

EXECUTIVE SUMMARY
AND
FINAL REPORT TO
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TECHNOLOGY AND TRAINING

TITLE OF RESEARCH GRANT: The role of microorganisms in the deterioration of atmospheric pollutants of stone used in historic buildings and monuments

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EXECUTIVE SUMMARY

The objectives of this grant were to determine the responses of the microflora to contamination of historic limestone materials with atmospheric pollutants. We also studied the effects of these interactions on the deterioration of the materials.

In the first year of this project we did a detailed comparative analysis of the effect of sulfur and hydrocarbons on the microflora of limestone tombstones in a polluted and unpolluted area of Massachusetts. We found that the populations of bacteria and fungi were significantly smaller on limestone in the polluted area. Similarly, the diversity of the microflora was much smaller on the stone in the polluted area. Conversely, the populations of bacteria capable of utilizing sulfur compounds or hydrocarbons were much larger on the stone from the polluted areas, presumably as a result of the presence of a plentiful supply of sulfur and hydrocarbons deposited from air pollution on the limestone. We found that these bacteria were capable of utilizing very small quantities of atmospheric pollutants and producing significant quantities of acid.

During the second year we investigated the corrosive processes in detail. We isolated and identified the predominant microorganisms growing on the stone in the polluted area. The predominant bacteria belong to the genera *Bacillus*, *Vibrio* and *Xanthomonas*. The major groups of fungi include *Aureobasidium* and *Cladosporium*. We inoculated these predominant microorganisms onto sterilized limestone, and exposed the samples to sulfur and hydrocarbons in our environmental chamber at a temperature of 30 degrees and 80% relative humidity. In these accelerated tests we found that the predominant microorganisms grow to large populations on the limestone at low

concentrations of sulfur and hydrocarbons. Significant quantities of corrosive acids were produced in less than a month in these experiments.

We carried out an extensive scanning electron microscope study of the growth of these organisms on the limestone. Both sulfur and hydrocarbon degrading populations were investigated for their ability to attack limestone. We found that a complex interaction of fungi and bacteria is involved in the limestone attack by both the sulfur and hydrocarbon-utilizing microfloras. Our data indicate that the initial penetration is by fungi that grow into the pores of the stone. However, these fungi act as Trojan horses, carrying a large population of bacteria into the interior of the stone.

During the final year of the project we tested a new innovative method of quantitative analysis of the effects of pollutants on limestone. We used a new high power computer assisted tomography instrument at the Harvard Medical School. This instrument was designed to analyze solid materials non-destructively. It has proved to be an excellent analytic tool in our research.

Our data showed that stone treated with products of acid rain increased in volume. When we exposed this stone to other microbial acids, no loss in volume or voids within the stone were detected. We concluded that the minerals formed by the interaction of sulfates with the limestone provided protection from the action of microbial acids. However, this does not suggest that acid rain provides protection to the stone. Ultimately gypsum minerals exfoliate and cause limestone deterioration.

Our research yielded an important discovery in terms of the microbial ecology of these materials. We frequently detected very small striated microorganisms in our samples. These bacteria were less than one micrometer in size. They were not

culturable. We did not find any previous references to their occurrence. These observations suggest that limestone exposed to atmospheric pollutants may harbor an unusual microflora either capable of living on the resultant minerals or involved in their formation.

INTRODUCTION

Deposition of sulfur dioxide on historic buildings and monuments is well documented in both Europe and the United States (Yerrapragada *et al.*, 1994). The resulting acidity has been shown to cause severe decay. For example, Gauri and Holdner (1981) observed that increased levels of SO₂ in the atmosphere was responsible for deterioration of marble monuments in both Athens and Chicago.

Urban air pollutants are also rich in both aliphatic and aromatic hydrocarbons. Saiz-Jimenez demonstrated that the black crusts coating buildings in European cities where the air pollution is high are rich in both aliphatic and polycyclic aromatic hydrocarbons (Saiz-Jimenez, 1992). These chemicals were found to be trapped in the mineral matrices of the buildings.

Microorganisms have been implicated in the attack of both natural limestone materials and concrete by sulfur compounds. The chemolithotrophic thiobacilli have been shown to cause severe damage to concrete sewer pipes exposed to volatile sulfur compounds (Sand & Bock, 1991). We have observed that, in addition to Thiobacillus, the fungus Fusarium plays an important role in concrete deterioration (Gu, Ford, Berke & Mitchell, 1998). It is probably that a combination of thiobacilli and fungi, forming biofilms on the material surface, are involved in degradation of historic limestone materials.

There is extensive evidence for the involvement of a hydrocarbon utilizing microflora in the biodeterioration of historic buildings. Chemoorganotrophic microorganisms isolated from rocks have been shown to utilize hydrocarbons as sole

carbon sources and to produce significant quantities of organic acids (Warscheid, Oelting & Krumbein, 1991). In the current investigation we have compared the biofilm microflora on limestone gravestones in two locations. One set of gravestones is in a highly polluted urban environment, while the other is in a less polluted rural location. In a laboratory study, we have determined the capacity of the biofilm community, isolated from limestone gravestones in the polluted habitat, to produce acidity and attack limestone.

MATERIALS AND METHODS

Sampling method

Gravestones were selected in two cemeteries. The polluted location was Harvard Square, Cambridge, Massachusetts. The cemetery is located close to the urban center where there is heavy continuous traffic. The gravestones in this cemetery date from the seventeenth century. For our study, we selected limestone gravestones dating from the mid-nineteenth century. For our less polluted location we chose a cemetery in Lexington, Massachusetts. The cemetery, approximately 15 km from Cambridge, is in an area with minimal exposure to urban pollution. We sampled from limestone gravestones dating from the mid-nineteenth century.

In all cases we prepared cotton swabs by dipping them in 10ml of sterile distilled water containing one drop of the non-toxic surfactant Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). The gravestones were swabbed with the damp cotton over one square centimeter areas. The swabs were homogenized in 10 ml of sterile distilled water before microbial analysis.

Enumeration of biofilm microorganisms

All microorganisms were enumerated by plate counts following one week of incubation at 30°C. Heterotrophic bacteria were enumerated on nutrient agar (Difco Lab., Detroit, MI, USA). Chemolithotrophic bacteria were enumerated on the following medium: (gL⁻¹) NH₄Cl, 1.0g; MgSO₄, 0.5g; K₂PO₄, 0.5g; KH₂PO₄, 0.5g; Fe₂(SO₄)₃, 0.5g; Na₂S₂O₃, 1.0g.

For growth of thiobacilli the medium used consisted of (g L^{-1}) $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 10.0g; NH_4Cl , 1.0g; MgCl_2 , 0.5g; $\text{K}_2\text{HP0}_4$, 0.6g; KH_2PO_4 , 0.4g; FeCl_3 , 0.02g; chlorophenol red, 0.08g; agar (Difco Lab, Detroit, MI, USA), 1.0g.

Fungi were enumerated following growth on agar plates containing malt extract, 30g L^{-1} and agar 15g L^{-1} (Difco Lab, Detroit, MI, USA). Penicillin G, $97,500\text{ U L}^{-1}$ and bacitracin 6500 U L^{-1} were added to the medium to inhibit bacterial growth.

Identification of microorganisms

Samples from the gravestones were inoculated to nutrient agar plates for identification of heterotrophic bacteria and to the fungal growth medium described above for identification of fungi. Following one week of incubation at 30°C , the individual colonies were sub-cultured and purified. Characterization of the bacteria was achieved using the Biolog identification system (Biologic Inc., Hayward, CA, USA). Fungi were identified microscopically.

Scanning electron microscopy

Limestone samples were prepared for electron microscopy by inoculating ten square centimeter cubes of marble with mixed populations of microorganisms obtained by swabbing the gravestones. We incubated the limestone in the presence of either one ppm of sodium thiosulfate or one ppm of kerosene in an environmental chamber at 30°C and 80% relative humidity. Following four weeks of incubation samples were prepared for scanning electron microscopy.

A diamond cutter was used to prepare surface layers of the limestone for examination. These pieces were rinsed lightly in sterile water to remove non-biofilm bacteria and then were fixed for 12 hours in 3% gluteraldehyde-0.2M sodium cacodylate, previously filtered through a 0.2 um pore size polycarbonate membrane filter (Gelman Science, Ann Arbor, MI, USA). After washing in cacodylate and dehydration in a series of increasing ethanol concentrations, the samples were critical point dried in liquid carbon dioxide (Samdri PV T-3B, Tousimis Research Co., Rockville, MD, USA). Immediately after drying, the specimens were coated with gold-palladium and viewed under an AMR 1000 scanning electron microscope.

Micro-Computer Tomography (μ CT) Analysis

Samples for μ CT analysis were prepared as follows. Stone blocks (average weight 0.06g) were glued with silicone adhesive (DAP, Inc., Baltimore, MD) onto a plastic cover slip (Dispo Slips, American Scientific Products, McGraw Park, IL). After a 24 hour drying period at 70°C, the stones were scanned using a desktop μ CT imaging system (μ CT 20, Scanco Medical AG, Bassersdorf, Switzerland).

A microfocus X-ray tube with a focal spot of 10 μ m was used as an X-ray source. The filtered 40 kVp X-ray spectrum was peaked at 25 keV, allowing excellent stone-air contrast due to the pronounced photoelectric effect. The source produces a fan beam that is detected by a charge coupled device (CCD) array with 1024 elements. Measurements were obtained by mounting the unprocessed specimen on a turntable that could be shifted automatically in the axial direction. Six hundred projections were taken over 216° (180° plus half the fan angle on either side). A standard convolution-backprojection procedure

with a Shepp and Logan filter was used to reconstruct the CT images in 1024 x 1024 pixel matrices. The spatial resolution of the system was defined by the 10% contrast level in the modulation transfer function (MTF) resulting in a spatial resolution of 28 μm . This micro-tomographic system was originally designed specifically for the nondestructive measurement and analysis of unprocessed surgical bone biopsies and small animal bones. It can be used equally well for other materials with similar X-ray absorption profiles.

For each sample, a total of 51 to 102 micro-tomographic slices, using a slice increment of 17 μm , were acquired depending on the height of each sample (0.9 – 1.7 mm). Measurements were stored in three-dimensional image arrays with an isotropic voxel size of 17 μm . A constrained three-dimensional Gaussian filter was used to partly suppress the noise in the volumes. Stone samples were segmented from background using a global thresholding procedure. In addition to the visual assessment of the three-dimensional images, morphometric indices such as total stone volume and total stone surface were determined from the micro-tomographic data sets using direct three-dimensional morphometry. After scanning, the stone samples were transferred into Eppendorf tubes containing 1 ml of distilled water (control), 250 mM nitric acid and 250 mM sulfuric acid.

RESULTS AND DISCUSSION

The concentrations of atmospheric pollutants in urban environments in the United States have increased dramatically during the past quarter century. Sulfur dioxide is a major pollutant in most cities. Concentrations range from 20 to 200 ppb hr⁻¹ depositing on surfaces, in urban environments, compared to less than 10 ppb hr⁻¹ in rural areas (Seinfeld, 1986). Similarly, organic pollutants, particularly hydrocarbons, are present in high concentrations in the urban atmosphere. Hydrocarbons typically deposit at rates of 500-1500 ppb h⁻¹ in major United States cities. This compares with rates of less than 100ppb h⁻¹ in less polluted habitats (Seinfeld, 1986). These pollutants deposit and accumulate on limestone materials, providing nutrients for a biofilm community on the surface. We compared the microflora on limestone gravestones in cemeteries at two locations in Massachusetts, USA. One location, in Cambridge, was exposed to high concentrations of air pollution from motor vehicles. The other sampling area, a cemetery in Lexington, was exposed to a minimal level of air pollution. We sampled gravestones in both cemeteries from the mid-nineteenth century.

In order to investigate the biofilm microflora on the limestone gravestones we swabbed their surfaces with sterile distilled water. The swabs were used as inocula for both quantitative and qualitative comparisons of the biofilms on the gravestones in the polluted and less polluted locations.

We analyzed for differences in the population size of fungi, heterotrophic and chemolithotrophic bacteria in biofilms on the limestone at the two locations. Figure 1 shows the data. The fungal population was suppressed to a minor degree on the stone in

the polluted city. However, the bacterial biofilms were dramatically different. Both the heterotrophic and chemolithotrophic bacteria on the stone in the less polluted area were orders of magnitude larger than on the stone in the cemetery in the polluted city. All three groups of microorganisms, fungi, heterotrophic and chemolithotrophic bacteria were suppressed in the polluted location, presumably by the air pollutants emitted by the motor vehicles.

When we compared the predominant populations of microorganisms in the biofilms at the locations, we found that there were twice as many different genera of both bacteria and fungi present in the biofilms on gravestones in the less polluted city.

Xanthomonas, *Vibrio* and *Bacillus* were very common in both biofilms. A number of species of *Pseudomonas*, found in biofilms in the less polluted biofilms, were absent from biofilms in the polluted city. Among the fungi, *Penicillium*, *Cladosporium*, *Fusarium*, and *Aureobasidium* were predominant in both locations. *Epicoccum* and *Alternaria* species, common in Lexington biofilms, were rarely found in Cambridge biofilms. The data suggest that air pollution causes a suppression in the species diversity of both the bacterial and fungal communities living in limestone biofilms.

As a means of determining the effect of sulfur pollution on limestone biofilms, we measured the percentage of sulfur-utilizing bacteria in the chemolithotrophic community. Figure 2 shows that only 20% of the chemolithotrophic bacteria in the biofilms on limestone in Lexington, the less polluted location, were capable of utilizing sulfur. Apparently 80% of the biofilm bacteria utilize other inorganics in the stone as sole energy sources. In contrast, we found that 50% of the biofilm chemolithotrophic community on the limestone in Cambridge, the location contaminated by air pollution, used sulfur

compounds as their sole energy source. The data indicate that, despite the suppression of the total population of chemolithotrophic bacteria by air pollutants, there is an enrichment of sulfur oxidizers. Presumably this selective enrichment results from the accumulation of sulfur compounds on the stone.

We carried out a parallel investigation of the effect of air pollution on the hydrocarbon-utilizing bacteria in the biofilms. We compared the populations of bacteria growing on a complete medium and a minimal medium containing hydrocarbon utilizing bacteria in the biofilms on limestone in Cambridge relative to those in less polluted Lexington, the differences were minor. Almost 90% of the heterotrophic bacteria in the biofilms from both locations were capable of using kerosene as a sole carbon source. The results are not surprising, since we would expect that even in the absence of contamination of the stone by hydrocarbons, a proportion of the indigenous heterotrophic bacterial population would be capable of utilizing hydrocarbons at relatively low concentrations of the chemicals. We found no evidence of an enrichment of fungi in the biofilms exposed to air pollutants.

It is probable that, in Massachusetts, where there is frequent precipitation throughout the year, the concentration of sulfur compounds and hydrocarbons depositing and then remaining on the limestone in the cemeteries remains quite low. We studied the effect of sulfur and hydrocarbon concentrations on the biofilm bacterial population from the polluted city to estimate the ability of the microflora to utilize low concentrations of the pollutants.

We inoculated bacteria from the limestone surface to a minimal liquid medium containing different concentrations of sulfur from one ppm to 50 ppb. We counted the numbers of sulfur oxidizing bacteria after fifteen days of incubation. In all cases we found populations in excess of 10^6 cm² growing in the medium. We concluded that the biofilm population was capable of utilizing and developing on sulfur concentrations as low as 50 ppb.

As part of our study of the biofilm sulfur-utilizing population, we measured the production of acidity by the microflora growing at different concentrations of sulfur. At all concentrations, including 50 ppb, acidity began to develop after ten days of incubation. After fifteen days the pH had declined to 6. Even at low concentrations of sulfur, the biofilm microflora is capable of producing damaging sulfuric acid.

We also tested the ability of hydrocarbon degrading bacteria from the biofilms on stone in the polluted location to utilize low concentrations of hydrocarbons. We inoculated microorganisms from gravestones in Cambridge to a minimal liquid medium containing hydrocarbons as the sole carbon source. We inoculated media containing hydrocarbons at concentrations between 10 ppm and 1 ppb.

Following fifteen days of incubation, we counted the number of bacteria in the media at the different hydrocarbon concentrations. Our data showed that there were more than 10^6 cm² hydrocarbon degrading bacteria present at all concentrations tested. At hydrocarbon concentrations as low as 1 ppb there were more than a million hydrocarbon-degrading bacteria present in biofilms per square centimeter of the gravestone.

In a parallel study, we determined the ability of these hydrocarbon degraders in the biofilm to produce acidity. In all cases the pH had declined from seven to six in

fifteen days. Since the acids produced by these bacteria are organic, it is surprising to find such a rapid production to compensate for the probability that the bacteria were producing such weak acids as acetic, propionic and butyric acids.

The presence of large populations of sulfur and hydrocarbon degrading bacteria in biofilms on limestone gravestones in polluted areas suggested that these bacteria were capable of penetrating the limestone and acceleration deterioration. We inoculated both the hydrocarbon-degrading and sulfur-utilizing bacteria to limestone and incubated the samples in an environmental chamber at 25°C and 80% relative humidity for 30 days. Following incubation, the stone samples were critical point dried and prepared for scanning electron microscopy. Within 30 days a thick biofilm had formed on the surface. We observed that the biofilm had penetrated into the pores of the limestone. It is probable that growth was controlled by the depth of penetration and availability of the hydrocarbon in the pores of the limestone. Similar observations were observed in experiments with biofilms of the sulfur oxidizing bacteria. As in the case of the hydrocarbon degraders, our scanning electron microscopic analysis showed that the sulfur-oxidizers in the biofilm penetrated into the limestone pores. It is likely that this microflora produces sufficient acid to attack the limestone, and release calcium, causing dissolution of the stone.

We analyzed changes on the surface of the marble samples and loss of material using μ CT. After being exposed to 250 mM nitric and sulfuric acid solutions, the stones were analyzed. Figure 3 shows composites of the data. Following four days of exposure in distilled water, no apparent change in the surface composition of the stone was detected (Figure3a). However, our data showed that the stone samples exposed to nitric

acid was severely eroded (Figure 3b). It appeared that the nitric acid had dissolved the surface layers with which it came into contact. Figure 3c shows a composite of the μ CT data of a marble sample exposed to sulfuric acid. A newly formed superficial layer with a different density, presumably gypsum, can be observed. We analyzed the μ CT data to determine the effects of the acids on the interior of the limestone samples. Nitric acid caused interior pitting that can be clearly seen as black pockets in Figure 4. Sulfuric acid and deionized water caused no apparent interior dissolution.

The compelling three-dimensional images are reconstructions of numeric data. These data can also be used to quantify the internal and external morphometry of the stone. Quantification of surface area and volume is useful for the investigation of stone. Figure 4 shows that four days of immersion in distilled water marginally affected surface area and volume of the stone sample. Nitric acid, however, caused a major decrease in the volume due to solubilization of the stone, while sulfuric acid had little effect. Conversely, sulfuric acid caused a major increase in surface area, most likely due to the formation of gypsum.

The deposition of acid precipitation on limestone is well documented. Guari and his colleagues have demonstrated the presence of sulfates on gravestones that had been installed less than five years earlier (Gauri & Holdren, 1981). The potential for thiobacilli to produce corrosive sulfuric acid in limestone biofilms had been demonstrated by Sand and Bock (1991). They observed severe corrosion of concrete sewage pipes exposed to sulfur compounds originating in the sewage. They found that the degree of corrosion was proportional to the size of the population of thiobacilli. Recently, we observed that a fungus growing in the concrete biofilm facilitates the attack by thiobacilli

(Gu, Ford, Berke & Mitchell, 1998). The fungus, identified as a *Fusarium* species, significantly increased both calcium release and weight loss from the limestone.

In the current study, we found no evidence that the biofilms in the presence of sulfur compounds contained fungi. We did not observe, in our scanning electron microscopic study, that the thiobacilli penetrating the limestone was associated with fungal hyphae. In contrast to sewage pipes which are exposed to high concentrations of organic matter, the limestone in the current investigation was not heavily contaminated with organic chemicals likely to be utilized by fungi.

Both aliphatic and polycyclic aromatic hydrocarbons are found in the black crusts of monuments (Saiz-Jimenez, 1997). He suggests that exposed building materials act as non-selective surfaces, passively entrapping deposited air borne particulate matter and organic compounds. A number of investigators have suggested that fungi play an important role in the degradation of hydrocarbons (Saiz-Jimenez, 1992; de la Torre, Gomez-Alarcon, Vizcaino & Garcia, 1993). However, no evidence was obtained in the current investigation linking biofilm development on limestone in the presence of hydrocarbons with a fungal population. Neither our comparison of microbial populations in polluted and less polluted locations nor our investigation of the specific hydrocarbon utilizing microbial population yielded evidence of stimulation of a fungal community in the biofilms. It is probable that the porosity of the limestone favored the preferential development of a community of hydrocarbon-degrading bacteria.

Our data showed that the presence of atmospheric pollutants inhibited both the size and diversity of the biofilm microbial community. These observations provide new insights into the effects of stress on microbial community ecology. It is unusual for

pollution stress to suppress microbial activity. Typically, one population becomes dominant, at the expense of others. However, the total size of the community remains stable. In our study, the data suggest that the air pollutants suppress the total community of microorganisms.

The effect of pollution stress normally causes a reduction in the diversity of the microbial community. For example, effluents from coal-coking activities have been shown to reduce the diversity of the microbial community in marine sediments (Saylor *et al.*, 1982). Our investigation demonstrated a similar effect. The biofilm community on limestone impacted by air pollutants was much less diverse than in the less polluted location.

Despite the suppression of the total community of microorganisms by atmospheric pollutants, a specific population of bacteria capable of utilizing sulfur compounds was stimulated. The presence of a large population of these bacteria in limestone biofilms in the polluted location is a strong indication of the involvement of metabolic products of the sulfur bacteria in biodeterioration of historic limestone in habitats contaminated by atmospheric sulfur pollution. Hydrocarbon-degrading bacteria have also been demonstrated to excrete sufficient quantities of organic acids to damage stone materials in historic buildings (Warscheid, Oelting & Krumbein, 1991).

Warscheid *et al.* (1991) suggest that, in addition to organic acids, extracellular polymers produced by bacteria on stone in polluted environments act as surfactants permitting increased capillary action of water into the stone. In the current investigation we observed, in our scanning electron microscope investigations, that both the sulfur and hydrocarbon-utilizing bacteria penetrated from the surface biofilm into the interior of the

limestone. It is possible that surfactants in the bacterial extracellular polymers accelerate the growth of the biofilm into the limestone and facilitate the biodeterioration of the material.

CONCLUSIONS

The goal of this project was to investigate the effects of atmospheric pollutants on the microbial community living on the surface of historic limestone materials, and the resultant deterioration caused by the interaction of the pollutants with the microflora.

At the end of our study we reached the following conclusions:

1. We carried out a comparison of the biofilm communities on tombstones at a relatively unpolluted location and a heavily polluted area, and found that:
 - a) The populations of bacteria and fungi were significantly larger on limestone tombstones in the polluted location.
 - b) The microbial diversity was much smaller on limestone in the polluted area
 - c) The bacterial community on the stone capable of utilizing sulfur or hydrocarbons, was much larger in the location heavily polluted by atmospheric pollutants.
2. In a laboratory study we found that the sulfur and hydrocarbon utilizing bacteria were capable of utilizing very low concentrations of the pollutants, producing significant quantities of acid.
3. We identified the predominant microorganisms growing on the limestone in the polluted areas.
4. Some of our samples yielded very unusual, previously unreported, bacteria.

5. Scanning electron microscopy showed that, in the presence of atmospheric pollutants, biofilms of bacteria covered the surface of the stone and penetrated into the limestone pores.
6. We used a new analytic technique micro-computer-assisted-tomography (μ CT) to investigate the processes involved in microbial interactions with these pollutants on limestone.
7. Our data showed that the products of sulfur oxidation increased the stone volume, probably due to the formation of an external gypsum layer.
8. The μ CT analyses showed that no internal volume loss occurred as a result of the interaction of the limestone with acid products of sulfur oxidation.

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