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The great influenza pandemic of 1918

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Introduction

Severe acute respiratory syndrome (SARS) is currently causing alarm as speculation mounts that it could be the next global pandemic. Great pandemic diseases have swept the world ever since mankind ceased to live in small communities and began to breed domestic animals in close proximity to homes. Viruses in particular, crossing from one species to another, mutate to invade new hosts and can develop lethal characteristics. Man, pig and fowl form a dangerous domestic combination for the RNA virus of the orthomyxovirus group (Influenza virus).

SARS is not of this group, it is possibly a coronavirus (Common cold virus) that has mutated and can now cause a 10-15% death rate. Compared with influenza virus, SARS has low infectivity and low mortality. Influenza currently kills 4,000 people a year in the UK. In 1918 the mortality rate for influenza reached 60% in some communities and it caused more deaths than the whole of the 1914-18 war. SARS has demonstrated that rapid air travel can spread locally-constrained diseases across the world. To get the SARS outbreak into context it is necessary to re-examine the great influenza pandemic of 1918 when influenza swept across the globe and in less than a year it left some 40 million dead in its wake. The influenza virus was finally isolated and identified in 1930.

The origin of pandemics

Porter[1] considered that the era of human epidemics began as civilisation developed. Around 3000BC cities were arising in the Middle East with populations of scores of thousands. Such settlements required huge animal resources for food and animal byproducts eg., fur, leather. These large congregations of animals transferred lethal contagious pathogens (Zoonotic infections eg., smallpox, diphtheria, influenza, chickenpox, mumps and other devastating illnesses) which rapidly spread in the local population. Degrees of immunity developed in afflicted human populations but the increasing traffic of people between large settlements and the outreaches of explorers into new territories, continuously supplied new strains of infection and spread infections into new populations. Some epidemic diseases eventually became endemic, continuously infecting the young, culling the old and more susceptible individuals. Tropical endemic disease, which required intermediate hosts that could not survive in more temperate climates eg., malaria, yellow fever, remained in warmer latitudes.

Historical descriptions, based on symptoms, indicate that influenza epidemics have probably been around human populations since before 5000BC. It was in the 20th century, however, when large population migrations both before and during the 1914–18 War, that helped create the most mobile lethal pandemic the world has ever witnessed, as it circumnavigated the globe in 1918–19. The country that suffered the greatest number of deaths was India where it is suggested that 16 million people died.

Where did this pandemic originate?

Although influenza epidemics have been recorded for centuries, the spread of this disease was never charted. The normally unremarkable death rate of influenza was a pale shadow of Plague (*Yersinia pestis*) where the Justinian outbreak in the 2nd century AD can be tracked in written records from Asia across Europe. Plague reached Europe in the 14th century, killing 25 million people. It remained for the next three centuries, waxing and waning in local epidemics across Europe.

The pandemic may have arisen in troops at the Western Front and have been carried back to the USA in troopships arriving in 1918. The USA seems to have reported the first fatal cases from army barracks in Denver and South Carolina. It quickly spread into surrounding communities and a main wave of infection peaked in September-November 1918, killing over 10,000 people per week in US cities. Large proportions of the US population became ill (28%) with mortality rates >2.5% compared with <0.1% in previous influenza epidemics. An estimated total of 675,000 persons died in the USA from the 1918–19 pandemic. Another significant factor that differentiated this pandemic was that most deaths occurred among young adults. Influenza and pneumonia death rates for 15–34 year old persons were more than 20 times higher in 1918 than in previous years.

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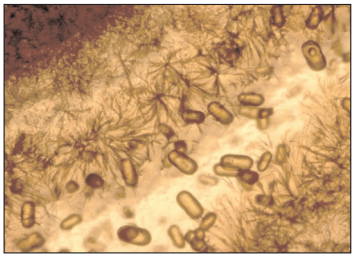
Microbes on building stone – for good or ill?

Eric May



Calcinogenic bacteria in laboratory culture, showing calcite crystals developing within colonies.

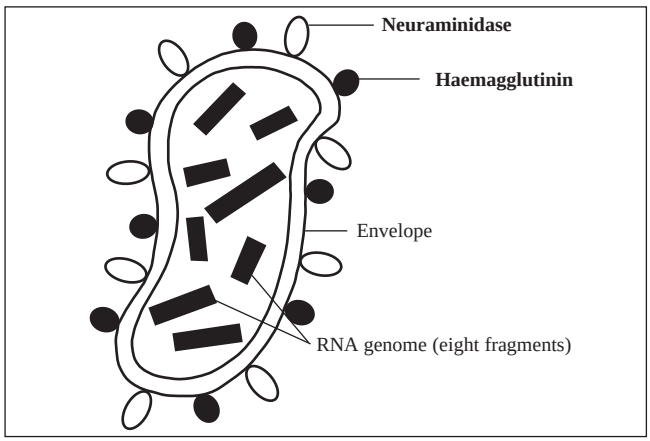


Figure 1. A schematic influenza viron showing RNA segments and H and N proteins on the envelope.

The influenza virus

Influenza is caused by an RNA virus of the orthomyxovirus group. Human influenza is transmitted from person to person through the air, primarily in droplets expelled during coughing and sneezing. The virus infects the mucous membranes of the upper respiratory tract and more occasionally invades the lungs. Recovery is usually spontaneous and rapid. The majority of the serious consequences of influenza infection are not caused by the virus but by secondary invasion of pathogenic bacteria. Thus bacterial pneumonia is especially prevalent in infants and the elderly.

Once a strain of influenza virus has passed through a population, the surviving majority are sufficiently immune to that strain to give 'herd' immunity protection to the non-immune minority. It is then unlikely that a strain of virus of similar antigenic type can cause another epidemic for 2–3 years. Antigenic 'drift' is responsible for recurrent epidemics of influenza to erupt in 2–3 year cycles.

Influenza virus was isolated from a human victim for the first time in 1933. The virus has two main types A and B, there is a type C but it does not cause serious disease. Type B virus shows less variation and infects humans only. It causes regional epidemics rather than pandemics. Type A is the more important form, causing pandemics and infecting pigs, horses, seals, whales and birds, although not all variant forms infect all species (only four variants have been found in humans).

The simplified diagram of the influenza viron (**Figure 1**) shows the viral RNA is present in a number of separate pieces (segments). This segmentation is an important cause of antigenic 'shift' because the host cell may be infected by two genetically distinct strains and the RNA segments from both strains may re-associate to create a new strain. The new strain may then be able to infect hosts that were immune to the two original strains. **Figure 1** also shows protein 'spikes' on the outside of the viron envelope; haemagglutinin (H) and neuraminidase (N). The function of these proteins is to adhere to the host nucleated cell and then facilitate the transfer of the viron into the cell. Haemagglutination of erythrocytes is a convenient measure of viral activity but the influenza virus has little interest in these non-nucleated cells. Immune antibodies, produced by infection or vaccination, are directed against these proteins and neutralise the virus by preventing ingress into cells with subsequent replication. It is considered to be most probable that these H and N proteins on the viron envelope are closely associated with infectivity and virulence. Type A virus has 15 H and 9 N variants giving rise to many different subtypes eg., H1N1, H1N2, H2N2 etc. Analyses of antibodies carried by survivors of the 1918 pandemic suggest that the strain was a classic swine H1N1 subtype influenza A virus. During the pandemic simultaneous outbreaks of influenza were reported in humans and pigs from around the world. Whether the virus passed from humans to pigs or *vice versa* has not been resolved. The natural reservoir for influenza virus is thought to be wild water fowl. Periodically, genetic material from avian strains is detected in human infections. Pigs can be infected with both avian and human forms of influenza virus and the pig could be an intermediate host.

Evidence so far suggests the H gene sequence of the 1918 strain is associated with strains that infect pigs and humans but not with strains that infect fowls. Little is known about the genetic features of influenza virulence. Virulent strains show an ability to infect various organs in the host, particularly spreading throughout the lungs to cause extensive, normally lethal damage.

A possible lead is that in some avian strains, a cleavage site mutation in the H protein can cause systemic disease in birds, instead of the normally benign infection of the gastro-intestinal tracts of host birds. These mutant strains are associated with exceptionally high mortality among infected birds. So far, there is no indication that the 1918 strain carried this mutation (one amino-acid change in the H protein) which has only been observed in H5 or H7 subtype viruses associated with infection in domestic poultry.

The search for evidence of the 1918 strain

As previously stated, in 1918 the influenza virus was unknown and virus study methodology was not available. Much later, opportunities were taken to get hold of tissue from victims of the pandemic buried in permafrost in the Arctic. Tissues could be expected to be preserved in these constant low temperatures for hundreds or possibly thousands of years. [A frozen mammoth body is currently being exhumed in Siberia that is alleged to be 10,000 years old.] Originally it was hoped that the influenza virus could be recovered but this proved impossible.

In 1951 a Swedish-born American pathologist, John Hultin from the University of Iowa, led a small party to exhume the bodies of victims who had died from the 1918 pandemic. These bodies had been buried in permafrost at Teller Mission, an Inuit fishing village on the Seward Peninsula of Alaska. This village suffered an extremely high mortality rate from influenza in November 1918. The disease spread through the village in five days and killed 72 people, about 85% of the adult population. No influenza virus could be recovered from the tissues. Molecular genetic analyses of the samples were impossible in 1951, the structure of DNA (the beginning of molecular genetics) was not determined until 1953.

In 1997, Hultin returned to Teller Mission and obtained frozen lung tissue from four more influenza victims. These were placed in formaldehyde for histology and viral RNA isolation. One piece of lung tissue showed evidence of massive pulmonary haemorrhage, typical of virulent influenza and it also contained RNA fragments of the virus.

Meanwhile in 1995, Jeffrey Taubenberger, Head of a Division of Molecular Pathology, Armed Forces Institute of Pathology (AFIP) Washington DC, turned to archival formalin-fixed, paraffin-embedded autopsy tissues of 1918 influenza victims stored in the AFIP. In total 78 autopsy cases from the pandemic were examined. The majority of the victims had died from secondary bacterial pneumonia. A small subset of these cases died within a week, with massive pulmonary oedema or haemorrhage. Death with these symptoms could occur in 48 hours.

Such rapid deaths also occurred in later epidemics of influenza but they were a cardinal feature of the pandemic in 1918–19. The first positive case was found in 1996 with lung sections showing acute focal bronchiolitis and alveolitis that are consistent with

primary influenzal viral pneumonia. Influenza RNA was recovered from the tissue blocks but it was fragmented into pieces not longer than 150 bases in length. The eight RNA fragments in living influenza virus vary from 900 to 2300 bases in length. A second case was identified in 1997 showing the same lung characteristics and RNA fragments no longer than 150 bases.

In 1998, John Oxford, professor of virology at the Royal London Hospital led an expedition to Spitzbergen in Norway to exhume the frozen bodies of six young coal miners who had died in the 1918 influenza pandemic. Tissues from various organs were removed, preserved and brought back to England. After months of testing at the National Institute of Medical research, Mill Hill, fragmentary genetic traces of viral RNA were found in lungs and other tissues.

This work continues in several centres but Taubenberger has two major goals for his investigation:

1. To determine the origin of the 1918 influenza virus ie., bird, pig or man and to discover how it can be transferred to humans.

2. To learn whether specific features of its nucleotide sequence can explain the virulence of this or any other strains.

This second goal was repeated by Professor John Oxford in a "Times" newspaper article on November 17, 1999. He told the reporter that once 'factor X' was identified, it will be possible to know what made this organism the most lethal virus ever known. It should be possible to detect the same gene sequence in recurring influenza epidemics and hopefully get an effective vaccine to prevent influenza returning as the Grim Reaper.

Contrast John Oxford's enthusiasm with Taubenberger's more cautious opinion to a reporter in the 'ASM News' 65:7. 1999. p.475. Taubenberger thought it unlikely that a 'smoking gun' specific genetic structure in a lethal virus will be found. Virulence may involve subtle changes in many genes. Host immune factors play a significant role in deciding the outcome

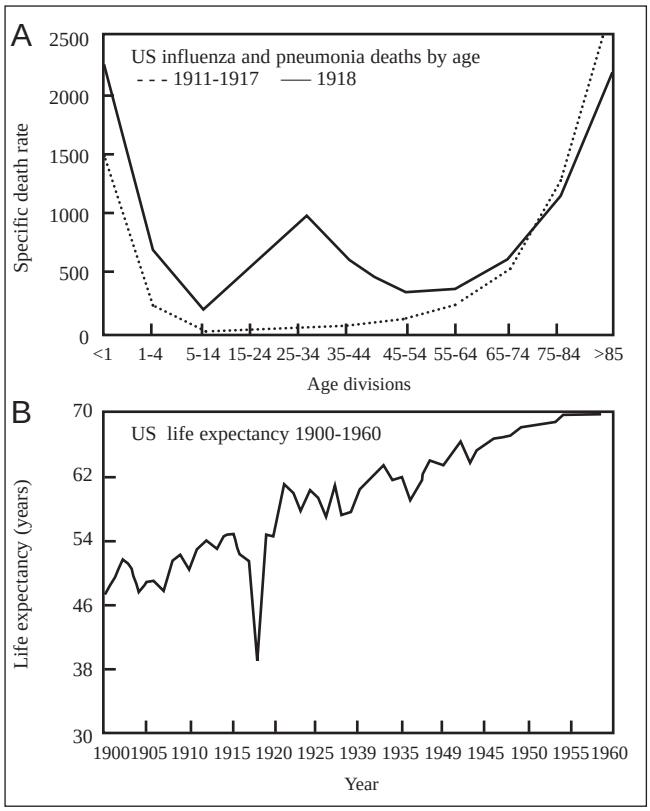


Figure 2. A. The rise in death rates of the 15-45 year age groups in the 1918-19 influenza pandemic compared with previous influenza epidemics. B The sharp fall in US life expectancy in 1918-19 from the exceptional influenza death rate (Taubenberger 1999).

of a serious infection. The unique massive movements of people in 1918–19 travelling or living in crowded conditions on troopships, in barracks or in cattle trucks may have been a critical factor in the spread and virulence of the pandemic across the world. An explanation is still required about the lethality of the 1918 strain for the 15–45 year age group (**Figure 2**).

Epidemiology of recent influenza epidemics

It was not until the pandemic of Asian influenza in 1957 that careful analysis of its worldwide progress was carried out. This influenza outbreak first appeared in central China in late February 1957. By early April it had reached Hong Kong and Australasia, spreading across to the USA. The USA epidemic reached a peak in October 1957. Meanwhile, it spread through South America and across to Europe where it met another wave of this epidemic from the Middle east and Africa.

In 1968, a Hong Kong influenza pandemic occurred which had a similar transglobal spread. In 1979 an avian influenza A virus entered the pig population in Northern Europe, forming a stable viral lineage.

Until 1997, there was no evidence that wholly avian influenza virus could infect humans. This evidence was provided in Hong Kong in 1997 when a new strain of influenza killed 4,500 chickens in Southern China. In Hong Kong 18 people were infected with this avian virus and six rapidly died. Millions of domestic fowls were slaughtered by the Hong Kong authorities to prevent the avian strain spreading, whilst the rest of the world held its breath!

Humans have no pre-existing immunity to avian subtypes and the prompt, vigorous action in Hong Kong may have stopped a calamitous epidemic. This avian strain was later shown to be unique in that it leapt from birds to humans but fortunately it appeared not to spread human to human. No doubt given more time it would have overcome this problem.

Discussions are being held about whether to include avian subtype strains in the human vaccine but this proposal is not without risk. Kolata[2] described an unfortunate chain of circumstances that led to one of America's worst public health catastrophes. In 1976, a USA soldier rapidly died from a swine type influenza infection. The implications of this single death heralding a major epidemic, grew from a remote possibility expressed in the army camps, up to projections of millions of deaths by the time it reached the White House. President Gerald Ford was persuaded by his medical advisors that this could be a rerun of the 1918 influenza pandemic. Ford ordered a population-wide vaccination programme with the slogan "Better a vaccine without an epidemic than an epidemic without a vaccine." He could not have been more wrong. The epidemic never materialised but the vaccine appeared to produce serious and sometimes lethal side-effects. The resulting litigation costs and compensation claims amounted to 3.5 billion US$.

Vaccination

Whilst new drug treatments for influenza are available or in the pipeline eg, 'Relenza' and 'Tamiflu', common sense suggests that prevention of the disease by vaccination is the most economic and effective method of treatment.

Unfortunately, antigenic 'drift' by the virus means that vaccination must be repeated every Autumn, using strains of influenza that are considered by global reference laboratories to be the greatest threat in the coming year. In 1999, the vaccine contained the Beijing and Sydney strains which were a threat in Britain to unvaccinated people over Christmas. In 2000 a *New Caledonia* strain of virus was included because it appeared to be an antigenic 'shift' from previous strains. Selection of the

correct strains is absolutely critical because it takes many months to prepare large stocks of vaccine and there is not time to repeat the exercise.

The public health disaster in the USA in 1976 should not be used as an excuse for avoiding vaccination. What happened on that occasion has not been fully explained. Attempts to rush production of adequate stocks of vaccine may have had a role in the consequences. Equally, recipients of the vaccine with pre-existing egg-product hypersensitivity, may not have been properly screened out. It was a unique unfortunate calamity which current changes in vaccine production may prevent ever happening again.

Influenza vaccine production

Influenza vaccines have been common in the USA since the 1940s. Vaccines against any given influenza variant strain take about six months to produce, test for safety and distribute. This is no match for a fast moving pandemic. Antigenic 'drift' is the gradual revision of the amino-acid sequence in the surface proteins. This drift is a particular characteristic of Type B influenza which evolves gradually in the human host to avoid recognition by the host immune system. Type A influenza can additionally undergo a more drastic antigenic 'shift'.

The change in the haemagglutinin or the neuraminidase proteins may be so great that the virus can evade the antibody repertoire of all the people in the world and that causes a pandemic. Such big changes do not occur through simple genetic mutation, it requires the mixing of two viral strains in one cell:

1918 pandemic	H1N1
1957 pandemic	H2N2
1968 pandemic	H3N2
1997 nearly epidemic	H5N1

The annual process of vaccine production begins each winter as influenza virus samples, collected by 110 surveillance sites around the world, are analysed. In February the WHO pinpoints three strains – two Type A and one Type B that seem likely to account for most of the influenza that will occur in the coming season (November to March in the Northern hemisphere). These three strains will comprise the new vaccine.

It would seem to be obvious that the next step must be to grow vast numbers of the selected strains, inactivate them so that they cannot cause infection and then combine them into a single vaccine. What seems to be obvious is not always realistic. The selected strains often grow slowly in the laboratory. To overcome this problem, the immune-stimulating proteins (haemagglutinin and neuraminidase – H and N) of the selected strains are inserted into an influenza strain that will grow rapidly in laboratory conditions. After infecting chick embryos with both selected and fast growing strains, the fast growth strains with the selected H and N proteins are isolated and delivered to the vaccine manufacturers. It is at this stage that mass production of the vaccine strains commences.

Future developments of influenza vaccine includes:

1. Use vaccines composed solely of H and N proteins.
2. Use vaccines made from weakened live influenza viruses, which can stimulate T lymphocytes. T cells can cope with viral antigenic drift. Live viruses can be delivered as a nasal spray. WHO, however, has issued a warning about the future use of live-cell vaccines because of the increasing number of HIV/AIDS patients that will be put at risk.
3. Use vaccines composed of other viral hosts, bearing selected H and N proteins, which grow readily in cultured cells (influenza viruses grow poorly in tissue culture cells). The high yield of encoded influenza proteins can then be purified for use in vaccines.
4. Use vaccines composed of DNA plasmids containing H and N genes. When the plasmids are injected subcutaneously, nearby cells will take them up to produce H and N proteins. These 'foreign' proteins will be detected by the host immune system which will then deploy antibodies and T cells to neutralise free virus and eradicate infected cells. Such 'naked DNA' influenza vaccines have worked well in animals but have yet to be tested in humans.

Conclusion

Eighty years after the Influenza pandemic of 1918–19 we are still no wiser about the cause of this most devastating killing disease but we are better informed about the circumstances.

Influenza pandemics occur every 30 years or so and unless substantial preventive measures are put into operation, there will be more to come. Whilst correct antibiotic treatment will reduce deaths from secondary bacterial pneumonia, the best primary defence is vaccination.

More work needs to be done to determine how the influenza virus cycles between the three major groups of hosts: birds, animals, humans and how often this occurs.

Influenza has been a well recognised disease for centuries and if a previous outbreak of such lethal dimensions had occurred, it would surely be written in the medical history of one or more countries. The English Sweate (1485 and four more visitations up to 1517) was well recorded, although the death rate was much smaller than the contemporary influenza epidemics[3].

There is a possibility that the 1918 influenza pandemic was an entirely unique event that could never return. It would not be wise, however, to take this possibility for granted.

References

1. Porter R. "The Greatest Benefit to Mankind." 1997; Harper Collins. London. pp. 22–25.
2. Kolata G. "FLU: the story of the Great Influenza Pandemic of 1918 and the search for the virus that caused it." 1999; Macmillan. London.
3. Bridson E. "The English 'Sweate' (*Sudor Anglicus*) and Hantavirus." *Med Sci History.* 1998; **14:** 20–32.

Further reading

Taubenberger JK. "Seeking the 1918 Spanish Influenza Virus." 1999; *ASM News.* **65:** 473–478.

Laver WG, Bischofberger N, Webster RG. "Disarming Flu Viruses." 1999; Scientific American. january. pp.55-65.

Collier R. "The Plague of the Spanish Lady." 1974; Atheneum Press.

Crosby AW. "America's forgotten pandemic." 1989; Cambridge University Press.

Kilbourne ED. "Influenza." 1987; Plenum Medical Book Company

Reid RH, Taubenberger JK. "The 1918 flu and other influenza epidemics 'over there' and back again." 1999; *Lab Invest.* **79:** 95–101.

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Microbes on building stone – for good or ill?

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Introduction

Microorganisms play a crucial role in mineral transformation in the natural environment, notably in the formation of soils from rocks and the cycling of elements such as nitrogen and sulphur. It is therefore not surprising that a wide variety of micro-organisms, especially bacteria and fungi, have been isolated from rocks and the stonework of historic monuments and buildings such as Portchester Castle (**Figure 1**). The complex interaction of numerous microbial types at a microscopic level in intimate association with the mineral substrate is readily observed[1] often reaching deeper than 3cm into the stone. Microorganisms can be on or inside stone, as endolithic communities. In some circumstances their long-term surface growth establishes a coloured, varied *patina*, which can sometimes be protective to the underlying stone. Often, however, some types of patina growth leads to damage caused by erosion, biopitting and exfoliation (**Figure 2**). Research has highlighted a possible role for microbes in stone deterioration due to one or more mechanisms: their presence as undesirable *surface growths* (aesthetic), *mechanical damage* (biogeophysical change) by biofilms or penetrating hyphae and *corrosive effects* (biogeochemical change) due to metabolic activity (**Table 1**). Scientific investigation can present severe problems with objects of cultural value. Phototrophic organisms such as higher plants, lichens and mosses, together with algae and cyanobacteria, cause obvious surface effects. The impact of most bacteria and fungi is more difficult to appreciate and separate from purely physical and chemical phenomena that are acknowledged threats to the integrity of building stone.

Influence of air pollution

There is extensive evidence to suggest that historic buildings may suffer damage as a result of microorganisms using hydrocarbons in air as a carbon source and producing corrosive organic acids[2]. It is well known that atmospheric combustion pollutants such as nitrogen oxides and sulphur dioxide are a primary cause of accelerated deterioration of exposed stone-works, The gases are oxidized in the air to nitric and sulphuric acid, form acid rain which is deposited onto the surface of stone where carbonates are converted into sulphates (gypsum) and highly soluble nitrates. The presence of dust, residual hydrocarbons and other organic pollutants in urban air leads to stone alterations such as black crust formation, nitratation, and sulphatation, and damage. Black crusts on buildings are the result of atmospheric particles (spores, pollen, dust, and heavy hydrocarbons) being trapped in a mineral matrix of gypsum and re-crystallized calcite minerals. Atmospheric hydrocarbons on artistic stone-works will be supplemented by organic matter related to inadequate past restoration and lysis of microbial cells originating from primary surface colonisation. Nitrate and sulphate pollution processes, accompanied by crust formation and incrustations with organic patina on stonework, induce accelerated weakening and deterioration of the stone matrix. The substrates for microbial activity are certainly present but other factors play a role in the stone deterioration and it is difficult to assess the precise contribution that microorganisms might make to this process. Consequently, damage to stone by microbial mechanisms is the least well understood and was not widely recognised by conservators as a problem to be addressed[3].

Stone colonisation and biofilms

The stone ecosystem is subject to harsh environmental change, especially temperature and moisture, exerting extreme selective pressure on any developing microbial community. The complex consortium of micoorganisms that exists on weathered building stone at any given time is the result of ecological successions and interactions that directly relate to fluctuating substrate availability and environmental conditions. Initially, the mineralogy and structure of stone in relation to its capacity to collect water, organics and particles will control its predisposition to biodeterioration, or bioreceptivity[4].

The ability of the stone-colonizing microflora to cover and even penetrate material surface layers by the excretion of organic extracellular polymeric substances (EPS) leads to the formation of complex biofilms in which the microbial cells are embedded. Phototrophic organisms usually initiate colonisation by establishing a visible, nutrient rich biofilm on new stone from which they can penetrate the material below to

Figure 1. Portchester Castle: a historic monument suffering stone biodeterioration.
Figure 2 (inset). Stone decay and crusts on decorative arches at Portchester Castle.

Table 1. Microbial activities associated with stone biodeterioration

Type of activity	Process
Aesthetic	Surface colour change Slime production
Biogeophysical	Biofilm formation Contraction and expansion of biofilms Blockage of pores Interaction with salts and water Growth/movement through stone
Biogeochemical	Excretion of inorganic acids Excretion of organic acids Enzyme attack of nutrients Chelation of minerals Mineral migration

seek protection from high light intensities or desiccation. Stone EPS trap aerosols, dust and nutrients, minerals, and organic compound complexes and take up water from air and release it under low RH conditions. Stone moisture and nutrients are thereby increased while porosity, water-uptake capacity and evaporation are reduced[5].

Notably rich and homogeneous biofilms, composed mostly of bacterial rods, are often observed on weathered stone substrates from sheltered areas (**Figure 3**). Microorganisms may degrade stone mechanically, chemically and aesthetically through metabolic activities and biomineralisation processes in these biofilms. The mechanical stress induced by shrinking and swelling of the colloidal biogenic slimes inside stone pores may damage stone and it may cause changes in the circulation of moisture to further enhance chemical dissolution and mineral loss from stone.

Interactions of microbes with stone salts

Salts acting on their own are very important decay agents and can attack stones, mainly mechanically in pore spaces during RH and temperature changes. Efflorescences present a niche for halotolerant and halophilic bacterial populations which are osmotically well-adapted to an extreme existence, such as members of Archaea. Media containing high concentrations of sodium chloride and magnesium sulphate (up to 25%) may be appropriate for studying efflorescences on stone monuments[6]. It has also been shown that microorganisms can enhance the physical or chemical processes by interacting with salts in stone[7]. When limestone has been subjected to both microbial and salt weathering, under different temperature/wet/dry cycling regimes, weight loss was higher with microbes alone (7.7%) than Na_2SO_4 alone (4.9%) but the two agents together more than doubled the additive effect and caused extensive exfoliation and fissure formation (**Figure 4**). Thus, by interacting with the effects of the salt, microbial biofilm growth can increase water content and enhance physical, mechanical pressures on stone during wet/dry cycling.

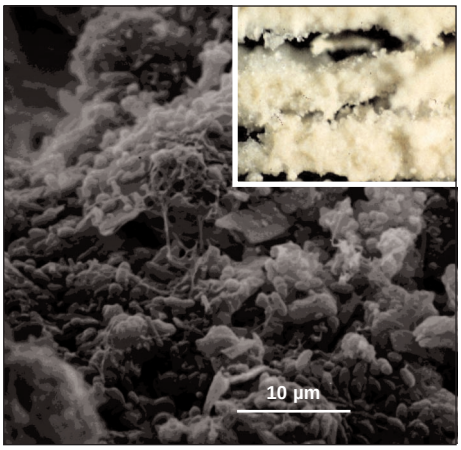

Figure 3. Biofilms on weathered stone.
Figure 4 (inset). Stone discs showing exfoliation after treatment with salts and mixed microbial populations.

Microorganisms associated with damage

Biodeterioration of stone is rarely associated with one group of microorganisms; weathering stone may support a balanced community whose members co-evolve with time to enable recycling of essential elements for activity and growth. Damage may thus be gradual through slow growth (biogenic *drift*) or be sudden and harmful stimulated by a dramatic change in environment, moisture or nutrients (biogenic *shift*). Microbial colonisation of building stones is characterised by a biological succession. Colonisation and conditioning of fresh stone by predominantly phototrophic types (*cyanobacteria, algae, lichens*) will enrich the stone so that chemorganotrophic *fungi, bacteria* and *actinomycetes* can grow on accumulated organic matter, from dead cells and trapped debris. Chemolithotrophs (*sulphur* and *nitrifying bacteria*) will become significant wherever inorganic nitrogen or sulphur compounds are available.

Algae are photosynthetic, developing on porous stone provided dampness, warmth and light are present. There are many instances where algae have caused fouling of stone surfaces or staining without surface changes (e.g. red discoloration of marble due to surface growth of *Haematococcus pluvialis*). Algal communities on stone are often embedded in surface slimy mats together with heterotrophic bacteria and these patinas undergo considerable volume changes through repeated wetting and drying and this has the effect of loosening the stone particles to promote decay. Although the main contributions to decay are to encourage water retention and facilitate succession by more aggressive microbes, corrosive acids have been shown to be produced on marble and limestone.

Cyanobacteria are oxygenic, phototrophic bacteria that can colonise rocks and stone in buildings and produce aesthetic changes due to stains, coloured biofilms and incrustations. They are considered to be pioneers in the colonisation process, along with other autotrophic types, but they may assist the damage process by supporting the growth of other more active decay types. Their tolerance to desiccation, water stress and varying light intensities help to explain their frequent occurrence on stone surfaces.

Lichens are 'microbial' in the sense that they have algal and fungal cells in close association, forming a visible thallus. They can tolerate extreme dehydration and nutrient limitation in the absence of algae or mosses although they are sensitive to air pollution. Growing slowly on (epilithic) and in (endolithic) stone, they are undoubtedly the cause of damage through mechanical and/or chemical means. Deterioration can be caused by the mechanical effect of substratum-penetrating fungal hyphae (bleaching, blistering or sloughing), excretion of oxalic acid and complexing and leaching of stone minerals by chelation.

Fungi are associated with the deterioration of stone and the mechanism of attack is thought to be both mechanical, due to hyphal growth, and chemical, as a result of acid secretion. Fungal mycelia are found penetrating many millimetres into porous stone. One group of fungi isolated from stone are the rock-inhabiting fungi consisting of black yeasts and meristematic fungi, a heterogeneous group of black-pigmented fungi that survive extreme conditions of humidity and sunlight. The latter group includes the Hyphomycetes and Coelomycetes that are more ubiquitous and widely distributed in soil and organic material.

Actinomycetes are filamentous bacteria that are often observed on stone surfaces during *in situ* studies and a large range of actinomycetes have been isolated from stone. Mechanical damage to stone by hyphal penetration of actinomycetes occurs and SEM analysis reveals an extended web of hyphae. These hyphae penetrate the stone material, producing patches of biofilm on stone particles and around the stone pores often interacting with salt crystals. The mycelial nature of actinomycetes (and fungi) gives them a greater capacity to penetrate the stone if it is friable. This may damage the stone directly as well as indirectly by increasing the surface area of biofilm production, which further enhances the stone damage. Laboratory investigations show that *Streptomyces* can greatly enhance the deterioration caused by salts to limestone[8]. *Nocardia restricta* has also been to be prevalent on decaying sandstone, detected by molecular probes[9].

Heterotrophic bacteria are readily isolated in large

numbers from decaying stone (**Figure 5**) but their deteriogenic activity was discounted because stone was thought to contain little organic nutrient to support their growth. However all stonework probably possesses sufficient organic matter from soil, dust and dirt to sustain heterotrophic activity. Moreover, many stone bacteria have a preference for low concentrations of organic nutrients and may even be oligotrophic. Population activity has been related to seasonal and climatic changes and isolated bacteria can produce acids that cause morphological alteration of the stone surface and elution of minerals.

Sulphur-oxidising bacteria are chemolithotrophs which convert inorganic sulphur compounds to sulphuric acid that can cause severe damage to mineral material. Bacteria such as *Thiobacillus thiooxidans*, *T. thiosporus* and other thiobacilli have been isolated from decayed sandstone buildings and marble monuments in urban and rural areas. *Thiobacillus* species have been implicated with concrete corrosion in the Melbourne and Hamburg sewer systems due to sulphuric acid formation. However, a role in stone decay is less certain since sulphuric acid and calcium sulphate in stone can originate from the direct action of atmospheric pollution and acid rain.

Nitrifying bacteria are chemolithotrophs which oxidise inorganic nitrogen compounds for energy and generate acidic end-products either nitrous acid or nitric acid. Ammonia may be carried onto stone in dust as ammonium salts while nitrite can originate from the automobiles, soil or industry. Nitrifying bacteria can be isolated from stone material but a role in stone decay will be favoured in buildings with an obvious source of ammonia or nitrite. Nitrifiers often exist in a biofilm on the surface and within the pores of the stone and *Nitrosomonas*, *Nitrospira* with *Nitrosovibrio* are commonly isolated[10].

Investigating stone populations

Although microbial activity is not always correlated with the numbers of microorganisms on stone, traditional counts of microbial populations have tended to dominate the literature. The traditional approach using artificial growth media has severe limitations due to inappropriate nutrient balance or quantity and inevitably neglects the important interactions between different stone micro-organisms[11]. It is clear that the distortion induced by the use of artificial media gives an unrepresentative estimate of the *in situ* population. Direct microscopic observation by SEM gives no indication of metabolically-active cells. Light microscopy, in combination with the use of fluorescent dyes or chemicals to detect dehydrogenase activity has been used to detect metabolically-active cells. This approach reveals far higher numbers of viable and active bacteria than plate counts and suggests substrate-accelerated death may be partially responsible for the apparent non-culturability of a high percentage of colony-forming units found on artificial media.

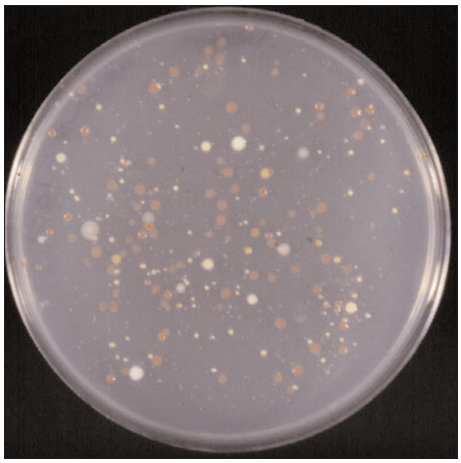

Figure 5. Heterotrophic bacteria recovered from stone on selective media.

Culture-independent techniques based on molecular biology have been used in the last ten years, initially for studying communities on biodegraded wall paintings[12] and extended to buildings and monuments by heritage microbiologists[13]. These methods of molecular ecology, based on extraction of DNA, amplification by PCR and identification by separation of marker sequences using DGGE, can characterise the entire microbial consortium on mineral materials, including the non-culturable majority and rare organisms. Recently Fluorescent *In Situ* Hybridisation (FISH) techniques have been used to detect bacteria and Archaea on stone monuments[14]. Thus target bacteria can be identified and it is possible to detect catabolic genes involved in biodeterioration such as those metabolic activities required for using aromatic hydrocarbon pollutants in air[15]. Molecular methods have been used successfully to assess biodiversity on stone and, as we suspected, our selective media are missing much microbial diversity. Heritage microbiologists are certainly interested in what is there but we especially want to know what they do. Much work is needed if molecular methods can quantify microbial activities that lead to damage. Until this can be done, a polyphasic approach, combining traditional isolation and culture practices with the discriminating power of molecular ecology, will provide the basis for investigating stone damage. Above all, perhaps, the need to understand what is there and how damage is caused must lead to a consideration of how to control the problem.

Controlling microbial growths

Ideally, control of stone biodeterioration should start with the environment (moisture, temperature and nutrients) that determines the growth of microbes. Direct intervention without such an understanding can sometimes lead to new problems[16]. Conservation techniques for stone include manual cleaning to remove biological growths, stains and soluble salts, chemical biocide washes and the application of water repellants and resins.

Microorganisms are most often associated with a visual disfigurement of buildings which can be physically removed by blasting with water or grit, or chemical cleaning. Unfortunately, it appears that such interventions remove only superficial layers and may only reduce microbial numbers for a short time so eradication of established growths requires toxic biocidal action.

Biocides have been widely used before and after conservation treatments, to remove existing microbes (possibly with hydrophobic compounds) and prevent re-growth of the restored surface. There have been concerns about safety in use, environmental effects and long-term effectiveness. Toxic chemical washes, such as quaternary ammonium compounds, are used to eradicate or remove unsightly biological growths from stone but they could be succeeded by other microbes or mosses and higher plants with greater damage potential. In Cambodia, treatment of Angkor Wat to remove a biopatina of algae and lichens led to extensive blackening of the treated stone due to growth of melanin-producing fungi in the absence of competition[16].

In recent years polymers and resins have been used in preservative treatments as waterproofing, consolidant or protective coating. The main types are silicone-based chemicals, inorganics, synthetic organic polymers and waxes/natural resins. Research has shown that some preservative treatments may actually act as a food source and unintentionally stimulate biodeterioration[17].

Bioremediation – microbes as restorers?

While microorganisms have usually been associated with

detrimental effects on stone, affecting mineral integrity or exacerbating powerful physical processes of deterioration, there had been growing evidence that some types can be used to reverse the deterioration processes on historic buildings and objects of art. Bacteria, such as *Pseudomonas* and *Desulfovibrio*, have shown potential to remove harmful salts such as nitrate and sulphate by denitrification and sulphate reduction[18] and to mineralize organic residues or pollutants like carbohydrates, waxes or hydrocarbons which commonly occur in crusts on stonework[19].

Bacteria are also known to precipitate calcium carbonate in their immediate environment (**Figure 6**) and encrust cells in the process of carbonatogenesis (**Figure 7**). This process of biomineral formation by calcinogenic bacteria occurs in the natural environment but recently it has been used on calcareous stones and decorative reliefs (as in **Figure 8**). *Bacillus cereus* has been shown to protect exposed mineral surfaces by the formation of sacrificial layers of calcite, vaterite or aragonite crystals, which may be dissolved in a polluted environments but can be renewed when necessary[20]. Non-sporing bacteria such as *Micrococcus xanthus* may also produce calcite or vaterite crystals which strongly adhere to the original stone and production can, be controlled by changing the environmental conditions[21].

Recently, the EU has funded projects to develop bioremedation processes for conservation. One such project, BIOBRUSH (www.biobrush.org), aims to initially treat damaging salt crusts with different bacteria that can remove sulphate, nitrate and organics (as gases in sulphate reduction, denitrification and respiration) and then consolidate the stone with calcinogenic bacteria using biocalcification. Research will aim to establish how the bacteria can be delivered to the stone surface and to identify the conditions favouring biomineralising activity. Therefore our understanding of how microbes might damage stone provides us with a basis for putting some types to work for us to restore stoneworks and control the damage to cultural heritage in European cities.

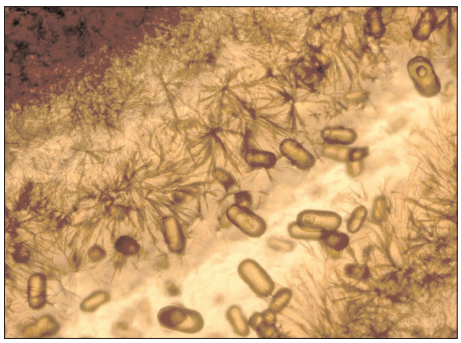

Figure 6. Calcinogenic bacteria in laboratory culture, showing calcite crystals developing within colonies.

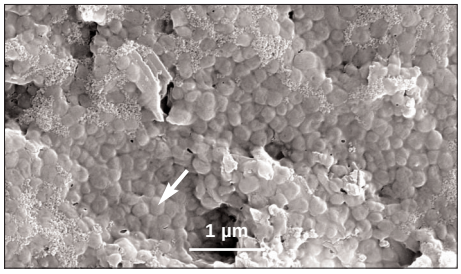

Figure 7. Encrustation of bacteria cells (arrowed) as a result of calcification during culture on stone.

Figure 8. Effect of conservation treatments using calcinogenic bacteria. (Courtesy of Dr Franz Möll, ars restauro, Germany)

Conclusions

Since microorganisms transform minerals in nature, it is no surprise to a microbiologist that many different groups of microbes exist on building stone and may be linked to stone deterioration. Alongside physical and chemical agents of decay, it is sometimes difficult to persuade conservators that biological mechanisms may be significant. Our understanding of the interaction between microorganisms and stone minerals has advanced greatly in the last 10 years, mainly because of dramatic improvements in methodology and research by multidisciplinary groups. Not surprisingly, metabolic diversity and versatility, combined with remarkable tolerance to extreme environmental conditions, characterise microbial communities on stone. However, through a combination of biomineralisation processes, we may be able to tap this versatility and put microbes to work to help us restore historic stonework.

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References

1. Krumbein WE. Microbial interactions with mineral materials. In: Biodeterioration 7. Houghton DR, Smith RN & Eggins HOW (eds), 1988; pp. 78–100. New York, US: Elsevier.
2. Mitchell R and Gu Ji-D. Changes in biofilm microflora of limestone caused by atmospheric pollutants. *International Biodeterioration & Biodegradation*, 2000; **46:** 299–303.
3. Schnabel L. The treatment of biological growths on stone: a conservator's viewpoint. *International Biodeterioration*, 1991; **28:** 125–131.
4. Guillette O. Bioreceptivity: a new concept for building ecology studies. *The Science of the Total Environment*, 1995; **167:** 215–220.
5. Warscheid T, Becker TW, Braams J *et al.* Studies on the temporal development of microbial infection of different types of sedimentary rocks and its effect on the alteration of the physico-chemical properties in building materials. In: Thiel M-J, (Ed.), Conservation of Stone and Other Materials, 1993; RILEM, vol. 1, pp. 303–310.
6. Saiz-Jimenez C and Laiz L. Occurrence of halo-tolerant/halophilic bacterial communities in deteriorated monuments. *Int. Biodeterioration & Biodegradation*, 2000; **46:** 319–326.
7. Papida S, Murphy W and May E. Enhancement of physical weathering of building stones by microbial populations. *Int. Biodeterioration & Biodegradation* 2000; **46**(4): 305–317.
8. May E, Papida S and Abdulla H. Consequences of the microbe-biofilm-salt interaction for stone in monuments. In R Koestler (ed.), Art, Biology and Conservation: Biodeterioration of Works of Art, 2003, In Press, Metropolitan Museum of Art, New York.
9. Palla E, Anello L, Pecorella S, Russo R and Damiani. Characterisation of bacterial communities on stone monuments by molecular biology tools. In: C. Saiz-Jimenez (ed.), Molecular Biology and Cultural Heritage, 2003, pp 115–118.
10. Bock E, Sand W, Meincke M *et al.* Biologically induced corrosion of natural stones – strong contamination of monuments with nitrifying organisms. In: Biodeterioration 7. Houghton DR, Smith RN, Eggins HOW (eds), 1988; pp. 436–440. New York, US: Elsevier.
11. Warscheid T, Petersen K and Krumbein WE. Physiological characterization of chemoorganotrophic bacteria isolated from sandstones. In: VIth International Congress on Deterioration and Conservation of Stone: Supplement, 1988; pp. 26-32. Torun, Poland: Nicholas Copernicus University Press Department.
12. Röllecke S, Witte A, Wanner G and Lubitz W. Identification of bacteria in a biodegraded wall painting by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied & Environmental Microbiology*, 1996; **62:** 2059–2065.
13. Laiz L, Piñar G, Lubitz W and Saiz-Jimenez C. The colonisation of building materials by microorganisms as revealed by culturing and molecular methods. In: C. Saiz-Jimenez (ed.), Molecular Biology and Cultural Heritage. 2003, pp 23–28.
14. Urzi C and Albertano P. Studying phototrophic and heterotrophic microbial communities on stone monuments. *Methods in Enzymology*, 2003; **336:** 340–355.
15. Daffonchio D, Borin S, Zanardini E *et al.* Molecular tools applied to the study of deteriorated artworks. In: Of Microbes and Art: The role of microbial communities in the degradation and protection of cultural heritage (ICMC 99, Florence), 2000; pp 21–38, Kluwer/Plenum.
16. Warscheid T. Integrated concepts for the protection of cultural artefacts against bio-deterioration. In: Of Microbes and Art: The role of microbial communities in the degradation and protection of cultural heritage (ICMC 99, Florence), 2000; pp 185–201, Kluwer/Plenum.
17. Koestler RJ and Santoro ED. Assessment of the susceptibility to biodeterioration of selected polymers and resins. GCI Scientific Program Report, 1988; pp. 56–66. New York, US: The Getty Conservation Institute.
18. Ranalli G, Chiavarini M, Guidetti V *et al.* The use of microorganisms for the removal of nitrate and organic substances on artistic stoneworks. Proceedings of the VIIth International Congress on Deterioration and Conservation of Stone, Berlin, 1996; pp1421–1427.
19. Saiz-Jimenez C. Biodeterioration vs Biodegradation: the Role of Microorganisms in the Removal of Pollutants Deposited on to Historic Buildings. *International Biodeterioration & Biodegradation*, 1997; **40:** 225–232.
20. Castanier S, Le Metayer-Levrel G, Orial G, Loubiere JF and Perthuisot JP. Bacterial carbonatogenesis and applications to preservation and restoration of historic property. In: Of Microbes and Art: The role of microbial communities in the degradation and protection of cultural heritage (ICMC 99, Florence), 2000; pp 246–252, Kluwer/Plenum.
21. Rodriquez-Navarro C, Rodriguez-Gallego M, Chekroun and Gonzalez-Munoz MT. Conservation of ornamental stone by Myxococcus xanthus-induced carbonate biomineralization. *Applied & Environmental Microbiology*, 2003; **69:** 2182–2193.

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