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16. Abstract

Inspections of bridge substructures in Texas identified surface deterioration of reinforced concrete columns on bridges continuously exposed water. Initial hypothesis were that the surface deterioration was a result of the acidity of the water in which the columns were exposed. However, evaluation of the water acidity indicated that the surrounding waters were only very slightly acidic and near neutral. Discussions between engineers from the Texas Department of Transportation (TxDOT) and researchers at Texas A&M University and the Texas Transportation Institute (TTI) hypothesized that the damage could be a result of microbial attack. Microbial attack is often identified as an acid attack because some microbes can produce sulfuric acid. This research investigated whether microbes were present at areas on the bridge that were exhibiting attack, determined if there was a correlation between degree of damage and number of microbes present, determined if these microbes were acid producing microbes, and identified the microbes present at the field sites. Results indicate that microbes are present at the bridge columns experiencing surface deterioration, that the number of microbes present is directly correlated with the degree of damage, and that these microbes are acid producing. The research identified five genera: these included *Bacillus*, *Brachybacterium*, *Flavobacterium*, *Lysinibacillus* and *Thiomonas*. The group with the largest numbers of representatives was *Bacillus*, which was composed of 17 strains. The second largest group was identified as *Thiomonas perometabolis,* which consisted of seven strains. The researchers concluded that the damage to the concrete bridge columns is microbial attack. Because some bridge structures are exhibiting significant microbial attack of the concrete cover and because the long-term performance of the columns (and hence bridges) are most sensitive to concrete cover, further research is needed on how to prevent and mitigate this attack.

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ANALYSIS AND ASSESSMENT OF MICROBIAL BIOFILM-MEDIATED CONCRETE DETERIORATION

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ABSTRACT

Inspections of bridge substructures in Texas identified surface deterioration of reinforced concrete columns on bridges continuously exposed water. Initial hypothesis were that the surface deterioration was a result of the acidity of the water in which the columns were exposed. However, evaluation of the water acidity indicated that the surrounding waters were only very slightly acidic and near neutral. Discussions between engineers from the Texas Department of Transportation (TxDOT) and researchers at Texas A&M University and the Texas Transportation Institute (TTI) hypothesized that the damage could be a result of microbial attack. Microbial attack is often identified as an acid attack because some microbes can produce sulfuric acid. This research investigated whether microbes were present at areas on the bridge that were exhibiting attack, determined if there was a correlation between degree of damage and number of microbes present, determined if these microbes were acid producing microbes, and identified the microbes present at the field sites.

Results indicate that microbes are present at the bridge columns experiencing surface deterioration, that the number of microbes present is directly correlated with the degree of damage, and that these microbes are acid producing. Total counts of microbial cells indicated a range from $5.27\pm0.88\times10^{6}$/g (slight deterioration) to $3.60\pm0.31\times10^{7}$/g (severely deteriorated). The research identified five genera: these included *Bacillus, Brachybacterium, Flavobacterium, Lysinibacillus* and *Thiomonas*. The group with the largest numbers of representatives was *Bacillus*, which was composed of 17 strains. The second largest group was identified as *Thiomonas perometabolis*, which consisted of seven strains. The researchers concluded that the damage to the concrete bridge columns is microbial attack. Because some bridge structures are exhibiting significant microbial attack of the concrete cover and because the long-term performance of the columns (and hence bridges) are most sensitive to concrete cover, further research is needed on how to prevent and mitigate this attack.

EXECUTIVE SUMMARY

The durability of our infrastructure system is critical. Significant costs are incurred when infrastructure systems have to be repaired, rehabilitated, or replaced early due to deterioration. In addition to the direct costs, indirect costs can be as high as 10 times more than the direct costs. Not considering costs, the disadvantages of having the traveling public delayed or rerouted are significant: taxpayers deserve a robust and durable infrastructure system. Significant research has been performed to investigate and mitigate deterioration in the infrastructure. Corrosion in reinforced concrete (RC) structures has been a long-term challenge, especially in areas where the structures are exposed to salts. Similar to corrosion damage, freeze-thaw damage has been a long-term challenge. More recently, alkali silica reaction (ASR) and delayed ettringite formation (DEF) have been identified as contributing to the early deterioration of RC structures. Even more recent has been an unknown attack of the concrete surface; in some cases attack of both the hydrated cement paste (HCP) and aggregate.

Initial inspection of this surface attack identified the attack as being a result of the surrounding water being acidic. However, some structures were exhibiting significant deterioration indicating that the surrounding water had to be fairly acidic. Evaluation of the surrounding water indicated that the water was very slightly acidic but near neutral, likely eliminating the cause of deterioration. Further investigations and discussions identified the likely cause of this deterioration of the concrete surface as possibly being microbial attack. Of particular concern is that the depth of concrete over the reinforcement is a critical parameter in determining the durability of the RC structure. Deterioration of the surface reduces the depth of cover, reducing the service life of the column and bridge.

Researchers from the Zachry Department of Civil Engineering and the Department of Plant Pathology and Microbiology at Texas A&M University sampled concrete from bridge columns

exhibiting this surface attack. A short-term research program was implemented to identify if microbes were present in the bridge column concrete, to determine if there was correlation between the microbe count and the degree of surface damage, to determine if the microbes were acid producing, and to identify the microbes. The researchers concluded that microbes are present at the bridge columns sampled, that the number of microbes present is directly correlated with the degree of damage, and that these microbes are acid producing. These microbes are deteriorating the concrete, reducing the cover as a function of time. Total counts of the microbial cells ranged from 5.27±0.88×106/g (slight deterioration) to 3.60±0.31×107/g (severely deteriorated). Five genera were identified: *Bacillus, Brachybacterium, Flavobacterium, Lysinibacillus* and *Thiomonas*. The group with the largest numbers of representatives was *Bacillus*, which was composed of 17 strains. The second largest group was identified as *Thiomonas perometabolis*, which consisted of seven strains.

The researchers concluded that the damage to the concrete bridge columns is microbial attack. Because some bridge structures are exhibiting significant microbial attack of the concrete cover and because the long-term performance of the columns (and hence bridges) are most sensitive to concrete cover, further research is needed on how to prevent and mitigate this attack.

INTRODUCTION

The Texas Department of Transportation (TxDOT) has nearly 50,000 bridges in its inventory. A large majority of these bridges are reinforced concrete structures. Over the past several years, structural deterioration has become a critical issue affecting the entire transportation infrastructure network. Reductions in structural capacity with time have been caused corrosion, alkali-silica reactions (ASR), freeze-thaw damage, concrete cracking and loss of bond, and other deterioration mechanisms. Non-fracture critical bridges are inspected every 2 years. In addition to the more common modes of deterioration, TxDOT inspectors recently identified a surface deterioration phenomena not commonly reported or observed in the past on bridge structures (Fig. 1).

Figure 1 (a) view of bridge; (b) close up view of deteriorated column (photo courtesy of K. Pruski, TxDOT)

Of particular concern is that the concrete cover is deteriorating. As the surface concrete deteriorates the depth of cover over the reinforcement is reduced. Reduced concrete covers make the structure more susceptible to corrosion of the reinforcement. The depth and integrity of the concrete cover is critical for the long-term service life of these bridges. Trejo and

Reinschmidt (2007) concluded that the durability of RC infrastructure systems were most sensitive to depth of concrete cover over the steel reinforcement – small changes in the cover depth lead to early corrosion and higher repair, rehabilitation, and replacement costs when compared to other parameters (chloride threshold of the steel reinforcement, reduction of the diffusion coefficient, etc.). Deterioration of the concrete cover could result inefficient use of resources: time, capital, and energy.

Although initial inspections identified the surface deterioration as being acid attack, evaluation of the acidity of the water indicated that the water was only slightly acidic and near neutral. Further discussions and inspections indicated that the damage could be a microbial induced deterioration (MID).

MID of materials constitutes a significant challenge in several industries, including the wastewater treatment, pipeline, and transportation industries (Islande et al 1991, Davis et al 1998, Peccia et al 2000, Vincke et al 2001, Okabe et al 2007). An understanding of the biological and physicochemical processes associated with microbiologically induced concrete deterioration (MICD) is emerging, predominantly from studies on the deterioration in concrete sewer pipes. First, when sufficient moisture and nutrients are available sulfur-oxidizing microbes can colonize on concrete surfaces. It is not well known whether the concrete surface has to be carbonated for these microbes to colonize. If the concrete surface is carbonated, it is believed that several microbe species can colonize. However, at areas where the concrete surface is not carbonated, it is believed that alkaline-tolerant microbes may participate in these early colonization events.

Second, in cases where the surface pH is high, biogenic oxidation of sulfur on the concrete surface lowers the pH and creates conditions for further microbial colonization by neutraphilic and/or acidophilic organisms. Typically, *Thiobacillus spp*. (including *T. thioparus, T. novellus, T. neapolitanus, T. intermedius and T. thiooxidans*) play key roles in these colonization events

(Rigdon and Beardsley, 1956; Mori et al., 1992). Fungal species may also participate in these processes (Cho and Mori, 1995; Gu et al., 1998; Nica et al., 2000).

Finally, microbial growth can further reduce the surface pH of the concrete, thereby leading to significant biogenic release of polythionic and sulfuric acid (Rigdon and Beardsley, 1956; Milde et al., 1983; Sand, 1987; Diercks et al., 1991; Islander et al., 1991). This biogenic release of acid degrades the cementitious material in concrete, generating gypsum ($CaSO_4$) of various hydration states (Mori et al., 1992) and possibly ettringite ($3CaO{\cdot}Al_2O_3{\cdot}CaSO4{\cdot}12H_2O$ or $3CaO{\cdot}Al_2O_3{\cdot}3CaSO_4{\cdot}31H_2O$). Gypsum is soluble and can wash away and ettringite can easily absorb water, resulting in surface expansion and deterioration of the concrete cover surface. The conversion of hydrated cement paste to gypsum and ettringite weakens the integrity of concrete cover, effectively reducing the cover and making the structure susceptible to corrosion.

MICD in sewer systems provides a good example of biogenic concrete deterioration. The pH of uncarbonated concrete is approximately 12; many believe that little microbial activity can occur at such a high pH levels (Sand et al 1997, Lens and Kuenen 2001). However, the pH of alkaline concrete surface can be gradually reduced by carbonation and neutralization of hydrogen sulfide, which buildup in the sewer systems (Zhang et al 2008, Nielsen et al 2005, Matos et al 1995, Lahav et al 2004). Biological oxidation of hydrogen sulfide can take place at the concrete surface exposed to sewer atmosphere (Vollertsen et al 2008). When the pH is reduced, different aerobic and autotrophic *Thiobacillus sp.* colonize on the concrete surface and contribute to a successive oxidation of reduced sulfur to dissociated sulfur acid (Devereus et al 1989, Islander et al 1991). This causes deterioration of the concrete surface. *T. thioparus* is thought to be the first to colonize at the concrete surface, and is believed to disappear as deterioration becomes more severe (Rigdon and Beardsley 1956). When the concrete surface pH is further reduced to moderate or weakly acidophilic conditions, *T. novellus*, *T. neapolitanus* and *T. intermedius* can establish on the surface of the concrete (Milde et al 1983, Sand 1987). At pH values of

approximately 5 or less, *T. thiooxidans* can grow and produce high amounts of sulfuric acid, reducing the pH to as low as 1.5 (Sand and Bock 1984).

A positive correlation between the cell number of *T. thiooxidans* and the level of deterioration has been noted under aggressive conditions (Milde et al 1983). As already noted, the sulfuric acid produced by *Thiobacillus sp* can react with the hydrated cement paste to form gypsum and ettringite, causing surface deterioration. In sewer systems, these products can be removed by sewer flow, accelerate the deterioration process (Mori et al 1992). For environments not in the sewer, higher water flows would be expected to lead to faster deterioration as these conditions would likely carry away the products at a faster rate.

Several approaches to addressing MICD have been pursued. For example, protective coatings that create a physical barrier between susceptible concrete and the biologically active environment have been employed. However, coatings can delaminate over time because of improper preparation of the concrete surface, or inadequate and improper application in the field. In addition, the installation of these coating are generally sensitive to the environment in which they are applied and could be costly. Moreover, deterioration inducing bacteria can penetrate inadequate coatings, thrive on the concrete surface beneath, and more rapidly attack the concrete. Rapid attack could lead to earlier and more costly repairs.

In addition to coatings, biocides could provide an alternative approach to mitigating the effects of MICD. However, common biocides can be toxic to humans and the environment, and are subject to regulatory scrutiny and restrictions. In addition, treating large bodies of water can be cost prohibitive. These issues have encouraged research into environmentally benign MICD control agents; however, an incomplete understanding of the molecular processes mediating MICD has stymied progress in this area.

Several methods for studying MICD have been employed. First, to characterize the population structure of microbial communities on deteriorated concrete, traditional cultivation methods for enrichment and isolation have been used (Islander et al 1991, Diercks et al 1991).

However, this method fails to provide a complete snapshot of all the bacteria that are associated with (and perhaps responsible for) deteriorated concrete (Amann et al 1995). Second, molecular techniques have proven useful for accurately describing the microbial communities in environmental samples. Specifically, a comparison of microbial 16S rRNA gene sequences to sequences present in the databases has been used as the basis for polygenetic analysis. In addition, the profiles of bacterial communities on deteriorated concrete surfaces have been analyzed by denaturing gradient gel electrophoresis (DGGE, Vincke et al 2001). However, both 16S rRNA gene library screening and DGGE are not reliable methods for quantitative population analysis, and bacterial relative abundance cannot be determined by these methods.

Fluorescent In Situ Hybridization (FISH) provides an alternative approach towards quantitative population analysis in these environments (Schrenk et al 1998, Edward et al 1999, Hernandez et al 2002, Okabe et al 2007, Peccia et al 2000), and studies using this approach have suggested that sulfur oxidizing microorganisms are likely responsible for promoting sulfuric acid production in sulfide rich environments. In this study the researchers used both traditional and molecular biology methods to characterize the active acidophilic bacteria growing on the concrete collected from the bridge column environment. Specifically, the results of this systematic analysis of the microbial contribution to concrete deterioration at a bridge site in Texas are reported here. The researchers found that microbial mediated activity likely plays an important and under-appreciated role in the concrete deterioration process of bridge columns in Texas. It should be noted that this deterioration has been identified in seven TxDOT districts.

MATERIALS AND METHODS

Sampling

Samples were collected from a bridge that spans the Navasota River near Bryan, Texas. Three samples were taken from this bridge at areas that exhibited slight to more severe surface deterioration (Fig. 2a). Samples of the deteriorated and non-deteriorated concrete were collected from the concrete surface by scraping the surface with a clean metal chisel and transferring it to a separate sterilized container. Concrete that was not exhibiting deterioration, the control sample for this research, was collected from top of the wall (Fig. 2b).

(a) *(b)*

Figure 2 Locations of sample collections

Sample Fixation

Immediately following the field collection samples were taken to the laboratory. Five grams (0.01 lbs) of each sample was transferred to a sterilized 50 ml (1.6 fl oz) centrifuge tube and fixed with 5 ml (0.16 fl oz) sterilized phosphate buffered saline (PBS) buffer (130 mM NaCl, 7

mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2) containing 4% paraformaldehyde. The samples were then vortexed and stored at 4 ℃ for subsequent treatment. For the total cell count and Fluorescent In Situ Hybridization (FISH) analyses, the tubes were vortexed thoroughly and 2 ml (0.07 fl oz) of each suspension was transferred to a new 2 ml (0.07 fl oz) microcentrifuge tube. After the large particles settled for 2 minutes, a supernatant was pipetted into a new 1.5 ml (0.05 fl oz) microcentrifuge tube and subjected to most probable number (MPN) and FISH analyses.

Total Microbial Cell Counts

The total cell number was determined by MPN technique (Schallenberg et al 1989). Each supernatant (50 ml [1.6 fl oz) was added to a 50+ ml (1.6+ fl oz) centrifuge tube containing 9.95 ml (0.34 fl oz) of filter sterilized water with 2 μg/ml (1.3 E-7 lb/fl oz) DAPI (4', 6'-diamidino-2-phenylindole). The tubes were vortexed and incubated in the dark for 30 minutes at room temperature. The stained solution was filtered onto a 25-mm- (1-inch-) diameter black polycarbonate filter (0.2 μm (0.008 mils) and rinsed with 2 volume of 10 ml (0.34 fl oz) filter sterilized water. The filter was mounted on a clean glass slide and quantified by direct count with fluorescence microscopy. Slides were examined using a fluorescence microscope. Particles that fluoresced bright blue and had well-defined edges of round or rod-shaped were counted as microorganisms. Each sample was counted by randomly choosing 20 fields per slide.

Fluorescent in situ hybridization

The bacteria 16S rRNA targeted oligonucleotide probes were used in this study as follows: probe EUB338 can hybridize with most bacteria (Amann et al 1990, Okabe et al 2007); probe Thio820 is specific for targeting *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* (Hernandez et al 2002, Peccia et al 2000); probe S-S-T.int-0442-a-A-18 is specific for targeting *Thiomonas intermedia* and *Thiomonas peromotabolis* (Okabe et al 2007, Katayama et al 2006); probe S-S-H.neap-635-a-A-19 is specific for targeting for *Thiobacillus neapolitanus* (Okabe et al 2007). The probes were labeled with CY3 and purified with High Purity Liquid Chromatography (HPLC).

FISH was performed according to Heidelberg et al (1993), Hernandez et al (2002) and Bertaux et al (2007) with modifications. Two hundred milliliters (6.7 fl oz) of each fixed cell was pelleted at 10,000 g acceleration for 10 minutes and washed with PBS, resuspended with 50 µl (0.0017 fl oz) PBS and added it to 2 ml (0.07 fl oz) microcentrifuge tube containing 450 µl (0.015 fl oz) of hybridization buffer (0.9 M NaCl, 0.1% SDS, 20 mM Tris pH 7.2). The tubes were preheated up to 45°C (113°F), and then added to the final concentration of 50 ng/ml (3.26 E-9 lbs/oz) of each probe, respectively. After 3 hours of hybridization, cells were then counter-stained with DAPI with a final concentration of 2 µg/ml (1.3 E-7 lbs/fl oz), the hybridization solution was diluted to 20 ml (0.68 fl oz), and then filtered onto a 25-mm- (1-inch-) diameter black polycarbonate filter (0.2 µm [0.008 mils]). To remove the unbound probe, the filter was washed with 20 ml (0.68 fl oz) of PBS. Filters were mounted on microscope slides for observation under a fluorescent microscope. The bacterial cells, which had both hybridization signals and DAPI fluorescence, were counted as hybridized cells. Relative abundance of each specific bacterial group was expressed as the ratio of hybridized cells to total DAPI-stained cells.

Enrichment and isolation of sulfur oxidizing microorganisms

Biogenic sulfuric acid corrosion is known as biodeterioration of concrete in sewer pipelines (Islander et al 1991, Diercks et al 1991). An enrichment method was used to recover the sulfuric acid producing bacteria as Davis et al (1998) and Nica et al (2000) described with modifications. Approximately 10 g (0.02 lbs) of deteriorated concrete was added to 50 ml (1.7 fl oz) of enrichment medium ($Na_2S_2O_3.5H_2O$ 10 g (0.02 lb), NH_4Cl 1 g, $MgCl_2.6H_2O$ 0.5 g (0.001 lb), KH_2PO_4 0.6 g (0.001 lb), K_2HPO_4, $FeCl_3$ 0.02 g (0.00004 lb), yeast extract 1 g (0.002 lb) in 1000 ml (33.8 fl oz) water), and shake incubated for 5 days at 30°C (86°F). Ten ml (0.34 fl oz) of the culture was transferred to a new shaking flask containing 50 ml (1.69 fl oz) of enrichment medium without yeast extract. A positive enrichment growth was indicated by the reduced pH.

After three successive enrichment cultivations, an obvious pH drop was observed. A volume of 50 µl (0.0017 fl oz) of the culture was spread on the enrichment medium agar plates,

and incubated for 7 to 10 days at 30°C (86°F). Different types of colonies in morphology were picked up and streaked onto fresh solid enrichment medium to obtain pure cultures. Acid producing bacteria can be efficiently resolved on solid medium using chlorophenol red as a pH indicator. All of the colonies were made glycerol and subjected to 16S rRNA gene sequence analysis.

DNA Extraction, Polymerase Chain Reaction Amplification, and Sequencing

Bacterial genomic DNA was extracted with a method described by Sambrook et al (1989). The 16S rDNA genes were amplified from genomic DNA using the primers 11f: GTTTGATCCTGGCTCAG and 1492r: TACCTTGTTACGACTT (Siripong and Rittmann 2007). The thermal cycling used the following program: 95 °C (203°F) for 5 minutes, 40 cycles of 50 seconds at 95 °C (203°F), 50 seconds at 45 °C (113°F)and 1.5 minute at 72 °C (162°F), followed by a final extension for 10 minutes at 72 °C (162°F). The Polymerase Chain Reaction (PCR) products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Sequences were determined by MCLAB (California, USA) with the 16S rDNA sequencing primers.

Polygenetic analyses

Phylogenetic affiliation of each 16S rDNA sequence was initially investigated with a Basic Local Alignment Search Tool (BLAST) search against a GenBank database. Sequences were aligned and phylogenetic trees were constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method using the DANMAN software (version 5.1, Lynnon Biosoft, Quebec, Canada). 16S rDNA sequences of phylogenetically related to sulfur oxidizing strains were chosen from GenBank as reference sequences.

RESULTS

Vertical distribution of bacteria on the deteriorated concrete

To assess whether the amount of bacteria found associated with concrete samples correlated with the amount of deterioration observed in the field, the researchers used the MPN method to count the vertical distribution of total bacteria (Fig. 3). Total counts of microbial cells varied between $5.27\pm0.88\times10^6$/g to $3.60\pm0.31\times10^7$/g. The largest amount of bacteria was found in the severely deteriorated concrete, while the slightly deteriorated concrete harbored significantly fewer bacteria. The data therefore indicated a positive correlation between the level of concrete deterioration and the total number of bacteria.

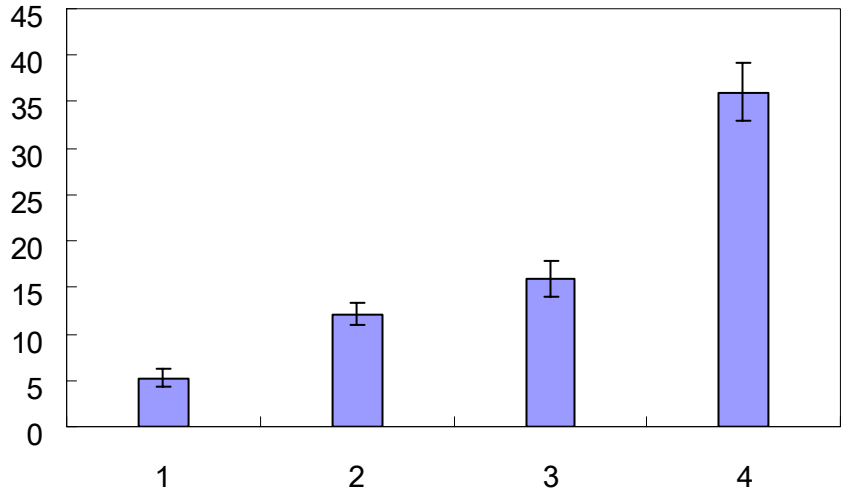

Figure 3 Vertical distribution of bacteria on the corroded concrete. Number on abcissa identifies sampling location, 1 is undeteriorated concrete, 2 is slightly deteriorated concrete, 3 is moderately deteriorated concrete, and 4 is more severely deteriorated concrete. The ordinate is the total number of cells ($\times10^6$ cells/g)

Enriched cultures contain acid-producing strains

Previous studies have demonstrated that sulfuric acid producing bacteria can be recovered from concrete (sewer) systems using enriched culture methods (Davis et al 1998, Nica N et al 2000). The researchers therefore modified this approach to recover and analyze microbial

consortia present in the deteriorated concrete samples collected from bridge. The acid producing activity of the collected consortia was very high. In fact, these microbes dramatically lowered the pH of culture medium from 6.5 to 2.5. Microbes cultured from the concrete material that did not exhibit deterioration failed to display similar levels of acid generation. Samples from the moderately deteriorated concrete, for example, harbored microbes that lowered the pH to 5.1. These data thereby provide further evidence that the deterioration in the concrete samples is a result of acid producing bacteria.

Isolation and characterization of acid-producing strains

In the enrichment studies, the researchers employed media that contained thiosulfate as the sole source of energy. After three successive enrichments, only the microbial consortia derived from severely deteriorated concrete possessed the ability to dramatically lower the pH of the medium. This consortium was spread on a selective medium containing chlorophenol red, a pH indicator dye. Microbes that failed to produce acid did not change the original color of the indicator dye in the medium (purple). However, microbes that produced acid changed the color of the indicator to yellow (Fig. 4). Using this assay, the researchers recovered 27 isolates that grow on selective medium, seven (or 39%) of which displayed dramatic acid producing properties. The pH measurements during growth of this stain on the enrichment medium are shown in Fig. 5. The cells grow quickly from OD 0 to OD 1.78, while the pH of the culture decreased continuously from pH 6.5 to as low as pH of 1.98 after 7 days.

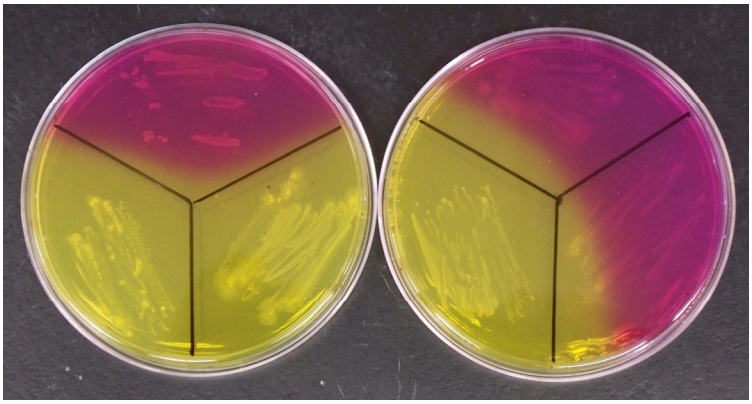

Figure 4 Acid producing strains growing on medium containing sodium thiosulfate pentahydrate and chlorophenol red, a pH indicator. Acid producing microbes cause the indicator dye to turn yellow. Non-acid producing microbes do not change the color of the indicator, and thus the medium appears purple.

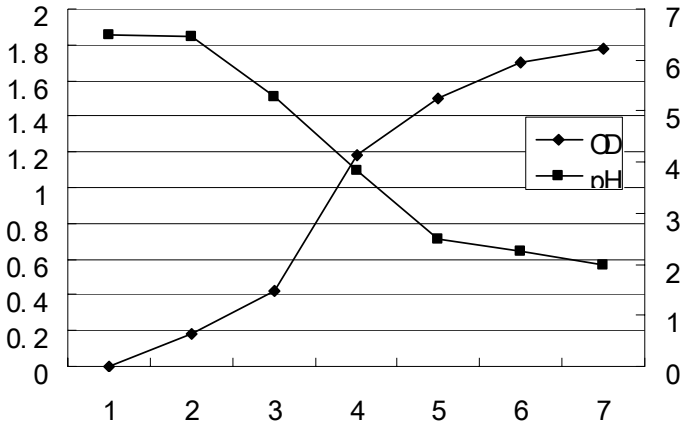

Figure 5 Growth curve and pH changes of *Thimonas perometablis* CBC3 as thiosulfate as substrate

Phylogenetic analysis of the enriched bacteria

Twenty-seven separate enriched isolates were identified by sequencing of 16S rDNA. These sequences were BLAST searched against the GenBank database using BLASTN program. Twenty-seven strains belonging to 5 genera were identified. These included *Bacillus*, *Brachybacterium*, *Flavobacterium*, *Lysinibacillus,* and *Thiomonas*. The group with the largest

numbers of representatives was *Bacillus*, which was composed of 17 strains, followed by another group comprised of 7 *Thiomonas perometabolis* strains. These seven strains shared the same 16S rDNA sequence, which was 99% identical to *Thiomonas perometabolis* (Table 1). To precisely determine the phylogenetic position of strain CBC3 the researchers compared the 16S rDNA sequence with those of other close relatives of sulfur oxidizing microorganisms from the Genbank. A rooted phylogenetic tree was constructed (Fig. 6). Strain CBC3 was found to cluster tightly with the members of group of *Thiomonas perometablis* and *Thiomonas intermedia* with 99.3 and 99 similarities, respectively. The 16S rDNA sequence analysis of strain CBC3 in this study strongly indicated that it was very close to the species of *Thiomonas perometablis.*

Table 1 Sequence similarities after sequencing of amplified 16S rDNA fragments for isolates from deteriorated bridge concrete consortia enriched with sulfate. Sequences were aligned to their closest relatives in Genbank database using BLAST program.

Strains No.	Closest type strains in GenBank database (Accession No.)	Length of Fragment for alignment analysis (bp)	Similarity (%)
11	Bacillus cereus DS16 (EU834245)	882	99
12	Bacillus cereus DS16 (EU834245)	989	99
13	Bacillus cereus FM-4 (EU794727)	1012	99
25	Bacillus cereus JL (EU871042)	1010	99
15	Bacillus luciferensis KSC_SF5b (DQ870692)	742	92
18	Bacillus marisflavi DS6 (EU835732)	890	100
6	Bacillus sp. TS2 (EU073068)	933	99
16	Bacillus sp. B14Ydz-xm (EU070393)	906	99
17	Bacillus sp. KDNB4 (EU835566)	966	99
20	Bacillus sp. BD-93 (AF199522)	627	82
21	Bacillus sp. LQC-4 (DQ219340)	933	99
22	Bacillus sp. C-24 (EU661792)	990	98
26	Bacillus sp. PH3 (EU563374)	874	99
10	Bacillus soli LMG21838 (AJ52513)	659	95
14	Bacillus thuringiensis me-9 (EU652060)	826	100
19	Bacillus thuringiensis me-9 (EU652060)	850	99
24	Bacillus thuringiensis B144 (EU240371)	1006	99
9	Brachybacterium sp. 10084 (EU432559)	657	88
27	Flavobacterium sp. CC-UTSB42218 (DQ072106)	860	98
23	Lysinibacillus sp. 631 (EU841531)	1003	99
1	Thiomonas perometabolis (AY455808)	977	99
2	Thiomonas perometabolis (AY455808)	972	99
3	Thiomonas perometabolis (AY455808)	978	99
4	Thiomonas perometabolis (AY455808)	970	99
5	Thiomonas perometabolis (AY455808)	1033	99
7	Thiomonas perometabolis (AY455808)	956	99
8	Thiomonas perometabolis (AY455808)	959	99

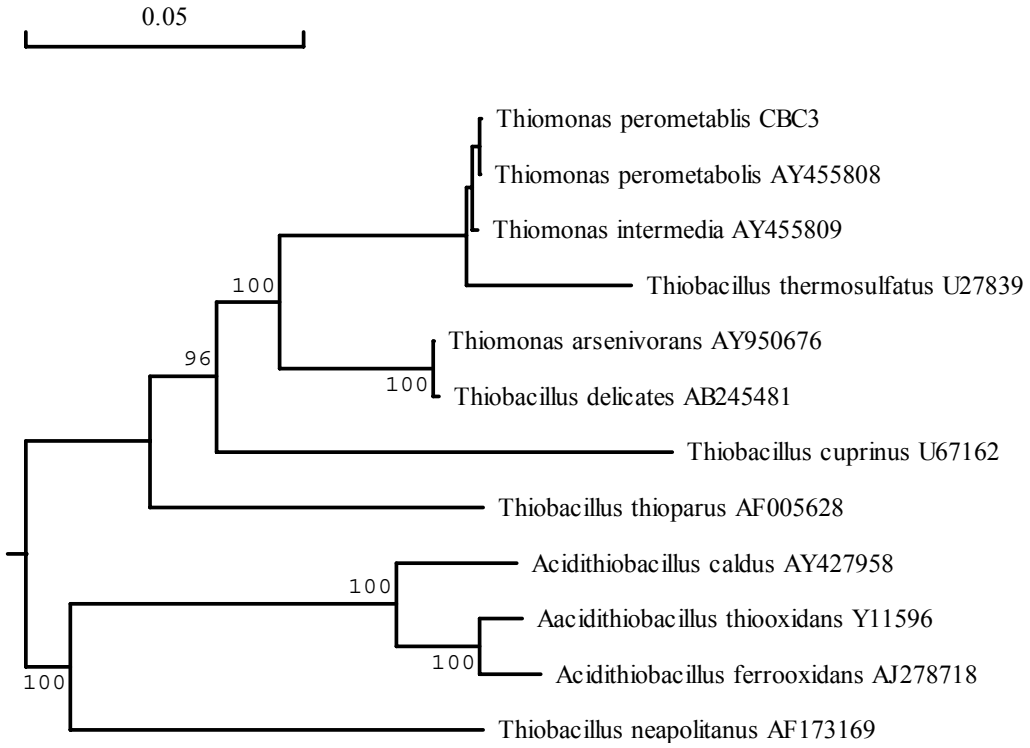

Figure 6 Phylogenetic tree based on 16S rDNA gene sequences showing the relationship of *Thiomonas perometablis* CBC3 to the other species of *Thiomonas*, *Acidithiobacillus* and *Thiobacillus*. The tree was generated by using approximately 1,400 bp of 16S rDNA genes and the neighbor joining method. Reference strains used in the polyogenetic tree can be retrieved by the database accession number following the strains name. The numbers at the branch nodes are bootatrp values based on 1000 re-samplings for maximum. Scale bar equals approximately 5% nucleotide divergence.

FISH Analysis Results

The concrete samples collected from the bridge were subjected to whole cell hybridization with the following four oligonucleotide probes: EUB338, S-S-T.int-0442-a-A-18, Thio820 and S-S-H.neap-635-a-A-19. Results of whole cell hybridizations are presented in Fig. 7. Cells of FISH stained bacteria with the universal probe EUB338 were counted and the number of cells per gram (or per 0.002 lb) was calculated. The researchers found that between $3.75\pm0.65\times10^{6}$

cells/g and 2.88±0.35×10^7cells/g were present in the samples. Moreover, 80% of total cells (i.e., the DAPI stained fraction) were hybridized with the probe EUB338.

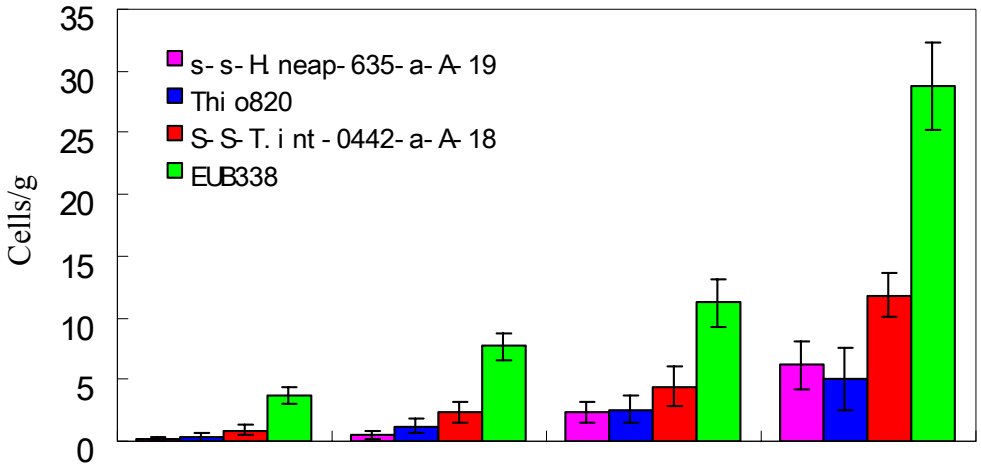

Figure 7 Cell abundance determined by FISH staining in the four sampling location with different level of deteriorated concrete. 1 is undeteriorated concrete, 2 is slightly deteriorated concrete, 3 is moderately deteriorated concrete, and 4 is more severely deteriorated concrete. The ordinate is the total number of cells (×10^6 cells/g)

To investigate the community structure of sulfate oxidizing bacteria, which are believed to be responsible for producing sulfuric acid and for deteriorating the concrete surface, the three remaining probes for detecting *Thiobacillus thiooxidans*, *Thiobacillus ferrooxidans*, *Thiomonas intermedia*, *Thiomonas peromotabolis* and *Thiobacillus neapolitanus* were used. The number of bacterial cells detected with all of the three probes increased with increasing amounts of deteriorated concrete. The cell number hybridized with S-S-T.int-0442-a-A-18 was highest among the detected sulfur oxidizing bacteria, and the amount ranged from 9.1±0.46×10^5 cells/g in the undeteriorated concrete to 1.19±0.18×10^7 cells/g in the deteriorated samples. The concrete that exhibited the most deterioration contained the largest number of cells that hybridized with probe S-S-H.neap-635-a-A-19 and Thio820. These probes can be used to detect *Thiobacillus neapolitanus*, *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans*, which accounted for 17%

and 14% of total bacteria, respectively. *Thiomonas intermedia* and *Thiomonas peromotabolis*, identified with S-S-T.int-0442-a-A-18 was the dominant sulfuric oxidizing bacteria and accounted for 32% of the total microbes.

DISCUSSION

Previous studies demonstrated that biogenic sulfuric attack of sewer concrete can cause severe concrete deterioration (Islande et al 1991, Davis et al 1998, Peccia et al 2000, Vincke et al 2001, Okabe et al 2007). This research shows, quite unexpectedly, that the phenomenon of concrete deterioration on bridge concrete can be nearly as severe as deterioration found in sewer pipes. However, the rate of deterioration is unknown. A conventional most possible number counting technique was used to evaluate the grade of corrosion by the bacteria communities growing on the bridge concrete. A positive correlation between the total cell number of bacteria and the level of deterioration was identified. These results agree with the data obtained by Vincke et al (2001).

Enrichment methods have proven to be an effective technique for isolating the dominant microorganisms in the environment. A variety of microorganisms, heterotrophic and autotrophic bacteria and fungi have been previously enriched and isolated from deteriorated concrete samples (Vincke et al 2001, Nica et al 2000, Gu et al 1998). The researcher's enrichment data include a variety of *Bacillus sp*., *Flavobacterium sp*., *Lysinibacillus sp*., and *Thiomonas sp*. Pure culture of these bacteria, however, demonstrated that only *Thiomonas sp*. was responsible for reduction of pH of the media to 1.98. It was also able to convert thiosulfate to sulfuric acid. Therefore, it is likely that it participates in the concrete deterioration process. By contrast, *T. thiooxidans* was found as the dominant bacteria in the deteriorated sewer concrete (Vincke et al 2001, Nica et al 2000). The other bacteria strains identified in this research failed to produce the acid and little is known about their metabolic products.

Phylogenetic analysis of 16S rDNA sequence of strain CBC3 showed that the strain was found to cluster tightly with the members of group of *Thiomonas perometablis* and *Thiomonas intermedia* with 99.3 and 99 similarities, respectively. The researchers provisionally designated this strain as *Thiomonas perometablis* CBC3. The 16S rDNA gene sequences of *Thiomonas intermedia* and *Thiomonas perometabolis* differ by only four nucleotides (Katayama et al 2006).

To precisely identify its species, other methods such as G+C genomic composition and interspecific DNA-DNA hybridization as well as physiological classification must be performed.

To get a more complete profile of the bacteria that are responsible for sulfur oxidation and pH reduction, a robust and widely applied method (FISH) was used to identify and enumerate bacteria in the concrete samples collected from the bridge. The higher the level of deteriorated concrete, the higher the amount of bacteria was detected by FISH. This result was similar with the report of Vincke et al (2001). When the universal probe EUB338 was hybridized to the cells, the detection ratio of FISH positive cells to total DAPI stained cells ranged from 70 to 80%. The dissimilar detection rates were due to the different activities of microorganisms or different physiological activity in samples. Previous studies showed that the production of sulfuric acid by certain acidophilic bacteria may cause the concrete corrosion in sewer pipes (Islande et al 1991, Davis et al 1998, Peccia et al 2000, Vincke et al 2001, Okabe et al 2007). Three specific probes for hybridization to acidophilic bacteria were used to detect the community structure in the sampled concrete. The abundance of *Thiomonas sp*. detected with probe S-S-T.int-0442-a-A-18 was highest from slightly deteriorated concrete to severely deteriorated concrete. These data thereby provide an explanation on why *Thiomonas sp*. was repeatedly enriched after three successive enrichment cultivations, while the other acidophilic bacteria were lost during the enrichment process due to its lower abundance.

SUMMARY AND CONCLUSIONS

The researchers successfully detected the active sulfur oxidizing bacteria in concrete samples sampled from a bridge exhibiting deterioration using FISH analysis. Through the use of enrichment methods, the research team successfully isolated one strain CBC3, which likely belongs to specie of *Thiomonas perometablis* as determined by 16S rDNA sequence analysis. It was found that this strain can also reduce the pH as low as 1.98. More details about the growth and physiological properties of strain CBC3 are needed to better understand its role in the deterioration bridge concrete.

The researchers found that microbes are present and likely causing surface deterioration to concrete bridge substructure elements continuously exposed to water. Because the durability and long-term performance of a RC structure is concrete cover is most sensitive to the depth of concrete cover and because the microbe attack reduces this cover with time, methods are needed to mitigate and prevent this damage. Future research is needed to identify the majority of microbes that result in deterioration of the concrete in Texas, identify the rate at which these microbes deteriorate the concrete, identify key environmental parameters that promote microbe activity and growth, and to identify methods that can mitigate and prevent this damage.

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