the agar plate based assay, just under two thirds of them, 9 out of 13, were acidified their envionment. Two of the acidifiers also produced alkalisation as part of the growth curve but not to a strong level. The remaining 4 were all alkalisers, with 2 of the 5 being weak. No species which were neutral for pH modification were shown to dissolve calcium carbonate in either the plate based assay or with direct observation under the SEM. Species such as *Staphylococcus xylosus* and *Pseudomonas putida* were observed to dissolve calcium carbonate in growth media and demonstrated pitting and etching of the limestone surfaces under the SEM, and yet showed no evidence of acidification of growth media, in fact both species strongly alkalised the environment. While it is possible that the growth of the bacteria in nutrient broth may suppress the metabolic pathways that these species use for organic acid production, or that acids and alkali were being produced simultaneously, it is also possible that the dissolution of calcium carbonate was being carried out by an alternative mechanism. The absorption of iron metal ions by bacterially produced siderophores, iron chelating molecules (Neilands, 1995) is well known, as is the existence of extra cellular calcium binding bacterial nucleases (Rigden *et al.*, 2003) so the chelation or binding of metal ions essential to bacterial functioning is a potential mechanism that would be interesting to pursue.

An interesting correlation between the hydrolysis of gelatin and the dissolution of calcium carbonate was also observed. While the production of gelatinase is unlikely to cause the dissolution of calcium carbonate, the presence of calcium carbonate has been shown to increase the production of gelatinase in some species (Merrill & Clark, 1928), which provides a viable link between the two.

Under the SEM a further 4 species were identified as showing biocorrosion when compared to the results from the calcium carbonate agar tests, again suggesting that either the growth medium

was suppressing biocorrosive activity or that mechanisms of dissolution not reported in the literature were at play. The 4 species identified were *Bacillus licheniformis* strain *PAG2D, Bacillus muralis* strain *OX2D8, Microbacteriaceae* sp*. PAG4D* and *Paenibacillus lautus*. Of these species all but *P. lautus* were identified as environmental acidifiers. *Paenibacillus lautus* was identified as a reasonably strong alkaliser which suggests that the metabolic pathways responsible for the etching observed were not active under the test conditions, or not related to acid production.

Species which under the SEM only showed calcification of the biofilm matrix, namely *A. calcoaceticus, B. sporothermodurans, S. maltophilia, S.* silvestris and *S.* sp. IMCC21906 had all been shown to dissolve calcium carbonate in the plate based assay and so while pitting or etching was not observed in these species under the SEM it is likely to be because the biofilm matrix was covering the evidence in these areas. Only *B. sporothermodurans* alkalised the media during testing, the other species all showed acidification of the environment.

While not all of the species isolated from the damaged surface were shown to cause damage under the SEM or with the calcium carbonate culture medium, all of them produced biofilm on the limestone surface and therefore have the potential to contribute to accelerated weathering.

The analysis of biodeteriorative potential provided by the current study provides key information necessary for an accurate understanding of biocorrosion caused by the bacterial microbiome present on limestone surfaces. Previous work looking at biodeterioration has been limited by only looking at damaged surfaces, something which this study has shown excludes some species with biodeteriorative potential. Looking at taxonomic levels such as family, class or even genus for causative factors in biodeterioration has also been shown to be like looking for a needle in the proverbial haystack, in this study differences in the biocorrosive capability have been shown down to the strain level, with *Bacillus safensis* strain *LCATB3* showing the capacity to dissolve calcium carbonate whereas strain *OX3U10* did not show this capability.

Of the bacterial species isolated from both damaged and undamaged surfaces, several were shown to have biocorrosive potential. The presence of *M. luteus* on both damaged (all sites sampled) and undamaged stone (6 of the 20 sites sampled) in urban areas suggests that it could be used as an early indicator for colonisation of species responsible for biodeterioration.

The analysis of the species associated with the surfaces combined with identification of their potential for biodeterioration allows, for the first time, the identification of species for targeted treatments such as bacteriophage therapy. The significant association between species such as *B. licheniformis,* which demonstrated biocorrosive capacity, and *P. psychrodurans,* an exceptionally

strong biofilm former, suggest that these would be viable targets for treatment. In addition to the potential for targeted therapy, the identification of species which are significantly associated with undamaged surfaces and who show no evidence of biodeteriorative potential, opens up the possibility of inoculating cleaned surfaces with a defined microbiome to help inhibit the recolonisation of the surface by harmful species.

Nineteen of the 24 species which were isolated in this study have also been identified in the literature as biocorrosive; the evidence in the literature for biocorrosion was based on the original biochemical characterisation of the type strain, this study has provided experimental evidence for these species. Of the remaining 5 species identified in the papers, *A. lwoffii, C. flaccumfaciens* and *I. variabilis* were said to be evidently causing biodeterioration due to their presence on biodeteriorated surfaces with no supporting evidence (De Leo *et al.*, 2012), in the case of *C. flaccumfaciens* this study has demonstrated a novel mechanism of biodeterioration through biofilm action. No claims were made regarding *P. fluorescens*, just a note regarding its presence (Dupont *et al.*, 2007; Dakal & Cameotra, 2012), and finally *A. agilis* was claimed to be oxidising iron, although no mechanism was suggested (Heyrman & Swings, 2001). In this study it has been shown to acidify its environment, dissolve calcium carbonate and cause pitting to limestone under the SEM. The production of acid is most likely the cause of the oxidation of iron shown in the previous studies and confirms their finding.

It is likely that the assumptions made in the literature about biofilm formation and its role in biodeterioration, have resulted in the physical damage to oolitic stone surfaces by biofilm remaining undiscovered until this study. SEM imaging identified a mechanism of damage by the biofilm matrix which is not related to environmental physical and chemical weathering, something which has not been previously considered, as biofilm matrix has only ever been seen as enhancing these processes. The mechanism displayed by the biofilm matrix in the biodeterioration of stone surfaces by the biofilm matrix of *C. flaccumfaciens* and *S. silvestris* had never been identified until this study. Figure 24, image E, in 5.4 SEM analysis of monoculture biofilms on limestone, shows the biofilm matrix growing through the oolitic structure of the stone and breaking off oolites from the surface. As a biodeteriorative process this is not dependent on changes in the environment which are associated with physical and chemical weathering, and can therefore be considered a purely biological process of physical weathering akin to plant root growth through the stone structure. It is likely that this novel mechanism will not just effect limestones, but all finely grained sedimentary stones providing they have a bioreceptivity suitable for supporting species whose biofilm matrix has mechanical properties relevant to this mechanism. The identification of this

mechanism demonstrates the strength of combining the 3 testing methods; identifying the production of organic acids using the Bromocresol purple assay or testing for the dissolution of calcium carbonate by culturing on the agar media would have resulted in this species being classified as non-damaging.

6 Biocide testing

6.1 Introduction

Having established that bacteria do cause damage, it was important to evaluate the effectiveness of the most common, current, method of cleaning limestone, which plays a key role in the conservation process. When the EU Biocidal Product Regulations were introduced in 2012 (HSE, 2017) many of the biocides described in the literature were discontinued, for example Mergal S97, Preventol A9D and Mancozeb.

To ensure that the biocides chosen for testing would be current, selection was carried out based on the results of a survey of stone conservators. As a result of the survey, 4 biocides were identified to test: Microtech by Wykamol (used by Lincoln Cathedral), Soluguard Multisurface Biocide (a quaternary ammonium compound, QAC, based biocide which uses the same chemical components as Preventol R80, a biocide used in stone conservation globally), Algo bio inhibitor (an alternative QAC) and Industrial Methylated Spirits.

From the literature, biocide treatment is one of the most common methods for removing microbiological growth from limestone due to the porous and soft nature of the stone. As mentioned in the introduction to the thesis, the majority of biocides do not make any claims about efficacy against bacteria. Of the 4 biocides identified for testing in this study only Algo bioinhibitor makes any claim about efficacy against bacteria, Microtech biocide and Soluguard multisurface biocide only claim efficacy against algae and fungi for microorganisms. While not explicitly marketed as a biocide IMS has long been identified as having a biocidal effect on bacteria, although the effect becomes less pronounced in many species below 50% concentration (Morton, 1950).

While Algo bioinhibitor states that it is bactericidal no information is provided about whether the testing was performed planktonically or against biofilm encapsulated cells and correspondence with the manufacturers was unable to clarify this; as the standard tests for antimicrobials against bacteria are against planktonic growth it must be assumed that this is the case. As a result no data is available regarding the biocidal activity of these biocides against the species isolated in this study when grown as a biofilm, matching their growth state in the natural environment. The majority of studies which look at antimicrobial effects against biofilms tend not to look at the effect of the antimicrobials (or agents) on the biofilm matrix (Sepandj *et al.*, 2004; Starner *et al.*, 2008; Harriott & Noverr, 2009) which is significant to the prevention of bacterial biodeterioration

and has been identified as a significant requirement in studies looking at resistance of biofilm encapsulated bacteria to antimicrobials (Frank *et al.*, 2009). This chapter of the study addresses the lack of information about efficacy against biofilm encapsulated bacteria while also addressing the action of the biocides against the biofilm matrix.

As conservators are concerned that the application of biocides can enhance salt weathering of the surfaces a small comparative study was carried out in collaboration with Dr. Lynda Skipper, of the University of Lincoln, School of History and Heritage. The aim of this study was to confirm whether the biocides measurably effected the physical appearance of the surface, using photographic analysis of treated limestone blocks, and whether the salts present in the biocides were identifiable which was analysed using SEM-EDX.

6.2 Spot plate testing of Microtech biocide and IMS

Spot testing allows for identification of breakthrough when the species recolonises the clearance zone but does not control evaporation of volatile components of biocides, is labour intensive and low throughput. As a result of this only Microtech and IMS were tested using this method with the testing for biocides being changed to the microplate format and carried out on a Bioscreen C.

Spot testing of Microtech was carried out using dilutions equivalent to the manufacturers recommended usage, and after incubation the plates were checked for bacterial growth. Where a zone of clearance was observed the isolate was considered susceptible to the biocide, where a lawn of growth was observed it was considered resistant (Figure 25 A, B and C). The highest dilution at which the isolate was susceptible to the biocide was recorded as the minimum inhibitory concentration (MIC).

Microtech showed an MIC greater than the manufacturers' recommended minimum concentration for 67 of the 70 isolates, the 2 isolates which showed resistance, *P.* sp. *HZ06* and *P. fluorescens* demonstrated an MIC of 1:150 dilution from stock, equivalent to $5m^2$ coverage using the recommended 1:24 dilution from stock. *S. microflavus* demonstrated an MIC of 1:96 dilution from stock, equivalent to $4m^2$ coverage using the recommended 1:24 dilution from stock.

Forty eight hours after the initial reading, the clearance zone for each biocide dilution was re-checked for the appearance of colonies within it, thus demonstrating breakthrough. Breakthrough was observed in 11 of the 70 isolates, see Figure 25 D for an example. *Acinetobacter lwoffii,*

Bacillus muralis strain *LCA1D6a, Bacillus subtilis* strain *LCA1U3, Exiguobacterium sibiricum, Isoptericola variabilis, Solibacillus silvestris,* and *Stentrophomonas maltophilia* all showed breakthrough at all biocide dilutions. *Bacillus safensis* strain *LCATB3* showed breakthrough between 1:37.5 and 1:150*, Lysinibacillus fusiformis* showed breakthrough between 1:54 and 1:150, *Micrococcus luteus* showed breakthrough between 1:121.5 and 1:150 and finally *Streptomyces microflavus* showed breakthrough between 1:96 and 1:150.

Spot testing of IMS incorrectly showed all species to be resistant to the IMS up to and including 100% concentration due to the volatility of the IMS. Evaporation was reducing the concentration down to levels where the isolates could metabolise the alcohols. This has been discussed in detail in chapter 4 section 5.

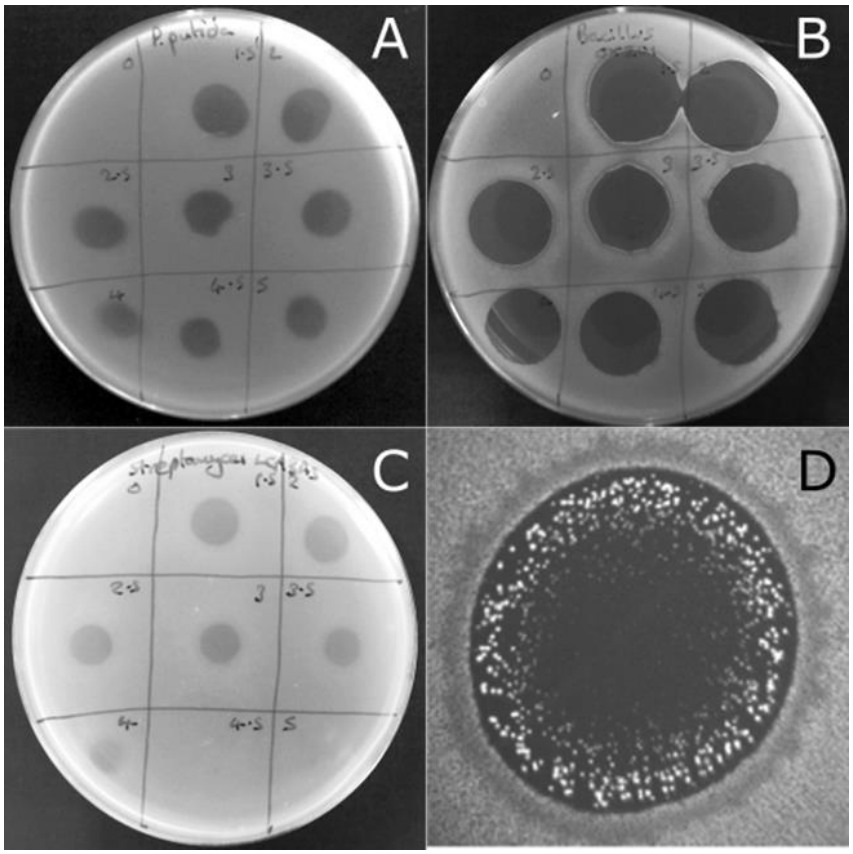


Figure 25: Microtech biocide activity against Pseudomonas putida (A), Bacillus thuringensis (B) and Streptomyces microflavus (C). Biocide dilutions from stock decrease from 1:18 through to 1:150 which are the equivalent of the manufacturers' standard dilution over the recommended treatment area (1.5 - 5m^2). Inhibition of bacterial growth by the biocide is observed as a zone of clearance, a dark disc in the images. Solibacillus silvestris (D) showed breakthrough into the zone of clearance at all concentrations.

6.3 Biocide testing against bacteria growing planktonically.

In order to provide evidence for manufacturers claims of efficacy, and to provide a baseline for testing the efficacy of the biocides against biofilm encapsulated bacteria, planktonic testing was carried out in a microdilution format on the 4 biocides. The microdilution method is the most commonly used high throughput methodology for testing biocides and antimicrobials against bacterial species in industry (Wikler, 2006). Retardation of the growth curve is classified as breakthrough of the isolate to the biocide in this assay format, demonstrating the biocide as actually being bacteriostatic rather than bacteriocidal.

IMS was tested for efficacy over a range between 80% to 16%. The other three biocides were tested at dilution ranges between 1:24 and 1:216 which spanned the manufacturers recommended range for all three, Microtech's highest recommended dilution being 1:150.

Of the 4 commercial biocides tested, Microtech, Algo bioinhibitor and Soluguard were all equally effective against the isolates when grown planktonically; having an MIC for all 70 isolates lower than the minimum manufacturers recommended concentration. This is demonstrated in Figure 26 showing the effects of the four biocides against *Pseudomonas brassicacearum.* In all cases the biocides were completely effective, the 1:216 dilution of Microtech biocide was below the manufacturers recommended range. None of the isolates tested showed evidence of breakthrough, demonstrating that Microtech, Algo bioinhibitor and Soluguard were all biocidal in their action against the isolates when grown planktonically (Table 23).

	Microtech		Algo bioinhibitor		Soluguard		IMS	
	Planktonic	Breakthrough	Planktonic	Breakthrough	Planktonic	Breakthrough	Planktonic	Breakthrough
No resistance	70	70	70	70	70	70	68	64
Resistance	0	0	0	0	0	0	2	6
Efficacy expressed as %	100	100	100	100	100	100	97.14	91.43

Table 23: Overview of planktonic biocide testing, Efficacy of the biocide is expressed as the percentage of isolates which showed no resistance.The commercial biocides all demonstrated 100% efficacy, IMS was less effective.

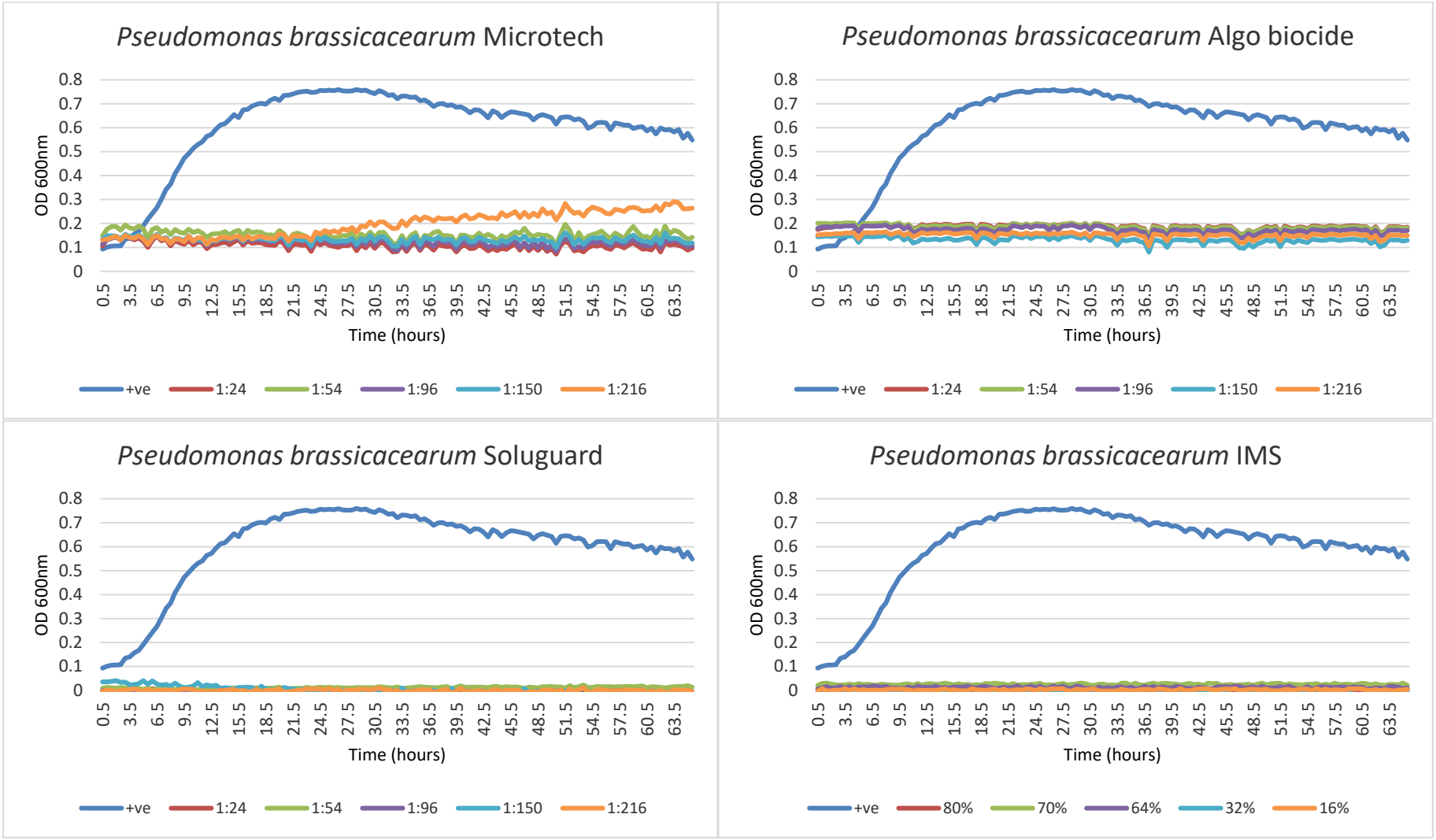


Figure 26: <u>Pseudomonas brassicacearum</u> growth curves against Microtech, Algo and Soluguard biocides and IMS. Data shown is average of n=3.

Based on Morton (1950), growth in IMS above 16% was considered evidence of resistance. *B. psychrosaccharolyticus* was capable of growth in the presence of 70% IMS and *M. roseus* in the presence of 64%. Six species, *Arthrobacter phenanthrenivorans, Brevibacillus brevis, Microbacterium ginsengisoli, Pseudomonas brenneri, Psychrobacillus psychrodurans* and *Sphingobacterium faecium* all showed signs of breakthrough after 24 hours at all concentrations of IMS, with a retarded growth curve when compared to the positive control.

Correlation testing of physical and biochemical characteristics against the biocide data was carried out and several correlations were identified. Resistance to IMS while in planktonic growth showed a significant (p-value = 0.01) correlation with catalase production. As the catalysis of hydrogen peroxide by catalase can also be used to oxidise ethanol resulting in an increased efficiency of alcohol metabolic pathways (Whittenbury, 1964) the correlation is to be expected. There was also a significant, p-value = 0.03, correlation, 0.26, between planktonic resistance to IMS and planktonic growth. As faster growing bacteria will have higher metabolic rates (Tweeddale *et al.*, 1998) the correlations between growth rate and catalase production reinforce each other. Finally for planktonic resistance to IMS there was a significant, p-value = 0.03, correlation, 0.26, to optimum growth pH; while there may be other explanations for this correlation, the optimum pH for catalase activity for *Escherichia coli* links in very nicely with the resistance being linked to catalase production (Semchyshyn *et al.*, 1999).

6.4 Biocide testing against biofilm matrix and encapsulated bacteria

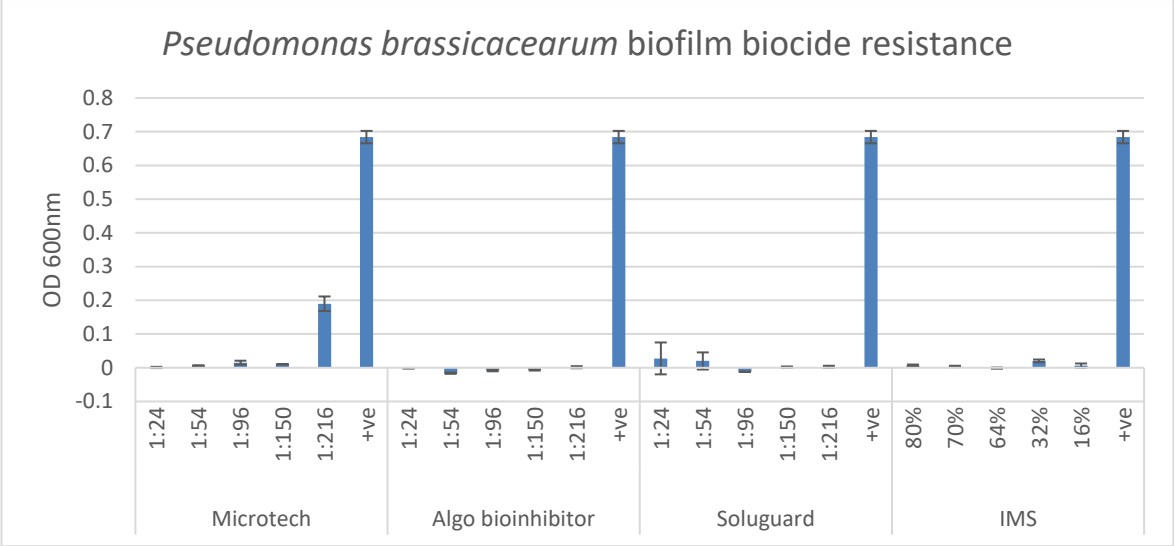
The current, standard, method for testing biocides against biofilm encapsulated bacteria is an endpoint experiment (Ceri *et al.*, 1999). Growth following biocide treatment is classified as resistance of the bacteria to the biocide and the retention of biofilm matrix on the “Calgary peg” plate lid as resistance of the matrix to the chemical cleaning effects of the biocide (Figure 27).

In this study, the resistance of biofilm encapsulated bacteria was carried out at the same concentrations as the planktonic testing.

Figure 27: Crystal violet stained biofilm coating the surface of a "Calgary peg" plate lid prior to the elution of the stain into the 96 well plate using absolute ethanol. Varying levels of biofilm retention can be measured using a colorimeter to determine the differing effects of biocide on the biofilm matrix of different species.

P. brassicacearum demonstrates the differences seen between resistance of the bacteria to biocides and the resistance of the biofilm matrix to chemical disruption by the biocide (Figure 28). It showed no resistance to Microtech across the manufacturers recommended range, 1:24 – 1:150, while demonstrating growth at the next dilution down, 1:216. The 1:216 dilution was included in the biocide range in order to allow testing for all 3 commercial biocides across the same range. Algo bioinhibitor, Soluguard and IMS were all equally as effective showing no growth at any dilution. When this is compared to the effects of the biocide on the biofilm matrix the lower dilutions of Soluguard are shown to only partially clean the peg and IMS demonstrates chemical fixing of the matrix to the peg at higher concentrations.

Moving on to the chemical removal of the biofilm matrix by the biocides, Microtech and Algo bioinhibitor are both equally as effective. Soluguard is less effective and shows increased biofilm remaining bound to the pegs at lower concentrations, although much lower than the biofilm bound to the pegs in the positive control. IMS in many cases showed the slight inverse measure of biofilm bound to IMS concentration, this was due to alcohol fixation of biofilm matrix to the pegs (Kwasny & Opperman, 2010). Readings due to alcohol fixation never exceeded 0.05 OD 580nm and so this was accepted as the lower limit for scoring biofilm removal using IMS.



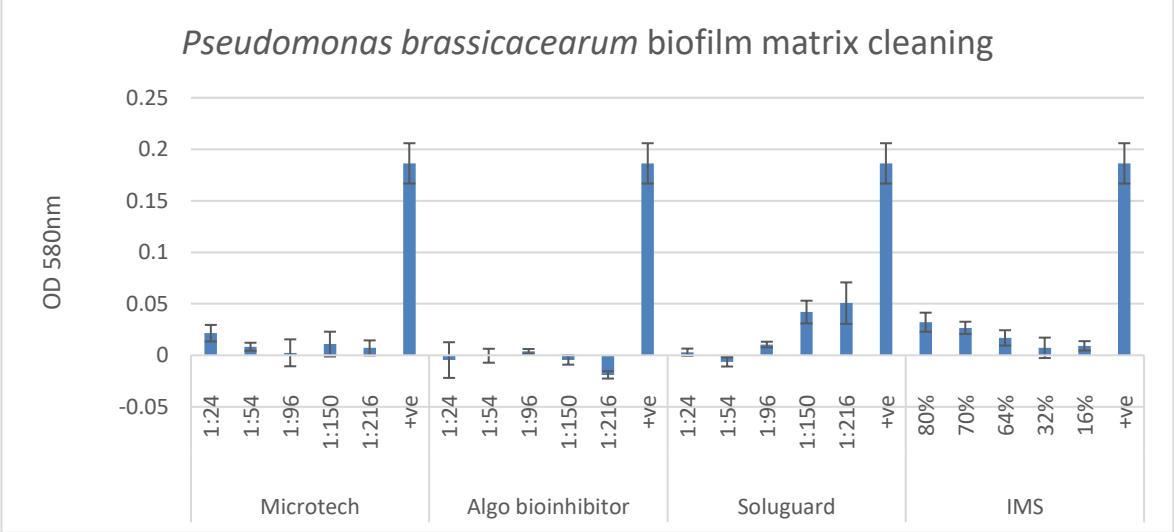


Figure 28: Biocide resistance of Pseudomonas brassicacearum encapsulated in biofilm matrix and the chemical cleaning effects of the biocides against the matrix. Biocide type and dilutions tested are shown along the x-axis along with the positive and negative controls. Data shown is average of n=3.

All biocides except Algo bioinhibitor proved generally less effective against the species when encapsulated in the biofilm matrix, Table 24 for overview, Table 25 for interpreted results from plate reader.

	Microtech		Algo bioinhibitor		Soluguard		IMS	
	Biofilm	Crystal violet	Biofilm	Crystal violet	Biofilm	Crystal violet	Biofilm	Crystal violet
No resistance (no. of strains)	68	45	70	53	67	43	54	49
Resistance (no. of strains)	1	25	0	17	3	27	16	21
Efficacy expressed as %	98.57	64.29	100	75.71	95.71	61.42	77.142857	70

Table 24: Overview of biofilm biocide testing, columns labelled biofilm show the efficacy of the biocide against biofilm encapsulated cells, columns labelled crystal violet show the efficacy of the biocide against the biofilm matrix. The final efficacy of the biocide is expressed as the percentage of isolates which showed no resistance. When compared to the planktonic testing efficacy is lower all but Algo bioinhibitor.

Resistance was observed with the Microtech biocide for *Stentrophomonas rhizophila,* which was isolated from both damaged and undamaged stone, at the 1:150 dilution.

Bacillus sporothermodurans, Brevibacillus brevis, both only found on damaged stone, and *Micrococcus roseus*, found on damaged and undamaged stone, all showed resistance across the whole range for Soluguard.

The species which showed resistance to IMS were as follows, *A. johnsonii, Bacillus muralis* strain *WE2D1c, Microbacterium schleiferi, Paenibacillus lactis* and *Streptomyces microflavus* which were all found solely on damaged stone. *A. phenanthrenivorans, Bacillus* sp. *BC11, Bacillus* sp*. PVS08, Bacillus thuringensis, Solibacillus silvestris, Spongiibacter* sp. *IMCC21906* which were all found solely on undamaged stone. *A. agilis, Bacillus safensis* strain *OX3U, Bacillus safensis* strain *LCATB3, Escherichia coli,* and *Sporosarcina saromensis* which were all found on both damaged and undamaged stone. *Microbacterium schleiferi* and *Streptomyces microflavus* both had an MIC of >80% IMS with the other 14 species being capable of growth following a 24 hour immersion in any of the concentrations tested. As none of these species had shown significant resistance to IMS under planktonic conditions the biofilm must have been providing a protective growth environment.

	Microtech		Algo bioinhibitor		Soluguard		IMS	
Species	Biofilm cells	Biofilm matrix	Biofilm cells	Biofilm matrix	Biofilm cells	Biofilm matrix	Biofilm cells	Biofilm matrix
Acinetobacter baylyi	-	P	-	P	-	P	-	+
Acinetobacter calcoaceticus	-	+	-	+	-	+	-	+
Acinetobacter johnsonii	-	P	-	-	-	P	+	-
Acinetobacter lwoffii	-	-	-	+	-	+	-	+
Advenella kashmirensis	-	-	-	-	-	-	-	P
Arthrobacter agilis	-	-	-	-	-	P	+	P
Arthrobacter protophormiae	-	+	-	+	-	P	-	P
Arthrobacter phenanthrenivorans	-	+	-	+	-	+	+	P
Bacillus aerophilus	-	P	-	P	-	P	-	P
Bacillus cecembensis	-	+	-	+	-	+	-	+
Bacillus cereus	-	+	-	+	-	+	-	+
Bacillus mycoides	-	+	-	+	-	+	-	+
Bacillus foraminis	-	P	-	P	-	P	-	P
Bacillus infantis	-	+	-	+	-	+	-	+
Bacillus licheniformis strain *PAG2D*	-	P	-	+	-	+	-	+
Bacillus licheniformis strain *LCA3A*	-	+	-	+	-	+	-	+
Bacillus muralis strain *LCA1D6a*	-	+	-	+	-	+	-	+
Bacillus muralis strain *WE2D1c*	-	+	-	+	-	+	+	+
Bacillus muralis strain *OX2D8*	-	P	-	+	-	P	-	P
Bacillus niacini	-	P	-	+	-	P	-	+
Bacillus psychrosaccharolyticus	-	+	-	+	-	+	-	+
Bacillus pumilis	-	P	-	P	-	+	-	P
Bacillus pumilis strain *PAG4D2*	-	-	-	P	-	P	-	P
Bacillus safensis strain *OX3U*	-	-	-	+	-	-	+	+
Bacillus safensis strain *LCATB3*	-	+	-	+	-	+	+	P
Bacillus simplex	-	+	-	+	-	+	-	+
Bacillus sp. *BC11*	-	+	-	+	-	+	+	+
Bacillus sp. *PVS08*	-	+	-	+	-	+	+	+
Bacillus sporothermodurans	-	P	-	+	P	+	-	+
Bacillus subtilis strain *LCA1D*	-	+	-	+	-	+	-	+
Bacillus subtilis strain *LCA1U3*	-	+	-	+	-	+	-	+
Bacillus thuringensis	-	+	-	+	-	P	+	+
Brevibacillus brevis	-	+	-	+	P	P	-	+
Curtobacterium flaccumfaciens	-	-	-	-	-	-	-	-
Enterococcus hirae	-	+	-	+	-	+	-	+
Escherichia coli	-	P	-	+	-	P	+	P
Exiguobacterium sibiricum	-	+	-	+	-	+	-	+

Isoptericola variabilis	-	+	-	+	-	P	-	+
Lysinibacillus fusiformis	-	+	-	+	-	+	-	+
Lysinibacillus parviboronicapiens	-	+	-	+	-	+	-	+
Microbacteriaceae sp. *PAG4D*	-	+	-	+	-	+	-	+
Microbacterium ginsengisoli	-	+	-	+	-	+	-	+
Microbacterium pseudoresistens	-	+	-	P	-	P	-	P
Microbacterium schleiferi	-	P	-	+	-	P	+	P
Microbacterium thalassium	-	+	-	+	-	+	-	+
Micrococcus halobius	-	+	-	+	-	+	-	+
Micrococcus luteus	-	+	-	+	-	+	-	+
Micrococcus roseus	-	+	-	+	P	P	-	+
Paenibacillus lactis	-	-	-	+	-	-	+	+
Paenibacillus lautus	-	+	-	+	-	+	-	+
Paenibacillus pabuli	-	P	-	+	-	+	-	+
Paenibacillus polymyxa	-	+	-	+	-	+	-	+
Paenibacillus sp.*1105*	-	+	-	+	-	+	-	+
Pseudomonas brassicacearum	-	+	-	+	-	P	-	+
Pseudomonas brenneri	-	P	-	+	-	P	-	+
Pseudomonas fluorescens	-	P	-	+	-	+	-	+
Pseudomonas putida	-	+	-	P	-	+	-	+
Pseudomonas sp. *HZ06*	-	P	-	P	-	P	-	P
Pseudomonas stutzeri	-	+	-	+	-	+	-	+
Psychrobacillus psychrodurans	-	+	-	+	-	+	-	+
Psychrobacter faecalis	-	+	-	+	-	P	-	+
Solibacillus silvestris	-	+	-	P	-	+	+	P
Sphingobacterium anhuiense	-	+	-	-	-	P	-	P
Sphingobacterium faecium	-	+	-	P	-	+	-	P
Spongiibacter sp. *IMCC21906*	-	P	-	P	-	P	+	P
Sporosarcina saromensis	-	+	-	+	-	+	+	+
Staphylococcus xylosus	-	+	-	+	-	+	-	+
Stentrophomonas maltophilia	-	+	-	+	-	+	-	+
Stentrophomonas rhizophila	P	P	-	+	-	+	-	+
Streptomyces microflavus	-	-	-	-	-	-	+	-

Table 25: Biofilm resistance testing data for all 70 isolates with biocide cleaning efficacy. Biofilm cells column contains data regarding the level of efficacy of the biocide against the cells, biofilm matrix column contains data on the level of efficacy of the biocide against the biofilm matrix. - is no resistance or for biofilm matrix no cleaning, + is effective for biocidal activity or cleaning, P is partially effective for biocidal activity or cleaning.

Efficacy of the biocides against the biofilm matrix as a chemical cleaner, i.e. chemically disrupting the biofilm matrix and removing it from the polystyrene peg, was lower than the biocidal effect. In some cases the biofilm matrix was partially removed when compared to the readings for the positive control.

IMS was the most generally effective biocide for chemically disrupting the biofilm matrix, completely removing the matrix from 49 of the 70 isolates and partially cleaning a further 18. Only 3 of the isolates had biofilm matrix which resisted the cleaning activity, *Acinetobacter johnsonii, Curtobacterium flaccumfaciens* and *Streptomyces microflavus*.

Algo bioinhibitor proved the most effective for completely removing biofilm matrix from 53 of the 70 isolates. Of the remaining 17 isolates 11 showed a partial cleaning and Algo bioinhibitor had no cleaning activity against the matrix for the remaining 6, *Acinetobacter johnsonii, Advenella kashmirensis, Arthrobacter agilis, Curtobacterium flaccumfaciens, Sphingobacterium anhuiense* and *Streptomyces microflavus*.

Soluguard was the next most effective for cleaning with 5 species, *Advenella kashmirensis, Bacillus safensis* strain *OX3U10, Curtobacterium flaccumfaciens, Paenibacillus lactis* and *Streptomyces microflavus* being completely resistant to the cleaning effects. Only 43 of the 70 isolates demonstrated complete removal of the biofilm matrix with 22 showing partial removal.

Microtech had no discernible effect on the matrix of 8 of the isolates, *Acinetobacter lwoffii, Advenella kashmirensis, Arthrobacter agilis, Bacillus pumilis* strain *PAG4D2, Bacillus safensis* strain *OX3U10, Curtobacterium flaccumfaciens, Paenibacillus lactis* and *Streptomyces microflavus*. Biofilm matrix of 45 of the isolates were completely removed with the remaining 17 being partially removed.

None of the biocides tested had any effect on the biofilms of *C. flaccumfaciens* or *S. microflavus* and *A. kashmirensis* biofilm matrix was only removed by IMS.

In order to determine whether there was a link between the physical and biochemical characteristics of the isolates and biocide resistance when growing in a biofilm, or resistance of the biofilm matrix to chemical cleaning by the biocide, correlation testing was carried out.

Gram negative species also showed a positive correlation to the biofilm matrix demonstrating resistance to Microtech biocide, 0.32 p-value = 0.01, and Algo biocide, 0.23 p-value = 0.05. For both Microtech and Algo biocide there was a significant, p-value = 0.003 for Microtech and p-value = 0.004 for Algo biocide, positive correlation, 0.35 and 0.34 respectively, between resistance

to cleaning and biofilm formation. Resistance of the biofilm matrix to Microtech biocide also showed a significant (p-value = 0.02) correlation, 0.28, with catalase production which is interesting as biofilm formation by species also showed a similar correlation (0.23, p-value = 0.05).

There was no significant correlation between the resistance of the biofilm matrix to the cleaning effects of IMS and the cell wall chemistry.

The resistance of biofilm matrix to IMS demonstrated a significant, p-value = 0.04, negative correlation, -0.24, with the ability to ferment xylose. There was also a significant, p-value = 0.04, correlation, 0.24, between biofilm matrix resistance to IMS and optimum growth pH. The negative correlation between the ability to ferment xylose and resistance of the matrix to IMS suggests that there is specific chemical activity which results in the removal of biofilm matrix by IMS. The correlation of optimum growth pH to cleaning can be seen to support this as the pH of the environment would affect chemical interactions between the IMS and the biofilm matrix.

When comparing biofilm matrix resistance for the 4 biocides to isolates recovered from damaged surfaces, undamaged surfaces and those recovered from both there was a significant negative correlation between resistance and isolates from undamaged surfaces for Microtech, -0.285 p-value = 0.02, showing that it is significantly less effective at removing biofilm matrix from undamaged surfaces. Algo bioinhibitor showed a significant negative correlation for isolates from damaged surfaces, -0.299 p-value = 0.02, and for isolates from both surfaces, -0.32 p-value < 0.01, again showing that the biocide was less effective against the matrix produced by isolates from these surfaces.There was a significant negative correlation between IMS cleaning of the matrix and isolates from both surfaces, -0.24 p-value = 0.05, showing it was less effective against them. No significant correlation for Soluguard multisurface biocide was observed.

There was a significant positive correlation between resistance of the biofilm matrix encapsulated cell to the biocide and the isolates which were found on undamaged surfaces for IMS showing that the isolates on the undamaged surfaces were significantly more likely to show resistance to IMS as a biocide.

In order to determine which biocides would have enhanced efficacy when used serially on a surface a correlation test was run between the results for all 4 biocides. All 4 biocides showed significant correlations with each other, as they were all killing the majority of the isolates. The lowest significant correlation was between Microtech and IMS, giving a positive correlation of 0.39 p-value = 0.0007. Assessing the data for biocidal activity for both biocides a combination treatment would be effective against all isolates for biocidal activity with a 97.14% efficacy against

biofilm matrix, just leaving biofilm matrix for *Curtobacterium flaccumfaciens* and *Streptomyces microflavus* which none of the biocides are able to remove.

6.5 Analysis of salt deposition from biocides.

The results in this section are from collaborative work carried out with Dr. Lynda Skipper. Dr. Skipper carried out the salt and biocide applications on the stone, SEM imaging and EDX testing of all samples in the study with standard photography and analysis of all of the results being carried out by the author. Sodium chloride was selected for testing as it is one of the most common salts detected in weathering (Rodriguez-Navarro & Doehne, 1999).

Characterisation of visual changes in the limestone block was performed using histogram averaging, allowing the comparison of the area which had been treated with biocide to the untreated control (Figure 29).

Measureable colour alteration was observed for all 3 commercial biocides when comparing the treated block to the untreated control block, Figure 30. Application of Microtech biocide and Soluguard resulted in an increase in all 3 colour components, shifting the overall colour mostly towards blue. Algo bioinhibitor showed a higher increase in mean red pixels when compared to the control. IMS showed small changes with a reduction in red and green and a slight increase in blue, possibly due to soluble salt transport to the surface. While in the literature some biocides have been identified as causing iron staining no evidence of this was observed in this study.

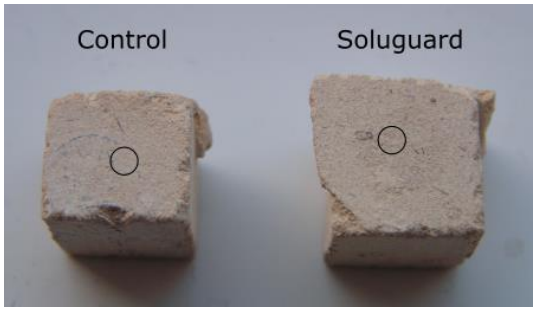


Figure 29: Control (deionized water) and Biocide (Soluguard) treated limestone blocks. An area of each block in the image was selected and analysed to look for variation in the level of red, green and blue pixels. The black circles on each block indicate the 12269 pixel area in the original image where histogram analysis was applied to measure the mean number of red, green and blue pixels present in the area.

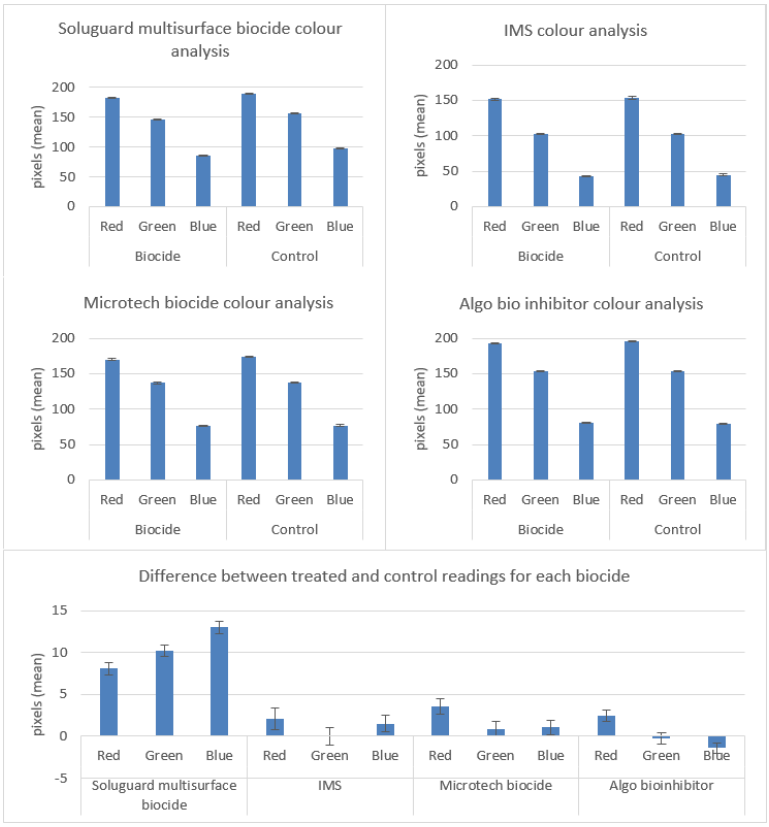


Figure 30: RGB histogram analysis of limestone blocks treated with Algo bio inhibitor, IMS, Microtech biocide and Soluguard multisurface bioinhibitor. Data shown is average of 12269 sample points. The charts show the mean of red, green and blue pixels within the measured area of the block allowing comparison of the differences between the colours of the limestone surfaces between biocide treated and untreated stone. The bottom graph shows the difference between the biocide treated samples and the controls in the first four graphs demonstrating that while there was minor variation in the colour for IMS, Microtech and Algo bioinhibitor, these were not visible to the naked eye, there was a much larger difference for Soluguard which was visibly apparent on the treated block.

Analysis of the samples by SEM-EDX confirmed that the level of sodium and chlorine ions introduced to the surface by the biocides would be below the level of detection by EDX, Table 26. The addition of a known amount of sodium chloride, plus the biocide, brought the level of sodium and chlorine ions in the biocides up to detectable levels The percentage weight of sodium and chlorine ions in the Control +NaCl sample were then compared to levels which would be expected of pure NaCl using a Chi Squared test and no significant difference was found between the control and pure NaCl. Any alterations to this ratio in the biocide treated samples may therefore be due to the effect of the applied biocides.

In order to determine whether the level of sodium and chlorine ions detected in the biocide treated samples deviated significantly from the biocide free control a Chi Squared test was carried out using the control data as the expected result. Algo, IMS and Soluguard showed no significant

difference in the levels of sodium or chlorine atoms present at the surface when compared to sodium chloride.

In contrast Microtech biocide showed a higher level of sodium atoms present on the surface than could be explained by the presence of the sodium chloride alone. This result was a highly significant deviation with a p-value of 0.001 and suggests that Microtech biocide could play a role in enhanced salting on surfaces.

While the Microtech and Soluguard results appear to show a high level of carbon when compared to the control, when compared to the calcium readings for the same samples this is proportionally lower than the levels seen in freshly cut stone in Chapter 3. The absence of carbon in the other samples is most likely due to the high levels of salt crystals on the surface, these block the reading as the carbon signal is prone to being swamped by high readings for other elements (Jonker *et al.*, 2015).

During EDX analysis it was observed that the surface of the samples treated with Soluguard multisurface biocide showed the presence of a waxy coating when compared to naïve stone or the stone treated with other biocides (Figure 31). To confirm this observation surface roughness analysis was carried out using the ImageJ analysis package (Schneider *et al.*, 2012). Surface characteristics, specifically the average height between peaks and valleys, the ratio of peaks to valleys and valley depth in the surface roughness testing, for the sample treated with Soluguard multisurface biocide were shown to be different by greater than two standard deviations than those obtained from untreated stone (Table 27).

	Ca	O	Si	Na	Cl	C
Control	58.28	41.23	4.00	0.00	0.00	0.00
Control + NaCl	18.92	66.00	0.00	35.65	55.83	0.00
IMS	54.00	44.33	11.00	0.00	0.00	0.00
IMS + NaCl	0.00	0.00	0.00	40.57	68.25	0.00
Algo bioinhibitor	60.40	39.60	0.00	0.00	0.00	0.00
Algo bioinhibitor + NaCl	16.50	36.50	28.00	27.34	56.03	0.00
Soluguard multisurface biocide	45.45	42.59	5.78	0.00	0.00	66.00
Soluguard multisurface biocide + NaCl	18.00	47.00	0.00	27.25	45.67	0.00
Microtech biocide	39.75	46.21	39.00	0.00	0.00	68.00
Microtech biocide + NaCl	30.67	63.67	0.00	31.20	43.33	0.00

Table 26: Average detection of atoms by percentage weight for each sample, n>3, together with sodium to chlorine ratio for each sample. In the samples without added sodium chloride the presence of the atoms was below the level of detection for the SEM-EDX hence the 0 reading. With the addition of a known level of sodium chloride the salts present in the stone and the biocides are brought up past the level of detection. Levels of sodium or chlorine atoms which differ from the ratio expected for sodium chloride provide evidence for the presence of salts in the biocide which are being deposited onto the stone surface, for example Microtech shows a significantly higher level of sodium deposited on the surface.

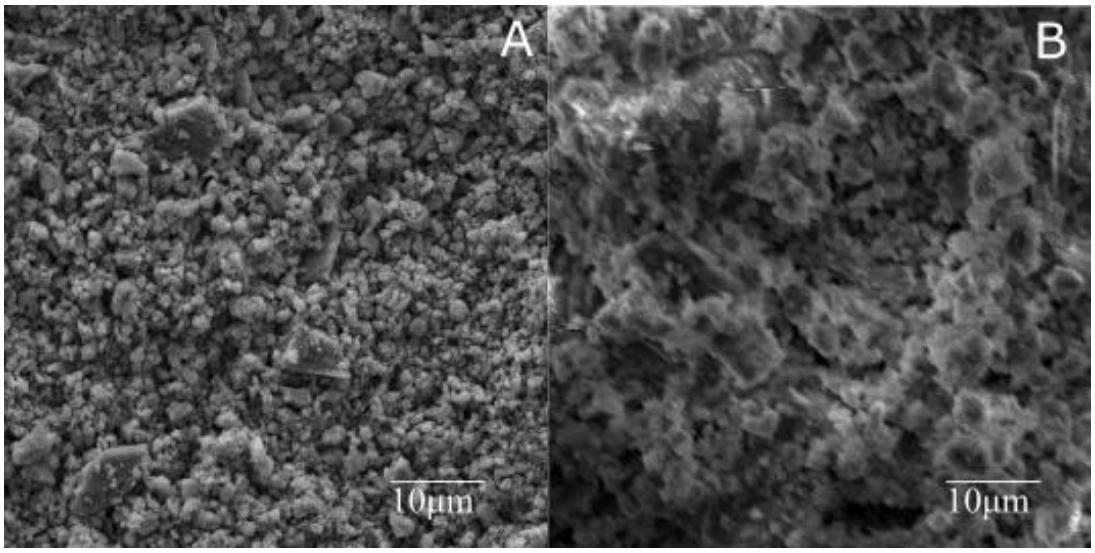


Figure 31: SEM images of untreated stone (A) and Soluguard multisurface biocide treated stone surfaces (B).

	Untreated	Microtech	Algo	IMS	Soluguard
Ra	93.321	57.431	138.679	98.764	71.461
Rsk	1.104	1.164	1.104	1.127	1.269
Rku	1.285	1.499	1.296	1.36	1.81
Highest Peak	204	196	255	248	254
Lowest Valley	7	13	10	12	0
Total height	211	209	265	260	254

Table 27: Surface roughness analysis of SEM images of biocide treated limestone blocks using ImageJ.

Ra, the roughness average, shows a reduction in the peaks and valleys in the Soluguard and Microtech measurements and an increase for Algo bioinhibitor. Of specific interest to determining whether the biocides are coating the surfaces is the Rsk, skewness, which was higher for Microtech, Algo bioinhibitor and Soluguard, indicating a higher proportion of peaks than valleys and thus showing that a coating is filling in the valleys. For Soluguard the Rsk analysis was also supported by the lowest valley measurement which was reduced to zero suggesting that all of the pores had been closed.

The Rku measure, kurtosis, defines the Soluguard multisurface biocide and Microtech samples as having sharper peak profiles than those observed for the other 3 samples.

A property of Soluguard, which is not identified in the manufacturer's literature, therefore is a surface coating which has been shown to block the valleys in the surface and therefore has the potential to block the pores.

While Microtech and Algo bioinhibitor also appear to alter the surface when compared to the control and IMS samples, they do not result in the lowest value reading being reduced suggesting that the coating does not block the pores.

6.6 Discussion

The role of biocides in the protection of surfaces from biodeterioration is twofold. As inferred from the generic name, the biocide should kill the microorganisms growing on the treated surface. The second role is one claimed by the manufacturer's literature namely cleaning the surface of biological growth products, in this case biocide matrix. The commercial biocides tested all made this joint claim of cleaning and killing.

A third requirement is imposed by the conservators who are end users of the biocides, that of minimal intervention as codified in the Venice charter (Gazzola *et al.*, 1964). In the case of biocides the broad spectrum effects cannot be minimised and so the requirement is that the biocide itself should not damage or modify the stone in any fashion. The testing for enhanced salting and discolouration carried out in this study was designed to address these considerations.

When considering the biocidal activity of the formulations, the initial testing carried out by spotting the surface of inoculated agar plates with the Microtech biocide has identified 10 species associated with damaged stone which, under these test conditions, are either partially resistant or capable of quickly recolonising stone work after treatment with biocides. A further two species were resistant to the lowest manufacturers recommended dose of Microtech biocide but are associated with undamaged stone. Spot testing, while being the laboratory test which was closest to the application of biocides in the environment, was shown to have innate limitations regarding the volatility of the compounds when testing effectiveness of IMS. The volatility of IMS resulted in sufficient loss through evaporation that the concentration applied to the bacterial cells was reduced to tolerable or metabolisable levels before any biocidal effects could be observed. As dodecylbenzenesulfonic acid, one of the components of Microtech biocide, has a similar boiling point to IMS, 82°C compared with 78°C, it is possible that the rapid regrowth observed was a result of the evaporation of this component of the biocide.

An alternative testing method, planktonic microplate testing, was carried out as it reduced the potential for evaporation of volatile products and was more closely related to the "Calgary Peg

System" testing method used for analysing the efficacy of biocides against biofilm encapsulated microorganisms. While all of the commercial biocides demonstrated 100% efficacy in the microplate system, this test does not reflect the natural, biofilm encapsulated, environment in which the species grow. For the purposes of this study the planktonic testing gave a baseline of activity to compare against.

The most effective biocide tested was Algo bioinhibitor, which was the only biocide to be effective against all of the isolates in both planktonic and biofilm growth states. Microtech, Soluguard and IMS all proved less effective against cells encapsulated in biofilm.

Hypothesis 8, that biofilm production will result in reduced efficacy of biocides was only partially supported by this data, the results from Algo bioinhibitor support the null hypothesis, namely that the efficacy of the biocides will not be effected by the presence of biofilm. The efficacy of the other 3 biocides was reduced when compared to the planktonic testing so hypothesis 8 cannot be fully rejected.

Of the 4 biocides, Algo bioinhibitor and Microtech were the most effective for biocidal activity against biofilm encapsulated bacteria. Algo biocide was biocidal against all isolates tested even at the lowest recommended concentration, Microtech proved slightly less effective with *S. rhizophila* showing resistance to the highest dilution, 1:150, which was equivalent to the lowest recommended concentration. In the biofilm formation testing *S. rhizophila* was the second highest producer of biofilm matrix; it is likely that the high level of biofilm production is responsible for the resistance observed in this case with the biocide being either diluted or inactivated by the matrix components. Studies have shown that resistance of biofilm encapsulated cells to antimicrobials can be due to dilution effects resulting from reduced penetration of the antimicrobial into the biofilm matrix, from the higher surface area within matrix, and chemical interactions with the matrix neutralising the antimicrobials (Stickler, 1999; Sutherland, 2001; Davies, 2003).

S. rhizophila, did not demonstrate any evidence of biocorrosive potential, Chapter 5, but the high level of biofilm production implies a strong link to enhanced physical weathering. As this species has been isolated from both damaged and undamaged surfaces this is of concern, especially given that protective polymicrobial interactions to antimicrobials have been observed in the literature (Peters *et al.*, 2012). It would be advisable, based on this, to approach the treatment of built heritage with Microtech at an increased concentration of biocide in order to prevent the potential protective effects which could lead to the development of resistance in other organisms.

Soluguard was the third most effective of the biocides for killing biofilm encapsulated cells. Resistance was observed in *B. sporothermodurans, B. brevis* and *M. roseus*. All 3 species were completely resistant to Soluguard at the lowest manufacturers recommended dilution and were not considered weak biofilm formers. Given the high level of resistance it is likely that a component of the biofilm matrix in these species was responsible for inactivating the biocidal effect. Both *B. sporothermodurans* and *M. roseus* were identified as biocorrosive in this study.

IMS was the least effective of the biocides against biofilm encapsulated cells. Of the 16 isolates which showed resistance to IMS when grown as biofilms, only *B. muralis* strain *WE2D1c* and *S. saromensis* were considered weak biofilm formers. With weak biofilm formers a likely explanation for the development of resistance when grown within a biofilm matrix is chemical inactivation of the biocidal activity by matrix components. The remaining 14 isolates which were resistant to IMS, when grown as biofilms, all showed a higher level of matrix production. With these isolates the explanation of the development of resistance is most likely a combination of all 3 mechanisms, reduced penetration, dilution by surface area and chemical inactivation. Interestingly the two species which showed resistance to IMS in planktonic growth, *B. psychrosaccharolyticus* and *M. roseus* showed no resistance to IMS when encapsulated in biofilm. Several species have been shown in the literature to utilise different metabolic pathways when encapsulated than when in planktonic growth (Shin *et al.*, 2009; Stahlhut *et al.*, 2012) and it is possible that in this case the pathways which allowed *B. psychrosaccharolyticus* and *M. roseus* to resist the biocidal effects of IMS in planktonic growth were not active in the biofilm growth mode.

Correlation testing of the biocide data identified several associations between resistance and physical or biochemical characteristics of the isolates. Catalase production correlated with resistance to IMS while in planktonic growth, as discussed earlier this will be due to its ability to oxidise ethanol. There was also a correlation between planktonic resistance to IMS and planktonic growth rate as well as optimum growth pH. The only correlation seen between biocidal activity and biofilm encapsulated cells showed that IMS was less effective against isolates solely found on undamaged surfaces.

As the majority of biocides are sold as biocidal cleaning agents with no data regarding their efficacy against bacterial encapsulated in biofilm testing was carried out to confirm the manufacturers' claims, hypothesis 9. Efficacy for complete removal of the biocide matrix was shown against 61.4% of isolates for Soluguard multisurface biocide, 64.3% of isolates for Microtech, 70% of isolates for IMS and 75.7% of isolates for Algo bioinhibitor.

When taking into account the species where the biocide also partially removed the matrix the efficacy was increased to 88.6% for Microtech, 91.4% for Algo biocide, 92.9% for Soluguard multisurface biocide and 95.7%, IMS. Thus the null hypothesis for hypothesis 9, that biocides will be ineffective for chemical removal of biofilm matrix, can be rejected.

The efficacy of Microtech and Algo biocide has been demonstrated to be reliant on the level of biofilm present on the surface, as there are significant correlations between removal of matrix and biofilm formation.

IMS showed a correlation which was most likely associated with the chemical composition of the biofilm, as opposed to the level of production. Species which have metabolic pathways for the fermentation of xylose are significantly less likely to produce biofilm which is resistant to IMS cleaning, presumably due to the lack of a component of the matrix which requires that pathway.

While IMS was the least effective of the biocides against biofilm encapsulated cells it has proven the most effective over all for reducing the levels of biofilm bound to the peg surface.

Acinetobacter johnsonii, Curtobacterium flaccumfaciens and *Streptomyces microflavus* were the only isolates where IMS could not completely or partially remove the biofilm matrix and it is of note that none of the biocides tested were effective against the biofilm matrix of *C. flaccumfaciens* and *S. microflavus*.

Eight of the isolates which demonstrated resistance to biocides while encapsulated in biofilm also showed complete removal of the biofilm matrix from the Calgary peg system. Due to the fact that there was planktonic growth from surviving cells released from the post treatment biofilm matrix, we can conclude that removal of the matrix was not due to breaking up the matrix, but disruption of the components which anchored the matrix to the surface. When transferred from the IMS to the growth media to test for surviving cells the matrix detached from the surface and the isolates had insufficient time to recolonize the surface and form a biofilm.

Ten of the isolates which demonstrated biocide resistance showed partial removal of the matrix, when compared to the biocide free positive control. While a similar mechanism to that postulated for those where the biofilm matrix was completely removed may be in place, it would be likely that the biofilm matrix for these species utilised additional anchoring components resulting in a disrupted matrix rather than a completely removed one.

Based on these results the selection of biocide would depend on the required outcome, Algo bioinhibitor and Microtech would both give effective biocidal activity against biofilm encapsulated

bacteria but are less effective at removing the biofilm matrix, in the case of Algo bioinhibitor it is less effective against the matrix produced by isolates found on damaged surfaces which is an indication that, in spite of its highly effective biocidal activity, it may be less suitable for cleaning limestone. IMS is the most effective at removing the matrix but suffers from its volatility. The volatility of the IMS could be overcome with the use of poulticing (Long & Young, 2016) to reduce evaporation of the IMS.

Where selection of the biocides is based on environmental and safety concerns for usage IMS while the least effective biocidally is also the least harmful, being classified as an irritant and flammable. Soluguard's components are classified as harmful, corrosive, flammable and irritant. Algo bioinhibitor is corrosive, irritant, harmful and harmful to aquatic organisms, and may cause long-term adverse effects in the aquatic environment. Microtech's components are classified as harmful, corrosive, mutagenic and harmful to aquatic organisms with the potential for long term adverse effects in the aquatic environment.

IMS and Microtech were shown to be the most effective combination based on correlations of efficacy. A combination treatment regime would be effective against all isolates for biocidal activity with a 97.14% efficacy against biofilm matrix, just leaving biofilm matrix for *Curtobacterium flaccumfaciens* and *Streptomyces microflavus* which none of the biocides are able to remove.

Resistance to the biocidal activity of all biocides was not specific to any single location sampled. As no biocide treatments have been applied to Saint Peters-at-Gowt in recent history (Personal communication, Revd. Jeremy Cullimore, 2014) when compared to Lincoln Cathedral and the other sites this indicates that the genes or mechanisms which enable the species to resist the effects of the biocide are already present in the built environment. This raises the probability of horizontal gene transfer of resistance mechanisms between species (de Vries & Wackernagel, 2002; Rumbo *et al.*, 2011; Tribble *et al.*, 2012) with the misuse or overuse of biocides.

With the possibility of resistance to biocides in the natural environment being transferred to the clinical environment (Morton *et al.*, 1998; Chapman, 2003; Knapp *et al.*, 2011) and the requirements under the Venice charter for minimal intervention (Gazzola *et al.*, 1964) new, targeted, approaches are needed for the prevention of biodeterioration. With the identification of the species which are causing biodeterioration this becomes possible.

In order to address the final concern related to the modification of the stone by the biocides, salt testing and surface analyses were carried out using SEM-EDX, SEM imaging and analysis of colour

photographs. Of the 4 biocides tested only IMS demonstrated no modification of the physical or chemical nature of the stone. Microtech biocide increased the level of soluble sodium ions which could result in enhanced salt damage.

The higher level of sodium atoms detected in the Microtech biocide treated sample is most likely due to the chemical composition of the biocide, with the MSDS reporting <5% sodium hydroxide.

Of course in all cases the biocide manufacturers are not required to identify components of the product which are classified as non-harmful in the MSDS, so there could be additional sources of sodium ions in addition to those listed.

The visual modification of the sample, Figure 30, measured for Soluguard multisurface biocide is in direct contradiction of their claims that the product will not discolour treated surfaces, (Safeguard Europe, 2016), and supports the modification of surface roughness which was detected using the SEM images which were taken as part of the EDX analysis. The chemical components in the MSDS should not cause surface modification of calcium carbonate which suggests that a non-hazardous component of the biocide has been included which is coating the surface. While the manufacturers do not state that the biocide coats surfaces (Safeguard Europe, 2016), they do make the claim that it will minimise future growth, presumably because of the surface modification detected. Given that the low surface roughness and high capillary coefficient of Lincoln limestone already causes issues with enhanced frost damage (Personal communication, Jane Cowan, Head of Conservation, Lincoln Cathedral Works Department, 2016) the potential for Soluguard multisurface biocide to block pores and increase water retention of the stone should not be ignored should its use as a biocide be under consideration.

While Microtech and Algo bioinhibitor also altered the surface colour measurably they did not affect the physical surface in the same way.

7 Discussion

Previous research into bacterial biodeterioration of limestone in Europe has significant limitations. This body of research aims to clarify aspects of the current literature and fill in the some of the gaps. While many of the results in literature are supported by this study, the assumption that species isolated from damaged surfaces must be damaging did not stand up to scrutiny. Similarly the assumption that if a species can produce organic acids it is a source of biocorrosion has also been shown to be fallacious.

The conservation of built heritage is important for social, cultural and historical reasons and the enhanced understanding of biodeterioration delivered by this study will help to preserve this heritage for future generations.

7.1 Conclusions

7.1.1 Bioreceptivity of Lincoln limestone

In the literature the bioreceptivity of a surface is often assumed, in fact there is only one paper which directly characterises the limestones in that study for bioreceptivity (Miller *et al.*, 2009). There is no literature on the bioreceptivity of Lincoln limestone and in fact this study is the first to fully characterise the bioreceptivity of any stone in the United Kingdom.

As discussed in the introductory chapter of this thesis, the chemical characterisation of Lincoln limestone available in the literature could, at best, be described as poor. Characterisation of the chemical composition of Lincoln limestone in this study using SEM-EDX gave an atomic profile similar to that seen by Miller et al. (2009); and showed none of the strontium or vanadium which were erroneously identified as the key components of Lincoln limestone in the literature (Barber, 1974). The chemical composition also showed strong similarities to the M9 salts minimal media used to grow bacterial species confirming that the minerals present would support growth.

This study also contributes new information through the measurement of surface roughness and capillary coefficient of Lincoln limestone. With a high capillary coefficient and low surface roughness, Lincoln limestone has a higher capacity for water retention than any of the limestones in Miller *et al.* (2009). The high water retention combined with the chemical composition means that the limestone surface has a good level of bioreceptivity and that therefore the species isolated from it are most likely to be ones which have colonised the stone rather than transient inhabitants. The identification of bacteria inhabiting a biofilm matrix on all, bar one, of the sample locations provides direct evidence of the bioreceptivity of the surface. The site which showed no

evidence of biofilm was number 4 at Lincoln Cathedral, which as discussed previously had strong copper staining which has been shown to be biocidal and to inhibit biofilm production, as well as one of the isolates being known to inhibit biofilm production through the secretion of biosurfactants.

7.1.2 Microbiome

This study is unique in its approach, it is the only study to date to have analysed the limestone microbiome on both damaged and undamaged surfaces. This has, for the first time, allowed an accurate representation of the damaged microbiome by identifying which of the species identified in past studies are not unique to the damaged microbiome. Analysis of the sampling has also demonstrated for the first time that the damaged and undamaged microbiomes show differences between rural and urban environments, something which should be pursued as future work.

There were significant differences in the bacterial species present on damaged and undamaged limestone in both the direct sampling and culturing of species and the data obtained by the 16S rRNA metagenomics study. Both studies showed compatible results including the isolation of *B. licheniformis* and *B. aerophilus* solely from damaged surfaces. Several species, such as *M. luteus, P.* sp.*1105* and *P. psychrodurans,* while found on both surfaces, were significantly more likely to be isolated from damaged stone. The data from this analysis has been made available in Appendix C to support future studies. At the OTU level, taxonomic class rather than species, there were also statistically significant differences between the two surfaces.

The sampling results conflict with some of the claims in the literature, but in each case the conflict can be explained due to the fact that the studies in the literature were only looking at damaged stone, and therefore were working with only half the data set compared to this study. Based on the sampling, culture and identification of species there were initially 5 conflicts with the literature, *B. thuringensis, P. polymyxa, P. fluorescens, P. stutzeri* and *S. maltophilia* were all isolated from undamaged surfaces when the literature (Heyrman & Swings, 2001; Saarela *et al.*, 2004; Nuhoglu *et al.*, 2006; Dupont *et al.*, 2007; Banks *et al.*, 2010; Dakal & Cameotra, 2012; De Leo *et al.*, 2012; Dhami *et al.*, 2014) stated that they were significantly associated with damaged surfaces. With the addition of the metagenomic data it became clear that they were associated with both environments, in fact *P. fluorescens* is part of the core microbiome, although within the scope of this study it was not possible to state whether they were significantly associated with either surface.

The metagenomics study also resolved conflicts in the literature, many studies have claimed that *Bacillus* species are associated with damaged surfaces while others stated a significant association with undamaged surfaces (Di Bonaventura *et al.*, 1999; Mitchell & Gu, 2001; Cappitelli *et al.*, 2007; Piñar *et al.*, 2009; Banks *et al.*, 2010; De Leo *et al.*, 2012). In this study, by comparing damaged and undamaged surfaces, it has been possible to demonstrate that many of the *Bacillus* species identified showed no significant preference for damaged stone. In the original studies for these papers, and also for *Pseudomonas* species, authors did not carry out comparisons to undamaged areas (Videla *et al.*, 2000; Mitchell & Gu, 2001; Cappitelli *et al.*, 2007; De Leo *et al.*, 2012) as the studies focused solely on damaged surfaces. This study show that many of these species are not significantly associated with either, and indeed some have been shown to make up the core of the microbiome.

While there were only two rural samples in the metagenomic sampling, one damaged and one undamaged, the data from both the direct sampling and the 16S rRNA metagenomics supports there being significant differences between the rural and urban environments. Both methods showed compatible results including a significant association for *M. luteus* and *P.* sp*. 1105* both being significantly (p-value < 0.05) associated with the urban environment, and *B. safensis* being significantly (p-value < 0.05) associated with the rural environment. At the OTU level the differences between rural and urban microbiomes were distinct enough that classes of bacteria were significantly associated with urban damaged surfaces, rural damaged surfaces, urban undamaged surfaces and rural undamaged surfaces.

The higher total estimated population on damaged surfaces compared to undamaged surfaces suggests that the damaged surface is more amenable to bacterial growth, either through increased availability of minerals or the increased surface roughness, resulting in a higher surface area and different microclimate compared to the undamaged surface. The fact that there are species which are solely isolated from the damaged surfaces suggests that the damage, either physical or biological, allows these species to grow on what would otherwise be an inimical surface to them. Whether it is the change in the surface, or the presence of the species that change the surface, which promotes the presence of these damage specific species is a topic for future investigation.

The potential for transition to a damaging population was addressed by looking for species with the potential to engineer the pH of their environment. *A. baylyi,* pH 5-6.6, *M. halobius,* pH 4.6-7.6, *P. stutzeri,* pH 4.6-5.6, *P. psychrodurans,* pH 4.6-7.6, and *S. rhizophila*, pH 5.6-7.6, all demonstrated the capacity to grow at a wide range of pH's. In the tests for acid production only

M. halobius was identified as an acidifier, *P. stutzeri* and *P. psychrodurans* showed no measurable change in pH during growth and *A. baylyi* and *S. rhizophila* demonstrated alkalisation of the environment. This data set has identified the potential for some species being involved in environmental engineering, specifically having the potential to acidify the environment in order to gain a selective advantage over the other species present in the microbiome. *M. halobius* in particular from this set of experiments has shown the capacity for acidification of the environment as well as the capacity to grow optimally across a wide pH range. It is therefore reasonable to speculate that the introduction of *M. halobius* to an undamaged stone surface would result in it acidifying the environment to create selective pressure against the other species present. While the same cannot be said about the other 4 species identified with the capacity to grow across a wide range of pH's, the results of acidification and alkalization of the environment during the growth cycle are dependant on growth media, and therefore further work is needed to elucidate the full potential of these species.

7.1.3 Biodeterioration

This study detected four mechanisms of biodeterioration during the testing of the isolates, enhanced physical and geochemical weathering of the stone through the presence of high levels of biofilm matrix, biocorrosion, biochemical modification of the stone matrix and biomechanical weathering of the stone by biofilm matrix. Half of the species identified in this study demonstrated biodeteriorative capacity through one or more of these mechanisms.

7.1.3.1 Enhanced physical weathering by biofilm matrix

Of the 64 species tested only *Sporosarcina saromensis* was classified as not producing biofilm, therefore 98.5% of the species tested were biofilm formers. Eighteen of the isolates were classed as weak biofilm formers, due to strain to strain variation in biofilm formation this works out at 25% of species tested; these species are therefore unlikely to substantially contribute to the enhancement of physical weathering through biofilm formation. The species which are the strongest biofilm formers (Table 28) are most likely to rapidly enhance physical weathering due biofilm secretion; the key contributors identified in this study being *P. fluorescens, B. muralis* and *P. psychrodurans* based on their prevalence at the sampling sites and the high levels of biofilm formed. The analysis of biofilm formation by species, based on the surface they were sampled from, has provided a direct link between species which show a higher production of biofilm matrix and damaged surfaces. This provides experimental evidence supporting the link between biofilm matrix and biodeterioration through the enhancement of physical weathering which was previously missing in the literature.

Surface	Species	Prevalence % sites sampled	physical weathering Biofilm	biomechanical weathering Biofilm	biochemical modification calcification	Biocorrosion	Environmental engineering pH modification	Biocides efficacy against biofilms	
								bacterial resistance	matrix resistance
Both	*Arthrobacter agilis*	100				X		I	S,I
	Micrococcus luteus	100				X			
	Pseudomonas fluorescens	87	X						M
	Acinetobacter calcoaceticus	62			X	X			
	Pseudomonas stutzeri	37				X	X		
	Bacillus cereus	25				X			
	Curtobacterium flaccumfaciens	25	X	X					M,A,S,I
	Sphingobacterium faecium	25	X						A,I
	Stentrophomonas maltophilia	25			X	X			
	Micrococcus roseus	13				X		S	S
	Acinetobacter baylyi	12	X				X		M,A,S
	Bacillus safensis	12			X	X		I	I
	Pseudomonas putida	12				X			A
	Staphylococcus xylosis	12				X			
	Stentrophomonas rhizophila	12	X				X	M	M
	Bacillus pumilis	6	X						M,A,S,I
	Bacillus thuringensis	6	X					I	S
Undamaged	*Advenella kashmirensis*	25	X						I
	Pseudomonas brenneri	25	X			X			M,S
	Solibacillus silvestris	25		X	X	X			A,I
	Microbacterium thalassium	6	X						
	Spongiibacter sp. *IMCC21906*	6	X		X	X			M,A,S,I
Damaged	*Bacillus licheniformis*	100				X			M
	Bacillus muralis	50	X			X			M,S,I
	Psychrobacillus psychrodurans	50	X				X		
	Bacillus aerophilus	25	X						M,A,S,I
	Bacillus sporothermodurans	25			X	X		S	M
	Paenibacillus lautus	25				X			
	Bacillus foraminis	6	X						M,A,S,I
	Microbacteriaceae sp. *PAG4D*	6				X			
	Microbacterium pseudoresistens	6	X						A,S,I
	Pseudomonas sp. *HZ06*	6	X						M,A,S,I

Table 28: Summary of the properties of species associated with biodeterioration and biocorrosion, together with their prevalance across the sample sites. Species which are shown as enhancing physical weathering through biofilm production all produced high levels of matrix. Biophysical weathering through biofilm identifies the species where the biofilm matrix was disrupting the stone structure as opposed to enhancing physical weathering. Biocides are denoted as M for Microtech, A for Algobioinhibitor, S for Soluguard and I for IMS.

7.1.3.2 Biocorrosion

Species capable of biocorrosion (Table 28) made up a much higher proportion of the damaged population, and the population found on both surfaces, than the undamaged population. A higher percentage of the population were shown to solely acidify the environment as part of their growth curve under these test conditions compared to the undamaged population. There was also a much higher proportion of the damaged population capable of acidifying and alkalising the environment at different stages of the growth phase than the undamaged population or the population isolated from both.

The combination of analysing isolates for acidification of their environment and biocorrosive potential has demonstrated that the production of organic acids is insufficient evidence for biocorrosion, as several species which produced acid in the Bromocresol purple tests showed no experimental evidence of biocorrosion or dissolution of calcium carbonate. This highlights a problem with the current literature on biodeterioration, where the species capacity for biocorrosion has not been directly tested but assumed based on literature searches for organic acid production.

Of the 18 species identified as dissolving calcium carbonate in this study, 8 of them, *A. calcoaceticus, B. safensis, M.* sp*. PAG4D, P. lautus, P. brenneri, S. silvestris, S.* sp. *IMCC21906* and *S. xylosus* had not been identified in the literature as damaging to the limestone surface. Three of the above species were only isolated from undamaged surfaces; of these *Spongiibacter* sp*. IMCC21906* and *P. brenneri* were not found in the metagenomics study but *S. silvestris* was identified in the metagenomics study as well as the direct sampling and only isolated from undamaged surfaces. The only isolates in this study which were isolated solely from a damaged surface which dissolved calcium carbonate were *Bacillus licheniformis* and *Microbacteriaceae* sp*. PAG4D*, a new species. Based on these numbers somewhere between 16-45% of the species which have the potential to cause biodeterioration through biocorrosion are missing from the literature due to previous studies solely focussing on damaged surfaces.

Only 3 of the species identified as strong biofilm formers were demonstrated to cause biocorrosion, *P. brenneri, S.* sp. *IMCC21906* and *B. muralis* (Table 28). It is possible that the remaining 15 species are using biocorrosion to engineer the environment into one which better fits their growth requirements.

Based on prevalence in the sampling *A. agilis, A. calcoaceticus, B. licheniformis, B. muralis, M. luteus* and *P. stutzeri* should all be considered key contributors to biocorrosion as they were present in 30% or more of the sites sampled.

7.1.3.3 Biochemical modification of the stone matrix

While the sample numbers are too small to determine the significance it is interesting to note that of the 3 species identified as actively dissolving calcium carbonate on undamaged stone, two were also calcifying their biofilm matrix. When this is compared to the isolates found on both surfaces only 3 of the 10 isolates produced calcification, and only 1 of the 5 isolates which dissolved calcium carbonate on the damaged surface also calcified the environment. This suggests that the action of species which dissolve calcium carbonate on visibly undamaged surfaces could be more likely to result in re-deposition of the calcium carbonate. The dissolution and redeposition of the stone around the biofilm matrix will result in alteration of the physical characteristics of the stone surface; whether in this case it is harmful, with the flexible matrix resulting in a more friable surface, or beneficial as the deposited calcium carbonate will penetrate the stone surface in a similar fashion to the structural pins used in stone conservation, is beyond the scope of the current study.

7.1.3.4 Biomechanical weathering by the biofilm matrix

The final mechanism of biodeterioration identified in this study is also novel. In the literature the weathering caused by the biofilm matrix is only ever assumed to be caused by enhancing the effects of physical weathering, expansion and contraction of the matrix with changes in relative humidity and temperature accelerating the effect which these environmental changes would have on the stone structure anyway. The observation that the biofilm matrix of *Curtobacterium flaccumfaciens* and *Solibacillus silvestris* was growing through the oolitic matrix of the limestone and removing oolites from the surface has resulted in the identification of a new mechanism of biomechanical weathering.

7.1.3.5 Detection of biodeterioration

The environmental testing which was carried out at each sample site showed that there was a significant difference in surface pH between the damaged and undamaged stone surfaces which is most likely associated with the microbiomes. The increased acidity levels suggests that taking pH measurements of stone may be a marker for biodeterioration, with a measured change in acidity suggesting a need for conservation treatment before damage is visible to the eye. This also provides a measure of surface damage which is not based purely on physical weathering.

M. luteus and *B. licheniformis* are both significantly associated with damaged stone as well as having been shown to cause biodeterioration; as such they have the potential to act as biomarkers. Regular monitoring of the presence of *M. luteus* to determine whether the levels on a surface are increasing, or looking for the appearance of *B. licheniformis* in a microbiome would

both demonstrate that the population present had moved towards one where biodeteriorative processes were active.

7.1.4 Biocides

Efficacy of the biocides against biofilm encapsulated bacteria was high, Algo bioinhibitor still showing 100% efficacy against the bacteria when they were protected by the biofilm matrix. The other biocides tested showed a reduction in efficacy which was expected based on the literature.

Testing the biocides for efficacy against the biofilm matrix, chemical cleaning, showed lower efficacy than bactericidal activity but supported the manufacturers claims of cleaning. Cleaning efficacy was above 60% for total removal of biofilm matrix and between 88-96% for partial removal of biofilm matrix. If only the species which showed potential to cause biodeterioration are considered, 69% of the species had biofilm matrix which was resistant to chemical attack by some or all of the biocides tested (Table 28). Unfortunately none of the biocides were effective against the biofilm matrix of *C. flaccumfaciens*, a species where clear evidence of the matrix causing biomechanical damage to the oolitic composition of the limestone was observed.

The biocides used in this study are those currently in use in the conservation profession, studies into biocide efficacy have not been carried out since the changes in legislation removed access to the majority of biocides in the literature. This study characterised them as generally effective and capable of removing biofilm matrix. This comes at a cost with the most effective biocides being harmful to the environment, and potentially harmful to the stone surface which they are being used to treat. Concerns regarding the effect of volatility of biocide components on their efficacy have also been identified as part of this study; IMS and Microtech both showed reduced efficacy in spot testing compared to the other tests because of this. Where these biocides are being used in environmental conditions the use of poulticing to prevent evaportation of the active components would be recommended.

7.2 Future work

As would be expected from any major study, questions have been raised from the analysis of the results which are external to the initial remit. These are framed below as work which could be carried out to enhance the general understanding of bioreceptivity and biodeterioration of stone surfaces.

The identification of three potential markers for biodeterioration, lower surface pH on damaged stone, the presence of *B. licheniformis* and a higher level of *M. lutetus*, opens the possibility for

rapid testing. As the latter two, both biomarkers, would require a more complex skill set than the surface measurement of pH they are more applicable for research studies than general monitoring. The significantly lower pH found on damaged stone suggests that taking pH measurements of the stone surface would provide a rapid method of monitoring biodeterioration, with a drop in pH towards or below pH 5.5 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 and discover whether it is specific to Lincoln limestone or whether it can be generically applied.

An interesting correlation was observed between gelatin hydrolysis and dissolution of calcium carbonate. Further research should be carried out to determine whether this provides another biomarker which could result in a relatively simple test for microbiomes which will cause biocorrosion.

Having the chemical composition of Lincoln limestone provides a baseline for an alternative method for measuring biodeterioration and chemical weathering of the stone. By tracking chemical conversion and change in the balance of minerals through salting, damage can be quantified using techniques such as XRF.

To date nothing has been published about the early bacterial colonisation of naïve surfaces. Identifying early colonisers will be useful for testing whether species on found on surfaces which have been treated with biocide are newly introduced from the local or regional environment. As this study is based on further characterisation of the microbiome, a metagenomic approach would be acceptable, preferably identifying the complete microbiome, bacterial, fungal and algal.

The microbiome is likely to show seasonal variation which was not addressed as part of this study. A larger survey of urban and rural sites, possibly including coastal sampling, with seasonal sampling looking at 16S and 18/26S rRNA to cover whole microbiome rather than just bacteria/archaebacterial would address this. This would also enhance our understanding of the differences in the damaged microbiome in the rural environment when compared to the urban environment.

The potential discovery of a new species in *Microbacteriaceae* sp. *PAG4D* requires further study, in order to fully characterise the species and to determine which genus it belongs to.

The current methods for the identification of species using metagenomic analysis have been shown to suffer from the short reads required by the established technology. Nanopore technologies are becoming available which do not have the current read length limitations.

Developing 16S metagenomics using nanopore technology would allow sequencing of the whole 16S gene to give more accurate results, it should be noted that this still would not overcome false reading from heterogeneous copies of the 16S gene in single organisms.

As part of this study initial evidence has been gathered showing that some species are potentially engineering the environment by acidification to provide an environment where they have a competitive advantage. Further laboratory testing using polyspecies biofilms containing *A. baylyi, S. rhizophila, M. halobius* or *P. stutzeri* would provide further evidence for this.

Based on comparrisons between growth rate and alkalization of the growth environment, it is possible that the slower growing species on the undamaged stone help to regulate the pH of their environment limiting the damage caused by the faster growing species. Further work is needed to confirm this observation as the inoculation of damaged surfaces with these species could provide a mechanism for the bioremediation of damaged stone surfaces.

Identification of species as being capable of biocorrosion in the current literature is most commonly based on their capacity to produce organic acids when biochemical profiling of the species is carried out. This study has shown that the production of organic acids does not necessarily directly relate to active biocorrosion of limestone, and therefore further work is required to test all the species in the literature which have not already been covered by this study to see whether they are actually involved in biodeterioration.

In this study only the primary bioreceptivity was analysed for Lincoln limestone, bioreceptivity of damaged stone (secondary bioreceptivity) and stone which has undergone conservation treatment (tertiary bioreceptivity) could be carried out to complete the picture. This study would also help to clarify whether it is the damage to the surface which allows the damage specific species to grow or whether it is the development of the community of bacteria which promotes damage which makes the surface supportive of these species.

The literature on bioreceptivity shows a distinct lack of characterisation of UK stones, a larger study into the bioreceptivity of different UK building stones using standardised testing would overcome this lack and allow truly comparative results.

Currently the role of biofilm formation for the individual species in the microbiome is poorly understood, based on the results from further analysis of the microbiome and characterisation of early colonisers, experimental studies should be undertaken to clarify this area which will increase the understanding of the role of species specific biofilms in biodeteriorative processes.

This study identified a novel mechanism of bacterial biofilm damage, resulting in direct physical removal of smaller oolites by the biofilm matrix. Further investigation is needed into the biomechanical damage caused by biofilm matrix on fine grained sedimentary stones by species like *C. flaccumfaciens*.

The calcification of biofilm matrix has been observed in several species as part of this study on the physical properties of stone. While calcium carbonate precipitation has been considered as a positive aspect in the literature as it has been shown to promote surface cohesion (Dhami *et al.*, 2014) these studies have not examined the effects on the physical strength of the stone and assume the precipitation is only associated with the stone rather than being wrapped in a thin layer around a flexible biofilm matrix as was observed in this study. Further research into the alteration of the physical properties of the stone structure of bioprecipitated calcium carbonate is clearly required.

An aspect of the study which could also be pursued, as it opens the possibility of developing more effective biocides, is the correlation observed between gram negative cell wall morphology and the resistance of the biofilm matrix to chemical cleaning.

With the commercially available biocides having the potential to enhance salt weathering of limestone surfaces there is a clear requirement for alternatives to biocides. The following 3 studies would provide alternatives to biocidal treatments.

The highly alkaline environment produced by fresh mortar has been shown to have biocidal properties (Doehne & Price, 2010) which suggests that application of alkaline solutions could provide an alternative to chemical biocides, especially as calcium hydroxide has been used in past attempts to consolidate the limestone surface and is therefore already acceptable to the conservation community, although considered fairly ineffective as a consolidant.

Another alternative to biocide treatment is phage 'therapy'. With the enhanced understanding of biodeterioration provided by this study, bacteriophage could be isolated which would specifically target the damaging organisms only. If this was combined with the inoculation of species which have been demonstrated as having no role in the biodeterioration of surfaces this would allow the artificial engineering of the microbiome to produce a more protective community.

Finally, while studies have looked at the potential for natural biocides instead of chemical ones for controlling surface colonisation (Sasso *et al.*, 2016; Silva *et al.*, 2016) the use of biosurfactants to inhibit biofilm production is not one which has been addressed as a potential tool for the conservation of stone. *P. polymyxa* has been identified in the literature as producing

biosurfactants which inhibit the development of bacterial biofilms, something which is supported by the absence of biofilm on the surface from which it was isolated. Further work on the potential of biosurfactants to provide non-toxic biofilm inhibition has, in the author's opinion, a great deal of potential.

In addition to the peer reviewed conference papers already published, the author plans to publish the following papers from the research carried out in this study.

Characterisation of the microbiome of damaged limestone in an oceanic climate.

This paper will cover the results of the metagenomic and direct sampling work along with the characterisation of the species for the type of damaged caused. The paper will also cover the species identified in past publications allowing confirmation of general presence specifically on damaged stone and screening the results of those studies for species which are common to damaged and undamaged.

The intention is to publish in Nature Scientific Reports or Frontiers in Microbiology

Characterisation of bioreceptivity of Lincoln limestone surfaces

This paper will cover the results of the physical and chemical characteristics of Lincoln limestone, as covered in chapter 3. The intention will be to publish in Science of the Total Environment or International Biodeterioration & Biodegradation.

Biocide efficacy against the bacterial species specific to damaged limestone

Two papers are envisaged from the data set covered in Chapter 6, a research based publication in either Heritage Science or Journal of Cultural Heritage and a biocide usage guidance publication in the Journal of the Institute of Conservation.

Effects of biocide components on the salting of limestone surfaces.

This paper will cover the SEM-EDX and photographic analysis of the changes to the surface salt levels following the application of biocides discussed in section 5 of Chapter 6, and has been submitted to the Journal of the Institute of Conservation.

References

Aibéo, C. (2012) 'EU-PANNA project Plasma And Nano for New Age "soft" conservation - Development of a Portable Plasma Torch for Cleaning multi Surfaces and Coating Deposition', in *2nd European Workshop on Cultural Heritage Preservation*. Staatliche Museen zu Berlin: Rathgen-Forschungslabor. Available at: http://www.nadir-tech.it/dloads/PANNA/Aibeo_EWCHP_2012.pdf (Accessed: 6 December 2016).

Alan, V., Macaskie, L. E. and Callow, M. E. (1999) 'Development of a pH gradient within a biofilm is dependent upon the limiting nutrient', *Biotechnology Letters*, 21(5), pp. 407–413. doi: 10.1023/A:1005401926320.

Allsopp, D., Seal, K. and Gaylarde, C. (2004) *Introduction to biodeterioration*. Second, *International Biodeterioration*. Second. Cambridge: Cambridge University Press. doi: 10.1016/0265-3036(88)90076-0.

Ascaso, C. *et al.* (2002) 'In situ evaluation of the biodeteriorating action of microorganisms and the effects of biocides on carbonate rock of the Jeronimos Monastery (Lisbon)', *International Biodeterioration and Biodegradation*, 49(1), pp. 1–12. doi: 10.1016/S0964-8305(01)00097-X.

Ashall, G. and Webb, M. (2013a) *Testing of Ancaster Limestone*.

Ashall, G. and Webb, M. (2013b) *Testing of Lincolnshire Limestone*.

Audrain, B. *et al.* (2015) 'Role of bacterial volatile compounds in bacterial biology', *FEMS Microbiology Reviews*, 39(2), pp. 222–233. doi: 10.1093/femsre/fuu013.

Auras, M. *et al.* (2016) 'THE BLACK SURFACES OF THE PORTA NIGRA IN TRIER (GERMANY) AND THE QUESTION OF CLEANING', in Hughes, J. and Howind, T. (eds) *Science and Art : A Future for Stone Volume II*. Paisley: University of the West of Scotland, pp. 1077–1087.

Babauta, J. T. *et al.* (2012) 'pH, redox potential and local biofilm potential microenvironments within Geobacter sulfurreducens biofilms and their roles in electron transfer.', *Biotechnology and bioengineering*, 109(10), pp. 2651–62. doi: 10.1002/bit.24538.

Baer, N. S. (Norbert S. and Snethlage, R. (1997) *Saving our architectural heritage : the conservation of historic stone structures : report of the Dahlem Workshop on Saving our Architectural Heritage--the Conservation of Historic Stone Structures, Berlin, March 3-8, 1996*. J. Wiley.

Bak, J. *et al.* (2009) 'Dose requirements for UVC disinfection of catheter biofilms.', *Biofouling*, 25(April 2013), pp. 289–296. doi: 10.1080/08927010802716623.

Banks, E. D. *et al.* (2010) 'Bacterial Calcium Carbonate Precipitation in Cave Environments: A Function of Calcium Homeostasis', *Geomicrobiology Journal*, 27(5), pp. 444–454. doi: 10.1080/01490450903485136.

Barber, C. (1974) 'Major and trace element associations in limestones and dolomites', *Chemical Geology*, 14(4), pp. 273–280.

Bartoli, F. *et al.* (2014) 'Biological colonization patterns on the ruins of Angkor temples (Cambodia) in the biodeterioration vs bioprotection debate', *International Biodeterioration and Biodegradation*, 96. doi: 10.1016/j.ibiod.2014.09.015.

Bell, E. (2012) *Life at extremes : environments, organisms, and strategies for survival*. CAB

International.

Berdoulay, M. and Salvado, J. C. (2009) 'Genetic characterization of microbial communities living at the surface of building stones.', *Letters in applied microbiology*, 49(3), pp. 311–316. doi: 10.1111/j.1472-765X.2009.02660.x.

Berner, M., Wanner, G. and Lubitz, W. (1997) 'A comparative study of the fungal flora present in medieval wall paintings in the chapel of the castle Herberstein and in the parish church of St Georgen in Styria, Austria', *International Biodeterioration & Biodegradation*, 40(1), pp. 53–61. doi: 10.1016/S0964-8305(97)00062-0.

Bioscreen C MBR (2017). Available at: http://www.bioscreen.fi/bioscreencmbr.html (Accessed: 3 January 2017).

Di Bonaventura, M. P. *et al.* (1999) 'Microbial Formation of Oxalate Films on Monument Surfaces: Bioprotection or Biodeterioration?', *Geomicrobiology Journal*, 16(1), pp. 55–64. doi: 10.1080/014904599270749.

Booth, I. R. R. (1985) 'Regulation of cytoplasmic pH in bacteria.', *Microbiological reviews*, 49(4), pp. 359–378. doi: 10.1016/S1569-2582(97)80142-0.

British-Geological-Survey (2013) *British Geological Survey*, *Lincolnshire Building Stone Atlas.* Available at: http://www.bgs.ac.uk/mineralsuk/buildingStones/StrategicStoneStudy/EH_atlases.html (Accessed: 18 December 2013).

British Geological Society (2016) *BGS Lexicon of Named Rock Units -Oxwich Head Limestone Formation*. Available at: http://www.bgs.ac.uk/lexicon/lexicon.cfm?pub=OHL (Accessed: 19 August 2016).

Cacchio, P. *et al.* (2003) 'Calcium Carbonate Precipitation by Bacterial Strains Isolated from a Limestone Cave and from a Loamy Soil', *Geomicrobiology Journal*, 20(2), pp. 85–98. doi: 10.1080/01490450303883.

Cadw (2012) *Oxwich Castle — Conservation Action*, *Cadw website*. Available at: http://cadw.gov.wales/historicenvironment/conservation/conservationcadwsites/oxwichcastlecasestudy/?lang=en (Accessed: 15 September 2016).

Caporaso, J. G. *et al.* (2012) 'Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms', *The ISME Journal*, 6(8), pp. 1621–1624. doi: 10.1038/ismej.2012.8.

Cappitelli, F. *et al.* (2007) 'Bacterial and fungal deterioration of the Milan Cathedral marble treated with protective synthetic resins.', *The Science of the total environment*, 385(1–3), pp. 172–81. doi: 10.1016/j.scitotenv.2007.06.022.

Ceri, H., Olson, M. and Stremick, C. (1999) 'The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms', *Journal of clinical …*, 37(6), p. 1771. Available at: http://jcm.asm.org/content/37/6/1771.short (Accessed: 10 June 2014).

Cezar, T. M. (1998) 'Calcium Oxalate: A Surface Treatment for Limestone', *Journal of Conservation and Museum Studies*, 4(0), p. 6. doi: 10.5334/jcms.4982.

Chakravorty, S. *et al.* (2007) 'A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria.', *Journal of microbiological methods*, 69(2), pp. 330–9. doi: 10.1016/j.mimet.2007.02.005.

Chang, S. M. and Tien, M. (1969) 'Effects of Heavy Metal Ions on the Growth of Microorganisms.', *Bull. Inst. Chem.*, 16, pp. 29–39.

Chapman, J. S. (2003) 'Disinfectant resistance mechanisms, cross-resistance, and co-resistance', *International Biodeterioration & Biodegradation*, 51(4), pp. 271–276. doi: 10.1016/S0964-8305(03)00044-1.

Cherblanc, F., Berthonneau, J. and Bromblet, P. (2016) 'ROLE OF HYDRO-MECHANICAL COUPLING IN THE DAMAGE PROCESS OF LIMESTONES USED IN HISTORICAL BUILDINGS', in Hughes, J. and Howind, T. (eds) *Science and Art: A Future for Stone. Proceedings of the 13th International Congress on the Deterioration and Conservation of Stone - Volume I*. Paisley: University of the West of Scotland, pp. 49–56.

Chinga, G. and Dougherty, B. (2002) *Roughness Calculation plugin for Image J*. Available at: https://imagej.nih.gov/ij/plugins/roughness.html (Accessed: 23 February 2017).

Chmielewski, F. M. and Rotzer, T. (2001) 'Response of tree phenology to climate change across Europe', *Agricultural and Forest Meteorology*, 108(2), pp. 101–112. doi: 10.1016/S0168-1923(01)00233-7.

St. Clair, L. L. and Seaward, M. R. D. (2004) *Biodeterioration of stone surfaces : lichens and biofilms as weathering agents of rocks and cultural heritage*. Kluwer Academic Publishers.

Concha-Lozano, N. *et al.* (2013) 'Color thresholds for aesthetically compatible replacement of stones on monuments', *Color Research & Application*, 38(5), pp. 356–363. doi: 10.1002/col.21729.

Cooper, M. (1998) *Laser cleaning in conservation : an introduction*. Butterworth-Heinemann.

Cotter, P. D. and Hill, C. (2003) 'Surviving the acid test: responses of gram-positive bacteria to low pH.', *Microbiology and molecular biology reviews : MMBR*, 67(3), p. 429–53, table of contents. doi: 10.1128/mmbr.67.3.429-453.2003.

Croes, S. *et al.* (2009) 'Staphylococcus aureus biofilm formation at the physiologic glucose concentration depends on the S. aureus lineage', *BMC Microbiol*, 9(1), p. 229. doi: 10.1186/1471-2180-9-229.

Cutler, N. *et al.* (2012) 'Non-destructive sampling of rock-dwelling microbial communities using sterile adhesive tape', *Journal of microbiological …*, 91(3), pp. 391–398. doi: 10.1016/j.mimet.2012.09.022.

Dakal, T. and Cameotra, S. (2012) 'Microbially induced deterioration of architectural heritages: routes and mechanisms involved', *Environmental Sciences Europe*, 24(1), p. 36. doi: 10.1186/2190-4715-24-36.

Davies, D. (2003) 'Understanding biofilm resistance to antibacterial agents.', *Nature reviews. Drug discovery*, 2(2), pp. 114–22. doi: 10.1038/nrd1008.

Davies, D. G. *et al.* (1998) 'The involvement of cell-to-cell signals in the development of a bacterial biofilm.', *Science (New York, N.Y.)*, 280(5361), pp. 295–8. doi: 10.1126/SCIENCE.280.5361.295.

Defra (2016) *UK Ambient Air Quality Interactive Map - Defra, UK*. Department for Environment, Food and Rural Affairs (Defra), Nobel House, 17 Smith Square, London SW1P 3JR helpline@defra.gsi.gov.uk.

Delgado, J. M. P. Q. (2016) *New Approaches to Building Pathology and Durability*. Edited by J. M.

P. Q. Delgado. Singapore: Springer Singapore (Building Pathology and Rehabilitation). doi: 10.1007/978-981-10-0648-7.

Dhami, N. K., Reddy, M. S. and Mukherjee, A. (2014) 'Application of calcifying bacteria for remediation of stones and cultural heritages.', *Frontiers in microbiology*, 5, p. 304. doi: 10.3389/fmicb.2014.00304.

Diaz-Herraiz, M. *et al.* (2014) 'Deterioration of an Etruscan tomb by bacteria from the order Rhizobiales.', *Scientific reports*, 4, p. 3610. doi: 10.1038/srep03610.

Doehne, E. and Price, C. A. (2010) *Stone Conservation: An Overview of Current Research*. 2nd edn. Getty Publications.

Dornieden T., Gorbushina, A. A. and W.E., K. (2000) 'Patina', in Ciferri, O., Tiano, P., and Mastromei, G. (eds) *Of microbes and art : the role of microbial communities in the degradation and protection of cultural heritage.* New York London: Kluwer Academic/Plenum, pp. 105–119.

Dupont, J. *et al.* (2007) 'Invasion of the French paleolithic painted cave of Lascaux by members of the Fusarium solani species complex.', *Mycologia*, 99(4), pp. 526–533. doi: 10.3852/mycologia.99.4.526.

Edmunds, W. M. (1996) 'Bromine geochemistry of British groundwaters', *Mineralogical Magazine*, 60, pp. 275–284.

Eklund, J. A. (2013) *Biological Growth on Masonry : Identification and Understanding*. Edinburgh. Available at: http://conservation.historic-scotland.gov.uk/bio-growth-masonry-inform.pdf (Accessed: 12 September 2016).

Emery, D. *et al.* (1988) 'The origin of late spar cements in the Lincolnshire Limestone, Jurassic of central England', *Journal of the Geological Society*, 145(4), pp. 621–633. doi: 10.1144/gsjgs.145.4.0621.

English Heritage (2013) *Listed Buildings.* Available at: http://www.english-heritage.org.uk/caring/listing/listed-buildings/ (Accessed: 20 December 2013).

EUCAST (2000) 'EUCAST Definitive Document E.DEF 3.1, June 2000: Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution.', *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, 6(9), pp. 509–15.

Flores, M., Lorenzo, J. and Gómez-Alarcón, G. (1997) 'Algae and bacteria on historic monuments at Alcala de Henares, Spain', *International Biodeterioration & Biodegradation*, 40(2–4), pp. 241–246. doi: 10.1016/S0964-8305(97)00058-9.

Frank, D. N. *et al.* (2009) 'Culture-independent microbiological analysis of foley urinary catheter biofilms.', *PloS one*, 4(11), p. e7811. doi: 10.1371/journal.pone.0007811.

Frederick, J. R. *et al.* (2001) 'Factors affecting catalase expression in Pseudomonas aeruginosa biofilms and planktonic cells.', *Applied and environmental microbiology*, 67(3), pp. 1375–9. doi: 10.1128/AEM.67.3.1375-1379.2001.

Garrett, T. R., Bhakoo, M. and Zhang, Z. (2008) 'Bacterial adhesion and biofilms on surfaces', *Progress in Natural Science*, 18(9), pp. 1049–1056. doi: 10.1016/j.pnsc.2008.04.001.

Gazzola, P. *et al.* (1964) 'International Charter for the conservation and restoration of monuments

and sites (The Venice Charter 1964)', *IInd International Congress of Architects and Technicians of Historic Monuments*, pp. 1–4. Available at: http://www.icomos.org/charters/venice_e.pdf (Accessed: 12 September 2016).

Gerdwilker, C. *et al.* (2016) 'THE IMPACT OF SCIENCE ON CONSERVATION PRACTICE : SANDSTONE CONSOLIDATION IN SCOTTISH BUILT HERITAGE', in Hughes, J. and Howind, T. (eds) *Science and Art : A Future for Stone Volume II*. Paisley: University of the West of Scotland, pp. 769–776.

Ghasemi, A. and Zahediasl, S. (2012) 'Normality tests for statistical analysis: a guide for non-statisticians.', *International journal of endocrinology and metabolism*, 10(2), pp. 486–9. doi: 10.5812/ijem.3505.

Gilbert, J. A., Jansson, J. K. and Knight, R. (2014) 'The Earth Microbiome project: successes and aspirations', *BMC Biology*, 12(1), p. 69. doi: 10.1186/s12915-014-0069-1.

Godet, M. *et al.* (2016) 'LASER YELLOWING OF HEMATITE-GYPSUM MIXTURES : A MULTI SCALE CHARACTERISATION', in Hughes, J. and Howind, T. (eds) *Science and Art : A Future for Stone Volume II2*. Paisley: University of the West of Scotland, pp. 785–792.

Goodwin, S., McPherson, J. D. and McCombie, W. R. (2016) 'Coming of age: ten years of next-generation sequencing technologies', *Nature Reviews Genetics*, 17(6), pp. 333–351. doi: 10.1038/nrg.2016.49.

Gorbushina, A. A. *et al.* (2004) 'Bacterial and fungal diversity and biodeterioration problems in mural painting environments of St. Martins church (Greene-Kreiensen, Germany)', *International Biodeterioration and Biodegradation*, 53(1), pp. 13–24.

Grammatikakis, G. *et al.* (2015) 'Laser-assisted removal of dark cement crusts from mineral gypsum (selenite) architectural elements of peripheral monuments at Knossos', *Studies in Conservation*, 60(sup1), pp. S3–S11. doi: 10.1179/0039363015Z.000000000201.

Griffiths, K. J., Shand, P. and Marchant, P. (2006) *Baseline Report Series : 23. The Lincolnshire Limestone*.

Gross, J. and Ligges, U. (2015) *nortest: Tests for Normality*. Available at: https://cran.r-project.org/package=nortest (Accessed: 5 November 2016).

Guillitte, O. (1995) 'Bioreceptivity: A new concept for building ecology studies', in *Science of the Total Environment*, pp. 215–220.

Guillitte, O. and Dreesen, R. (1995) 'Laboratory chamber studies and petrographical analysis as bioreceptivity assessment tools of building materials', *Science of the Total Environment*, 167(1–3), pp. 365–374. doi: 10.1016/0048-9697(95)04596-S.

Guiry, M. D. and Guiry, G. M. (2017) *AlgaeBase*, *World-wide electronic publication, National University of Ireland, Galway.* Available at: http://www.algaebase.org (Accessed: 8 September 2017).

Hallmann, C. *et al.* (2011) 'Microbial diversity on a marble monument: a case study', *Environmental Earth Sciences*, 63(7–8), pp. 1701–1711. doi: 10.1007/s12665-010-0772-3.

Harriott, M. M. and Noverr, M. C. (2009) 'Candida albicans and Staphylococcus aureus form polymicrobial biofilms: effects on antimicrobial resistance.', *Antimicrobial agents and chemotherapy*, 53(9), pp. 3914–22. doi: 10.1128/AAC.00657-09.

Harrison, D. J. *et al.* (2005) *Mineral resource information in support of national, regional and local planning : Lincolnshire*. Available at: https://www.bgs.ac.uk/downloads/start.cfm?id=2558 (Accessed: 20 October 2016).

Herrera, L. K. and Videla, H. a. (2009) 'Surface analysis and materials characterization for the study of biodeterioration and weathering effects on cultural property', *International Biodeterioration & Biodegradation*, 63(7), pp. 813–822. doi: 10.1016/j.ibiod.2009.05.002.

Heyrman, J. and Swings, J. (2001) '16S rDNA Sequence Analysis of Bacterial Isolatesfrom Biodeteriorated Mural Paintings in the Servilia Tomb (Necropolis of Carmona, Seville, Spain)', *Systematic and applied microbiology*, 24(3), pp. 417–422. doi: 10.1078/0723-2020-00048.

Hirai, Y. (1991) 'Survival of bacteria under dry conditions; from a viewpoint of nosocomial infection', *Journal of Hospital Infection*, 19(3), pp. 191–200. doi: 10.1016/0195-6701(91)90223-U.

HSE (2017) *The EU Biocides Regulation 528/2012 (EU BPR) - Biocides - HSE*, *hse.gov.uk*. Available at: http://www.hse.gov.uk/biocides/eu-bpr/ (Accessed: 9 January 2017).

Hsueh, Y.-H. *et al.* (2006) 'Biofilm formation by Bacillus cereus is influenced by PlcR, a pleiotropic regulator.', *Applied and environmental microbiology*, 72(7), pp. 5089–92. doi: 10.1128/AEM.00573-06.

Hugh, R. and Leifson, E. (1953) 'The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria.', *Journal of bacteriology*, 66(1), pp. 24–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13069461 (Accessed: 31 December 2016).

Ismail, N. I. A. *et al.* (2016) 'Associations of water quality and bacteria presence in cage cultured red hybrid tilapia, Oreochromis niloticus×O. mossambicus', *Aquaculture Reports*, 4, pp. 57–65. doi: 10.1016/j.aqrep.2016.06.004.

Jacobs, J. A. and Frickel, S. (2009) 'Interdisciplinarity: A Critical Assessment'. doi: 10.1146/annurev-soc-070308-115954.

Janda, J. M. and Abbott, S. L. (2007) '16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls', *Journal of Clinical Microbiology*, 45(9), pp. 2761–2764. doi: 10.1128/JCM.01228-07.

Jonker, J.-L. *et al.* (2015) 'The chemistry of stalked barnacle adhesive (Lepas anatifera).', *Interface focus*, 5(1), p. 20140062. doi: 10.1098/rsfs.2014.0062.

Jørgensen, S. E., Xu, F.-L. and Costanza, R. (2010) *Handbook of ecological indicators for assessment of ecosystem health*. CRC Press/Taylor & Francis.

Jost, L. (2007) 'PARTITIONING DIVERSITY INTO INDEPENDENT ALPHA AND BETA COMPONENTS', *Ecology*, 88(10), pp. 2427–2439. doi: 10.1890/06-1736.1.

Kearse, M. *et al.* (2012) 'Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data.', *Bioinformatics*, 28(12), pp. 1647–1649. doi: 10.1093/bioinformatics/bts199.

Keevil, C. W. (2004) 'The physico-chemistry of biofilm-mediated pitting corrosion of copper pipe supplying potable water.', *Water science and technology : a journal of the International Association on Water Pollution Research*, 49(2), pp. 91–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14982168 (Accessed: 6 December 2016).

Kimball, S. and Mattis, P. (2016) 'GNU Image Manipulation Program'. Available at: https://www.gimp.org/.

Knapp, C. W. *et al.* (2011) 'Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils.', *PloS one*, 6(11), p. e27300. doi: 10.1371/journal.pone.0027300.

Korkanç, M. and Savran, A. (2015) 'Impact of the surface roughness of stones used in historical buildings on biodeterioration', *Construction and Building Materials*, 80. doi: 10.1016/j.conbuildmat.2015.01.073.

Kostaki, M. *et al.* (2012) 'Differential biofilm formation and chemical disinfection resistance of sessile cells of listeria monocytogenes strains under monospecies and dual-species (with Salmonella enterica) conditions.', *Applied and environmental microbiology*, 78(8), pp. 2586–95. doi: 10.1128/AEM.07099-11.

Kusumi, A. *et al.* (2013) 'Bacterial communities in pigmented biofilms formed on the sandstone bas-relief walls of the Bayon Temple, Angkor Thom, Cambodia', *Microbes Environ*, 28(4), pp. 422–431. doi: 10.1264/jsme2.ME13033.

Kwasny, S. M. and Opperman, T. J. (2010) 'Static biofilm cultures of Gram-positive pathogens grown in a microtiter format used for anti-biofilm drug discovery.', *Current protocols in pharmacology*, Chapter 13, p. Unit 13A.8. doi: 10.1002/0471141755.ph13a08s50.

Lakretz, A., Ron, E. Z. and Mamane, H. (2011) 'Biofilm control in water by a UV-based advanced oxidation process.', *Biofouling*, 27(3), pp. 295–307. doi: 10.1080/08927014.2011.561923.

De Leo, F. *et al.* (2012) 'Chemoorganotrophic bacteria isolated from biodeteriorated surfaces in cave and catacombs', *International Journal of Speleology*, 41(2), pp. 125–136. doi: 10.5038/1827-806X.41.2.1</p>.

Lewis, K. I. M. (2001) 'Riddle of Biofilm Resistance MINIREVIEW Riddle of Biofilm Resistance', *Antimicrobial agents and chemotherapy*, 45(4), pp. 999–1007. doi: 10.1128/AAC.45.4.999.

Li, Q. *et al.* (2016) 'Distribution and diversity of bacteria and fungi colonization in stone monuments analyzed by high-throughput sequencing', *PLoS ONE*, 11(9). doi: 10.1371/journal.pone.0163287.

Long, E. S. and Young, D. A. (2016) 'TRIALS OF BIOCIDE CLEANING AGENTS ON ARGILLACEOUS SANDSTONE IN A TEMPERATE REGION', in Hughes, J. and Howind, T. (eds) *Science and Art : A Future for Stone Volume II*. Paisley: University of the West of Scotland, pp. 863–870.

Lottermoser, B. G. (2016) *Environmental indicators in metal mining*. Springer.

Maciel, B. M. *et al.* (2009) 'Simple DNA extraction protocol for a 16S rDNA study of bacterial diversity in tropical landfarm soil used for bioremediation of oil waste.', *Genetics and molecular research : GMR*, 8(1), pp. 375–388.

Mansch, R. and Bock, E. (1998) 'Biodeterioration of natural stone with special reference to nitrifying bacteria', *Biodegredation*, 9, pp. 47–64. Available at: http://download.springer.com/static/pdf/30/art%3A10.1023%2FA%3A1008381525192.pdf?auth66=1404394398_a08244000459268ce3551690db7dd9a1&ext=.pdf (Accessed: 1 July 2014).

Marasović, J. *et al.* (1976) 'Diocletian's Palace: Report on Joint Excavations in South-East Quarter.', *The Classical Review (New Series)*, 26(2), pp. 255–256.

May, E. (2003) ‘Microbes on building stone - for good or ill?’, *Culture*, 24(2), pp. 5–8.

Merrill, A. T. and Clark, W. M. (1928) ‘SOME CONDITIONS AFFECTING THE PRODUCTION OF GELATINASE BY PROTEUS BACTERIA.’, *Journal of bacteriology*, 15(4), pp. 267–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16559306 (Accessed: 19 December 2016).

Meulemans, C. C. E. (1987) ‘The Basic Principles of UV–Disinfection of Water’, *Ozone: Science & Engineering*, 9(4), pp. 299–313. doi: 10.1080/01919518708552146.

Mille, Y., Beney, L. and Gervais, P. (2005) ‘Compared tolerance to osmotic stress in various microorganisms: Towards a survival prediction test’, *Biotechnology and Bioengineering*, 92(4), pp. 479–484. doi: 10.1002/bit.20631.

Miller, A. Z. *et al.* (2009) ‘The influence of inherent properties of building limestones on their bioreceptivity to phototrophic microorganisms’, *Annals of Microbiology*, 59(4), pp. 705–713. doi: 10.1007/BF03179212.

Miller, A. Z. *et al.* (2010) ‘Primary bioreceptivity of limestones used in southern European monuments’, *Geological Society, London, Special Publications*, 331(1), pp. 79–92. doi: 10.1144/SP331.6.

Miller, A. Z. *et al.* (2012) ‘Bioreceptivity of building stones: a review’, *Science of the total …*, 426, pp. 1–12. doi: 10.1016/j.scitotenv.2012.03.026.

Mitchell, C. (2011) ‘High purity limestone quest’, *Industrial Minerals*, pp. 48–51. Available at: http://nora.nerc.ac.uk/15887/1/Reconnaissance_assessment_of_high-purity_limestone_plus_references.pdf (Accessed: 12 January 2017).

Mitchell, R. and Gu, J. (2001) ‘Changes in the biofilm microflora of limestone caused by atmospheric pollutants’, *International Biodeterioration & Biodegradation*, 46(2000), pp. 299–303.

Moisan, M. *et al.* (2002) ‘Plasma sterilization. Methods and mechanisms’, *Pure and Applied Chemistry*, 74(3), pp. 349–358. doi: 10.1351/pac200274030349.

Morrell, R. M. and Wasilauskas, B. L. (1992) ‘Tracking Laboratory Contamination by Using a Bacillus cereus Pseudoepidemic as an Example’, *JOURNAL OF CLINICAL MICROBIOLOGY*, 30(6), pp. 1469–1473.

Morton, H. E. (1950) ‘THE RELATIONSHIP OF CONCENTRATION AND GERMICIDAL EFFICIENCY OF ETHYL ALCOHOL’, *Annals of the New York Academy of Sciences*, 53(1), pp. 191–196. doi: 10.1111/j.1749-6632.1950.tb31944.x.

Morton, L. H. G. *et al.* (1998) ‘Consideration of some implications of the resistance of biofilms to biocides’, *International Biodeterioration & Biodegradation*, 41(3), pp. 247–259. doi: 10.1016/S0964-8305(98)00026-2.

NCBI Resource Coordinators, N. R. (2013) ‘Database resources of the National Center for Biotechnology Information.’, *Nucleic acids research*, 41(Database issue), pp. D8–D20. doi: 10.1093/nar/gks1189.

Neilands, J. B. (1995) ‘Siderophores: structure and function of microbial iron transport compounds.’, *The Journal of biological chemistry*, 270(45), pp. 26723–6. doi: 10.1074/JBC.270.45.26723.

Normandin, K. C. and Slaton, D. (2015) ‘Cleaning Techniques’, in Henry, A. (ed.) *Stone*

Conservation: Principles and Practice. Routledge, pp. 127–155. Available at: https://books.google.co.uk/books?id=s0seCwAAQBAJ&dq=pressure+washing+stone+conservation&source=gbs_navlinks_s.

Nuhoglu, Y. *et al.* (2006) 'The accelerating effects of the microorganisms on biodeterioration of stone monuments under air pollution and continental-cold climatic conditions in Erzurum, Turkey', *Science of the Total Environment*, 364(1–3), pp. 272–283. doi: 10.1016/j.scitotenv.2005.06.034.

Ochman, H., Elwyn, S. and Moran, N. A. (1999) 'Calibrating bacterial evolution.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(22), pp. 12638–43. doi: 10.1073/pnas.96.22.12638.

Odgers, D. (2013) *Steam Cleaning*, *The Building Conservation Directory*. Tisbury. Available at: http://www.buildingconservation.com/articles/steam-cleaning/steam-cleaning.htm (Accessed: 11 November 2016).

Ortega-Calvo, J. J. *et al.* (1995) 'Factors affecting the weathering and colonization of monuments by phototrophic microorganisms', *Science of The Total Environment*, 167(1–3), pp. 329–341. doi: 10.1016/0048-9697(95)04593-P.

Ortega-Morales, B. B. O. . B. B. B. O. . B. *et al.* (2013) 'Implications of colonizing biofilms and microclimate on west stucco masks at North Acropolis, Tikal, Guatemala', *Heritage Science*, 1(1), p. 32.

Papida, S., Murphy, W. and May, E. (2000) 'Enhancement of physical weathering of building stones by microbial populations', *International Biodeterioration and Biodegradation*, 46(4), pp. 305–317. doi: 10.1016/S0964-8305(00)00102-5.

Park, H. D. and Shin, G. H. (2009) 'Geotechnical and geological properties of Mokattam limestones: Implications for conservation strategies for ancient Egyptian stone monuments', *Engineering Geology*, 104(3–4), pp. 190–199. doi: 10.1016/j.enggeo.2008.10.009.

Parsek, M. R. and Singh, P. K. (2003) 'Bacterial biofilms: an emerging link to disease pathogenesis.', *Annual review of microbiology*, 57, pp. 677–701. doi: 10.1146/annurev.micro.57.030502.090720.

Parte, A. C. (2013) *List of prokaryotic names with standing in nomenclature*. Available at: http://www.bacterio.net/-generalinformation.html (Accessed: 20 September 2013).

Pasteur, L. (1878) 'Théorie des germes et ses applications à la médecine et à la chirurgie.', in. G. Masson.

Paulson, J. N. *et al.* (2013) 'Differential abundance analysis for microbial marker-gene surveys', *Nature Methods*, 10(12), pp. 1200–1202. doi: 10.1038/nmeth.2658.

Paulson, J. N. *et al.* (2013) 'metagenomeSeq: Statistical analysis for sparse high-throughput sequencing.' Available at: http://cbcb.umd.edu/software/metagenomeSeq.

Peel, B. L., Finlayson, B. L. and McMahon, T. a. (2007) 'Updated world map of the Köppen-Geiger climate classification.pdf', *Hydrology and Earth System Sciences*, 11, pp. 1633–1644. doi: 10.5194/hessd-4-439-2007.

Peraza Zurita, Y. *et al.* (2005) 'Microalgae associated with deteriorated stonework of the fountain of Bibatauín in Granada, Spain', *International Biodeterioration & Biodegradation*, 55(1), pp. 55–

61. doi: 10.1016/j.ibiod.2004.05.006.

Peters, B. M. *et al.* (2012) 'Polymicrobial interactions: impact on pathogenesis and human disease.', *Clinical microbiology reviews*, 25(1), pp. 193–213. doi: 10.1128/CMR.00013-11.

Pevsner, N., Harris, J. and Antram, N. (2002) *Lincolnshire*. Yale University Press.

Philips Electronics N.V. (2006) *Ultraviolet Purification Application Information*. Roosendaal. Available at: http://www.philips.com/uvpurification (Accessed: 31 December 2016).

Piñar, G. *et al.* (2009) 'The micro-biota of a sub-surface monument the medieval chapel of St. Virgil (Vienna, Austria)', *International Biodeterioration & Biodegradation*, 63(7), pp. 851–859. doi: 10.1016/j.ibiod.2009.02.004.

Pinna, D. (2014) 'Biofilms and lichens on stone monuments: do they damage or protect?', *Frontiers in microbiology*, 5, p. 133. doi: 10.3389/fmicb.2014.00133.

Prakash, A. (2012) *Microorganisms in sustainable agriculture and biotechnology*. Edited by T. Satyanarayana and B. N. Johri. Springer. Available at: https://books.google.co.uk/books?id=nMlHHCXVP0EC&source=gbs_navlinks_s (Accessed: 1 October 2017).

Prothero, D. R. and Schwab, F. L. (Frederic L. . (2004) *Sedimentary geology : an introduction to sedimentary rocks and stratigraphy*. W.H. Freeman.

R Core Team (2016) *R: A Language and Environment for Statistical Computing*. Vienna, Austria. Available at: https://www.r-project.org/ (Accessed: 3 March 2016).

Radaelli, a *et al.* (2004) 'Identification, molecular biotyping and ultrastructural studies of bacterial communities isolated from two damaged frescoes of St Damian's Monastery in Assisi.', *Letters in applied microbiology*, 38(6), pp. 447–453. doi: 10.1111/j.1472-765X.2004.01514.x.

Rainey, F. *et al.* (2015) *Bergey's Manual of Systematics of Archaea and Bacteria*. Edited by W. B. Whitman. Chichester, UK: John Wiley & Sons, Ltd. doi: 10.1002/9781118960608.

Rédei, G. P. (2008) 'M9 Bacterial Minimal Medium', in *Encyclopedia of Genetics, Genomics, Proteomics and Informatics*. Dordrecht: Springer Netherlands, pp. 1135–1135. doi: 10.1007/978-1-4020-6754-9_9692.

Rigden, D. J., Jedrzejas, M. J. and Galperin, M. Y. (2003) 'An extracellular calcium-binding domain in bacteria with a distant relationship to EF-hands.', *FEMS microbiology letters*, 221(1), pp. 103–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12694917 (Accessed: 7 December 2016).

Rodriguez-Navarro, C. and Doehne, E. (1999) 'Salt weathering: influence of evaporation rate, supersaturation and crystallization pattern', *Earth Surface Processes and Landforms*, 24(3), pp. 191–209. doi: 10.1002/(SICI)1096-9837(199903)24:3<191::AID-ESP942>3.0.CO;2-G.

Rousk, J. *et al.* (2010) 'Soil bacterial and fungal communities across a pH gradient in an arable soil', *Isme Journal*, 4(10), pp. 1340–1351. doi: 10.1038/ismej.2010.58.

Rumbo, C. *et al.* (2011) 'Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in Acinetobacter baumannii.', *Antimicrobial agents and chemotherapy*, 55(7), pp. 3084–90. doi: 10.1128/AAC.00929-10.

Saarela, M. *et al.* (2004) 'Heterotrophic microorganisms in air and biofilm samples from Roman

catacombs, with special emphasis on actinobacteria and fungi', *International Biodeterioration and Biodegradation*, 54(1), pp. 27–37. doi: 10.1016/j.ibiod.2003.12.003.

Sadekuzzaman, M. *et al.* (2015) 'Current and Recent Advanced Strategies for Combating Biofilms', *Comprehensive Reviews in Food Science and Food Safety*, 14(4), pp. 491–509. doi: 10.1111/1541-4337.12144.

Safeguard Europe (2016) *SoluGuard Multi-Surface Biocide*. Available at: http://www.safeguardeurope.com/products/soluguard/soluguard-multi-surface-biocide#product-information (Accessed: 17 November 2016).

Saikia, R. (2008) *Microbial Biotechnology*. New India Publishing. Available at: http://books.google.com/books?id=RXZVvuhEpw8C&pgis=1 (Accessed: 14 August 2014).

Saiz-Jimenez, C. (1984) 'Weathering and colonization of limestones in an urban environment', in *Soil biology and Conservation of the biosphere 2*. Budapest: Akadémiai Kiadó, pp. 757–767.

Saiz-Jimenez, C. *et al.* (2012) 'Uncovering the origin of the black stains in Lascaux Cave in France', *Environmental Microbiology*, 14(12), pp. 3220–3231. doi: 10.1111/1462-2920.12008.

Sakr, A. A., Ghaly, M. F. and Ali, M. F. (2013) 'The relationship between salts and growth of Streptomyces isolated from mural paintings in some ancient Egyptian tombs', *Conservation Science in Cultural Heritage*, pp. 313–330. doi: 10.6092/issn.1973-9494/4190.

Salminen, R. (2016) *Foregs Geochemical Atlas of Europe*. Espoo: Geological Survey of Finland. Available at: http://weppi.gtk.fi/publ/foregsatlas/.

Sandars, N. K. (1995) *Prehistoric art in Europe*. Yale University Press.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) 'DNA sequencing with chain-terminating inhibitors.', *Proceedings of the National Academy of Sciences of the United States of America*, 74(12), pp. 5463–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/271968 (Accessed: 21 September 2016).

Sant'Ana, A. S., Franco, B. D. G. M. and Schaffner, D. W. (2012) 'Modeling the growth rate and lag time of different strains of Salmonella enterica and Listeria monocytogenes in ready-to-eat lettuce', *Food Microbiology*, 30(1), pp. 267–273. doi: 10.1016/j.fm.2011.11.003.

Sarró, M. I. *et al.* (2006) 'Biodeterioration of the Lions Fountain at the Alhambra Palace, Granada (Spain)', *Building and Environment*, 41(12), pp. 1811–1820. doi: 10.1016/j.buildenv.2005.07.029.

Sasso, S. *et al.* (2016) 'Potential of natural biocides for biocontrolling phototrophic colonization on limestone', *International Biodeterioration & Biodegradation*, 107, pp. 102–110. doi: 10.1016/j.ibiod.2015.11.017.

Schaffer, R. J. (1932) *The Weathering of Natural Building Stones*. London.

Schloss, P. D. *et al.* (2016) 'Status of the Archaeal and Bacterial Census: an Update.', *mBio*, 7(3), pp. e00201-16. doi: 10.1128/mBio.00201-16.

Schloss, P. D. and Handelsman, J. (2004) 'Status of the microbial census.', *Microbiology and molecular biology reviews : MMBR*, 68(4), pp. 686–91. doi: 10.1128/MMBR.68.4.686-691.2004.

Schloss, P. D. and Westcott, S. L. (2011) 'Assessing and Improving Methods Used in Operational Taxonomic Unit-Based Approaches for 16S rRNA Gene Sequence Analysis', *Applied and Environmental Microbiology*, 77(10), pp. 3219–3226. doi: 10.1128/AEM.02810-10.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012) 'NIH Image to ImageJ: 25 years of image analysis', *Nature Methods*, 9(7), pp. 671–675. doi: 10.1038/nmeth.2089.

Schrader, C. *et al.* (2012) 'PCR inhibitors - occurrence, properties and removal', *Journal of Applied Microbiology*, 113(5), pp. 1014–1026. doi: 10.1111/j.1365-2672.2012.05384.x.

Schulz, S. and Dickschat, J. S. (2007) 'Bacterial volatiles: the smell of small organisms', *Natural Product Reports*, 24(4), p. 814. doi: 10.1039/b507392h.

Semchyshyn, H. M., Dyl'ovyĭ, M. V and Lushchak, V. I. (1999) 'pH-dependency of Escherichia coli catalase activity under modified culture conditions.', *Ukrains'kyi biokhimichnyi zhurnal (1999)*, 74(5), pp. 34–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12916153 (Accessed: 16 November 2016).

Sepandj, F. *et al.* (2004) 'Minimum inhibitory concentration (MIC) versus minimum biofilm eliminating concentration (MBEC) in evaluation of antibiotic sensitivity of gram-negative bacilli causing peritonitis.', *Peritoneal dialysis international : journal of the International Society for Peritoneal Dialysis*, 24(1), pp. 65–7.

Setunge, S. *et al.* (2009) 'Leaching of Alkali from Concrete in Contact with Waterways', *Water, Air, & Soil Pollution: Focus*, 9(5–6), pp. 381–391. doi: 10.1007/s11267-009-9234-x.

Sgobbi, M. *et al.* (2010) 'Surface stratigraphy on limestone of venetian palaces', *Journal of Architectural Conservation*, 16(3), pp. 51–70. doi: 10.1080/13556207.2010.10785075.

Shannon, C. E. (1948) 'A Mathematical Theory of Communication', *Bell System Technical Journal*, 27(3), pp. 379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x.

Shin, J.-H. *et al.* (2009) 'Proteomic analysis of Acinetobacter baumannii in biofilm and planktonic growth mode.', *Journal of microbiology (Seoul, Korea)*, 47(6), pp. 728–35. doi: 10.1007/s12275-009-0158-y.

Siano, S. *et al.* (2012) 'Laser cleaning in conservation of stone, metal, and painted artifacts: state of the art and new insights on the use of the Nd:YAG lasers', *Applied Physics A*, 106(2), pp. 419–446. doi: 10.1007/s00339-011-6690-8.

Silva, M. *et al.* (2016) 'Toxicological assessment of novel green biocides for cultural heritage', *Internation Journal of Conservation Science*, 7(1), pp. 265–272.

Smith, B. J. J. *et al.* (2005) 'Salt-weathering simulations under hot desert conditions: Agents of enlightenment or perpetuators of preconceptions?', *Geomorphology*, 67(1–2 SPEC. ISS.), pp. 211–227. doi: 10.1016/j.geomorph.2004.03.015.

Spormann, A. M. (2008) 'Physiology of Microbes in Biofilms', in. Springer, Berlin, Heidelberg, pp. 17–36. doi: 10.1007/978-3-540-75418-3_2.

Stahlhut, S. G. *et al.* (2012) 'Biofilm formation of Klebsiella pneumoniae on urethral catheters requires either type 1 or type 3 fimbriae.', *FEMS immunology and medical microbiology*, 65(2), pp. 350–9. doi: 10.1111/j.1574-695X.2012.00965.x.

Staley, J. T. and Konopka, A. (1985) 'Measurement of in Situ Activities of Nonphotosynthetic Microorganisms in Aquatic and Terrestrial Habitats', *Annual Review of Microbiology*, 39(1), pp. 321–346. doi: 10.1146/annurev.mi.39.100185.001541.

Stanley, B., Luxford, N. and Downes, S. (2016) 'Mould Attacks! a Practical and Effective Method of

Treating Mould Contaminated Stonework', *Science and Art : A Future for Stone Volume II*. Edited by J. J. Hughes and T. Howind, pp. 963–970.

Starner, T. D. *et al.* (2008) 'Subinhibitory concentrations of azithromycin decrease nontypeable Haemophilus influenzae biofilm formation and Diminish established biofilms.', *Antimicrobial agents and chemotherapy*, 52(1), pp. 137–45. doi: 10.1128/AAC.00607-07.

Sterflinger, K. and Piñar, G. (2013) 'Microbial deterioration of cultural heritage and works of art - Tilting at windmills?', *Applied Microbiology and Biotechnology*, 97(22), pp. 9637–9646. doi: 10.1007/s00253-013-5283-1.

Stewart, E. J. (2012) 'Growing unculturable bacteria.', *Journal of bacteriology*, 194(16), pp. 4151–60. doi: 10.1128/JB.00345-12.

Stewart, J., More, A. and Simpson, P. (2008) 'TH E USE O F ULTRAVIOLET ERRADLVTION T O CONTROL MICROBIOLOGICAL GROWTH O N MOSAIC PAVEMENTS: A PRELIMINARY ASSESSMENT AT NEWPORT ROMAN VILLA', in Michaelides, D. (ed.) *THE 10 TM CONTERENCE OF THE INTERNATIONAL COMMITTEE FOR THE CONSERVATION OF MOSAICS (I C C M) CONSERVATION : AN ACT OF DISCOVERY*. Palmero: International Committee for the Conservation of Mosaics, pp. 296–305.

Stickler, D. (1999) 'Biofilms', *Current Opinion in Biosciences*, 2, pp. 270–275. doi: 10.1016/S1369-5274(99)80047-2.

Summerfield, M. A. (2014) *Global Geomorphology.* Routledge, Taylor & Francis Group.

Sutherland, I. W. (2001) 'Biofilm exopolysaccharides: a strong and sticky framework', *Microbiology*, 147(1), pp. 3–9. doi: 10.1051/lait:2001108.

Thirst, M. L. (1957) 'Gelatin Liquefaction: a Microtest', *Journal of General Microbiology*, 17, pp. 396–400.

Tribble, G. *et al.* (2012) 'Natural competence is a major mechanism for horizontal DNA transfer in the oral pathogen Porphyromonas gingivalis', *MBio*, 3(1), pp. 1–8. doi: 10.1128/mBio.00231-11.Invited.

Tweeddale, H., Notley-McRobb, L. and Ferenci, T. (1998) 'Effect of slow growth on metabolism of Escherichia coli, as revealed by global metabolite pool ("metabolome") analysis.', *Journal of bacteriology*, 180(19), pp. 5109–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9748443 (Accessed: 16 November 2016).

Underwood, N. and Doermann, A. H. (1947) 'A Photoelectric Nephelometer', *Review of Scientific Instruments*, 18(9), pp. 665–669. doi: 10.1063/1.1741024.

Urzi, C. E. *et al.* (1993) 'Are colour changes of rocks caused by climate, pollution, biological growth, or by interactions of the three?', in Thiel, M. J. (ed.) *Conservation of Stone and Other Materials*. London: E&FN Spon, pp. 279–286.

Urzi, C. E., Krumbein, W. E. and Warscheid, T. (1992) 'On the question of biogenic colour changes of mediterranean monuments. In: (eds.)', in Decrouez, D., Chamay, J., and Zezza, F. (eds) *Proceedings of the Second International Symposium on: The Conservation of Monuments in the Mediterranean Basins. Geneva: Ville de Gèneve*, pp. 397–420.

Urzì, C., De Leo, F. and Schumann, P. (2008) 'Kribbella catacumbae sp. nov. and Kribbella sancticallisti sp. nov., isolated from whitish-grey patinas in the catacombs of St Callistus in Rome,

Italy.', *International journal of systematic and evolutionary microbiology*, 58(Pt 9), pp. 2090–2097. doi: 10.1099/ijs.0.65613-0.

Urzì, C. and Leo, F. De (2001) 'Sampling with adhesive tape strips: an easy and rapid method to monitor microbial colonization on monument surfaces', *Journal of microbiological methods*, 44(1), pp. 1–11. doi: 10.1016/S0167-7012(00)00227-X.

Větrovský, T. and Baldrian, P. (2013) 'The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses', *PLoS ONE*, 8(2), pp. 1–10. doi: 10.1371/journal.pone.0057923.

Videla, H. *et al.* (2000) 'Biodeterioration of Mayan archaeological sites in the Yucatan Peninsula, Mexico', *International biodeterioration & …*, 46(4), pp. 335–341. doi: 10.1016/S0964-8305(00)00106-2.

Videla, H. A. and Characklis, W. G. (1992) 'Biofouling and microbially influenced corrosion', *International Biodeterioration & Biodegradation*, 29(3–4), pp. 195–212. doi: 10.1016/0964-8305(92)90044-O.

Viles, H. A. and Gorbushina, A. A. (2003) 'Soiling and microbial colonisation on urban roadside limestone: a three year study in Oxford, England', *Building and Environment*, 38(9), pp. 1217–1224. doi: 10.1016/S0360-1323(03)00078-7.

de Vries, J. and Wackernagel, W. (2002) 'Integration of foreign DNA during natural transformation of Acinetobacter sp. by homology-facilitated illegitimate recombination.', *Proceedings of the National Academy of Sciences of the United States of America*, 99(4), pp. 2094–9. doi: 10.1073/pnas.042263399.

Waelkens, M. *et al.* (1992) *Ancient stones : quarrying, trade and provenance : interdisciplinary studies on stones and stone technology in Europe and Near East from the prehistoric to the early Christian period*. Leuven University Press.

Walbert, C. *et al.* (2016) 'FREEZING-THAWING PHENOMENA IN LIMESTONES AND CONSEQUENCES FOR THEIR PHYSICAL AND MECHANICAL PROPERTIES', in Hughes, J. and Howind, T. (eds) *Science and Art: A Future for Stone. Proceedings of the 13th International Congress on the Deterioration and Conservation of Stone - Volume I*. Paisley: University of the West of Scotland, pp. 173–180.

Walters, W. *et al.* (2015) 'Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys', *mSystems*, 1(1).

Wangler, T., Sanchez, A. A. and Peri, T. (2016) 'RAPID DEGRADATION OF STYLOLITIC LIMESTONES USED IN BUILDING CLADDING PANELS', in Hughes, J. and Howind, T. (eds) *Science and Art: A Future for Stone. Proceedings of the 13th International Congress on the Deterioration and Conservation of Stone - Volume I*. Paisley: University of the West of Scotland, pp. 181–188.

Warscheid, T. and Braams, J. (2000) 'Biodeterioration of stone: A review', *International Biodeterioration and Biodegradation*, 46(4), pp. 343–368.

Webster, M. (2016) *Biofilm: Pollution, slime and cultural heritage*. University of Lincoln.

Wei, S. *et al.* (2013) 'Microbiologically induced deterioration of concrete--a review.', *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, 44(4), pp. 1001–7. doi: 10.1590/S1517-83822014005000006.

Whittenbury, R. (1964) 'Hydrogen Peroxide Formation and Catalase Activity in the Lactic Acid

Bacteria', *J . gen. Microbiol*, 35, pp. 13–26.

Wikler, M. A. et al (2006) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Seventh Edition*. Seventh. Clinical and Laboratory Standards Institute.

Wright, D. B. and Herrington, J. A. (2011) 'Problematic standard errors and confidence intervals for skewness and kurtosis', *Behavior Research Methods*, 43(1), pp. 8–17. doi: 10.3758/s13428-010-0044-x.

Wright, J. P., Jones, C. and G. (2006) 'The Concept of Organisms as Ecosystem Engineers Ten Years On: Progress, Limitations, and Challenges', *Bioscience*, 56(3), pp. 203–209. doi: 10.1641/0006-3568(2006)056[0203:TCOOAE]2.0.CO;2.

Wu, Y. *et al.* (2015) 'Profiling bacterial diversity in a limestone cave of the western Loess Plateau of China.', *Frontiers in microbiology*, 6(March), p. 244. doi: 10.3389/fmicb.2015.00244.

Yoda, I. *et al.* (2014) 'Effect of surface roughness of biomaterials on Staphylococcus epidermidis adhesion.', *BMC Microbiology*, 14(1), p. 234. doi: 10.1186/s12866-014-0234-2.

Young, P. (1996a) 'Pollution-Fueled "Biodeterioration" Threatens Historic Stone', *Environmental Science and Technology*, 30(5), p. 206A–208A. doi: 10.1021/es962227t.

Young, P. (1996b) 'Pollution - Fueled " Biodeterioration " Threatens Historic Stone', *Environmental Science and Technology*, 30(5), pp. 206–208.

Zammit, G., Sánchez-Moral, S. and Albertano, P. (2011) 'Bacterially mediated mineralisation processes lead to biodeterioration of artworks in Maltese catacombs', *Science of the Total Environment*, 409(14), pp. 2773–2782. doi: 10.1016/j.scitotenv.2011.03.008.

Zhang, W. *et al.* (2014) 'Nutrient depletion in Bacillus subtilis biofilms triggers matrix production', *New Journal of Physics*, 16(1), p. 015028. doi: 10.1088/1367-2630/16/1/015028.

Ziuzina, D. *et al.* (2015) 'Cold Plasma Inactivation of Bacterial Biofilms and Reduction of Quorum Sensing Regulated Virulence Factors', *PLOS ONE*. Edited by T. Coenye, 10(9), p. e0138209. doi: 10.1371/journal.pone.0138209.

Appendix A: Data from M. Webster Thesis

BIOFILM ON-SITE SAMPLING FORM 20.

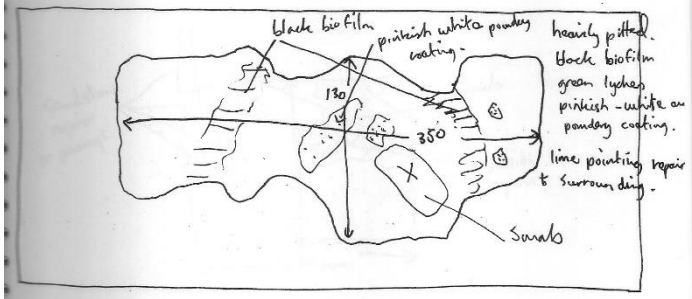
SITE:
SITE CONTACT:
DATE: 23·6·13
TIME: 15·05
SITE CODE: MO-1-D

PERSONS PRESENT: Mary Webster

STONE TYPE: Tufa
ASPECT OF WALL: S facing.

WEATHER CONDITIONS: Cloudy / light showers + some sun.
WIND DIRECTION: SW
↳ COASTAL SITES OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:
TEMP: 16°C
R.H: 73·4
UV: 22
LUX: 22552
pH: 8.5
MOISTMETER H20%: 40%

HEIGHT FROM GROUND: 150
SURROUND / FOLIAGE: Grass, 1 fir tree 8m away
COLOUR OF SAMPLE MATERIAL:

NOTES:

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

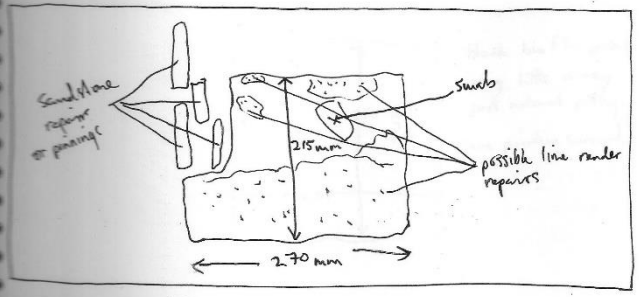
SITE:
SITE CONTACT:
DATE: 23.6.13
TIME: 15.30
SITE CODE: MO-2-D

PERSONS PRESENT:

STONE TYPE: Tufa
ASPECT OF WALL: W facing

WEATHER CONDITIONS: wind / cloud
WIND DIRECTION: SW
↳ COASTAL SITES OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:
TEMP: 15·9
R.H: 75
UV: 952
LUX: 14649
pH: 8
MOISTMETER H20%: 30%

HEIGHT FROM GROUND: 170 cm
SURROUND / FOLIAGE: grass on ground
COLOUR OF SAMPLE MATERIAL:

NOTES: lime repairs to V parts of stone. lower

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

PERSONS PRESENT:

SITE CONTACT: Rev Sally Barnes.

DATE: 23/04/2013

TIME: 16.00

SITE CODE: MO 3 UB (undamaged + block.)

STONE TYPE: Tufa

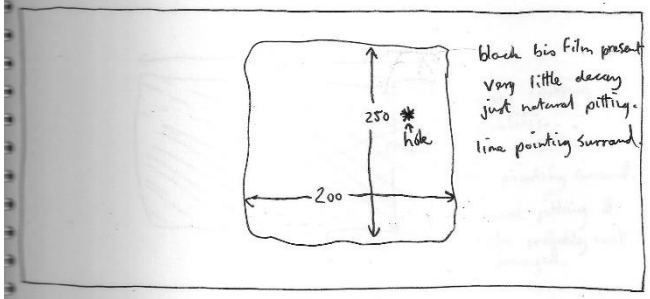
ASPECT OF WALL: NW

WEATHER CONDITIONS: Cloudy/sun spells/very light rain

WIND DIRECTION: SW.

↳ COASTAL SITES OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 16.5

R.H: 75.2

UV: 1014

LUX: 11119

pH: 8

ROTIMETER H2O%: 8.5

HEIGHT FROM GROUND: 115.5 cm

SURROUND / FOLIAGE: Trees within 6m grass on ground.

COLOUR OF SAMPLE MATERIAL:

NOTES: 13% H2O in hole * (variable, depending on location

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BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

PERSONS PRESENT:

SITE CONTACT:

DATE: 22.6.13

TIME: 16.55

SITE CODE: OX-1-O

STONE TYPE:

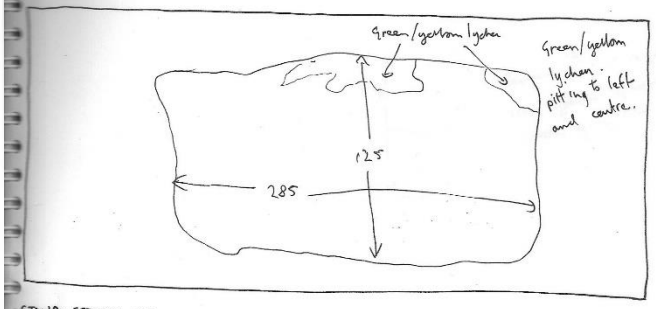
ASPECT OF WALL: NW facing

WEATHER CONDITIONS: rainy/windy/sunny intervals

WIND DIRECTION: Sheltered

↳ COASTAL SITES OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 15.6

R.H: 85.4

UV: 1241

LUX: 3052

pH: 8

ROTIMETER H2O%: 15.5

HEIGHT FROM GROUND: 106

SURROUND / FOLIAGE: Trees 5m away

COLOUR OF SAMPLE MATERIAL:

NOTES: Sheltered aspect surrounded by trees. within ½ mile of sea on top of a hill

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

SITE CONTACT:

DATE: 22.6.13

TIME: 15.05

SITE CODE: OX-01-U

PERSONS PRESENT:

STONE TYPE:

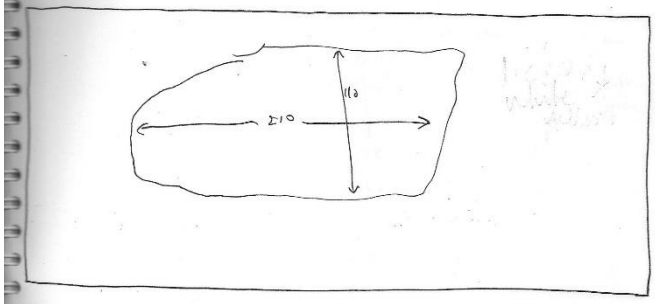
ASPECT OF WALL: NW Facing.

WEATHER CONDITIONS: cloudy/windy.

WIND DIRECTION: sheltered

↳ COASTAL SITES OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 14.7

R.H: 87.8

UV: 1236

LUX: 2475

pH: 7.5

ROTIMETER H20 %: 16.5

HEIGHT FROM GROUND: 120

SURROUND / FOLIAGE:

COLOUR OF SAMPLE MATERIAL:

NOTES:

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

SITE CONTACT:

DATE: 22/6/13

TIME:

SITE CODE: OX 2 O

PERSONS PRESENT:

STONE TYPE:

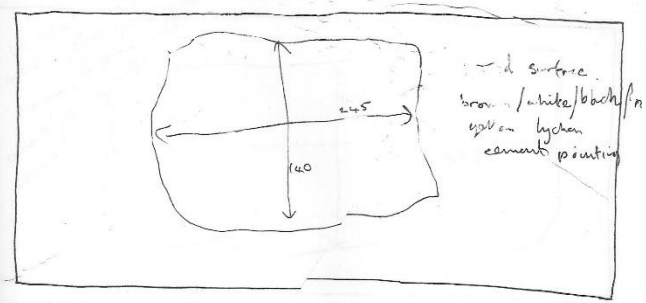
ASPECT OF WALL: SW Facing

WEATHER CONDITIONS: rain/wind

WIND DIRECTION: Westerly

↳ COASTAL SITES OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 13.8

R.H: 89.5

UV: 1082

LUX: 5006

pH: 7.5

ROTIMETER H20 %: 13

HEIGHT FROM GROUND: 120 cm

SURROUND / FOLIAGE: None

COLOUR OF SAMPLE MATERIAL:

NOTES:

MARY WEBSTER. JUN 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

SITE CONTACT:

DATE: 22.6.13

TIME:

SITE CODE: 0* 2 4

PERSONS PRESENT:

STONE TYPE:

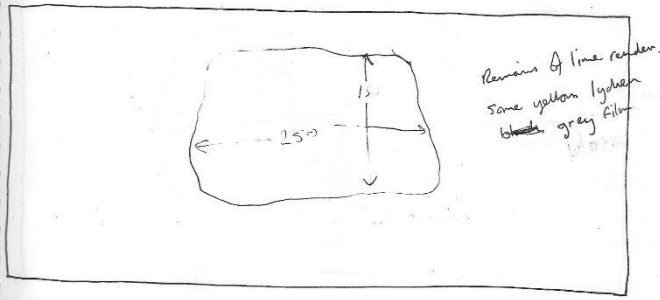
ASPECT OF WALL: SW.

WEATHER CONDITIONS: Wind/Rain.

WIND DIRECTION: N ~~SW~~ damaged.

↳ COASTAL SITES
OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 13.1

R.H: 93

UV: 34

LUX: 17468

pH: 8

ROTIMETER H2O %: 15

HEIGHT FROM GROUND: Same as damaged

SURROUND / FOLIAGE:

COLOUR OF SAMPLE MATERIAL:

NOTES:

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

SITE CONTACT:

DATE: 22.6.13

TIME: 18.15

SITE CODE: 0x 03 U

PERSONS PRESENT:

aggressive clearance zones on this agar plate

STONE TYPE:

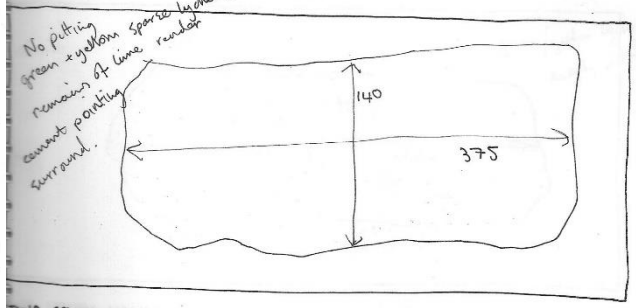
ASPECT OF WALL: SE facing

WEATHER CONDITIONS: cloudy windy

WIND DIRECTION: sheltered

↳ COASTAL SITES
OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 16.9

R.H: 91.4

UV: 1147

LUX: 12445

pH: 7

ROTIMETER H2O %: 17

HEIGHT FROM GROUND: 147cm

SURROUND / FOLIAGE:

COLOUR OF SAMPLE MATERIAL:

NOTES:

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE: WEOBLEY CASTLE

SITE CONTACT:

DATE: 22-06-2013

TIME: 14.45

SITE CODE: WEST WE-1-D

PERSONS PRESENT:
MARY WEBSTER
JONNY BRADFORD

STONE TYPE: LIMESTONE

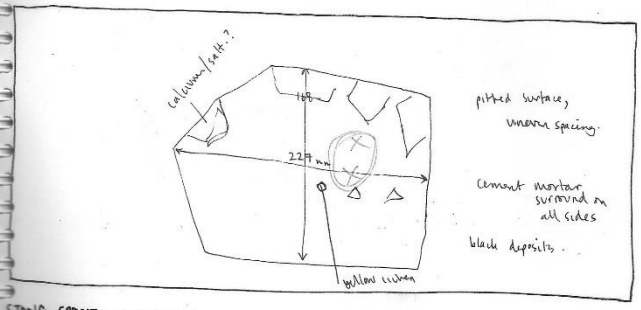
ASPECT OF WALL: WEST FACING.

WEATHER CONDITIONS: DRY, WINDY, VARIABLE

WIND DIRECTION: SSE.

↳ COASTAL SITES
OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:
TEMP: 15.6 °C
R.H: 80.2
IR: 882
UV: 16544
pH: 6.0
MOISTURE METER H2O %: 37

HEIGHT FROM GROUND: 144 cm

SURROUND / FOLIAGE: — . (no tree cover).

COLOUR OF SAMPLE MATERIAL: black/greenish

NOTES:

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE: WEOBLEY CASTLE.

SITE CONTACT:

DATE: 22/06/2013.

TIME: 15.16

SITE CODE: WE-1-U

PERSONS PRESENT:

STONE TYPE: LIMESTONE

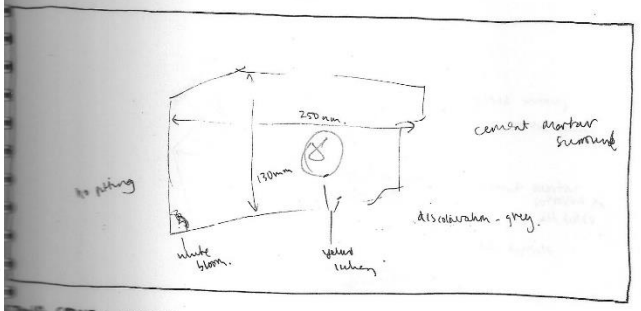
ASPECT OF WALL: WEST

WEATHER CONDITIONS: light rain

WIND DIRECTION:

↳ COASTAL SITES
OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:
TEMP: 16.6
R.H: 74.9
IR: 861
UV: 17084
pH: 6.0
MOISTURE METER H2O %: 16

HEIGHT FROM GROUND: 163

SURROUND / FOLIAGE:

COLOUR OF SAMPLE MATERIAL:

NOTES:

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

TE: WE-2-●D

PERSONS PRESENT:

E CONTACT :

TE: 22·6·13

E :

CODE :

E TYPE : LIMESTONE.

CT OF WALL : NORTH FACING

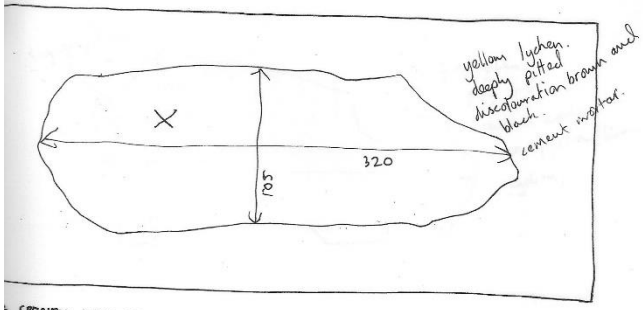
WEATHER CONDITIONS : cloudy/windy.

WIND DIRECTION : Southerly

↳ COASTAL SITES

OFFSHORE / ONSHORE

ORD DRAWING OF BLOCK / DETERIORATION :



E SPECIFIC MEASUREMENTS :

: 16·7

81

938

13906

d 6

ETER H2O % : 12·5

Y WEBSTER. JUNE 2013.

HEIGHT FROM GROUND : 190cm

SURROUND / FOLIAGE : Above marshland and trees.

COLOUR OF SAMPLE MATERIAL :

NOTES :

BIOFILM ON-SITE SAMPLING FORM 2013

SITE :

PERSONS PRESENT:

SITE CONTACT :

DATE : 22-6-13

TIME : 15·51

SITE CODE : WE-2-U

STONE TYPE : limestone

ASPECT OF WALL : North facing

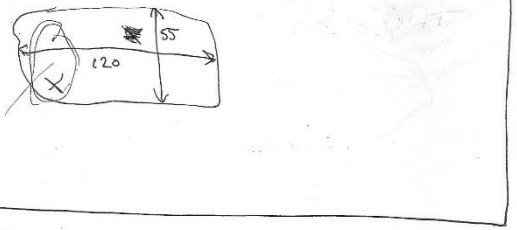
WEATHER CONDITIONS :

WIND DIRECTION :

↳ COASTAL SITES

OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION :



STONE SPECIFIC MEASUREMENTS :

TEMP: 16.6

R.H: 83.8

UV: 991

x: 15 524

H: 8

METER H2O % : 16

MARY WEBSTER. JUNE 2013.

HEIGHT FROM GROUND : 180cm.

SURROUND / FOLIAGE : marshland/trees + grass.

COLOUR OF SAMPLE MATERIAL :

NOTES :

BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

PERSONS PRESENT:

SITE CONTACT:

DATE: 22/6/13

TIME: 16·00

SITE CODE: WE - 3 - D

STONE TYPE: Limestone

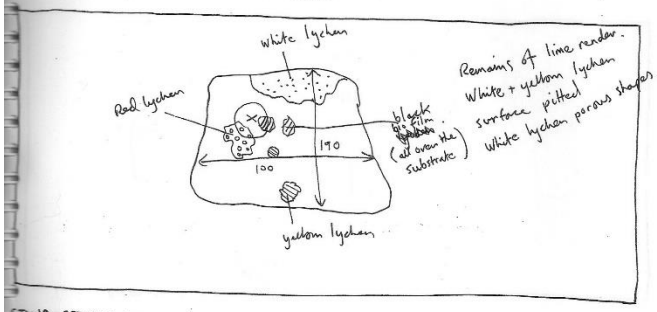
ASPECT OF WALL: SSE Facing

WEATHER CONDITIONS: cloudy / ~~with~~ rain / wind

WIND DIRECTION: sheltered by South East facing elevation.

↳ COASTAL SITES
OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 16·5

R.H: 82·2

UV: 1001

LUX: 7664

PH: 8·5

PROTIMETER H20 %: 18

HEIGHT FROM GROUND: 44cm

SURROUND / FOLIAGE: Trees Sw to East.

COLOUR OF SAMPLE MATERIAL: Red/black

NOTES: Area prone to South Westerly wind crossing arable land.

MARY WEBSTER. JUNE 2013.

BIOFILM ON-SITE SAMPLING FORM 2013

SITE:

PERSONS PRESENT:

SITE CONTACT:

DATE: 22-6-13

TIME:

SITE CODE: WE - 3 - U

STONE TYPE: limestone.

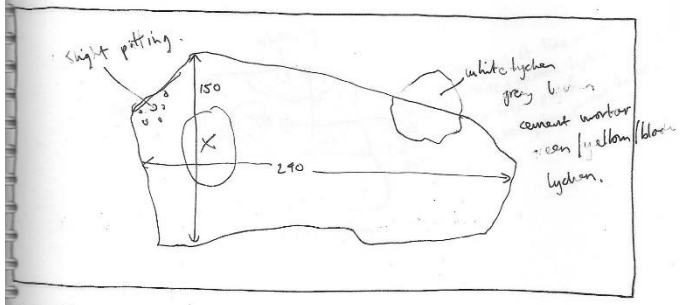
ASPECT OF WALL: SSE Facing

WEATHER CONDITIONS: cloudy / windy

WIND DIRECTION: Sheltered.

↳ COASTAL SITES
OFFSHORE / ONSHORE

RECORD DRAWING OF BLOCK / DETERIORATION:



STONE SPECIFIC MEASUREMENTS:

TEMP: 15·9

R.H: 86·2

UV: 973

LUX: 3551

PH: 8·5

PROTIMETER H20 %: 10

HEIGHT FROM GROUND: 53cm.

SURROUND / FOLIAGE:

COLOUR OF SAMPLE MATERIAL:

NOTES:

MARY WEBSTER. JUNE 2013.

Appendix B: Köppen – Geiger definitions.

The following information is taken from Peel, Finlayson and McMahon (2007).

The Köppen climate classification uses monthly temperature and precipitation for the 12 months, usually averaged over a long period of time. Climate types are represented by a two or three letter combination in which the first letter defines the major type. The major types can be further divided into subtypes based on the precipitation pattern (second letter, except for the E type) and the temperature (third letter).

Subtypes satisfy the criterion of their parent type(s). There can only be one climate type in a region and it is determined using the following hierarchy: ET, EF, BSh, BSk, BWh, BWk, Af, Am, As, Aw, Csa, Csb, Csc, Cwa, Cwb, Cwc, Cfa, Cfb, Cfc, Dsa, Dsb, Dsc, Dsd, Dwa, Dwb, Dwc, Dwd, Dfa, Dfb, Dfc, Dfd. So, for example, if the climate satisfies the criterion of both ET and BW, it is classified as ET.

The studies in the limestone microbiome literature were conducted in climate types Am, Aw, BWh, Cfa, Cfb, Csa, Csb, Dfa, Dfb and Dfc. These are described briefly below with the full classification system following the descriptions.

Am is a tropical monsoon climate. Annual rainfall is high, but most of the precipitation falls in the 7 to 9 hottest months. During the dry season very little rainfall occurs.

Aw, the tropical wet and dry or savanna has an extended dry season during winter. Precipitation during the wet season is usually < 1000 millimeters, and only during the summer season.

BWh is a subtropical true desert climate. It covers 12% of the Earth's land surface and is dominated by xerophytic vegetation.

Cfa : The humid subtropical climate has hot muggy summers and frequent thunderstorms. Winters are mild and precipitation during this season comes from mid-latitude cyclones. For simplicity, the criterion for each Köppen type is described using variables, which are described below.

Cfb marine climates are found on the western coasts of continents. They have a humid climate with short dry summer. Heavy precipitation occurs during the mild winters because of the continuous presence of mid-latitude cyclones.

Mediterranean climates (Csa and Csb) receive rain primarily during winter season from the mid-latitude cyclone. Extreme summer aridity is caused by the sinking air of the subtropical highs and may exist for up to 5 months.

Dfa, Dfb and Dfc are moist continental mid-latitude climates have warm to cool summers and cold winters. The location of these climates is pole ward of the C climates. The average temperature of the warmest month is > 10° Celsius, while the coldest month is < -3° Celsius. Winters are severe with snowstorms, strong winds, and bitter cold from Continental Polar or Arctic air masses. Summers are hot (Dfa), warm (Dfb) or cool (Dfc) depending on the subtype.

Variables and subscripts

[1] The threshold is defined as follows: if $P_w \geq \frac{2}{3} P_{ann}$ then $P_{th} = (2\ T_{ann})$, else if $P_s \geq \frac{2}{3} P_{ann}$ then $P_{th} = 2\ T_{ann} + 28$ °C, else $P_{th} = 2\ T_{ann} + 14$ °C.

[2] For example, $4\ T_{mon} < +10$ °C means "at least 4 months with mean temperature below 10 °C."

[3] The summer months are April through September (AMJJAS) on the Northern Hemisphere and the winter months are October through March (ONDJFM), and vice versa for the Southern Hemisphere.

Variable	Description
T	Air temperature
P	Precipitation
Subscript	
min	Minimum monthly value for the whole year
max	Maximum monthly value for the whole year
ann	Annual mean value
th	Threshold value [1]
mon	Number of months satisfying the criterion [2]
smin	Minimum monthly value for the summer months [3]
wmin	Minimum monthly value for the winter months [3]
smax	Maximum monthly value for the summer months [3]
wmax	Maximum monthly value for the winter months [3]
s	Mean value for the summer months [3]
w	Mean value for the winter months [3]

First and second letter

Type	Description	Criterion
A	Tropical climates	$T_{min} \geq +18$ °C
Af	Tropical rain forest	$P_{min} \geq 60$ mm
Am	Tropical monsoon	$P_{ann} \geq 25(100 - P_{min})$ mm
As	Tropical savannah with dry summer	$P_{min} < 60$ mm in summer
Aw	Tropical savannah with dry winter	$P_{min} < 60$ mm in winter
B	Dry climates	$P_{ann} < 10\ P_{th}$
BW	Desert (arid)	$P_{ann} \leq 5\ P_{th}$
BS	Steppe (semi-arid)	$P_{ann} > 5\ P_{th}$
C	Mild temperate	-3 °C $< T_{min} < +18$ °C
Cs	Mild temperate with dry summer	$P_{smin} < P_{wmin}$, $P_{wmax} > 3\ P_{smin}$, $P_{smin} < 40$ mm
Cw	Mild temperate with dry winter	$P_{smax} > 10\ P_{wmin}$, $P_{wmin} < P_{smin}$
Cf	Mild temperate, fully humid	Not Cs or Cw
D	Snow	$T_{min} \leq -3$ °C
Ds	Snow with dry summer	$P_{smin} < P_{wmin}$, $P_{wmax} > 3\ P_{smin}$, $P_{smin} < 40$ mm
Dw	Snow with dry winter	$P_{smax} > 10\ P_{wmin}$, $P_{wmin} < P_{smin}$
Df	Snow, fully humid	Not Ds or Dw
E	Polar	$T_{max} < +10$ °C
ET	Tundra	$T_{max} \geq 0$ °C
EF	Frost	$T_{max} < 0$ °C

Third letter

Type	Description	Criterion
h	Hot arid	$T_{ann} \geq +18$ °C
k	Cold arid	$T_{ann} < +18$ °C
a	Hot summer	$T_{max} \geq +22$ °C
b	Warm summer	$T_{max} < +22$ °C, $4\ T_{mon} \geq +10$ °C
c	Cool summer	$T_{max} < +22$ °C, $4\ T_{mon} < +10$ °C, $T_{min} > -38$ °C
d	Cold summer	$T_{max} < +22$ °C, $4\ T_{mon} < +10$ °C, $T_{min} \leq -38$ °C

Appendix C: Species identified in metagenomic analysis

species	% discovery across samples	Isolation surface	rural/ urban	species	% discovery across samples	Isolation surface	rural/ urban	species	% discovery across samples	Isolation surface	rural/ urban	species	% discovery across samples	Isolation surface	rural/ urban
Gemmiger prausnitzii	100	Both	Both	*Corynebacterium pyruviciproducens*	37.5	Both	Both	*Cardiobacterium hominis*	12.5	Damaged	Urban	*Ruminococcus callidus*	12.5	Damaged	Urban
Propionibacterium granulosum	100	Both	Both	*Ornithinibacter aureus*	37.5	Both	Both	*Streptococcus mitis*	12.5	Damaged	Rural	*Lachnospiracea incertae sedis torques*	12.5	Undamaged	Urban
Acinetobacter lwoffii	100	Both	Both	*Microbacterium humi*	37.5	Both	Both	*Leptotrichia* sp.*PTE15*	12.5	Damaged	Urban	*Bifidobacterium angulatum*	12.5	Undamaged	Rural
Collinsella aerofaciens	100	Both	Both	*Kineococcus lusitanus*	37.5	Both	Urban	*Aggregatibacter segnis*	12.5	Undamaged	Urban	*Couchioplanes caeruleus*	12.5	Damaged	Urban
Micrococcus luteus	100	Both	Both	*Corynebacterium striatum*	37.5	Both	Both	*Rhodoplanes* sp.*JA527*	12.5	Damaged	Urban	*Pseudomonas azotoformans*	12.5	Undamaged	Urban
Micrococcus terreus	100	Both	Both	*Deinococcus reticulitermitis*	37.5	Both	Both	*Acinetobacter parvus*	12.5	Damaged	Rural	*Bifidobacterium gallicum*	12.5	Damaged	Urban
Arthrobacter agilis	100	Both	Both	*Rhizobium skierniewicense*	37.5	Both	Both	*Caenimonas* sp.*TSX9-5*	12.5	Damaged	Urban	*Carnobacterium mobile*	12.5	Undamaged	Rural
Streptococcus thermophilus	100	Both	Both	*Campylobacter ureolyticus*	37.5	Both	Urban	*Providencia* sp.*DF1SB*	12.5	Damaged	Urban	*Planifilum fimeticola*	12.5	Damaged	Urban
Finegoldia magna	100	Both	Both	*Sphingomonas paucimobilis*	37.5	Both	Both	*Solibacillus PM-38*	12.5	Damaged	Urban	*Microlunatus ginsengisoli*	12.5	Undamaged	Rural
Blautia glucerasea	100	Both	Both	*Advenella mimigardefordensis*	37.5	Both	Urban	*Propionibacterium* sp.*434-HC2*	12.5	Damaged	Urban	*Loktanella atrilutea*	12.5	Damaged	Urban
Actinophytocola sediminis	100	Both	Both	*Streptococcus constellatus*	37.5	Undamaged	Both	*Turicibacter sanguinis*	12.5	Damaged	Urban	*Parabacteroides johnsonii*	12.5	Undamaged	Urban
Pseudomonas fluorescens	87.5	Both	Both	*Peptoniphilus grossensis*	37.5	Both	Both	*Micrococcus flavus*	12.5	Damaged	Urban	*Agrococcus citreus*	12.5	Damaged	Urban
Flavobacterium lindanitolerans	87.5	Both	Both	*Salinibacterium soli*	37.5	Both	Urban	*Granulicatella para-adiacens*	12.5	Damaged	Urban	*Microbacterium lacus*	12.5	Undamaged	Rural
Arthrobacter tumbae	87.5	Both	Both	*Chryseobacterium nakagawai*	37.5	Both	Both	*Sphingomonas* sp.*14*	12.5	Damaged	Urban	*Glaciibacter superstes*	12.5	Undamaged	Urban
Blastococcus jejuensis	87.5	Both	Both	*Martelella radicis*	37.5	Both	Both	*Anaerovorax bacterium*	12.5	Damaged	Urban	*Sphingomonas japonica*	12.5	Undamaged	Rural
Bifidobacterium pseudocatenulatum	87.5	Both	Both	*Sphingomonas kyeonggiensis*	37.5	Both	Both	*Enterococcus viikkiensis*	12.5	Undamaged	Urban	*Cellvibrio mixtus*	12.5	Undamaged	Rural
Blautia wexlerae	87.5	Both	Both	*Rhodococcus soli*	37.5	Both	Both	*Rhizobacter* sp.*CR2*	12.5	Damaged	Urban	*Acidovorax anthurii*	12.5	Damaged	Urban
Corynebacterium tuberculostearicum	87.5	Both	Both	*Pantoea colletis*	37.5	Both	Both	*Bradyrhizobium liaoningense*	12.5	Damaged	Urban	*Arthrobacter phytoseiuli*	12.5	Undamaged	Rural
Streptococcus sanguinis	87.5	Both	Both	*Ornithinimicrobium tianjinense*	37.5	Both	Both	*Sphingomonas humi*	12.5	Damaged	Urban	*Providencia vermicola*	12.5	Damaged	Urban
Arthrobacter subterraneus	87.5	Both	Both	*Massilia eurypsychrophila*	37.5	Both	Both	*Pantoea ananatis*	12.5	Damaged	Urban	*Devosia subaequoris*	12.5	Undamaged	Rural
Propionibacterium acnes	87.5	Both	Both	*Rhizobium binae*	37.5	Both	Both	*Haemophilus haemolyticus*	12.5	Damaged	Rural	*Agrococcus jejuensis*	12.5	Undamaged	Rural
Peptococcus niger	87.5	Both	Both	*Methylocapsa palsarum*	37.5	Both	Urban	*Ornithinimicrobium pekingense*	12.5	Damaged	Urban	*Actinomycetospora chiangmaiensis*	12.5	Damaged	Urban
Flavobacterium caeni	87.5	Both	Both	*Streptococcus anginosus*	25	Both	Urban	*Nocardioides aquiterrae*	12.5	Damaged	Urban	*Rhodobacter maris*	12.5	Damaged	Urban
Neisseria oralis	87.5	Both	Both	*Cardiobacterium* sp.*Auto5*	25	Both	Urban	*Devosia* sp.*Axs16*	12.5	Undamaged	Urban	*Chryseobacterium gregarium*	12.5	Undamaged	Rural

Ralstonia insidiosa	75	Both	Both	*Spirosoma escalantus*	25	Damaged	Both	*Morganella morganii*	12.5	Undamaged	Urban	*Kribbella catacumbae*	12.5	Undamaged	Rural
Rhodobacter sphaeroides	75	Both	Both	*Delftia acidovorans*	25	Both	Urban	*Arthrobacter bacterium*	12.5	Damaged	Urban	*Deinococcus aquatilis*	12.5	Damaged	Urban
Actinoplanes bacterium	75	Both	Both	*Coprococcus catus*	25	Both	Both	*Roseococcus* sp.*JNUS-15*	12.5	Damaged	Urban	*Roseburia faecis*	12.5	Undamaged	Urban
Actinobacillus bacterium	75	Both	Both	*Blastococcus ginsenosidimutans*	25	Undamaged	Urban	*Turicibacter* sp.*HGA0205*	12.5	Damaged	Urban	*Cellulomonas denverensis*	12.5	Damaged	Urban
Methylobacterium populi	75	Both	Both	*Aerococcus* sp.*C216*	25	Both	Urban	*Prevotella* sp.*HGA0217*	12.5	Damaged	Urban	*Ornithinimicrobium kibberense*	12.5	Undamaged	Rural
Subdoligranulum bacterium	75	Both	Both	*Lapillicoccus jejuensis*	25	Damaged	Both	*Devosia glacialis*	12.5	Damaged	Urban	*Mycobacterium aubagnense*	12.5	Undamaged	Urban
Flavobacterium sp.*JSC-P2-223-10*	75	Both	Both	*Cronobacter sakazakii*	25	Both	Urban	*Quadrisphaera* sp.*THG-DM1*	12.5	Undamaged	Urban	*Nocardioides dubius*	12.5	Damaged	Urban
Klebsiella sp.*HGA0187*	75	Both	Both	*Bacillus muralis*	25	Both	Urban	*Bradyrhizobium elkanii*	12.5	Damaged	Urban	*Brevundimonas kwangchunensis*	12.5	Undamaged	Urban
Pseudomonas indica	75	Both	Both	*Rhodobacter* sp.*BC14248*	25	Both	Urban	*Anaerococcus hydrogenalis*	12.5	Damaged	Urban	*Leifsonia naganoensis*	12.5	Undamaged	Urban
Dokdonella ginsengisoli	75	Both	Both	*Streptococcus australis*	25	Damaged	Urban	*Kytococcus sedentarius*	12.5	Undamaged	Urban	*Nocardioides furvisabuli*	12.5	Undamaged	Urban
Subtercola sp.*Lor40*	75	Both	Both	*Bacteroides bacterium*	25	Both	Urban	*Thermomonas brevis*	12.5	Undamaged	Urban	*Methylobacterium jeotgali*	12.5	Undamaged	Urban
Staphylococcus hominis	75	Both	Both	*Terrabacter* sp.*2APm3*	25	Both	Urban	*Luteimonas terricola*	12.5	Damaged	Urban	*Salinibacterium xinjiangense*	12.5	Damaged	Urban
Blastococcus sp.*13-106*	75	Both	Both	*Stenotrophomonas maltophilia*	25	Both	Both	*Acidocella facilis*	12.5	Damaged	Urban	*Skermanella aerolata*	12.5	Damaged	Urban
Afipia sp.*42S5*	75	Both	Both	*Corynebacterium genitalium*	25	Damaged	Urban	*Pseudomonas proteobacterium*	12.5	Undamaged	Urban	*Nocardioides insulae*	12.5	Damaged	Urban
Chryseobacterium sp.*C15(2016)*	75	Both	Both	*Balneimonas flocculans*	25	Undamaged	Urban	*Mycobacterium phocaicum*	12.5	Undamaged	Urban	*Pseudonocardia endophytica*	12.5	Undamaged	Rural
Staphylococcus epidermidis	75	Both	Both	*Pseudorhodoferax* sp.*KJ016*	25	Both	Urban	*Paenibacillus darwinianus*	12.5	Damaged	Urban	*Methylobacterium iners*	12.5	Undamaged	Rural
Anaerococcus nagyae	75	Both	Both	*Bradyrhizobium* sp.*alfa7-PCA-E3-2*	25	Damaged	Both	*Craurococcus* sp.*HM28-1*	12.5	Damaged	Urban	*Corynebacterium massiliense*	12.5	Damaged	Urban
Kocuria palustris	75	Both	Both	*Escherichia/Shigella flexneri*	25	Damaged	Both	*Bacillus aerophilus*	12.5	Damaged	Urban	*Marmoricola bigeumensis*	12.5	Damaged	Urban
Gordonia polyisoprenivorans	75	Both	Both	*Serratia* sp.*AMF2811*	25	Damaged	Both	*Rhizobacter* sp.*7B-213*	12.5	Undamaged	Urban	*Nocardioides dilutus*	12.5	Damaged	Urban
Pseudomonas aeruginosa	75	Both	Both	*Negativicoccus* sp.*S5-A15*	25	Both	Urban	*Leifsonia bacterium*	12.5	Damaged	Urban	*Knoellia aerolata*	12.5	Damaged	Urban
Okibacterium fritillariae	75	Both	Both	*Lysobacter* sp.*YC6270*	25	Both	Both	*Roseibacterium* sp.*HME9693*	12.5	Damaged	Urban	*Nocardioides fonticola*	12.5	Undamaged	Urban
Brevundimonas bullata	75	Both	Both	*Neisseria* sp.*2466a*	25	Damaged	Both	*Amorphus* sp.*JL1095*	12.5	Damaged	Urban	*Deinococcus radiomollis*	12.5	Damaged	Urban
Micrococcus sp.*BAB-4450*	75	Both	Both	*Pontibacter* sp.*D14*	25	Both	Urban	*Pelomonas puraquae*	12.5	Damaged	Urban	*Deinococcus claudionis*	12.5	Damaged	Urban
Escherichia/Shigella sp.*CCMICS*	75	Both	Both	*Hassallia* sp.*C76*	25	Damaged	Urban	*Klebsiella pneumoniae*	12.5	Damaged	Urban	*Massilia niastensis*	12.5	Damaged	Urban
Gemella morbillorum	75	Both	Both	*Hansschlegelia* sp.*CHL1*	25	Damaged	Urban	*Peptostreptococcus stomatis*	12.5	Damaged	Urban	*Brevibacterium aurantiacum*	12.5	Undamaged	Rural
Actinomycetospora sp.*CB*	75	Both	Both	*Proteus penneri*	25	Damaged	Urban	*Rothia dentocariosa*	12.5	Undamaged	Urban	*Desemzia incerta*	12.5	Damaged	Urban
Peptoniphilus sp.*EL1*	75	Both	Both	*Lewinella* sp.*UA-AR0336*	25	Both	Both	*Wautersiella* sp.*SCU-B169*	12.5	Undamaged	Urban	*Legionella worsleiensis*	12.5	Damaged	Urban
Microvirga soli	75	Both	Both	*Haloactinopolyspora* sp.*Pao15*	25	Both	Urban	*Beijerinckia* sp.*Pao66*	12.5	Damaged	Urban	*Aeromicrobium fastidiosum*	12.5	Damaged	Urban

Lachnospiracea incertae sedis sp.*AT12*	75	Both	Both	*Altererythrobacter* sp.*M0322*	25	Damaged	Urban	*Actinotalea fermentans*	12.5	Damaged	Urban	*Methylobacterium extorquens*	12.5	Undamaged	Rural
Bifidobacterium longum	75	Both	Both	*Hyphomicrobium* sp.*ColF*	25	Undamaged	Urban	*Bradyrhizobium palustris*	12.5	Damaged	Urban	*Rhizobium fabrum*	12.5	Undamaged	Rural
Ornithinicoccus hungaricus	75	Both	Both	*Afipia carboxidovorans*	25	Damaged	Both	*Alistipes inops*	12.5	Damaged	Urban	*Terriglobus saanensis*	12.5	Undamaged	Urban
Jannaschia rubra	75	Both	Both	*Nocardioides marinisabuli*	25	Both	Urban	*Arthrobacter echini*	12.5	Damaged	Rural	*Erythrobacter litoralis*	12.5	Undamaged	Rural
Blautia obeum	75	Both	Both	*Bacillus pumilus*	25	Undamaged	Urban	*Herbaspirillum* sp.*ES2-54*	12.5	Damaged	Urban	*Kribbella flavida*	12.5	Damaged	Urban
Corynebacterium urealyticum	75	Both	Both	*Kingella oralis*	25	Undamaged	Urban	*Pantoea* sp.*136A*	12.5	Damaged	Rural	*Intrasporangium calvum*	12.5	Damaged	Urban
Staphylococcus sp.*16S*	75	Both	Both	*Citrobacter* sp.*B10(2014)*	25	Both	Urban	*Nitrobacter* sp.*NKU*	12.5	Damaged	Urban	*Alicycliphilus denitrificans*	12.5	Damaged	Urban
Streptococcus sp.*3192A*	62.5	Both	Both	*Aeromicrobium panaciterrae*	25	Both	Both	*Acidovorax bacterium*	12.5	Damaged	Urban	*Brachybacterium faecium*	12.5	Undamaged	Rural
Pseudomonas tolaasii	62.5	Both	Both	*Microbacterium pumilum*	25	Undamaged	Both	*Bordetella* sp.*UASWS0941*	12.5	Damaged	Rural	*Sanguibacter keddieii*	12.5	Undamaged	Rural
Blautia obeum	62.5	Both	Both	*Flavisolibacter* sp.*ID1709*	25	Both	Urban	*Pusillimonas* sp.*UASWS0960*	12.5	Damaged	Urban	*Arthrobacter phenanthrenivorans*	12.5	Undamaged	Rural
Paracoccus sp.*AP27*	62.5	Both	Both	*Marisediminicola antarctica*	25	Both	Urban	*Enterococcus faecium*	12.5	Damaged	Urban	*Roseburia hominis*	12.5	Damaged	Rural
Nocardioides sp.*9_67*	62.5	Both	Urban	*Sphingobacterium nematocida*	25	Damaged	Urban	*Vogesella perlucida*	12.5	Damaged	Urban	*Actinobacillus succinogenes*	12.5	Undamaged	Rural
Acinetobacter calcoaceticus	62.5	Both	Both	*Prevotella bivia*	25	Damaged	Urban	*Patulibacter* sp.*R16*	12.5	Undamaged	Urban	*Escherichia/Shigella dysenteriae*	12.5	Undamaged	Urban
Actinomyces sp.*ICM47*	62.5	Both	Urban	*Enterococcus faecalis*	25	Damaged	Both	*Pedobacter luteus*	12.5	Damaged	Urban	*Selenomonas sputigena*	12.5	Undamaged	Urban
Branchiibius sp.*PSY066*	62.5	Both	Both	*Phenylobacterium aquaticus*	25	Undamaged	Urban	*Curvibacter fontanus*	12.5	Damaged	Urban	*Psychrobacter cryohalolentis*	12.5	Damaged	Urban
Phycicoccus sp.*GP0608*	62.5	Both	Both	*Roseateles* sp.*3HB-2p*	25	Damaged	Both	*Porphyrobacter mercurialis*	12.5	Damaged	Urban	*Pseudomonas entomophila*	12.5	Undamaged	Rural
Undibacterium oligocarboniphilum	62.5	Both	Both	*Caldimonas taiwanensis*	25	Both	Both	*Aquamicrobium populi*	12.5	Damaged	Urban	*Prevotella melaninogenica*	12.5	Undamaged	Rural
Flavobacterium sp.*CD13I-S2*	62.5	Both	Both	*Salinibacterium* sp.*MJAU*	25	Damaged	Urban	*Coenonia* sp.*LS-2015b*	12.5	Damaged	Urban	*Pantoea vagans*	12.5	Undamaged	Rural
Adhaeribacter sp.*120*	62.5	Both	Both	*Brochothrix thermosphacta*	25	Both	Both	*Salinarimonas* sp.*M016010*	12.5	Damaged	Urban	*Salmonella oxytoca*	12.5	Undamaged	Urban
Propionibacterium namnetense	62.5	Both	Both	*Bosea* sp.*BRJL2*	25	Both	Urban	*Mycobacterium litorale*	12.5	Damaged	Urban	*Streptococcus oligofermentans*	12.5	Damaged	Urban
Acinetobacter sp.*25_SBR2*	62.5	Both	Both	*Limnohabitans* sp.*Hippo3*	25	Damaged	Both	*Marmoricola* sp.*BN130122*	12.5	Undamaged	Rural	*Aeromicrobium halocynthiae*	12.5	Undamaged	Rural
Kineosporia mesophila	62.5	Both	Both	*Staphylococcus bacterium*	25	Both	Both	*Williamsia* sp.*ARP1*	12.5	Damaged	Urban	*Enterococcus gallinarum*	12.5	Undamaged	Rural
Chryseobacterium arthrosphaerae	62.5	Both	Both	*Cellulomonas hominis*	25	Damaged	Both	*Mycobacterium* sp.*27486-12*	12.5	Damaged	Urban	*Blautia hansenii*	12.5	Damaged	Urban
Achromobacter xylosoxidans	62.5	Both	Both	*Derxia bacterium*	25	Both	Urban	*Luteipulveratus* sp.*M20-45*	12.5	Damaged	Urban	*Pseudonocardia spinosa*	12.5	Damaged	Urban
Roseateles depolymerans	62.5	Both	Both	*Bacillus cereus*	25	Both	Both	*Janibacter cremeus*	12.5	Damaged	Urban	*Aerococcus viridans*	12.5	Undamaged	Rural
Sediminibacterium sp.*HSD04*	62.5	Both	Both	*Chryseobacterium taichungense*	25	Both	Urban	*Chryseobacterium hispalense*	12.5	Damaged	Urban	*Pseudonocardia alaniniphila*	12.5	Damaged	Urban
Kineococcus bacterium	62.5	Both	Both	*Salinibacterium aurum*	25	Both	Both	*Serinibacter* sp.*K3-2*	12.5	Damaged	Urban	*Actinomyces oris*	12.5	Undamaged	Urban
Janibacter anophelis	62.5	Both	Both	*Clostridium XVIII ramosum*	25	Both	Both	*Hoeflea* sp.*JSM*	12.5	Undamaged	Urban	*Providencia sneebia*	12.5	Undamaged	Urban

Roseateles sp.*K67*	62.5	Both	Both	*Microbacterium natoriense*	25	Both	Urban	*Fodinicola* sp.*I14A-00812*	12.5	Damaged	Urban	*Novosphingobium troitsensis*	12.5	Damaged	Urban
Escherichia/Shigella coli	62.5	Both	Both	*Geodermatophilus siccatus*	25	Damaged	Urban	*Agreia* sp.*I12A-02597*	12.5	Damaged	Rural	*Lysobacter korlensis*	12.5	Undamaged	Urban
Aurantimonas sp.*HC-3*	62.5	Both	Urban	*Ornithinimicrobium* sp.*CMT700*	25	Damaged	Both	*Rummeliibacillus* sp.*I13B-01798*	12.5	Damaged	Rural	*Methylobacterium phyllostachyos*	12.5	Damaged	Rural
Anaerococcus octavius	62.5	Both	Urban	*Gordonia paraffinivorans*	25	Both	Urban	*Trueperella* sp.*S350*	12.5	Damaged	Rural	*Pseudonocardia xishanensis*	12.5	Undamaged	Rural
Neisseria mucosa	62.5	Both	Both	*Gordonia* sp.*NIHHS105*	25	Both	Urban	*Parabacteroides* sp.*S449*	12.5	Damaged	Urban	*Leifsonia moechotypicola*	12.5	Undamaged	Urban
Bradyrhizobium japonicum	62.5	Both	Both	*Facklamia hominis*	25	Both	Urban	*Adlercreutzia* sp.*S45*	12.5	Damaged	Rural	*Williamsia phyllosphaerae*	12.5	Damaged	Urban
Arthrobacter flavus	62.5	Both	Both	*Lactobacillus curvatus*	25	Both	Urban	*Enterobacter adecarboxylata*	12.5	Damaged	Urban	*Ornithinimicrobium murale*	12.5	Damaged	Urban
Marinilactibacillus psychrotolerans	62.5	Both	Both	*Exiguobacterium sibiricum*	25	Both	Urban	*Bacillus thermoamylovorans*	12.5	Damaged	Urban	*Nocardioides ginsengisegetis*	12.5	Damaged	Urban
Friedmanniella capsulata	62.5	Both	Both	*Pseudomonas alcaligenes*	25	Damaged	Both	*Parabacteroides distasonis*	12.5	Damaged	Rural	*Nocardioides ginsengagri*	12.5	Undamaged	Rural
Brevundimonas sp.*0312MAR21U9*	62.5	Both	Both	*Rhizobium tumefaciens*	25	Both	Urban	*Amycolatopsis marina*	12.5	Damaged	Urban	*Agrococcus carbonis*	12.5	Damaged	Urban
Solirubrobacter bacterium	62.5	Both	Both	*Pectobacterium carotovorum*	25	Both	Urban	*Enterobacter xiangfangensis*	12.5	Damaged	Urban	*Acidovorax terrae*	12.5	Undamaged	Urban
Staphylococcus haemolyticus	62.5	Both	Both	*Zhihengliuella flava*	25	Both	Both	*Gillisia* sp.*M5A-3*	12.5	Damaged	Urban	*Pseudorhodoferax aquiterrae*	12.5	Damaged	Urban
Bifidobacterium adolescentis	62.5	Both	Both	*Micrococcus endophyticus*	25	Both	Rural	*Epilithonimonas* sp.*PDD-58b-23*	12.5	Damaged	Urban	*Rhodobacter halotolerans*	12.5	Undamaged	Rural
Paraprevotella bacterium	62.5	Both	Both	*Dietzia* sp.*CSC19*	25	Damaged	Urban	*Pseudomonas rhizosphaerae*	12.5	Damaged	Urban	*Leuconostoc pseudomesenteroides*	12.5	Damaged	Urban
Varibaculum sp.*Marseille-P2802*	62.5	Both	Both	*Enterococcus durans*	25	Damaged	Both	*Frondihabitans* sp.*PDD-63b-8*	12.5	Damaged	Urban	*Micromonospora abujensis*	12.5	Undamaged	Urban
Kocuria crystallopoietes	62.5	Both	Both	*Staphylococcus chromogenes*	25	Both	Both	*Frigoribacterium* sp.*PDD-69b-5*	12.5	Damaged	Urban	*Catellibacterium lanyuensis*	12.5	Undamaged	Rural
Pedobacter panaciterrae	62.5	Both	Both	*Leifsonia* sp.*215*	25	Damaged	Both	*Variovorax* sp.*PDD-69b-8*	12.5	Damaged	Urban	*Salinibacterium soli*	12.5	Undamaged	Rural
Segetibacter koreensis	62.5	Both	Both	*Clavibacter* sp.*300*	25	Damaged	Both	*Thermoactinomyces* sp.*T36*	12.5	Damaged	Urban	*Dyadobacter arcticus*	12.5	Undamaged	Rural
Bifidobacterium pseudolongum	62.5	Both	Both	*Sporosarcina* sp.*305*	25	Damaged	Urban	*Trichococcus pasteurii*	12.5	Damaged	Urban	*Hymenobacter saemangeumensis*	12.5	Undamaged	Urban
Humicoccus flavidus	62.5	Both	Both	*Haematobacter massiliensis*	25	Both	Both	*Serinicoccus* sp.*CR-19*	12.5	Damaged	Urban	*Paracoccus rhizosphaerae*	12.5	Damaged	Urban
Prevotella oris	62.5	Both	Both	*Sphingomonas* sp.*DUSK*	25	Undamaged	Urban	*Ureibacillus thermosphaericus*	12.5	Damaged	Urban	*Algoriphagus sdotyamensis*	12.5	Undamaged	Rural
Bifidobacterium bifidum	62.5	Both	Both	*Micrococcus yunnanensis*	25	Damaged	Both	*Acidovorax delafieldii*	12.5	Undamaged	Urban	*Tepidimonas fonticaldi*	12.5	Undamaged	Rural
Gemmiger formicilis	62.5	Both	Both	*Brevundimonas diminuta*	25	Both	Both	*Ignatzschineria* sp.*077229*	12.5	Damaged	Urban	*Luteolibacter yonseiensis*	12.5	Undamaged	Urban
Derxia mirabilis	62.5	Both	Both	*Cellvibrio* sp.*MVW-40*	25	Both	Urban	*Planococcaceae incertae sedis boronitolerans*	12.5	Damaged	Urban	*Aurantimonas jatrophae*	12.5	Undamaged	Urban
Marmoricola iriomotensis	62.5	Both	Both	*Proteus* sp.*SK3*	25	Both	Urban	*Bacillus oleronius*	12.5	Damaged	Urban	*Leifsonia aerilata*	12.5	Undamaged	Rural
Actinomyces massiliensis	62.5	Both	Both	*Paracoccus carotinifaciens*	25	Both	Both	*Streptomyces flavogriseus*	12.5	Damaged	Urban	*Roseomonas aerophila*	12.5	Undamaged	Urban
Blastococcus aggregatus	62.5	Both	Both	*Alkanindiges illinoisensis*	25	Undamaged	Both	*Stenotrophomonas humi*	12.5	Damaged	Rural	*Geodermatophilus taihuensis*	12.5	Undamaged	Rural
Rhodopseudomonas robiniae	62.5	Both	Both	*Arthrobacter gandavensis*	25	Both	Both	*Porphyrobacter* sp.*LB1-11*	12.5	Damaged	Urban	*Nocardioides salsibiostraticola*	12.5	Undamaged	Rural

Aciditerrimonas daechungensis	62.5	Both	Both	*Amaricoccus tamworthensis*	25	Both	Urban	*Rhizobium* sp.*Mol*	12.5	Damaged	Urban	*Lysobacter ginsengisoli*	12.5	Damaged	Urban
Ornithinicoccus hortensis	50	Both	Both	*Clostridium XlVa scindens*	25	Both	Urban	*Rhizobium giardinii*	12.5	Damaged	Urban	*Actinaurispora siamensis*	12.5	Undamaged	Rural
Lysobacter bacterium	50	Both	Both	*Staphylococcus auricularis*	25	Both	Rural	*Corynebacterium aurimucosum*	12.5	Undamaged	Urban	*Actinomycetospora chlora*	12.5	Undamaged	Rural
Curtobacterium sp.*227-FB*	50	Both	Both	*Nocardioides plantarum*	25	Both	Both	*Corynebacterium imitans*	12.5	Damaged	Urban	*Porphyromonas uenonis*	12.5	Undamaged	Urban
Pseudomonas libanensis	50	Both	Both	*Streptococcus agalactiae*	25	Both	Urban	*Pseudoxanthomonas* sp.*ALBL_054*	12.5	Undamaged	Urban	*Prevotella buccalis*	12.5	Undamaged	Urban
Corynebacterium durum	50	Both	Both	*Aquaspirillum arcticum*	25	Both	Urban	*Rhodopseudomonas* sp.*BRJL9*	12.5	Damaged	Urban	*Austwickia chelonae*	12.5	Damaged	Urban
Delftia tsuruhatensis	50	Both	Both	*Microbacterium pygmaeum*	25	Both	Both	*Arthrobacter bacterium*	12.5	Damaged	Urban	*Bifidobacterium biavatii*	12.5	Damaged	Urban
Methylophilus bacterium	50	Both	Both	*Pseudonocardia hydrocarbonoxydans*	25	Both	Urban	*Phycicoccus ochangensis*	12.5	Damaged	Urban	*Actinomycetospora iriomotensis*	12.5	Undamaged	Rural
Erythrobacter bacterium	50	Both	Both	*Roseburia inulinivorans*	25	Undamaged	Both	*Erwinia cedenensis*	12.5	Damaged	Urban	*Cellulomonas soli*	12.5	Damaged	Urban
Nocardioides rubriscoriae	50	Both	Both	*Arthrobacter monumenti*	25	Both	Both	*Ramlibacter* sp.*YS*	12.5	Damaged	Urban	*Dialister micraerophilus*	12.5	Undamaged	Rural
Lactobacillus gasseri	50	Both	Both	*Deinococcus marmoris*	25	Both	Both	*Ornithinimicrobium humiphilum*	12.5	Damaged	Urban	*Enhydrobacter osloensis*	12.5	Undamaged	Urban
Pedobacter sp.*2P1H2*	50	Both	Urban	*Sphingomonas mucosissima*	25	Both	Both	*Massilia* sp.*2PM3lan*	12.5	Damaged	Urban	*Brachybacterium paraconglomeratum*	12.5	Damaged	Urban
Eubacterium sp.*ICM62*	50	Both	Both	*Nocardioides hwasunensis*	25	Both	Both	*Sphingobium limneticum*	12.5	Damaged	Urban	*Capnocytophaga sputigena*	12.5	Damaged	Urban
Sorangium cellulosum	50	Both	Urban	*Pelomonas aquatica*	25	Undamaged	Both	*Chryseobacterium UYP8*	12.5	Damaged	Urban	*Brevundimonas vesicularis*	12.5	Damaged	Urban
Bacillus simplex	50	Both	Both	*Quadrisphaera granulorum*	25	Both	Both	*Kocuria carniphila*	12.5	Damaged	Urban	*Simplicispira psychrophila*	12.5	Undamaged	Rural
Lachnospiracea incertae sedis sp.*canine*	50	Both	Both	*Nocardioides lentus*	25	Both	Urban	*Rhodococcus fascians*	12.5	Undamaged	Urban	*Sphingobacterium mizutaii*	12.5	Damaged	Urban
Granulicatella elegans	50	Both	Both	*Rothia terrae*	25	Both	Urban	*Acinetobacter indicus*	12.5	Undamaged	Urban	*Pedobacter piscium*	12.5	Undamaged	Rural
Dyadobacter sp.*B2*	50	Both	Both	*Pedobacter insulae*	25	Undamaged	Both	*Paracoccus yeei*	12.5	Undamaged	Urban	*Sphingomonas adhaesiva*	12.5	Undamaged	Urban
Dialister sp.*S7MSR5*	50	Both	Both	*Nocardioides islandensis*	25	Both	Urban	*Pseudomonas taiwanensis*	12.5	Damaged	Urban	*Sphingomonas echinoides*	12.5	Damaged	Urban
Microlunatus sp.*H22*	50	Both	Both	*Microbacterium laevaniformans*	25	Both	Both	*Corynebacterium flavescens*	12.5	Damaged	Urban	*Paracoccus seriniphilus*	12.5	Damaged	Urban
Massilia plicata	50	Both	Both	*Rhodobacter sphaeroides*	25	Both	Both	*Chryseobacterium treverense*	12.5	Damaged	Urban	*Sphingomonas jaspsi*	12.5	Undamaged	Rural
Clostridium sensu stricto perfringens	50	Both	Both	*Kineococcus radiotolerans*	25	Undamaged	Both	*Microbacterium suwonense*	12.5	Damaged	Urban	*Rhizobium daejeonense*	12.5	Damaged	Urban
Friedmanniella sp.*Pao16*	50	Both	Urban	*Pseudomonas syringae*	25	Both	Both	*Leifsonia lichenia*	12.5	Damaged	Urban	*Sphingobium amiense*	12.5	Undamaged	Rural
Abiotrophia defectiva	50	Both	Both	*Lachnospiracea incertae sedis eligens*	25	Undamaged	Both	*Brevibacterium* sp.*AIC-5*	12.5	Damaged	Urban	*Oxalicibacterium solurbis*	12.5	Undamaged	Rural
Rothia sp.*B18*	50	Both	Urban	*Rothia mucilaginosa*	25	Undamaged	Both	*Curvibacter* sp.*S201*	12.5	Damaged	Urban	*Bradyrhizobium boonkerdii*	12.5	Undamaged	Rural
Pseudomonas poae	50	Both	Both	*Polaromonas* sp.*JS666*	25	Both	Both	*Lactobacillus coryniformis*	12.5	Damaged	Urban	*Acidovorax konjaci*	12.5	Undamaged	Urban
Aurantimonas sp.*4M3-2*	50	Both	Urban	*Calothrix* sp.*PCC*	25	Undamaged	Both	*Thermofilum archaeon*	12.5	Damaged	Urban	*Enterococcus hirae*	12.5	Undamaged	Rural
Friedmanniella aerolata	50	Both	Both	*Patulibacter rosea*	25	Undamaged	Both	*Lactobacillus agilis*	12.5	Damaged	Urban	*Microbacterium maritypicum*	12.5	Undamaged	Rural

Actinotalea sp.*734H3*	50	Both	Both	*Neisseria elongata*	25	Both	Urban	*Leuconostoc mesenteroides*	12.5	Damaged	Urban	*Acinetobacter baylyi*	12.5	Both	Urban
Ralstonia pickettii	50	Both	Urban	*Pseudonocardia seranimata*	25	Undamaged	Urban	*Erwinia rhapontici*	12.5	Undamaged	Urban	*Flavobacterium subsaxonicum*	12.5	Undamaged	Urban
Kocuria kristinae	50	Both	Both	*Janibacter badiiscoriae*	25	Both	Both	*Oerskovia* sp.*CON39-30*	12.5	Damaged	Urban	*Phenylobacterium mobile*	12.5	Undamaged	Rural
Streptococcus gastrococcus	50	Both	Urban	*Chryseobacterium yonginense*	25	Both	Urban	*Agrococcus baldri*	12.5	Damaged	Urban	*Solobacterium moorei*	12.5	Undamaged	Rural
Sporichthya polymorpha	50	Both	Both	*Novosphingobium dongtanensis*	25	Both	Both	*Streptococcus pasteurianus*	12.5	Undamaged	Urban	*Alkanindiges hongkongensis*	12.5	Damaged	Urban
Acinetobacter haemolyticus	50	Both	Both	*Saxeibacter panacisegetis*	25	Both	Urban	*Delftia lacustris*	12.5	Damaged	Rural	*Actinoplanes auranticolor*	12.5	Damaged	Rural
Arthrobacter cumminsii	50	Both	Both	*Blautia faecis*	25	Undamaged	Both	*Yimella* sp.*py1292*	12.5	Damaged	Urban	*Lysobacter dokdonensis*	12.5	Undamaged	Urban
Mesorhizobium mediterraneum	50	Both	Urban	*Gulosibacter chungangensis*	25	Undamaged	Both	*Curvibacter* sp.*DCY110*	12.5	Damaged	Urban	*Solibacillus isronensis*	12.5	Damaged	Urban
Rhodococcus sp.*320*	50	Damaged	Both	*Sphingomonas cynarae*	25	Both	Urban	*Salinispora* sp.*NHF45*	12.5	Undamaged	Urban	*Rhizobium soli*	12.5	Damaged	Urban
Phyllobacterium sp.*JCM*	50	Both	Urban	*Ralstonia psychrotolerans*	25	Both	Urban	*Macrochaete lichenoides*	12.5	Damaged	Urban	*Labrys wisconsinensis*	12.5	Undamaged	Rural
Modestobacter lapidis	50	Both	Both	*Dongia soli*	25	Both	Both	*Dermacoccus* sp.*CMT48*	12.5	Damaged	Rural	*Pseudonocardia saturnea*	12.5	Undamaged	Urban
Saxeibacter bacterium	50	Both	Both	*Mycobacterium arabiense*	25	Both	Both	*Aquamicrobium* sp.*342B3_12ECASO*	12.5	Damaged	Urban	*Phyllobacterium aestuarii*	12.5	Damaged	Urban
Anaerosphaera bacterium	50	Both	Both	*Friedmanniella lucida*	25	Damaged	Urban	*Rickettsia Rickettsia*	12.5	Damaged	Urban	*Jannaschia seohaensis*	12.5	Damaged	Urban
Dietzia cinnamea	50	Both	Both	*Porphyromonas catoniae*	25	Both	Both	*Corynebacterium variabile*	12.5	Damaged	Urban	*Streptococcus gordonii*	12.5	Undamaged	Rural
Brevundimonas faecalis	50	Both	Both	*Acidipila rosea*	25	Both	Urban	*Enterobacter asburiae*	12.5	Damaged	Urban	*Modestobacter marinus*	12.5	Damaged	Urban
Clostridium XlVa xylanolyticus	50	Both	Both	*Branchiibius cervicis*	25	Both	Urban	*Pseudomonas putida*	12.5	Undamaged	Urban	*Brevibacterium pityocampae*	12.5	Damaged	Urban
Veillonella atypica	50	Both	Both	*Dialister invisus*	25	Both	Both	*Pseudomonas fulva*	12.5	Damaged	Urban	*Serinicoccus profundi*	12.5	Damaged	Urban
Corynebacterium suicordis	50	Both	Both	*Sphingobacterium faecium*	25	Both	Both	*Clostridium sensu stricto novyi*	12.5	Undamaged	Urban	*Rhizobium rosettiformans*	12.5	Undamaged	Urban
Stenotrophomonas terrae	50	Both	Both	*Dolosigranulum pigrum*	25	Undamaged	Both	*Streptococcus tigurinus*	12.5	Damaged	Rural	*Microbacterium aurantiacum*	12.5	Damaged	Urban
Aurantimonas ureilytica	50	Both	Both	*Sphingomonas astaxanthinifaciens*	25	Both	Urban	*Atopobium parvulum*	12.5	Damaged	Urban	*Pseudonocardia autotrophica*	12.5	Damaged	Rural
Coprococcus comes	50	Both	Both	*Parvimonas micra*	25	Both	Urban	*Cosenzaea myxofaciens*	12.5	Damaged	Rural	*Sphingomonas hankookensis*	12.5	Damaged	Urban
Microbacterium kribbense	50	Both	Both	*Clostridium XVIII spiroforme*	25	Both	Urban	*Actinokineospora* sp.*R434*	12.5	Damaged	Urban	*Zhihengliuella salsuginis*	12.5	Damaged	Urban
Roseomonas frigidaquae	50	Both	Both	*Rhodoplanes piscinae*	25	Both	Both	*Rathayibacter tanaceti*	12.5	Damaged	Urban	*Sphingomonas hunanensis*	12.5	Undamaged	Rural
Porphyromonas bennonis	50	Both	Urban	*Anaerococcus murdochii*	25	Both	Both	*Pedobacter duraquae*	12.5	Damaged	Urban	*Eubacterium umeaense*	12.5	Damaged	Urban
Kocuria rhizophila	50	Both	Both	*Haemophilus parainfluenzae*	25	Undamaged	Both	*Labedella gwakjiensis*	12.5	Damaged	Urban	*Pseudonocardia adelaidensis*	12.5	Damaged	Urban
Proteus mirabilis	50	Both	Both	*Arenimonas oryziterrae*	25	Both	Both	*Acidovorax avenae*	12.5	Damaged	Urban	*Vibrio casei*	12.5	Damaged	Urban
Bifidobacterium breve	50	Both	Both	*Terrabacter aeriphilus*	25	Both	Both	*Lactobacillus crispatus*	12.5	Damaged	Urban	*Naxibacter suwonensis*	12.5	Undamaged	Urban
Blastococcus saxobsidens	50	Both	Both	*Arthrobacter halodurans*	25	Both	Both	*Pantoea* sp.*MDMC194*	12.5	Damaged	Rural	*Methylobacterium cerastii*	12.5	Damaged	Urban

Tepidimicrobium coagulans	50	Both	Both	*Deinococcus aerolatus*	25	Both	Both	*Chryseobacterium soldanellicola*	12.5	Damaged	Urban	*Anaerostipes hadrus*	12.5	Undamaged	Rural
Moraxella nonliquefaciens	50	Both	Urban	*Arthrobacter aestuarii*	25	Both	Both	*Cronobacter malonaticus*	12.5	Damaged	Urban	*Hymenobacter glaciei*	12.5	Damaged	Urban
Friedmanniella okinawensis	50	Both	Both	*Dietzia papillomatosis*	25	Both	Both	*Cedecea davisae*	12.5	Damaged	Urban	*Bacillus frigoritolerans*	12.5	Damaged	Urban
Psychrobacter immobilis	50	Both	Urban	*Negativicoccus succinicivorans*	25	Both	Urban	*Pseudochrobactrum asaccharolyticum*	12.5	Damaged	Urban	*Peptoniphilus tyrrelliae*	12.5	Undamaged	Rural
Kocuria phoenicis	50	Both	Both	*Nocardioides caricicola*	25	Damaged	Urban	*Bradyrhizobium faecalis*	12.5	Damaged	Urban	*Acinetobacter tandoii*	12.5	Undamaged	Rural
Rhodococcus cerastii	50	Both	Both	*Porphyromonas asaccharolytica*	25	Both	Both	*Pseudacidovorax intermedius*	12.5	Damaged	Urban	*Pseudomonas viridiflava*	12.5	Damaged	Urban
Methylobacterium bullatum	50	Both	Both	*Sphingomonas rubra*	25	Undamaged	Both	*Roseateles toxinivorans*	12.5	Damaged	Urban	*Sphingomonas ginsenosidivorax*	12.5	Undamaged	Rural
Dyadobacter sediminis	50	Both	Both	*Corynebacterium pilbarense*	25	Both	Both	*Variovorax ginsengisoli*	12.5	Damaged	Urban	*Fusobacterium periodonticum*	12.5	Damaged	Urban
Burkholderia metalliresistens	50	Both	Both	*Marmoricola korecus*	25	Undamaged	Both	*Geobacillus caldoxylosilyticus*	12.5	Damaged	Urban	*Microvirga lotononidis*	12.5	Damaged	Urban
Bacillus licheniformis	50	Damaged	Both	*Hymenobacter psychrophilus*	25	Both	Urban	*Exiguobacterium aurantiacum*	12.5	Undamaged	Urban	*Rhizobium tarimense*	12.5	Undamaged	Rural
Neisseria perflava	37.5	Both	Both	*Hymenobacter elongatus*	25	Both	Both	*Rhodococcus coeliaca*	12.5	Damaged	Urban	*Isoptericola nanjingensis*	12.5	Damaged	Urban
Corynebacterium glucuronolyticum	37.5	Undamaged	Urban	*Peptoniphilus coxii*	25	Both	Both	*Chryseobacterium M8*	12.5	Damaged	Urban	*Psychrobacter pulmonis*	12.5	Undamaged	Rural
Staphylococcus caprae	37.5	Both	Both	*Saxeibacter lacteus*	25	Undamaged	Both	*Rhizobium galegae*	12.5	Damaged	Urban	*Arenimonas metalli*	12.5	Undamaged	Urban
Microbacterium lacticum	37.5	Both	Both	*Sphingomonas roseiflava*	25	Undamaged	Both	*Anaplasma pipientis*	12.5	Undamaged	Urban	*Cellulomonas ferrariae*	12.5	Damaged	Urban
Kocuria bacterium	37.5	Both	Both	*Streptococcus dentisani*	25	Both	Both	*Bacillus kokeshiiformis*	12.5	Damaged	Urban	*Planococcus halocryophilus*	12.5	Damaged	Urban
Pseudonocardia zijingensis	37.5	Both	Urban	*Roseburia intestinalis*	25	Both	Both	*Staphylococcus vitulinus*	12.5	Damaged	Urban	*Massilia namucuonensis*	12.5	Damaged	Urban
Kineococcus sp.*psc3*	37.5	Both	Both	*Hymenobacter yonginensis*	25	Both	Rural	*Mycobacterium fortuitum*	12.5	Damaged	Urban	*Frondihabitans suwonensis*	12.5	Damaged	Urban
Paenibacillus sp.*1105*	37.5	Damaged	Urban	*Anaerococcus senegalensis*	25	Both	Both	*Psychrobacter nivimaris*	12.5	Damaged	Urban	*Sphingobium czechense*	12.5	Undamaged	Rural
Ralstonia proteobacterium	37.5	Both	Urban	*Propionibacterium avidum*	25	Both	Urban	*Kocuria salsicia*	12.5	Damaged	Urban	*Peptoniphilus timonensis*	12.5	Damaged	Urban
Prevotella salivae	37.5	Both	Urban	*Salmonella enterica*	25	Undamaged	Both	*Enhydrobacter* sp.*Td-10*	12.5	Damaged	Urban	*Novosphingobium lindaniclasticum*	12.5	Undamaged	Urban
Lachnospiracea incertae sedis Dorea	37.5	Both	Urban	*Neisseria cinerea*	25	Damaged	Urban	*Bradyrhizobium daqingense*	12.5	Damaged	Urban	*Anaerococcus obesiensis*	12.5	Damaged	Urban
Humicoccus bacterium	37.5	Both	Urban	*Frondihabitans sucicola*	25	Undamaged	Both	*Staphylococcus kloosii*	12.5	Damaged	Urban	*Aurantimonas phyllosphaerae*	12.5	Undamaged	Rural
Roseburia sp.*831b*	37.5	Both	Both	*Massilia kyonggiensis*	25	Both	Both	*Lactobacillus helveticus*	12.5	Damaged	Rural	*Phaeobacter gallaeciensis*	12.5	Undamaged	Rural
Hymenobacter perfusus	37.5	Both	Urban	*Hymenobacter qilianensis*	25	Both	Both	*Erwinia coffeiphila*	12.5	Undamaged	Urban	*Rathayibacter iranicus*	12.5	Undamaged	Rural
Arthrobacter arilaitensis	37.5	Both	Urban	*Demetria marinus*	25	Undamaged	Both	*Clostridium sensu stricto beijerinckii*	12.5	Damaged	Urban	*Mucilaginibacter calamicampi*	12.5	Damaged	Urban
Pseudomonas argentinensis	37.5	Damaged	Both	*Hymenobacter tibetensis*	25	Undamaged	Both	*Leuconostoc citreum*	12.5	Damaged	Urban	*Gordonia terrae*	12.5	Undamaged	Urban
Nocardioides jensenii	37.5	Both	Both	*Chryseobacterium arachidis*	25	Both	Urban	*Providencia rettgeri*	12.5	Damaged	Urban	*Bordetella trematum*	12.5	Undamaged	Rural
Corynebacterium tuscaniense	37.5	Both	Urban	*Flavobacterium plurextorum*	25	Undamaged	Both	*Actinomyces johnsonii*	12.5	Damaged	Urban	*Ralstonia solanacearum*	12.5	Damaged	Urban

Actinoplanes digitatis	37.5	Both	Both	*Hymenobacter arcticus*	25	Both	Both	*Providencia stuartii*	12.5	Damaged	Urban	*Sulfuricella denitrificans*	12.5	Undamaged	Rural
Mucilaginibacter sp.*G03*	37.5	Both	Both	*Hymenobacter kanuolensis*	25	Undamaged	Both	*Serratia* sp.*F3-1-11*	12.5	Damaged	Urban	*Micromonospora friuliensis*	12.5	Undamaged	Urban
Quadrisphaera bacterium	37.5	Both	Both	*Paenalcaligenes suwonensis*	25	Both	Urban	*Ralstonia* sp.*BAB-4439*	12.5	Damaged	Urban	*Stenotrophomonas rhizophila*	12.5	Damaged	Urban
Amaricoccus sp.*YIM125*	37.5	Both	Urban	*Pedobacter jejuensis*	25	Both	Both	*Escherichia/Shigella* sp.*BAB-5849*	12.5	Damaged	Urban	*Corynebacterium casei*	12.5	Damaged	Urban
Haemophilus influenzae	37.5	Damaged	Both	*Pedobacter glacialis*	25	Both	Both	*Staphylococcus saprophyticus*	12.5	Damaged	Urban	*Mycobacterium insubricum*	12.5	Undamaged	Rural
Bosea sp.*CC11C2*	37.5	Both	Urban	*Ralstonia syzygii*	25	Undamaged	Both	*Pantoea* sp.*S2*	12.5	Damaged	Rural	*Agaricicola taiwanensis*	12.5	Undamaged	Urban
Mesorhizobium sp.*ADC-19B*	37.5	Both	Urban	*Chelatococcus caeni*	25	Both	Urban	*Virgibacillus* sp.*SP-2.7*	12.5	Damaged	Urban	*Lactobacillus hominis*	12.5	Damaged	Urban
Aurantimonas glaciistagni	37.5	Both	Urban	*Clavibacter michiganensis*	25	Undamaged	Both	*Enterobacter vulneris*	12.5	Damaged	Urban	*Cellulomonas massiliensis*	12.5	Undamaged	Urban
Sphingomonas cucumeris	37.5	Both	Urban	*Frigoribacterium endophyticum*	25	Undamaged	Both	*Sphingomonas insulae*	12.5	Damaged	Urban	*Peptoniphilus obesi*	12.5	Undamaged	Urban
Rheinheimera tangshanensis	37.5	Both	Both	*Lysobacter mobilis*	25	Undamaged	Both	*Spirosoma fluviale*	12.5	Damaged	Urban	*Pseudonocardia atypica*	12.5	Undamaged	Rural
Methylibium petroleiphilum	37.5	Both	Both	*Roseomonas tokyonensis*	25	Undamaged	Both	*Bacteroides dorei*	12.5	Damaged	Urban	*Pontibacter rhizosphera*	12.5	Damaged	Urban
Roseateles chitosanitabida	37.5	Both	Both	*Porphyromonas pasteri*	25	Undamaged	Both	*Lactobacillus kitasatonis*	12.5	Damaged	Rural	*Kocuria assamensis*	12.5	Undamaged	Rural
Comamonas sp.*IDO2*	37.5	Both	Both	*Sphingomonas zeae*	25	Damaged	Both	*Spirilliplanes yamanashiensis*	12.5	Undamaged	Urban	*Pedobacter kyungheensis*	12.5	Undamaged	Urban
Rathayibacter sp.*JSM*	37.5	Both	Urban	*Nocardioides antarcticus*	25	Undamaged	Both	*Microbacterium saccharophilum*	12.5	Damaged	Urban	*Piscicoccus nakaumiensis*	12.5	Undamaged	Rural
Modestobacter sp.*I12A-02988*	37.5	Both	Urban	*Sphingomonas psychrolutea*	25	Both	Urban	*Lactobacillus delbrueckii*	12.5	Damaged	Urban	*Leucobacter kyeonggiensis*	12.5	Damaged	Urban
Gordonibacter sp.*S475*	37.5	Damaged	Both	*Mesorhizobium cantuariense*	25	Both	Urban	*Agromyces* sp.*ANK073*	12.5	Damaged	Urban	*Gemella taiwanensis*	12.5	Undamaged	Rural
Nesterenkonia sp.*CPA-95*	37.5	Both	Both	*Escherichia/Shigella agona*	25	Both	Both	*Xylophilus ampelinus*	12.5	Damaged	Urban	*Hymenobacter ruber*	12.5	Damaged	Urban
Kitasatospora sp.*1C-32*	37.5	Both	Urban	*Psychrobacillus psychrodurans*	25	Damaged	Urban	*Erwinia* sp.*JCM*	12.5	Damaged	Urban	*Ralstonia suwonense*	12.5	Damaged	Urban
Neisseria cerebrosus	37.5	Both	Both	*Curtobacterium flaccumfaciens*	25	Both	Both	*Kaistia soli*	12.5	Damaged	Urban	*Paracoccus communis*	12.5	Undamaged	Rural
Stenotrophomonas sp.*9Kp10a*	37.5	Both	Both	*Metascardovia* sp.*OB7196*	12.5	Damaged	Urban	*Planococcus donghaensis*	12.5	Damaged	Urban	*Sphingomonas daechungensis*	12.5	Undamaged	Urban
Delftia sp.*ALBL_009*	37.5	Both	Both	*Rubrivivax* sp.*YIT*	12.5	Damaged	Urban	*Clostridium XlVa* sp.*RK1P*	12.5	Undamaged	Urban	*Pedobacter pallidicorallinus*	12.5	Undamaged	Rural
Acidovorax sp.*ALBL_202*	37.5	Both	Both	*Virgisporangium ochraceum*	12.5	Undamaged	Urban	*Agaricicola* sp.*DMGB13*	12.5	Undamaged	Urban	*Klebsiella quasipneumoniae*	12.5	Damaged	Urban
Chryseobacterium indoltheticum	37.5	Both	Both	*Devosia submarina*	12.5	Undamaged	Urban	*Deinococcus radiophilus*	12.5	Damaged	Urban	*Pedobacter huanghensis*	12.5	Undamaged	Urban
Leifsonia sp.*2EM3*	37.5	Both	Urban	*Lachnospiracea incertae sedis hydrogenotrophica*	12.5	Damaged	Urban	*Sphingomonas yunnanensis*	12.5	Damaged	Urban	*Oceanicola sabulilitoris*	12.5	Undamaged	Rural
Chryseobacterium joostei	37.5	Both	Urban	*Clostridium sensu stricto celatum*	12.5	Damaged	Urban	*Rhizobacter* sp.*CB*	12.5	Damaged	Urban	*Thermomonas carbonis*	12.5	Undamaged	Rural
Sphingomonas canadensis	37.5	Both	Both	*Duganella* sp.*MsC-12-4CB4-02*	12.5	Damaged	Urban	*Terrimonas* sp.*CB*	12.5	Damaged	Urban	*Rhodopseudomonas gotjawalensis*	12.5	Damaged	Urban
Microbacterium trichothecenolyticum	37.5	Both	Urban	*Piscicoccus crocodyli*	12.5	Damaged	Urban	*Microvirga* sp.*CB*	12.5	Damaged	Urban	*Veillonella seminalis*	12.5	Damaged	Urban
Pedobacter suwonensis	37.5	Both	Urban	*Aestuariimicrobium thiooxidans*	12.5	Damaged	Urban	*Ameyamaea* sp.*C37*	12.5	Damaged	Urban	*Flavobacterium qiangtangense*	12.5	Damaged	Urban

Pantoea septica	37.5	Damaged	Urban	*Smithella* sp.*16S*	12.5	Undamaged	Urban	*Jiangella* sp.*1011TES3C79*	12.5	Damaged	Urban	*Blastococcus dictyosporus*	12.5	Undamaged	Rural
Bacteroides fragilis	37.5	Both	Both	*Nitrospira* sp.*clone*	12.5	Damaged	Urban	*Caulobacter mirabilis*	12.5	Damaged	Urban	*Microbacterium halimionae*	12.5	Undamaged	Rural
Prevotella timonensis	37.5	Both	Urban	*Escherichia/Shigella boydii*	12.5	Damaged	Urban	*Phycicoccus* sp.*URHC0019*	12.5	Damaged	Urban	*Olsenella scatoligenes*	12.5	Undamaged	Urban
Sphingomonas koreensis	37.5	Both	Both	*Serratia aquatilis*	12.5	Damaged	Urban	*Afipia bacterium*	12.5	Damaged	Urban	*Pontibacter humi*	12.5	Undamaged	Rural
Pantoea agglomerans	37.5	Both	Urban	*Pseudomonas synxantha*	12.5	Damaged	Urban	*Collinsella* sp.*GM6*	12.5	Undamaged	Urban	*Clostridium XI sedimentorum*	12.5	Damaged	Urban
Bacillus toyonensis	37.5	Both	Both	*Halococcus morrhuae*	12.5	Damaged	Urban	*Clostridium XlVa* sp.*AT9*	12.5	Damaged	Urban	*Sphingomonas gei*	12.5	Undamaged	Rural
Enterobacter cloacae	37.5	Both	Urban	*Serratia* sp.*NJ-71*	12.5	Damaged	Urban	*Aminobacter aminovorans*	12.5	Damaged	Urban	*Amnibacterium soli*	12.5	Damaged	Urban
Sphingobacterium sp.*FM2*	37.5	Both	Urban	*Bacillus longiquaesitum*	12.5	Damaged	Urban	*Staphylococcus pasteuri*	12.5	Undamaged	Urban	*Mesorhizobium jarvisii*	12.5	Damaged	Urban
Pseudomonas stutzeri	37.5	Both	Both	*Kribbella sancallistae*	12.5	Undamaged	Urban	*Variovorax boronicumulans*	12.5	Damaged	Urban	*Microbacterium proteolyticum*	12.5	Damaged	Urban
Psychrobacter sp.*44(2016)*	37.5	Both	Urban	*Nocardioides hungaricus*	12.5	Undamaged	Urban	*Rhodobacter* sp.*MST15SBBC*	12.5	Damaged	Urban	*Serratia chamberiensis*	12.5	Damaged	Urban
Serratia bacterium	37.5	Both	Urban	*Nitrosospira briensis*	12.5	Damaged	Urban	*Burkholderia* sp.*LMG*	12.5	Damaged	Urban	*Paracoccus sanguinis*	12.5	Damaged	Urban
Plantibacter sp.*H53*	37.5	Both	Urban	*Anaerorhabdus Hepatoplasma*	12.5	Damaged	Urban	*Planococcus* sp.*1304-W20*	12.5	Damaged	Urban	*Collimonas alpina*	12.5	Damaged	Urban
Actinoplanes lichenis	37.5	Both	Urban	*Corynebacterium appendicis*	12.5	Damaged	Urban	*Sanguibacter* sp.*1309-W7*	12.5	Damaged	Urban	*Staphylococcus petrasii*	12.5	Undamaged	Rural
Haemophilus parahaemolyticus	37.5	Both	Both	*Streptomyces lazureus*	12.5	Damaged	Urban	*Methanobrevibacter smithii*	12.5	Undamaged	Urban	*Paracoccus olei*	12.5	Damaged	Urban
Derxia sp.*CB*	37.5	Both	Urban	*Anaplasma Wolbachia*	12.5	Undamaged	Urban	*Lactobacillus ruminis*	12.5	Undamaged	Urban	*Intrasporangium nitratireducens*	12.5	Undamaged	Rural
Acidiphilium sp.*N29*	37.5	Both	Urban	*Sphingomonas trueperi*	12.5	Damaged	Urban	*Bacteroides uniformis*	12.5	Damaged	Urban	*Dactylosporangium cerinum*	12.5	Undamaged	Rural
Friedmanniella lacustris	37.5	Both	Both	*Kineococcus bacterium*	12.5	Damaged	Urban	*Parasutterella excrementihominis*	12.5	Undamaged	Urban	*Novosphingobium gossypii*	12.5	Damaged	Urban
Arthrobacter roseus	37.5	Both	Both	*Methylobacterium* sp.*14-324*	12.5	Damaged	Urban	*Actinoplanes consettensis*	12.5	Damaged	Urban	*Diaphorobacter polyhydroxybutyrativorans*	12.5	Undamaged	Rural
Lactobacillus iners	37.5	Both	Urban	*Hymenobacter bacterium*	12.5	Damaged	Urban	*Aquabacterium commune*	12.5	Undamaged	Rural	*Rhizobium lentis*	12.5	Undamaged	Urban
Hymenobacter soli	37.5	Both	Urban	*Arthrobacter cumminsii*	12.5	Damaged	Urban	*Subtercola boreus*	12.5	Damaged	Urban	*Tetrasphaera terrae*	12.5	Undamaged	Rural
Roseomonas aquatica	37.5	Both	Both	*Brevibacterium paucivorans*	12.5	Damaged	Rural	*Cellvibrio fulvus*	12.5	Damaged	Urban	*Paracoccus angustae*	12.5	Damaged	Urban
Chryseobacterium hominis	37.5	Both	Both	*Rhodobacter* sp.*D4028*	12.5	Damaged	Urban	*Arthrobacter albus*	12.5	Damaged	Urban	*Bifidobacterium ramosum*	12.5	Undamaged	Rural
Brevundimonas terrae	37.5	Both	Both	*Natroniella rupestris*	12.5	Damaged	Urban	*Pseudonocardia spinosispora*	12.5	Damaged	Urban	*Corynebacterium* sp.*16S*	12.5	Damaged	Urban
Piscinibacter aquaticus	37.5	Both	Both	*Legionella* sp.*legS052*	12.5	Damaged	Urban	*Haemophilus pittmaniae*	12.5	Undamaged	Rural	*Bacillus subtilis*	12.5	Undamaged	Urban
Chryseobacterium haifense	37.5	Both	Both	*Jejuia arisari*	12.5	Damaged	Urban	*Leifsonia pratensis*	12.5	Undamaged	Urban	*Solibacillus silvestris*	12.5	Undamaged	Rural
Streptococcus parasanguinis	37.5	Undamaged	Both	*Leptothrix* sp.*OTSz_A_252*	12.5	Damaged	Urban	*Pedobacter cryoconitis*	12.5	Undamaged	Urban	*Pseudomonas brenneri*	12.5	Undamaged	Both
Sphingomonas wittichii	37.5	Both	Both	*Mesorhizobium* sp.*OTSz_M_287*	12.5	Damaged	Urban	*Arsenicicoccus bolidensis*	12.5	Damaged	Urban	*Bacillus mycoides*	12.5	Both	Both
Exiguobacterium sp.*AT1b*	37.5	Both	Both	*Schlegelella aquatica*	12.5	Damaged	Urban	*Nocardioides aestuarii*	12.5	Damaged	Urban	*Brevibacillus brevis*	12.5	Damaged	Both

Solirubrobacter ginsenosidimutans	37.5	Undamaged	Both	*Advenella kashmirensis*	12.5	Undamaged	Urban	*Granulicatella adiacens*	12.5	Undamaged	Rural	Arthrobacter protophormiae	12.5	Damaged	Both
Methylobacterium gossipiicola	37.5	Both	Both	*Staphylococcus xylosus*	12.5	Damaged	Urban	*Actinobacillus porcinus*	12.5	Undamaged	Rural	Paenibacillus lactis	12.5	Damaged	Both
Blastococcus endophyticus	37.5	Both	Urban	*Micrococcus halobius*	12.5	Undamaged	Urban	*Turicella otitidis*	12.5	Undamaged	Urban	Isoptericola variabilis	12.5	Damaged	Both
Friedmanniella flava	37.5	Both	Both	*Altererythrobacter* sp.*CTDB1*	12.5	Damaged	Urban	*Agromyces ramosus*	12.5	Undamaged	Rural	Sporosarcina saromensis	12.5	Both	Both
Pseudonocardia antitumoralis	37.5	Both	Both	*Ilumatobacter* sp.*T2-YC6790*	12.5	Damaged	Urban	*Micrococcus lylae*	12.5	Undamaged	Rural	Bacillus sporothermodurans	12.5	Damaged	Both
Friedmanniella sagamiharensis	37.5	Damaged	Both	*Gemmata* sp.*Br1-2*	12.5	Undamaged	Urban	*Dermabacter hominis*	12.5	Undamaged	Rural	Bacillus sp.BC11	12.5	Undamaged	Both
Actinomycetospora cinnamomea	37.5	Both	Both	*Aeromicrobium* sp.*CNRD02*	12.5	Damaged	Urban	*Corynebacterium riegelii*	12.5	Undamaged	Rural	Paenibacillus lautus	12.5	Damaged	Both
Piscicoccus intestinalis	37.5	Both	Both	*Brevinema flagrans*	12.5	Damaged	Urban	*Facklamia ignava*	12.5	Damaged	Rural	Bacillus safensis	12.5	Both	Both
Paracoccus kocurii	37.5	Both	Both	*Massilia* sp.*sptzw26*	12.5	Damaged	Urban	*Clostridium sensu stricto disporicum*	12.5	Undamaged	Rural	Bacillus sp.PVS08	12.5	Undamaged	Both
Sporosarcina luteola	37.5	Both	Urban	*Veillonella dispar*	12.5	Damaged	Urban	*Rothia amarae*	12.5	Undamaged	Rural	Paenibacillus pabuli	12.5	Damaged	Both
Psychrobacter adeliensis	37.5	Both	Both	*Veillonella parvula*	12.5	Undamaged	Urban	*Pseudomonas salomonii*	12.5	Undamaged	Urban	Bacillus niacini	12.5	Damaged	Both
Hansschlegelia plantiphila	37.5	Both	Both	*Fusobacterium nucleatum*	12.5	Damaged	Urban	*Lactobacillus gastricus*	12.5	Damaged	Urban	Lysinibacillus fusiformis	12.5	Damaged	Both
Thalassobius gelatinovorus	37.5	Both	Both	*Eubacterium brachy*	12.5	Undamaged	Urban	*Nesterenkonia lutea*	12.5	Damaged	Urban	Paenibacillus sp.19783	12.5	Damaged	Both
Hymenobacter algoricola	37.5	Both	Urban	*Streptococcus cristatus*	12.5	Damaged	Urban	*Bifidobacterium saeculare*	12.5	Damaged	Urban	Bacillus coagulans	12.5	Damaged	Both

Appendix D: Species solely found in the rural environment.

Acinetobacter parvus, Acinetobacter tandoii, Actinaurispora siamensis, Actinobacillus porcinus, Actinobacillus succinogenes, Actinomycetospora chlora, Actinomycetospora iriomotensis, Actinoplanes auranticolor, Adlercreutzia sp.*S45, Aerococcus viridans, Aeromicrobium halocynthiae, Agreia* sp.*I12A-02597, Agrococcus jejuensis, Agromyces ramosus, Algoriphagus sdotyamensis, Anaerostipes hadrus, Aquabacterium commune, Arthrobacter echini, Arthrobacter phenanthrenivorans, Arthrobacter phytoseiuli, Aurantimonas phyllosphaerae, Bifidobacterium angulatum, Bifidobacterium ramosum, Blastococcus dictyosporus, Bordetella* sp.*UASWS0941, Bordetella trematum, Brachybacterium faecium, Bradyrhizobium boonkerdii, Brevibacterium aurantiacum, Brevibacterium paucivorans, Carnobacterium mobile, Catellibacterium lanyuensis, Cellvibrio mixtus, Chryseobacterium gregarium, Clostridium sensu stricto disporicum, Corynebacterium riegelii, Cosenzaea myxofaciens, Dactylosporangium cerinum, Delftia lacustris, Dermabacter hominis, Dermacoccus* sp.*CMT48, Devosia subaequoris, Dialister micraerophilus, Diaphorobacter polyhydroxybutyrativorans, Dyadobacter arcticus, Enterococcus gallinarum, Enterococcus hirae, Erythrobacter litoralis, Facklamia ignava, Gemella taiwanensis, Geodermatophilus taihuensis, Granulicatella adiacens, Haemophilus haemolyticus, Haemophilus pittmaniae, Intrasporangium nitratireducens, Kocuria assamensis, Kribbella catacumbae, Labrys wisconsinensis, Lactobacillus helveticus, Lactobacillus kitasatonis, Leifsonia aerilata, Marmoricola* sp.*BN130122, Methylobacterium extorquens, Methylobacterium iners, Methylobacterium phyllostachyos, Microbacterium halimionae, Microbacterium lacus, Microbacterium maritypicum, Micrococcus lylae, Microlunatus ginsengisoli, Mycobacterium insubricum, Nocardioides ginsengagri, Nocardioides salsibiostraticola, Oceanicola sabulilitoris, Ornithinimicrobium kibberense, Oxalicibacterium solurbis, Pantoea* sp.*136A, Pantoea* sp.*MDMC194, Pantoea vagans, Parabacteroides distasonis, Paracoccus communis, Pedobacter pallidicorallinus, Pedobacter piscium, Peptoniphilus tyrrelliae, Phaeobacter gallaeciensis, Phenylobacterium mobile, Piscicoccus nakaumiensis, Pontibacter humi, Prevotella melaninogenica, Pseudomonas entomophila, Pseudonocardia atypica, Pseudonocardia autotrophica, Pseudonocardia endophytica, Pseudonocardia xishanensis, Psychrobacter pulmonis, Rathayibacter iranicus, Rhizobium fabrum, Rhizobium tarimense, Rhodobacter halotolerans, Rhodococcus cerastii, Roseburia hominis, Rothia amarae, Rummeliibacillus* sp.*I13B-01798, Salinibacterium soli, Sanguibacter keddieii, Simplicispira psychrophila, Solobacterium moorei, Sphingobium amiense, Sphingobium czechense, Sphingomonas gei, Sphingomonas ginsenosidivorax, Sphingomonas hunanensis, Sphingomonas japonica, Sphingomonas jaspsi, Staphylococcus petrasii, Stenotrophomonas humi, Streptococcus gordonii, Streptococcus mitis, Streptococcus tigurinus, Sulfuricella denitrificans, Tepidimonas fonticaldi, Tetrasphaera terrae, Thermomonas carbonis,* and *Trueperella* sp.*S350.*