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Microbial mediated deterioration of reinforced concrete structures

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A B S T R A C T

Biogenic sulfuric acid corrosion is often a problem in sewer pipelines, compromising the structural integrity by degrading the pipeline's concrete components. We investigated the microbial populations in deteriorated bridge concrete, with samples taken from bridge concrete both above the water level and in adjacent soils. Total counts of microbial cells indicated a range of $5.3 \pm 0.9 \times 10^6$ to $3.6 \pm 0.3 \times 10^7$ per gram of concrete. These values represent the range from slightly to severely deteriorated concrete. From severely deteriorated concrete samples, we successfully enriched and isolated one sulfur-oxidizing bacterium, designated strain CBC3. This strain exhibited strong acid-producing properties. The pH of the pure culture of CBC3 reached as low as 2.0 when thiosulfate was used as the sole energy source. 16S rDNA sequence analysis revealed that the isolated strain CBC3 was close to members of *Thiomonas perometablis* with 99.3% identity. Fluorescent In Situ Hybridization (FISH) analysis of significant numbers of sulfur-oxidizing bacteria from deteriorated concrete indicated that *T. perometablis* was the dominant acidophilic bacterium, comprising 32.0% of the total active bacteria in the severely deteriorated concrete. Semi-continuous cultures of *T. perometablis* CBC3 and *Thiomonas intermedia* were used to evaluate the biodegradation of cement samples. A weight loss of up to 5.7% was observed after 3 months, compared with a weight loss of 0.3% in non-inoculated control.

1. Introduction

Concrete corrosion has an enormous economic impact worldwide, since most wastewater systems and bridges are concrete. Corrosion severely compromises the structural integrity of these concrete components, costing millions in repairs. Understanding of the fundamental corrosion process and the causative agents will help us develop a corresponding strategy to inhibit the concrete corrosion process.

Microbiologically Induced Deterioration (MID) of materials constitutes a significant problem in several industries, including the wastewater treatment and transportation industries (Islander 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 has begun to emerge. First, when sufficient moisture and nutrients are available sulfur-oxidizing microbes colonize concrete surfaces. Alkaline-tolerant microbes participate in these early colonization events because the pH of non-deteriorated concrete surfaces is high (pH 12). Second, biogenic oxidation of sulfur on concrete lowers the surface pH and creates conditions for further microbial colonization by neutrophilic and/or acidophilic organisms. Typically, *Thiobacillus spp*. (including *Thiobacillus thioparus*, *Thiobacillus novellus*, *Thiobacillus neapolitanus*, *Thiobacillus intermedius* and *Thiobacillus 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 further reduces the surface pH of 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, thereby generating gypsum ($CaSO_4$ of various hydration states) (Mori et al., 1992), and possibly ettringite ($3CaO\cdot Al_2O_3\cdot CaSO_4\cdot 12H_2O$ or $3CaO\cdot Al_2O_3\cdot 3CaSO_4\cdot 31H_2O$), which possess expansive properties. The conversion of concrete to gypsum and ettringite weakens the

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structural integrity of concrete, and eventually can lead to structural failure.

MID of the concrete of sewer systems provides the most thoroughly studied example of biogenic concrete deterioration. The pH of undeteriorated concrete is approximately 12; little microbial activity occurs at such a high pH (Sand et al., 1987). However, the pH of alkaline concrete surface is gradually reduced by carbonation and neutralization of hydrogen sulfide, which buildup in the sewer systems (Zhang et al., 2008; Nielsen et al., 2005; Matos and Aires, 1995; Lahav et al., 2004). Biological oxidation of hydrogen sulfide can occur at the sewer surface exposed to sewer atmosphere (Vollertsen et al., 2008). When the pH approaches neutral, different aerobic and autotrophic *Thiobacillus sp*. colonize the concrete surface and contribute to a successive oxidation of reduced sulfur to dissociated sulfuric acid (Devereux et al., 1989; Islander et al., 1991), which causes corrosion of the concrete. *T. thioparus* is the first to colonize new pipe surface, but disappear as corrosion becomes severe (Rigdon and Beardsley, 1956). When the pH continues to fall to moderate or weakly acidic conditions, *T. novellus*, *T. neapolitanus* and *T. intermedius* become established on the surface of concrete (Milde et al., 1983; Sand, 1987). At pH below 5, *T. thiooxidans* start to grow and produce high amounts of sulfuric acid, causing the pH drop as low as 1.5 (Sand and Bock, 1984). A positive correlation between the cell number of *T. thiooxidans* and the level of corrosion has been noted under these conditions (Milde et al., 1983). The sulfuric acid produced by *Thiobacillus sp*. reacts with calcium hydroxide presenting in concrete to form gypsum and ettringite. The ettringite is an expansive product that leads to rising internal pressure that results in cracking. With the removal of the deteriorated materials by sewage flow, the concrete corrosion accelerates (Mori et al., 1992).

Several approaches to addressing MID of concrete have been pursued. For example, protective coatings that create a physical barrier between corrosion susceptible concrete and the biologically active environment have been employed. However, coatings can delaminate over time because of either improper preparation of the concrete surface or inadequate and improper application in the field. Moreover, deterioration inducing bacteria can penetrate inadequate coatings, thrive on the underlying concrete surface, and thereby further destroy the bond between the coating and the concrete. This results in costly repairs. In addition, biocides provide an alternative approach to mitigating the effects of MID. However, common biocides can be toxic to humans and the environment and are subject to regulatory scrutiny and restrictions. These issues have encouraged research into environmentally benign MID control agents; however, an incomplete understanding of the molecular processes mediating MID has stymied progress in this area.

Several methods for studying MID have been developed. 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, neither 16S rRNA gene library screening nor DGGE are 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; Edwards 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 likely are responsible for promoting sulfuric acid production in sulfide rich environments. To visualize the biodeterioration, a chemical method is often used to assay the released gypsum (Minteny et al., 2000; Vincke et al., 1999; Aviam et al., 2004), and a simulation chamber is used to assay the weight lost (Sand et al., 1987; Mori et al., 1992; Vincke et al., 1999). Here, we used both traditional and molecular biology methods to characterize the active acidophilic bacteria growing on concrete found in a deteriorated bridge support environment. Specifically, we report the results of our systematic analysis of the microbial contribution to concrete deterioration at a bridge site in Texas. We find that the acid-producing bacteria likely play an important and underappreciated role in the concrete deterioration process.

2. Materials and methods

2.1. Site selection and characterization

A bridge that spans the Navasota River in Texas (N30°52′10″ and E96°11′33″) was selected as a case study. The field work included collecting three concrete samples (concrete is made of mixing cement sand and water at weight ratio of 2:2:1) from the bridge exhibiting slight to moderate surface deterioration (Fig. 1). The deteriorated and non-deteriorated concrete samples were collected from the surface of the wall by scraping with a clean metal chisel and transferring them to separate, sterilized containers. Non-deteriorated concrete was collected from top of the wall as a control. Samples were taken in May, 2008 to take into account variations in temperature.

2.2. Sample fixation

Immediately after samples were taken to the laboratory, five grams of each sample were transferred to a sterilized 50 ml centrifuge tube and fixed with 5 ml sterilized PBS buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2) containing 4% paraformaldehyde. Samples were then vortexed and stored at 4 °C for subsequent treatments. For total cell count and FISH (Fluorescent In Situ Hybridization) analysis, the samples were placed in

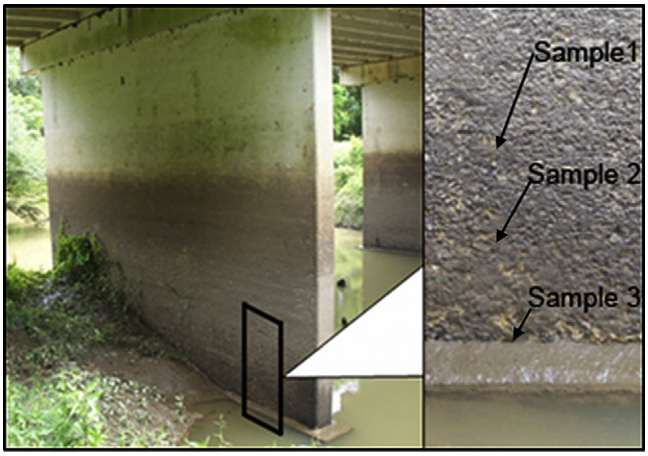

Fig. 1. Bridge support sample sites exhibiting slight (1), moderate (2) and severe (3) corrosion.

5 ml of PBS in a 50 ml tube and vortexed thoroughly, 2 ml of each suspension was transferred to a new 2 ml microcentrifuge tube. Large particles were allowed to settle for 2 min, and the supernatant was pipetted into a new 1.5 ml microcentrifuge tube and subjected to total microbial cell counts and FISH analysis.

2.3. Total microbial cell counts

The total cell number was determined by using the method of Schallenberg et al. (1989). Fifty microliters of each of the above supernatant were added to a 50 ml centrifuge tube containing 9.95 ml of filter-sterilized 2 μg/ml DAPI (4′, 6′-diamidino-2-phenylindole). The tubes were vortexed and incubated in the dark for 30 min at room temperature. The stained solution was filtered onto a 25-mm diameter black polycarbonate filter (0.2 μm, Whatman) and rinsed twice with 10 ml of filter-sterilized water. The filter was mounted onto a clean glass slide and quantified by direct counting under a fluorescence microscope (OLYMPUS BX51®). Particles that had well-defined round or rod-shaped edges and that fluoresced bright blue were counted as microorganisms. Twenty random fields were counted for each sample slide to obtain a valid average.

2.4. Fluorescent in situ hybridization

The bacteria 16S rRNA targeted oligonucleotide probes were used in this study as follows: probe EUB338 hybridizes with most bacteria (Amann et al., 1990; Okabe et al., 2007); probe Thio820 is specific for targeting *T. thiooxidans*, *Acidithiooxidans thiooxidans* and *Thiobacillus ferrooxidans* (Hernandez et al., 2002; Peccia et al., 2000; Okabe et al., 2007); probe S-S-T.int-0442-a-A-18 is specific for targeting *Thiomonas intermedia* and *Thiomonas perometabolis* (Okabe et al., 2007; Katayama et al., 2006); and probe S-S-H.neap-635-a-A-19 is specific for targeting *T. neapolitanus* (Okabe et al., 2007). The probes were labeled with CY3 at their 5′ end and purified with HPLC (Integrated DNA Technologies, Inc. Coralville, IA).

FISH was performed according to Heidelberg et al. (1993), Hernandez et al. (2002) and Bertaux et al. (2007) with modifications. Two hundred milliliters of each fixed sample were pelleted at 10,000*g* for 10 min, washed with PBS, resuspended with 50 μl PBS and added to 2 ml microcentrifuge tubes containing 450 μl of hybridization buffer (0.9 M NaCl, 0.1% SDS, 20 mM Tris pH7.2). The tubes were preheated to 45 °C, and then 50 ng/ml of each probe were added to the tubes. After 3 h of hybridization, cells from each tube were counter-stained with 2 μg/ml DAPI and then the hybridization solutions were diluted to 20 ml and filtered onto a 25-mm-diameter black polycarbonate filters (0.2 μm, Whatman). To remove the unbound probes, the filters were washed with 20 ml of PBS. Filters were mounted on microscope slides for observation under the 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.

2.5. 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 according to Davis et al. (1998) and Nica et al. (2000) with modifications. Approximately 10 g of the concrete sample was added to 50 ml of the *Thiobacillus* enrichment medium ($Na_2S_2O_3 \cdot 5H_2O$ 10 g, NH_4Cl 1 g, $MgCl_2 \cdot 6H_2O$ 0.5 g, KH_2PO_4 0.6 g, K_2HPO_4 0.4 g, $FeCl_3$ 0.02 g and yeast extract 1 g in 1000 ml water), and cultured for 5 days at 30 °C with shaking (150 rpm). Ten milliliters of the enrichment broth were transferred to a new shaking flask containing 50 ml of *Thiobacillus* enrichment medium without yeast extract. A positive enrichment growth was indicated by the reduced pH as determined by pH indicator.

After three successive enrichment cultivations, an obvious pH drop was observed. Fifty microliters of the culture were dilution-plated on the *Thiobacillus* enrichment medium agar plates amended with pH indicator (0.8% chlorophenol red) and incubated for 7 to 10 days at 30 °C. Acid-producing bacteria could be identified as they produced a yellow zone. Colonies with different morphologies were single-colony purified by streaking onto the selective media.

Representative colonies were subjected to 16S rRNA gene sequence analysis. Stocks for all colonies were maintained at −80 °C in a *Thiobacillus* enrichment medium amended to 15% glycerol (final concentration).

2.6. DNA extraction, PCR 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 program used was as follows: 95 °C for 5 min, 40 cycles of 50 s at 95 °C, 50 s at 45 °C and 1.5 min at 72 °C, followed by a final extension for 10 min at 72 °C. The 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.

2.7. Polygenetic analyses

Phylogenetic affiliations of each 16S rDNA sequence was initially investigated by BLAST search against 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). Phylogenetically related 16S rDNA sequence of *T. perometabolis* CBC3 (GenBank accession No.: HM856178) to sulfur-oxidizing strains were chosen from GenBank as reference sequences (Fig. 5).

2.8. Preparation of cement samples and gravimetric determination of cement

Biodegradation of HCP (Hardened Cement Past, the binder in the concrete system) in natural environments is often a slow process. To accelerate the biodegradation of the HCP, we developed a HCP deterioration assay system by submerging HCP samples in semi-continuous cultures of sulfur-oxidizing bacteria grown in *Thiobacillus* enrichment media. This setup allowed the HCP samples to be continuously submerged in a chemically oligotropic medium with *Thiobacillus*. HCP disks (diameter 6 cm; thickness 0.5 cm) with 0.5 cm holes in the center made of a water/Portland cement weight ratio of 0.5 were prepared.

The HCP deterioration setup consists of a covered plexiglass tank (39.4 × 22.8 × 14 cm) partitioned into three equal separate continuous flow chambers that permitted exposure of chemically and gravimetrically defined concrete samples (Fig. 2). Each chamber contained three HCP disks that were evenly spaced horizontally using Teflon spacers and anchored to the bottom of the tank. A peristaltic pump was used to circulate *Thiobacillus* selective medium alone (control), liquid medium containing a known cement deteriorating isolate (*T. intermedia*) (positive control), or liquid medium containing *T. perometabolis* CBC3. The semi-continuous cultures

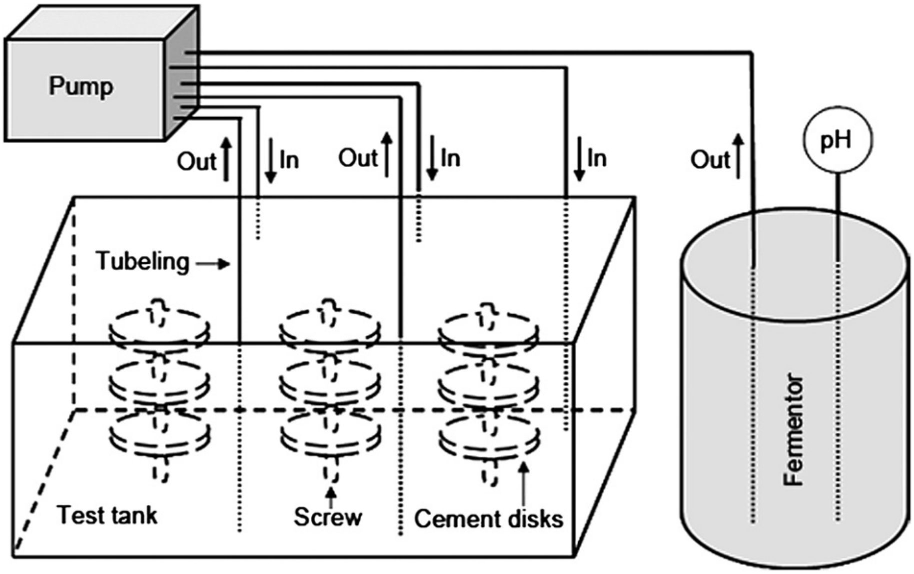

Fig. 2. Diagram of a simulation system flow through chamber for HCP biodeterioration assay. Each of the three tanks contained 9 HCP disks. Fermentors were used to cultivate *T. intermedia* or *T. perometabolis*. Bacteria cultures were pumped into corresponding tanks till the pH of the cultures reached 2. The cultures in the tanks were replaced every week.

were kept running for 3 months at 30 °C at a flow rate of 100 ml/min and monitored for pH. The biodegradation of cement was evaluated by measuring the gravimetric weight loss of the HCP samples. The surface precipitates were carefully removed (gypsum was formed on the surface) with a brush, and the disks were washed with running distilled water, dried, and then weighed.

3. Results

3.1. Vertical distribution of bacteria on deteriorated reinforced concrete bridge support

We obtained samples from a bridge support system that displayed a typical concrete deterioration pattern: that is, the amount of deterioration decreased with the vertical distance from the point at which the support entered the ground (Fig. 1). To assess whether the amount of bacteria associated with concrete material correlated with the amount of deterioration observed, we used a microscopy direct counting method to count the vertical distribution of total bacteria (Fig. 3). Total counts of microbial cells varied between $5.3 \pm 0.9 \times 10^6$ cells/g to $3.6 \pm 0.3 \times 10^7$ cells/g of sample. The largest number of bacteria were found in the severely deteriorated concrete sample (lowest part of the bridge), whereas slightly deteriorated concrete harbored significantly fewer bacteria (higher part of the bridge). The data therefore indicated a positive correlation between the level of visual concrete surface deterioration and the total number of concrete-associated bacteria.

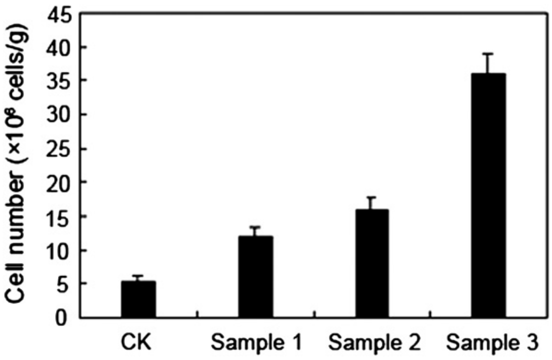

Fig. 3. Microbial load and distribution of bridge concrete samples determined by DAPI staining. CK: sample showing no corrosion; Sample1: slight corrosion; Sample 2: moderate corrosion; Sample 3: severe corrosion. Physical attributes of slight (1), moderate (2) and severe (3) corrosions were shown in Fig. 1.

3.2. Enriched cultures contain acid-producing isolates

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 et al., 2000). We therefore modified this approach to recover and analyze microbial consortia present in deteriorated concrete samples collected from a bridge support. 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 in 7 days when grown in *Thiobacillus* enrichment medium. Microbes cultured from non-deteriorated material did not produce similar levels of acid in broth medium and only reduced the pH of the medium from 6.5 to 6.4. Moderately deteriorated concrete, for example, harbored microbes that lowered the pH to 5.1 over the time course of these experiments (7 days). The data indicate a correlation with the presence of acid-producing bacteria in deteriorated concrete samples with the amount of deterioration observed.

3.3. Isolation and characterization of acid-producing strains

In our enrichment studies, we employed a medium 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 (from pH 6.5 to pH 2.5). This consortium was dilution-plated on a *Thiobacillus* enrichment 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. Using this method, we recovered 27 isolates that grew on the selective medium, seven of which (39%) display acid-producing properties.

The change in pH during growth of a representative *Thiobacillus* isolate, designated CBC3, in the *Thiobacillus* enrichment medium is shown in Fig. 4. The cells grew from OD_{600} 0 to OD_{600} 1.8, and the pH of the culture decreased continuously from 6.5 to 2.0 after 7 days.

3.4. *Phylogenetic analysis of the enriched bacteria*

Twenty-seven separately enriched isolates were identified and characterized by sequencing of the 16S rDNA. These sequences were BLAST searched against the GenBank database using the BLASTN program. Twenty-seven isolates belonging to 5 genera were identified. These included *Bacillus*, *Brachybacterium*, *Flavobacterium*, *Lysinibacillus* and *Thiomonas*. The group with the largest number of representatives was *Bacillus*, which comprised 17 isolates, followed by a group comprised of 7 *Thiomonas* isolates. These seven *Thiomonas* isolates shared the same 16S rDNA sequences, which displayed 99% identity to *T. perometabolis* (Table 1.). We designated one of these isolates CBC3. To precisely determine the phylogenetic position of isolate CBC3, we compared the 16S rDNA sequence with the sequences of other close sulfur-oxidizing relatives (as annotated in from GenBank). Isolate CBC3 was found to phylogenetically cluster tightly with the members of group of *T. perometabolis* and *T. intermedia* with 99.3% and 99.0% similarities, respectively (Fig. 5). Therefore, our 16S rDNA sequence analysis of isolate CBC3 strongly indicated that it was very close to the species of *T. perometabolis*.

3.5. *FISH analysis sheds light on the population structure of bacteria present in deteriorated concrete*

The collected samples from the bridge concrete were subjected to whole cell hybridization with oligonucleotide probes that recognized DNA fragments in eubacteria (EUB338). In addition, probes that hybridized to bacteria and are reported to be responsible for both producing sulfuric acid and deteriorating concrete in sewer systems were employed. These probes recognized *T. intermedia* and *T. perometabolis* (S-S-T.int-0442-a-A-18), *T. thiooxidans* and *Thiobacillus ferooxidans* (Thio820), and *Thiobacillus neopolitanus* (S-S-H.neap-635-a-A-19).

The number of cells that hybridized to the universal eubacterial probe (EUB338) per gram of collected samples was determined. We found that bacterial cells ranging from $3.8 \pm 0.7 \times 10^6$ cells/g (sample 1) to $2.9 \pm 0.4 \times 10^7$ cells/g (sample 3) were present in the collected samples (Fig. 6). Moreover, 80% of the total cells (i.e., the DAPI stained fraction) hybridized to probe EUB338 (Fig. 6), and thus were of eubacterial origin. The number of sulfur-oxidizing bacterial cells rose as the level of deterioration in the concrete samples increased. *T. intermedia* and *T. perometabolis* were the most

Table 1
Sequence similarities of amplified 16S rDNA fragments from representative bacteria enriched from deteriorated bridge concrete samples[a].

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

[a] Sequences were aligned to 16S rDNA of their closest relatives in Genbank database using the BLASTN program.

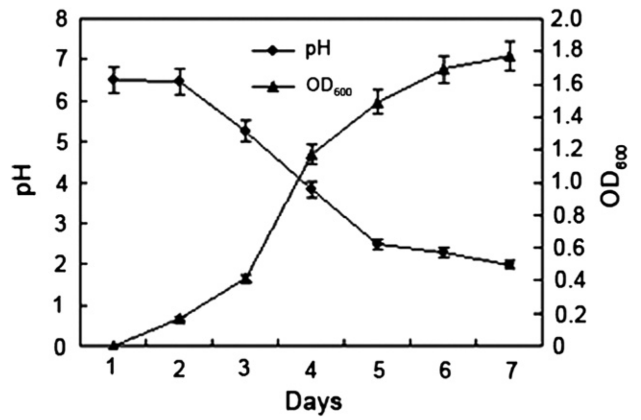

Fig. 4. Growth curve of *Thiomonas perometabolis* CBC3 and pH changes of *Thiobacillus* selective medium.

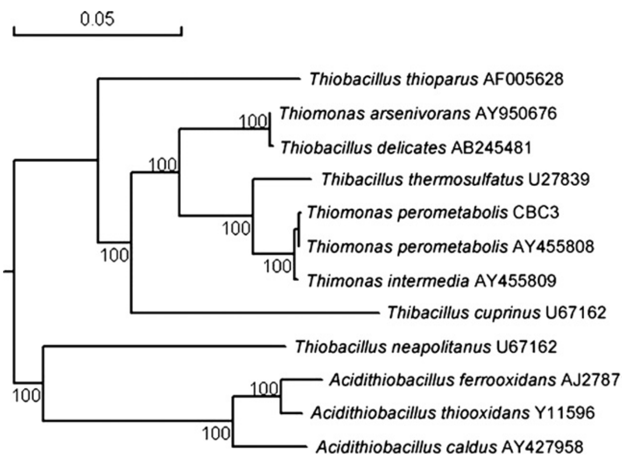

Fig. 5. Phylogenetic tree based on 16S rDNA gene sequences showing the relationship of *Thiomonas perometabolis* CBC3 to the other species of *Thiomonas*, *Acidithiobacillus* and *Thiobacillus*. The phylogenetic tree was generated using approximately 1,400 bp of 16S rDNA genes by the neighbor joining method. Reference strains used in the tree can be retrieved with their accession numbers. The numbers at the branch nodes are bootstrap values based on 1,000 re-samplings for maximum. Scale bar equals approximately 5% nucleotide divergence.

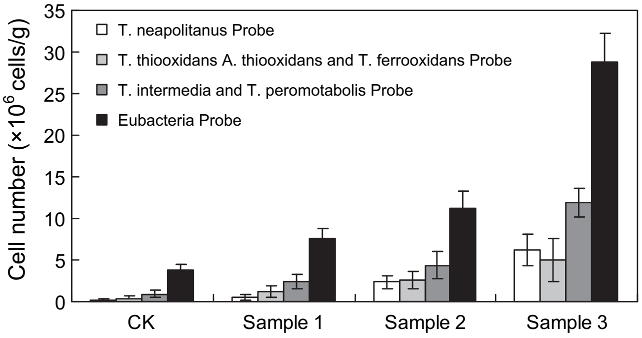

Fig. 6. Cell abundance determined by FISH-staining in the four sampling locations with different levels of deterioration using *T. neapolitanus* Probe (S-S-H.neap-635-a-A-19), *T. thiooxidans*, *A. thhiooxidans* and *T. ferrooxidans* Probe (Thio820), *T. intermedia* and *T. perometabolis* Probe (S-S-T.int-0442-a-A-18), as well as Eubacteria Probe (EUB338).

prevalent sulfur-oxidizing bacteria in all of the examined samples, accounting for 32.0% of the total in sample 3. The cell numbers of these two bacterial species ranged from $9.1 \pm 0.5 \times 10^5$ cells/g in the non-deteriorated concrete samples to $1.2 \pm 0.2 \times 10^7$ cells/g in the most deteriorated samples (e.g., sample 3). Severely deteriorated concrete contained the largest number of cells that hybridized with the probe S-S-H.neap-635-a-A-19 and Thio820, which detected *T. neapolitanus*, *Acidthioibacillus thiooxidans* (syn. *T. thiooxidans*) and *T. ferrooxidans*. These probes hybridized to 17.1% and 14.1% of the total bacteria, respectively.

3.6. Gravimetric determination of HCP deterioration in a laboratory model

To directly assess the role of *T. perometabolis* in mediating the deterioration of concrete material, we developed a laboratory model that mimicked salient aspects of the bridge support environment (Fig. 2). Specifically, HCP samples of known composition were submerged in laminar flow chambers filled with defined amount of *Thiomonas sp.* (See Materials and Methods for details). HCP samples submerged in sterile *Thiobacillus* selective medium or *Thiomonas sp.* in these chambers were weighed before and after 3 months of operation (Fig. 7). No significant weight loss was observed for the control HCP samples that were exposed to sterile medium. However, the weight losses of the HCP disks exposed to *T. intermedia* or *T. perometabolis* (CBC3) averaged 1.2 ± 0.2 g (5.6%) and 1.2 ± 0.2 g (5.7%), respectively.

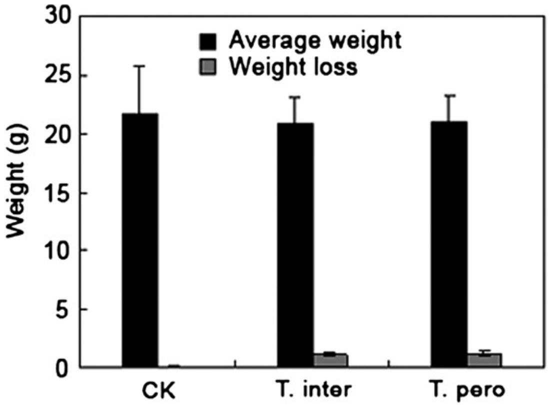

Fig. 7. The average weight of HCP disks before experiment and weight loss after submerged in semi-continuous cultures of *T. intermedia* (T. inter) and *T. perometabolis* CBC3 (T. pero). The HCP disks were submerged in liquid medium (control).

4. Discussion

Previous studies demonstrated that biogenic sulfuric acid attack of sewer concrete causes severe concrete deterioration (Islander et al., 1991; Davis et al., 1998; Peccia et al., 2000; Vincke et al., 2001; Okabe et al., 2007). Our research showed that bacterial-mediated deterioration also occurs on reinforced concrete bridge piles. A conventional microscopy direct counting technique was used to correlate the degree of corrosion and the presence of the bacterial communities on the concrete supports. A positive correlation between the total bacterial cell number and the level of deterioration was obtained. Our results agreed with the data obtained by Vincke et al. (2001), in which correlation of the severity of concrete deterioration to microbial abundance and activity was established in sewer systems.

Enrichment methods have proven to be an effective technique for isolating the dominant microorganisms in the environment. A variety of microorganisms, both 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). Our enriched bacteria included a variety of *Bacillus sp.*, *Flavobacterium sp.*, *Lysinibacillus sp.*, and *Thiomonas sp.* An analysis of pure cultures of these bacteria, however, demonstrated that only *Thiomonas sp.* was capable of significantly reducing the pH of the *Thiobacillus* enrichment media. It was also able to convert thiosulfate to sulfuric acid. Therefore, *Thiomonas sp.* likely participated in the concrete deterioration process. In contrast, *T. thiooxidans* was found to be the dominant bacteria in deteriorated sewer concrete (Vincke et al., 2001; Nica et al., 2000). Other isolates uncovered in our studies failed to produce acid and little is known about their metabolic products or activities.

Phylogenetic analysis of 16S rDNA sequence showed that isolate CBC3 clustered tightly with *T. perometabolis* and *T. intermedia* with 99.3% and 99.0% sequence similarities, respectively. The 16S rDNA gene sequences of *T. intermedia* and *T. perometabolis* differ by only four nucleotides (Katayama et al., 2006). To precisely identify this species, other methods such as G + C genomic composition, interspecific DNA–DNA hybridization as well as physiological classification must be performed.

To generate a more complete profile of the bacteria that were responsible for sulfur oxidation and pH reduction, FISH, a robust and widely applied method, was used to identify and enumerate bacteria in the deteriorated concrete samples. In the bridge support system, the higher the level of deterioration of concrete, the larger the amount of bacteria detected by FISH. A similar phenomenon was also reported by Vincke et al. (2001) in concretes from sewer systems. The detection ratio of FISH positive cells hybridized to the universal probe EUB338 to the total DAPI stained cells ranged from 70% to 80%. The dissimilar detection rates were likely due to the different activities of microorganisms or different physiological activity in the samples. Previous studies showed that the production of sulfuric acid by certain acidophilic bacteria was responsible for concrete corrosion in sewer pipes (Islander et al., 1991; Davis et al., 1998; Peccia et al., 2000; Vincke et al., 2001; Okabe et al., 2007). Three specific probes for acidophilic bacteria were used to detect the community structure in deterioriated bridge support concrete. The abundance of *Thiomonas sp.* detected with probe S-S-T.int-0442-a-A-18 was the highest in slightly deteriorated concrete (sample 2) and severely deteriorated concrete (sample 3). These data thereby explained why *Thiomonas sp.* was repeatedly enriched after three successive enrichment cultivations, whereas other acidophilic bacteria, which are less abundant, were lost during the enrichment process.

Biodegradation of HCP in natural environments is expected to be a slow process. To accelerate the biodegradation of HCP, we developed a HCP deterioration assay system by incubating HCP samples in semi-continuous cultures of sulfur-oxidizing bacteria grown in a thiosulfate containing medium. This setup allowed continuous submersion of the HCP samples in the acidic cultures and resulted in rapid HCP weight loss of up to 5.7% in 3 months, whereas a weight loss of only 0.3% was seen in the non-inoculated control. Weight losses due to exposure of concrete to sulfur-oxidizing bacteria were reported previously. For example, Sand and Bock (1984), Sand et al. (1987) performed experiments to expose concrete to biogenic sulfuric acid over a period of 270 days. The severely deteriorated test blocks had a weight loss of 5.8%. A higher rate of weight loss of up to 16% after 39 days by Aviam et al. (2004) was also reported. The different degradation rates of the HCP among these experiments were attributed to different running systems and the surface/volume ratio of the HCP samples (Aviam et al., 2004).

In summary, we successfully detected active sulfur-oxidizing bacteria in concrete samples derived from a deteriorated bridge. The average weight of concrete disks exposed to *T. perometabolis* CBC3 culture in a semi-continuous laminar flow system reduced by 5.7% after 3 months. More studies on the growth and physiological properties of isolate CBC3 are needed to better understand its role in the deteriorated reinforced concrete bridge supports.

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